The Development of a Luminescence Bioassay for Thyroid Stimulators

A thesis submitted for the degree of
Doctor of Philosophy

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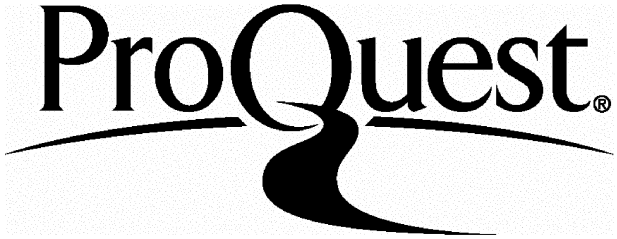

ABSTRACT

Graves' disease (GD) in an autoimmune thyroid disease caused by thyroid stimulating antibodies (TSAb). These mimic thyroid stimulating hormone (TSH) and result in hyperthyroidism. It has long been anticipated that an ability to quantitate TSAb in the circulation would improve the management of Graves' patients. However, to date, no entirely suitable bioassay has been developed. We aimed to exploit recent and on-going advances in both reporter and luminescence technologies to obtain a TSAb bioassay better suited for such clinical use. Ideally, it should be precise, sensitive, rugged and technically amenable so that it has a high sample throughput.

The bioassay was based upon target cells transfected to express both the human TSH receptor and also a cAMP-responsive luciferase reporter gene. An initial attempt to transfect JP_{26} CHO cells ourselves was unsuccessful. We therefore utilised the CHO25LUC cells which had been so transfected during the course of our work, at N.I.H.. These were used for a succession of 4 protocols (Bioassays A – D) aimed at progressively refining bioassay performance characteristics. The optimal within-assay precision of Bioassay D was ~3%, as demonstrated with imprecision profiles. This was achieved by a strategy of maximizing the magnitude of bioassay response and minimizing replicate errors. Bioassay conditions needed, however, to be strictly controlled to minimize between-assay variation, since the bioassay is not rugged. We exploited the favourable bioassay precision to demonstrate unequivocally that TSH and different TSAbs do not yield parallel dose-response curves. We subsequently, adapted the bioassay for use with unfractionated human sera (10μl) and finally, exploited the high throughput of the optimized bioassay in a preliminary screening of sera from Graves' patients (n = 231), comparing the results against relevant control groups.

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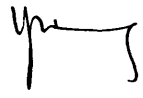
Finally, I would like to thank my parents and brother for their constant support and encouragement, and also friends, for without them, I would not have succeeded in fulfilling my dream.

DECLARATION OF INVOLVEMENT

I declare that this thesis has been composed by myself, and that the work contained herein was principally performed by myself.



Signed: Yerh Siu Wong

I declare that the conditions of the ordinance and regulation (Ph.D.) have been fulfilled.

Signed: Dr. Nicholas J Marshall

ABBREVIATIONS

ΔH	imprecision
AC	Adenylate cyclase
AM	Assay medium
AMP	Adenosine 5′-monophosphate
ATD	Antithyroid drug
ATG	Aurothioglucoase
ATP	Adenosine 5′-triphosphate
AVP	Arginine vasopressin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3′,5′-monophosphate
C (domain)	Constant (domain)
C-terminal	Carboxyl-terminal
(ϕ)CV	(mean) Coefficient of variation
Cys	Cysteine
CBZ	Carbimazole
CDRs	Complementary-determining regions
CHO	Chinese hamster ovarian
CRE	cAMP-response element
CREB	cAMP-response element binding (protein)
CREM	cAMP-response element modulator
CT	Calcitonin
CTLA-4	Cytotoxic T-lymphocyte-associated-4
D (segments)	Diversity (segments)
DAG	Diacylglycerol
DHI	Diagnostic Hybrids, Inc.
DIT	Diiodotyrosine
DMSO	Dimethylsulphoxide
EIA	Enzymeimmunoassay
ESTA	Eluted Stain Assay
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
GD	Graves' disease
GDP	Guanine 5′-diphosphate
GH	Growth hormone
GTP	Guanine 5′-triphosphate
hCG	Human chorionic gonadotropin
hGPHα	Human glycoprotein hormone α-subunit
hmB	Hygromycin B
hph	Hygromycin-B-phosphotransferase
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
HRE	Hormone response element
Ig	Immunoglobulin

IBMX	3-isobutyle-1-methylxanthine
ICER	Inducible cAMP early repressor
IL	Interleukine
IP_3	Inositol 1,4,5-triphosphate
J (segments)	Joining (segments)
LATS	Long-acting thyroid stimulators
LH	Luteinizing hormone
LRRs	Leucine-rich repeats
MHC	Major histocompatibility complex
MIT	Monoiodotyrosine
MMPs	Metalloproteinases
MTA	Microculture tetrazolium salt assay
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
nTRE	Negative thyroid hormone response element
N-terminal	Amino-terminal
N.I.H.	National Institute of Health
NIS	Sodium / iodide symporter
O.D.	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pit-1	Pituitary-specific transcription factor 1
P.I.	Potency index
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PLCβ	Phospholipase Cβ
PKA	Protein kinase A
PKC	Protein kinase C
PMS	Phenazine phosphodiesterase
PS	Patient serum
PTU	Propylthiouracil
rER	Rough endoplasmic reticulum
rT_3	Reverse T_3
R.L.U.	Relative light unit
S.D.	Standard deviation
T_o	Thyronine
T_3	Triiodothyronine
T_4	Thyroxine
Tg	Thyroglobulin
Tm	Melting temperature
TBAb	Thyroid blocking antibody
TBI (assay)	TSH-binding inhibition (assay)
TBG	Thyroxine-binding globulin
TGI	Thyroid stimulating immunoglobulin
TPO	Thyroid peroxidase
TR	Thyroid hormone receptor
TRAb	Thyroid receptor antibody

TRAPs	Thyroid hormone auxiliary proteins
TRE	Thyroid hormone response element
TRH	Thyrotropin releasing hormone
TSAb	Thyroid stimulating antibody
(h)TSH	(human) Thyroid stimulating hormone (i.e. thyrotropin)
TSHR	Thyroid stimulating hormone receptor
TSI	Thyroid stimulation index
TTF-1	Transcription factor-1
TTR	Transthyretin
UCH	University College Hospital
URE	Upstream regulatory element
V (domain)	Variable (domain)

CHAPTER 1

INTRODUCTION

Section 1

The Thyroid

1.1 Development of the thyroid

The thyroid gland has a dual embryonic origin, the median anlage and the two lateral anlagen, from two distinct regions of the endodermal pharynx. The median anlage arises from a thickening in the midline of the anterior pharyngeal floor and is located in between branchial arches 1 and 2 (Boyd JD, 1964; Nunez EA & Gershon MD, 1978). The two lateral anlagen, which are also called the ultimobranchial bodies, are caudal projections developed from the fourth or fifth pharyngeal pouch (Boyd JD, 1964; Nunez EA & Gershon MD, 1978; Merida-Velasco JA *et. al.*, 1989). These provide the precursors to the parafollicular cells.

The median anlage first appears in the human foetus around gestation days 16 and 17 and continues to proliferate rapidly and enlarge ventrally. The thyroglossal duct becomes the source of attachment of the anlage to the pharyngeal floor. Within the anlage, thyroid progenitors proliferate and result in the lateral expansion of the thyroid rudiment. This leads to the formation of the bilobe configuration. The rudiment descends interiorly and anteriorly to pass the hyoid bone on the anterior side and continues to descend into the neck after moving round the hyoid bone. This final caudal displacement is accompanied by the elongation of the thyroglossal duct, which eventually fragments and is followed by atrophy of the remnants (Boyd JD, 1964; Nunez EA & Gershon MD, 1978).

The ultimobranchial bodies as caudal projections begin their development when the median anlage commences its descent (Boyd JD, 1964; Merida-Velasco JA *et. al.*, 1989). The ultimobranchial bodies separate from the pharyngeal pouches and coalesce with the lateral portions of the thyroid lobes. This fusion is completed by the 8 or 9th week of gestation and the thyroid, by now, has taken on its definitive form. The gland is able to take up iodine by the twelfth week and secretes thyroid hormones soon after.

1.2 Thyroid Structure

1.2.1 Gross Structure

The thyroid gland is a bilobed endocrine gland located in the anterior of the neck, caudal to the larynx (Figure 1.1). It is the largest endocrine gland weighing ~20g in a human adult. The thyroid is attached to the trachea by the isthmus and consists of two asymmetrical lobes lying on either side of the trachea. Each lobe is in a flat oval shape and they are joined together by the isthmus which lies over the second to fourth tracheal rings. Thus, the gland resembles a butterfly shape. Although the lobes are similar in size (~ 5cm long x 3cm at the widest x 2cm thick), the right lobe is commonly larger than the left. Furthermore, a pyramidal lobe is often observed. It derives from the remnant of the thyroglossal duct which extends upward from the isthmus or the medial part of the lobes.

The thyroid is one of the most vascular organs. A normal thyroid gland has a flow rate of 5ml/g/min. There are four main arteries and veins. They include the paired superior

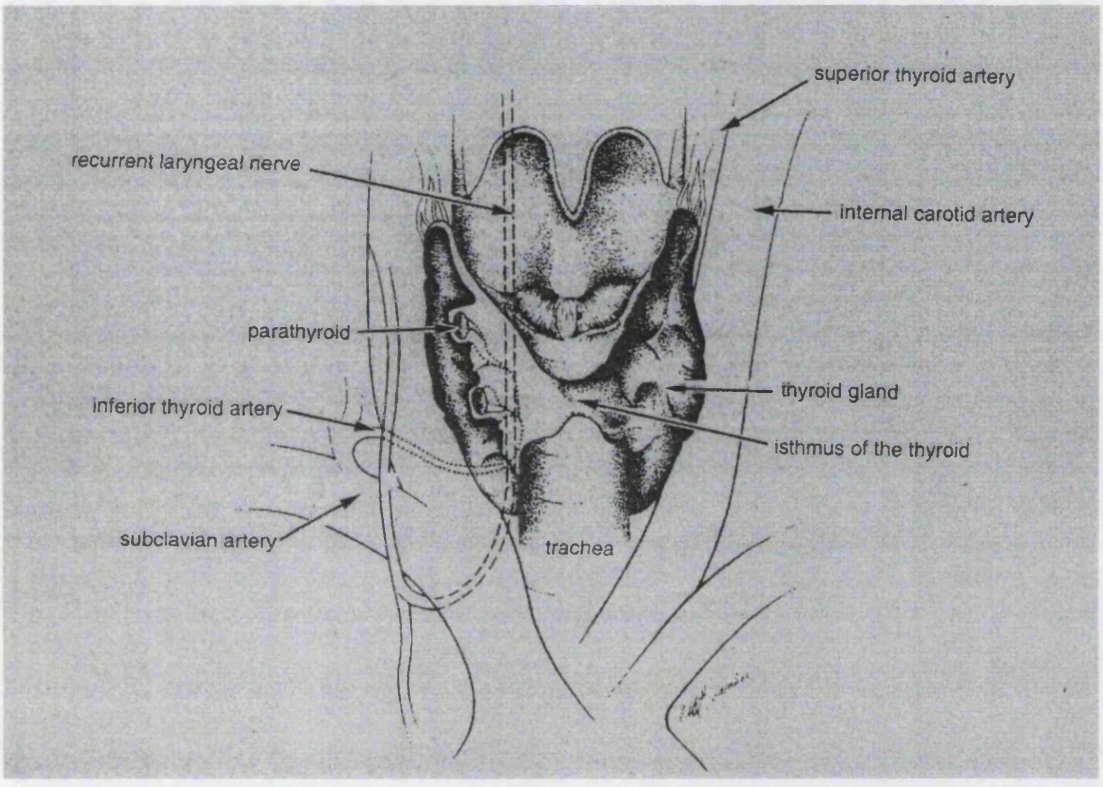


Figure 1.1 **The anatomy of the human thyroid gland**. (West JB. Physiological Basis of Medical Practice. Baltimore: Williams & Williams; 1991, p112)

thyroid artery/vein and inferior thyroid artery/vein. The superior artery, a branch from the external carotid artery, enters into the upper area of the lobes. The inferior artery is a branch of the subclavian artery which descends into the lower parts of the lobes. The paired veins, superior and inferior, are located in the same areas of the lobes as their counterpart arteries. They drain into the internal jugular vein and the left innominate vein respectively. In addition, a middle thyroid vein also drains into the internal jugular vein.

There is also a network of lymphatics following the veins. They communicate between the two lobes and drain into the internal jugular, pretracheal and anterosuperior mediastinal nodes. From the posterior areas of the lobes, the lymphatics drain into the retropharyngeal nodes.

1.2.2 Histology

The thyroid gland has a unique structure. It consists of thyroid follicles, C cells and a stroma that includes an extensive interfollicular capillary plexus that envelops the follicles (Figure 1.2A).

Thyroid follicles

Thyroid follicles are the most important functional units of the gland (Figure 1.2A). They are also called acini. A follicle is made up of a single layer of follicular epithelial cells surrounding the follicular lumen. The lumen contains the glycoprotein thyroglobulin which is sometimes referred to as "colloid". The follicles are produced

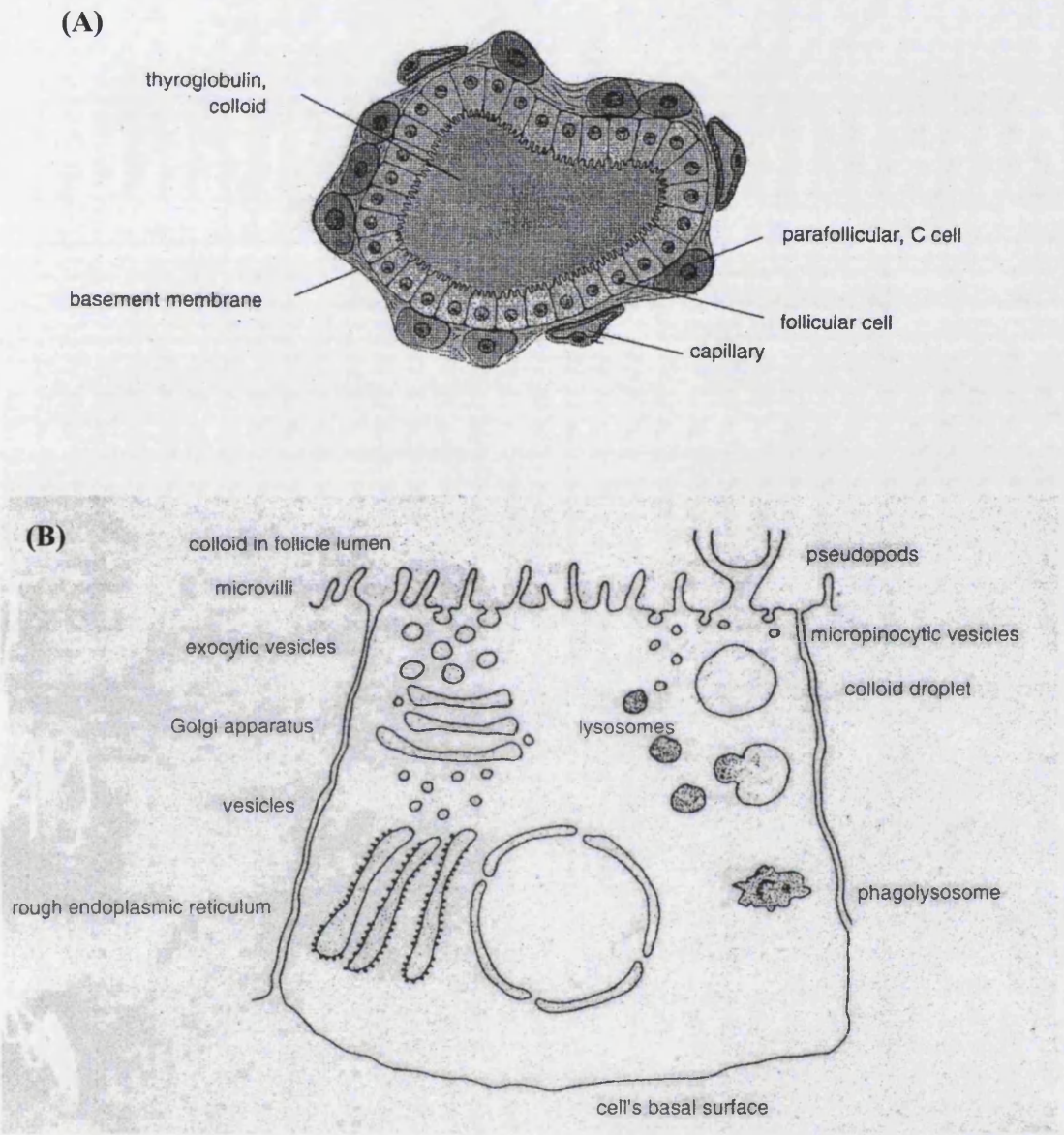


Figure 1.2 **Illustrations of (A) the histology of a thyroid follicle and (B) a thyroid follicular cell and its intracellular structures**. (Taken from: (A) Tortora GJ & Anagnostakos NP. Principles of Anatomy and Physiology, 6th Ed. New York: Harper & Row Pubs.; 1990, p514; (B) Ekholm R & Bjorkman U. Structural and functional intergration of the thyroid gland. In Greer MA, ed. The Thyroid Gland. New York: Raven Press; 1990, p39)

by the process of folliculogenesis. Initially, an intracytoplasmic cavity is developed within each cell. During development, the proliferation of component cells and the coalescence of adjacent colloid-containing microfollicles form these follicles (Collins WT & Capen CC, 1980; Toda S & Suglihara H, 1990). The follicles vary in size having an average diameter of between 200 - 300μm, but this can vary from 100 - 1,000μm.

Follicular cells

Follicular epithelial cells are cuboidal to columnar in shape (Figure 1.2B). Their size and shape are dependent on the level of thyroid activity. A typical cuboidal cell is approximately 10μm long. However, during stimulation, the cell can lengthen to ~20μm and becomes more columnar. These cells are structured specifically for their functions.

- The luminal surface that forms and surrounds the lumen is called the apical membrane. On the surface are numerous microvillar projections which protrude into the lumen. These serve to increase the surface area in contact with the colloid. This is important for the exchange of colloidal materials between the follicular cell cytoplasm and the lumen.
- The membrane at the opposite pole is referred to as the basal plasma membrane. It is responsible for the uptake of iodide and other substrates for the synthesis of the thyroid hormones.
- The intracellular components in the cytoplasm are equally important and definitive. These include:

  i) long profiles of rough endoplasmic reticulum (rER) for protein synthesis.

ii) prominent golgi apparatus for packaging of substantial amounts of protein to be transported into the lumen.

iii) numerous electron-dense lysosomal bodies which are required for the final processing and secretion of thyroid hormones.

iv) microtubules and microfilaments which are essential for the transport of materials within the cytoplasm.

Lumen

As mentioned above, the joining of adjacent apical membranes of the follicular cells forms the follicle lumen. It has two major functions, firstly as a store for newly synthesised thyroglobulin and secondly as a final assembly site for thyroid hormones. The latter is unique for the endocrine system. Consequently, the luminal size reflects the overall biosynthetic activity of a particular thyroid gland.

C cells

C cells or parafollicular cells are scarce in number in comparison with the follicular cells. They are located within the follicular wall proximate to the basal membrane or between follicular cells (Kalina M & Pearse AGE, 1971). They can also be found as a small group of cells amongst thyroid follicles. They do not therefore directly border the lumen. C cells secrete the peptide hormone calcitonin (CT). This is a polypeptide composed of 32 amino acid residues arranged in a straight chain. The hormone is contained within numerous small membrane-limited secretory granules in each C cell (DeGrandi PB *et. al.*, 1971). An increase in calcium ion (Ca^{++}) concentration in the

cytoplasm and extracellular fluid stimulates the release of CT directly into the interfollicular capillaries that surround the thyroid follicles.

The stroma

As mentioned before, the thyroid gland is acutely vascularised. Capillaries form an extensive inter- and intra-follicular capillary plexus. This provides the follicular cells with an abundant blood supply. This network lies in close proximity to the follicular basal plasma membranes. The follicular cells extract essential materials for the synthesis of the thyroid hormones via their basal membranes. When the thyroid gland is stimulated, thyroid hormones are released and diffuse into these capillaries thereby entering into the circulation. In addition to the capillary plexus, a lymphatic system and a number of nerve fibres can also be found. Most of the nerve fibres are sympathetic but a small number are parasympathetic. They either terminate on the blood vessels or in apposition to the follicular cells.

1.3 Thyroid Physiology

The thyroid gland is a functionally unique endocrine gland. It is the only gland which metabolises iodine. The thyroid extracts dietary iodide for the production of the two thyroid hormones: these are the iodothyronines triiodothyronine (T_3) and thyroxine (T_4). The metabolism of iodine, from uptake to the synthesis and secretion of the thyroid hormones can be summarised as follows:

i) active uptake and transport of iodide,

ii) iodination of tyrosyl residues of thyroglobulin (Tg),

iii) coupling of iodotyrosine molecules within Tg to form T_3 and T_4,

iv) proteolysis of Tg, release of free iodotyrosines and iodothyronines, and secretion of iodothyronines into the blood,

v) deiodination of iodotyrosines within the thyroid and the reuse of liberated iodide and,

vi) deiodination of T_4 to T_3 by deiodinase enzymes.

T_3 and T_4 are stored within the follicles until released in response to thyroid stimulating hormone (TSH). These hormones have a wide range of actions and, thus, an effective and efficient regulatory system is employed. This is detailed in the following sections.

1.3.1 Synthesis of Thyroid Hormones

The biosynthesis of T_3 and T_4 involves the uptake of inorganic iodide (I^-) which is incorporated, through a series of metabolic steps into thyroid hormones (Figures 1.3 & 1.4). This process takes place within the thyroid follicles.

Iodide uptake

Serum iodine is taken up in the form of I^- by the thyroid. This occurs from the follicular capillaries which are proximal to the basal membranes of the follicular cells. Uptake is an active transport process against a chemical and electrical gradient. The concentration of I^- inside each follicular cell is generally 20 - 40 times higher than that in serum.

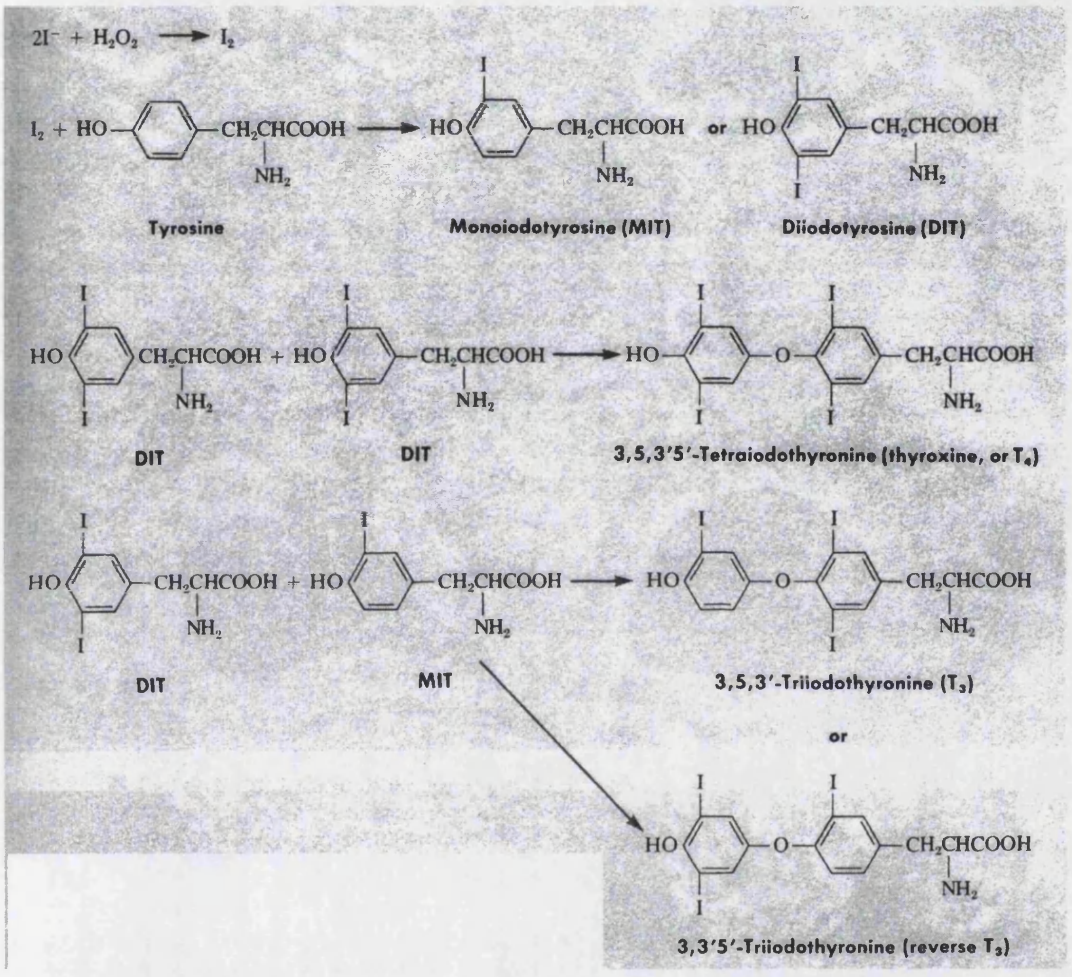


Figure 1.3 **The metabolic pathway of thyroid hormone synthesis**. Following the active uptake and transport of iodide (I^-) into the lumen, tyrosyl residues of thyroglobulin are iodinated to generate the iodotyrosine molecules: monoiodotyrosine (MIT) and diiodiotyrosine (DIT). Thyroid hormones, T_3, T_4, and rT_3, are then produced by the coupling of these iodotyrosine molecules as illustrated above. (Genuth SM. The Endocrine System – The Thyroid Gland. In Berne RM & Levy MN eds, Physiology, 3rd Ed. St Louis: Mosby – Year Book; 1993, p935).

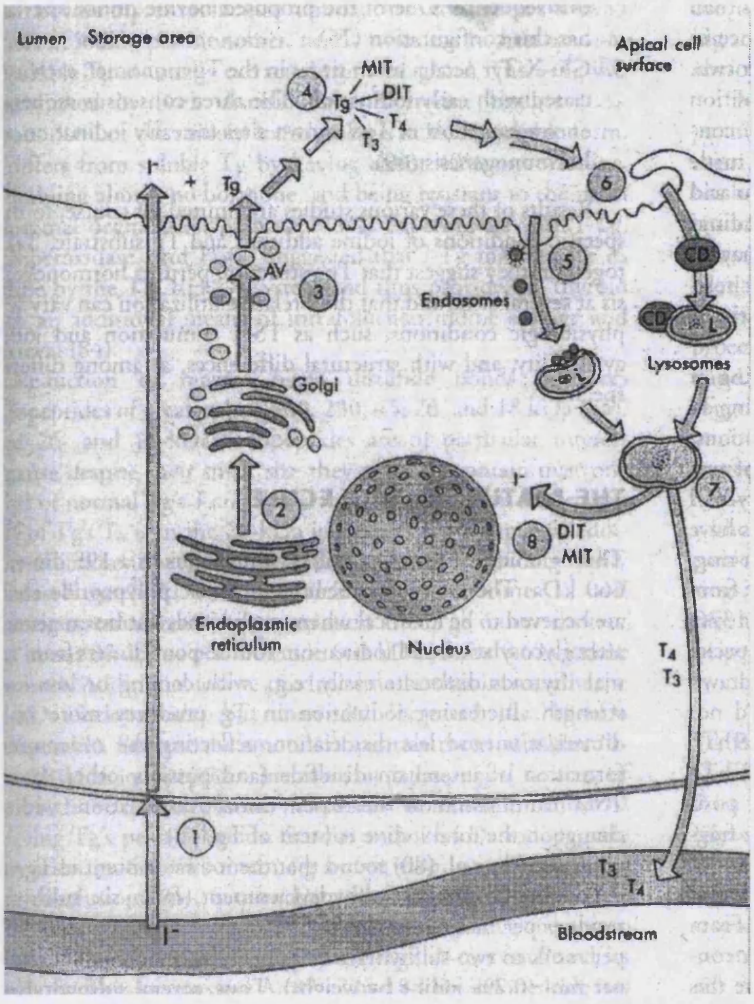


Figure 1.4 **The synthesis and secretion of thyroid hormones by the thyrocyte.** (1) Concentrated iodide (I^-), by the sodium/iodide symporter, at the basal plasma membrane is transported through the follicular cell into the lumen. (2) The synthesis of thyroglobulin (Tg) by the endoplasmic reticulum and (3) the transport of the newly formed Tg into the lumen via the golgi complex and apical vesicles. (4) The iodination of Tg and the coupling of the iodotyrosines to form T_3 and T_4. For the secretion of the thyroid hormones, pseudopods are first formed at the apical membrane. They are then internalised via two mechanisms: (5) micropinocytosis and (6) macropinocytosis. (7) The pseudopods then migrate to the lysosomes (L) for proteolysis of Tg. The thyroid hormones released are secreted into the bloodstream whereas (8) iodide released from iodotyrosines is recirculated. (Brody TM, Larner J & Minis KP, eds. Human Pharmacology: molecular to clinical, 3rd Ed. St. Louis: CV Mosby; 1998, p535)

This energy dependent active uptake is performed by a sodium/iodide (Na^+-I^-) symporter (NIS) which is located in the basal membranes. It transports two Na^+ together with one I^- into the cells, against their electrochemical gradients. Another transporter system, the sodium/potassium (Na^+-K^+) ATPase, generates the driving force to impel the NIS by the translocation of K^+ inwards at the expense of Na^+. A number of factors can consequently affect iodide transport. The most influential is the stimulatory hormone TSH. Others include cyclic adenosine 3',5'-monophosphate (cAMP), the mediator for the NIS, and an iodide autoregulation system (Ingbar SH, 1972; Pisarev MA, 1985). The latter is responsible for the inhibition of I^- uptake when there is excessive iodide. This system is thought to be mediated by different forms of organic iodide which are not yet identified (Braverman LE & Ingbar SH, 1963).

Once I^- is taken into the follicular cells, it is rapidly transported to the lumen and is immediately oxidised to I_2 by a thyroid peroxidase (or thyroperoxidase) located in the microvilli. Final iodination of the tyrosyl residues in the thyroglobulin occurs within the lumen, probably via a free radical intermediary of the iodide ion.

Thyroglobulin

Thyroglobulin (Tg) is a polypeptide which is unique to the thyroid. It provides the matrix for thyroid hormone synthesis. It is a high molecular weight (660kd) glycoprotein which consists of two identical subunits. Each molecule of Tg contains ~5,500 amino acid residues of which there are 120 - 130 tyrosine residues. Tg is synthesised by membrane-bound ribosomes on the rER in the follicular cells. Newly

synthesised Tg is packed into vesicles by the golgi complex and transported into the lumen via fusion with the apical membrane. Tg is the most abundant protein within the colloid.

Iodine (I_2) oxidised from I^- at the apical membrane is instantaneously incorporated into the tyrosyl residues of the Tg. Iodination which is catalysed by the enzyme thyroid peroxidase also occurs on the luminal side of the apical surface (Ekholm R & Wollman SH, 1975). Iodination of tyrosyl residues yields two iodotyrosines: monoiodotyrosine (MIT) and diiodotyrosine (DIT). MIT and DIT are produced by the substitution of one or two iodine atoms respectively on a tyrosyl residue (Figure 1.3). Each Tg contains approximately 7 - 10 MIT and 5 - 10 DIT residues. These are the precursors of the thyroid hormones. Coupling of MIT and DIT residues results in the formation of the biologically active iodothyronines. T_3 is formed by the combination of MIT with DIT while T_4 is the result of the association of two DIT molecules.

Thyroglobulin thereby provides the precursor of the thyroid hormones, and in addition acts as a storage vehicle. The extracellular storage of large amounts of Tg and the incorporated thyroid hormones is unique to the endocrine system. The protein concentration within the lumen can reach as high as 100 - 400mg/ml. The storage of such high concentrations in the lumen is made possible by the compaction and tight packaging of the molecules into globules (Herzog V *et. al.*, 1992) whose sizes range from 20 - 120μm in diameter. The thyroid gland regularly contains enough stores of thyroid hormones to ensure an adequate concentration in the circulation for 100 days.

Despite the potential to produce and store such a large amount of iodothyronines, the process of synthesis is however inefficient and extravagant as explained below:

i) the tyrosine content of each Tg molecule is comparatively low and thus abundant glycoprotein is required and synthesized,

ii) furthermore only several iodotyrosines are formed by each Tg although a larger number of tyrosyl residues are iodinated and,

iii) the majority of the incorporated iodine is in the form of inactive iodotyrosines and as a result, 1 highly iodinated Tg molecule forms only 2 - 4 molecules of T_4.

Thyroid Peroxidase

Thyroid peroxidase (TPO) or thyroperoxidase is the enzyme responsible for the oxidation of I^-. It also catalyses both the iodination of Tg and the coupling of iodotyrosines. TPO belongs to the same gene family of peroxidases as lactoperoxidase and myeloperoxidase. TPO is a glycosylated haemoprotein composed of 933 amino acids with a transmembrane domain (Kimura S *et. al.*, 1987). It has a molecular weight of ~110kd and there is evidence that TPO may exist as a dimer. The haeme iron in TPO is probably in the ferric form (Fe^{3+}) and may be involved in the iodination reaction.

TPO is a membrane bound enzyme. TPO has been located at numerous sites including intracellular components such as the rER, golgi apparatus, nuclear membrane and vesicles. Furthermore, TPO has also been localised on the microvilli of the apical membrane suggesting that the site of Tg iodination is at the apical surface (Ekholm R

& Wollman SH, 1975). However, the mechanism for iodination is not clear. The following are three likely possibilities:

i) via free radical intermediates - this mechanism proposes that there are two substrate sites within TPO which favour I^- and tyrosyl. These substrates undergo monoelectron oxidation to form the free radical forms of I^- and tyrosyl. These radicals, bound to TPO, react to form MIT which is then released from the enzyme. MIT itself may then also undergo oxidation becoming a radical to further react with an I^- free radical to yield DIT (Pommier J *et. al.*, 1973; Nunez J & Pommier J, 1982).

ii) with the iodinium ion (I^+) as an iodinating intermediate - this mechanism suggests that I^+ readily iodinates tyrosine in an electrophilic reaction to form TPO-I^+ as the iodinating intermediate (Ohtaki S *et. al.*, 1981 & 1982).

iii) with hypoiodite as the iodinating intermediate – the hypoiodite intermediate ($[EOI]^-$) is produced by the oxidation of I^- in two oxidation steps. This involves a two-electron change. Firstly, TPO (denoted as E) reacts with hydrogen peroxide (H_2O_2) to form EO, followed by the reaction with I^- to yield $[EOI]^-$ (Magnusson RP *et. al.*, 1984). According to this mechanism the iodination is therefore thought to occur entirely on the TPO enzyme (Morrison M & Bayse G, 1971; Morrsion M & Schonbaum, 1976; Dème D *et. al.*, 1978).

As mentioned above, TPO is also involved in the coupling of iodotyrosines to form iodothyronines within the structure of Tg. The proposed mechanisms for the coupling reaction entail iodotyrosine free radicals. The formation of T_4 via the generation of two DIT free radicals within the Tg molecule has also been suggested (Harrington CR,

1944 & 1951). The two DIT free radicals couple to form a quinol ether intermediate within the protein. The T_4 molecule is produced following the split from this intermediate. These reactions may be catalysed by TPO. For the formation of T_3, an MIT free radical substitutes one DIT free radical which then reacts with the other DIT free radical.

1.3.2 Secretion of Thyroid Hormones

The secretion process of thyroid hormones is complex (Figure 1.4). As synthesised hormones are stored linked to Tg, within the colloid, they first have to be released from Tg. The processes for the release and secretion of hormones are in outline:

i) retrieval of Tg into the follicular cells by internalisation procedures,

ii) proteolytic cleavage of iodothyronines from Tg followed by their release and

iii) deiodination of iodotyrosines released from degraded Tg and the reuse of I^-.

The initial visible response to TSH is the formation of pseudopods at the apical membrane of the thyroid follicular cells. These are elongations of microvilli which surround the colloid. The pseudopods are then internalised into the follicular cells by two internalisation mechanisms: macro- and micro-pinocytosis. Both are employed to varying extents depending on the species and the physiological conditions. Macropinocytosis is the non-specific absorption of large amounts of Tg. Pseudopods taken into follicular cells by this method generate large colloid droplets. In contrast micropinocytosis occurs either by receptor-mediated uptake or endocytosis. It involves the uptake of colloid via small vesicles which also become colloid droplets, but of a smaller size, within the follicular cells. The droplets then migrate towards the basal

membrane. Micropinocytosis have been observed in both intact thyroid tissue and cultures of reconstituted pig thyroid follicles (Bernier-Valentin F *et. al.*, 1990 & 1991). The vesicles migrated to the lysosomes for the proteolysis of Tg and thus, the release of the thyroid hormones, through an endosomal compartment. It has been suggested that micropinocytosis may play a more important role in the basal release of T_3 and T_4, whereas macropinocytosis only occurs following acute stimulation with a large dose of TSH.

Following the retrieval of Tg from the lumen, the thyroid hormones are then cleaved from the glycoprotein. This process is executed by proteolytic enzymes contained within lysosomes (Tokuyama T *et. al.*, 1987; Dunn AD *et. al.*, 1991a). The lysosomal proteases include: aspartic endopeptidase cathepsin D (Dunn AD & Dunn JT, 1982a), cysteine endopeptidases cathepsins B, L, and H (Dunn AD *et. al.*, 1991b; Nakagawa H & Ohtaki S, 1984), exopeptidase dipeptidyl peptidases I and II (Dunn AD & Dunn JT, 1982a & 1982b), lysosomal dipeptidase I-like enzyme (Loughlin RE & Trikojus VM, 1964) and a carboxyl exopeptidase (Dunn NW & McQuillan MT, 1971). These lysosomal bodies, situated in the cytosol, are then directed towards the internalized colloid droplets. Lysosomal movements are generated and regulated by intracellular cytoskeleton components, namely the microtubules and microfilaments. The colloid droplets which fuse with the lysosomes then form phagolysosomes. Tg is then degraded by the proteases into iodotyrosines and thyroid hormones within these phagolysosomes. This process appears to proceed in steps. First, discrete hormone-rich peptides are formed followed by the release of the two iodothyronines. T_4 is released more rapidly than T_3. Lastly, there is a general degradation of Tg into iodotyrosines. While the degradation of Tg occurs, phagolysosomes continue their

basal migration. This enables iodothyronines released into the cytosol to enter the circulation rapidly via the basal membrane and thus pass into the interfollicular capillary plexus.

The iodinated tyrosines generated by the degradation of Tg are deiodinated. This is performed by intracellular deiodinases. Liberated I^- is then recycled for the new iodination of Tg and thus the synthesis of the thyroid hormones.

1.3.3 Regulation of Thyroid Hormones

The secretion of T_3 and T_4 is stimulated by TSH which binds to receptors (TSHR) located on the basal membrane of the follicular cells. TSHR are G-protein linked and their interaction activates the α_s-subunit. This triggers the conversion of adenosine 5′-triphosphate (ATP) to cAMP by adenylate cyclase (AC). The intracellular accumulation of cAMP leads to the activation of the cAMP signal cascade. This results in both the release of thyroid hormones and an increase in their synthesis.

TSH is one of the two hormones involved in the hypothalamic-pituitary regulation of the thyroid gland (Figure 1.5). The other is a tripeptide hormone called thyrotropin-releasing hormone (TRH) which is secreted from the hypothalamus. Activation of the hypothalamic-hypophyseotrophic nuclei causes the release of TRH from dense core vesicles located at the ends of the nerve terminals. TRH then stimulates the thyrotrophs in the anterior pituitary to secrete TSH, a glycoprotein hormone, which acts to increase the synthesis and release of T_3 and T_4 as described above. As thyroid hormones have important and multiple functions, they are tightly regulated. This is

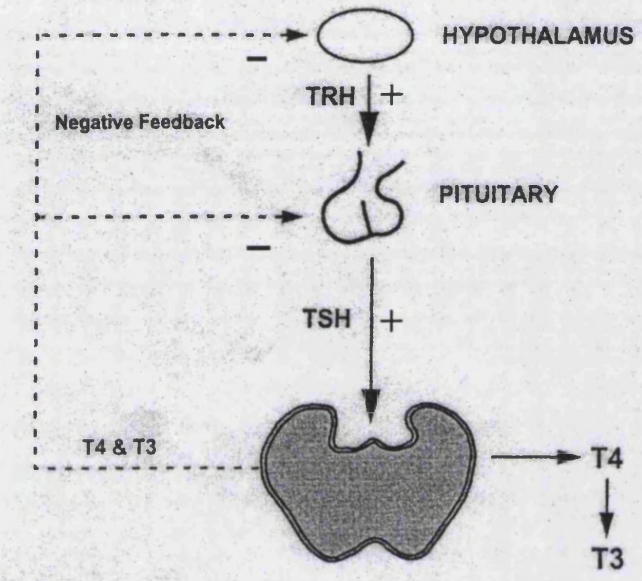


Figure 1.5 **The hypothalamic-pituitary regulation of the thyroid gland.** Thyrotropin-releasing hormone (TRH) is released from the hypothalamus which then stimulates the thyrotrophs in the anterior pituitary to secret thyroid stimulating hormone (TSH). TSH then acts on thyrocytes in the thyroid gland to stimulate the synthesis and secretion of the thyroid hormones T_3 and T_4. Furthermore, T_3 and T_4 in the circulation then act as negative regulators of both the hypothalamus and the pituitary gland in a negative feedback loop. (Neal JM. Basic Endocrinology – An Interactive Approach. Blackwell Science; 2000, p53)

achieved through negative feedback whereby T_3 and T_4 feedback in a negative manner at both the hypothalamus and the anterior pituitary. Thus, TRH and TSH together with the negative feedback loop complete the regulatory system governing the release of the thyroid hormones as illustrated in Figure 1.5.

However, the thyroid has several other well recognised autoregulatory mechanisms independent of the hypothalamic-pituitary axis. In the event of either a mild or severe deficiency in iodine, several effects including the enlargement of the thyroid gland, a decrease of the iodine concentration in the thyroid and an increase in MIT/DIT ratio are observed (Studer H & Greer M, 1966; Riesco G *et. al.*, 1977; Fukuda H *et. al.*, 1987). However, the most significant adaptation involves a shift from T_4 to T_3 formation in the thyroid. Overall, there is a reduction in serum T_4 whereas serum T_3 remains within the normal range.

In the event of excess iodine, the thyroid also adapts via several autoregulation mechanisms including:

- the reduction of I^- uptake into follicular cells which thus reduces the I^- availability for hormone synthesis,
- a mechanism that prevents excess hormone synthesis by inhibiting tyrosine iodination. This inhibitory effect is referred to as the acute Wolff-Chaikoff effect (Wolff J & Chaikoff IL, 1948). However, this mechanism depends upon reaching a critical level of intracellular I^- concentration (Raben MS, 1949). Furthermore, this is a transient effect and the "escape" from the inhibitory effect occurs when intracellular I^- concentrations return to normal (Wolff J *et. al.*, 1949). "Escape"

even occurs spontaneously when high levels of iodide are maintained and has been demonstrated to relate to adaptations of the iodide transport system (Braverman LE & Ingbar SH, 1963),

- excess I^- also reduces hormone secretion. The mechanism of this effect is not fully understood and
- in long-term chronic I^- excess, the above adaptations may not be adequate. Under this circumstance, there is an increase in I^- uptake and iodination of tyrosyl residues (Ingbar SH, 1972). However, there is no increase in T_4 release, but there is an increase in the release of I^-, referred to as the "iodide leak", from the thyroid, to compensate (DeGroot LJ *et. al.*, 1971; Wartofsky L & Ingbar SH, 1971; Nagataki S, 1974).

1.3.4 Transport of Thyroid Hormones in Serum

Only a minute fraction of thyroid hormones in the circulation is unbound (or "free"), the percentages of bound T_3 and T_4 being 99.7% and 99.97% respectively. The protein bound hormones are metabolically inactive. However, they can rapidly dissociate to become active hormones. There are three major transport proteins in the circulation namely thyroxine-binding globulin, transthyretin and albumin. These are all compact globular proteins of similar molecular weight which are synthesised in the liver.

Thyroxine-binding globulin

Thyroxine-binding globulin (TBG) is a glycoprotein α-globulin composed of a single polypeptide chain. It has a molecular weight of ~54kd and a half life of ~5 days. Although TBG is the least abundant of the three carrier proteins, it carries ~70% of the circulating T_4 and T_3. This is the result of its comparatively high affinity for the hormones, particularly T_4, which binds to TBG 10 - 20 fold more avidly than T_3. T_3 has a higher dissociation rate ($t_{1/2}$ = 4.2s) than T_4 ($t_{1/2}$ = 39s) (Hillier AP, 1971 & 1975). TBG appears to have only one iodothyronine binding site and T_4 is the preferred ligand. However, as the binding site lacks specificity, it will allow the binding of T_3, thyronine analogues (Korcek L & Tabachnik M, 1976) and a number of small molecules that partially resemble thyronines. TBG is relatively unstable. *In vitro*, it is irreversibly denatured by increased temperature (>50°C), diluted acid, guanidine and mechanical disturbances (Gershengorn MC *et. al.*, 1977; Johnson ML *et. al.*, 1980). TBG then loses its ability to bind the hormones. However, the stability of TBG is enhanced as a result of T_4 binding (Grimaldi S *et. al.*, 1982) which is accompanied by slight conformational changes. This may confer stability *in vivo*. TBG has no well established physiological function, other than the transport of thyroid hormones.

Transthyretin

Transthyretin (TTR), also known as thyroxine-binding prealbumin, is a tetramer (Blake CCF *et. al.*, 1974; 1978 & 1980; Blake CCF & Oatley SJ, 1977) with a half life of ~1 day. It consists of four polypeptide chains, each of which has a molecular weight of ~13.5kd. TTR has a high β-structure content which allows it to fold into an unusual

twofold symmetry around a cylindrical channel (Blake CCF & Oatley SJ, 1977) which contributes to the stability of the protein (Branch WT Jr *et. al.*, 1971 & 1972; Robbins J *et. al.*, 1978). This channel contains two identical T_4 binding sites which differ in their affinity for the hormone (Cheng S-Y *et. al.*, 1977; Robbins J *et. al.*, 1978). The first site binds to T_4 twice as avidly as the second site. Four well defined hydrophobic pockets allow the binding of T_4 in a specific configuration in which the pockets are occupied by the iodine atoms. T_3 binds in an alternative configuration and the proportion of serum T_3 carried by TTR is uncertain. Despite the two binding sites, only the first is normally occupied by T_4. As a result of the low occupancy and because of a lower affinity for the hormones, TTR only binds ~10% of the total iodothyronines in the circulation. TTR has a 10-fold lower affinity for T_3 than T_4 and the dissociation rates for both are ~5-fold higher than for TBG. As a consequence, TTR may be responsible for most of the delivery of T_4 and T_3 to their target cells. However, TTR is the major transport protein in cerebrospinal fluid where it distributes iodothyronines to the central nervous system. In addition, a third of TTR can complex with retinol-binding proteins for the transport of retinol, also known as vitamin A (Goodman DS, 1984; Soprano DR & Blaner WS, 1994).

Albumin

Albumin, like TBG, is also a monomer composed of a single polypeptide chain with a molecular weight of ~66kd (Steiner RF & Edelhoch H, 1962; Peters JT, 1985). Despite albumin having the largest capacity for thyroid hormone binding, only a small fraction transports T_3 and T_4. However, as a result of the abundance of albumin in serum, it carries ~15 - 20% of T_4 and possibly 30 - 50% of T_3 in the circulation

(Robbins J & Rall JE, 1979). Albumin has one high affinity binding site, which binds T_4 more avidly than T_3, and at least five other weaker binding sites. It also transports numerous other small molecules. Its dissociation rates for both T_4 and T_3 are even higher than those of TBG and TTR. Thus albumin is also responsible for delivering a large proportion of thyroid hormones to the target cells (Robbins J & Johnson ML, 1979; Pardridge WM, 1981).

In addition to the three major transport proteins, thyroid hormones can also be transported by lipoproteins. These are a group of complex molecules which include various lipids within a hydrophobic core, surrounded by phospholipids and apoproteins. They bind only a minor fraction of thyroid hormones. Although the distribution of thyroid hormones amongst these lipoproteins varies, high-density lipoproteins appear to be the major distributor binding to ~3% and ~6% of T_4 and T_3 respectively (Benvenga S *et. al.*, 1988). Other lipoprotein carriers of thyroid hormones include low-density lipoproteins. These contain the apolipoprotein B-100 which has binding sites for T_4 on three separate domains (Benvenga S *et. al.*, 1990). The binding sites of the lipoproteins contain β-structures which share amino acid homology with the binding domains of TBG, TTR and albumin (Benvenga S *et. al.*, 1994).

All the transport proteins described above deliver thyroid hormones to the target cells and are also responsible for maintaining a constant level of free hormones in serum. Changes in the levels of these transport proteins can alter this equilibrium. There are numerous factors that can lead to changes in their levels. These include genetic factors of inherited abnormalities, physiological inputs such as steroid hormones, pathological conditions and pharmacological manipulations with drugs. However, fluctuations in

the concentrations of thyroid hormones in the circulation, as a result of changes to the level of the binding proteins, will be detected by the anterior pituitary and be compensated for by the hypothalamic-pituitary-thyroid axis.

1.3.5 Metabolism of Thyroid Hormones

The step-wise conversion of T_4 to thyronine (T_0) is achieved via sequential monodeiodinations by deiodinase enzymes. The most functionally significant step in thyroid hormone metabolism is the conversion of T_4 to either T_3 or reverse T_3 (rT_3, Figure 1.6). There is then the subsequent degradation of both of these triiodothyronines. Although, thyroxine is the most abundant thyroid hormone, it has limited biological activity per se and is nowadays thought of as a prohormone. T_3 is several times more potent than T_4 and most of the actions of the thyroid hormones are now attributed to T_3. Hence, the mono-deiodination of thyroxine (with its 4 iodine atoms) to convert it to T_3 is biologically and functionally very significant. The conversion of T_4 to T_3 is achieved with the removal of an outer (phenolic) ring I^-. This 5′-monodeiodination is executed by the enzyme 5′-deiodinase. Furthermore, this enzyme is also responsible for any additional 5′-monodeiodination of I^- from the phenolic ring in the degradation of T_3 as shown in Figure 1.6. There are two forms of the enzyme 5′-deiodinase, referred to as type I and II, which are tissue specific. Type I is found in most tissues of the body but particularly in the liver, kidneys and muscle cells, whilst type II is selectively located in the pituitary, brown fat cells and selected areas of the brain.

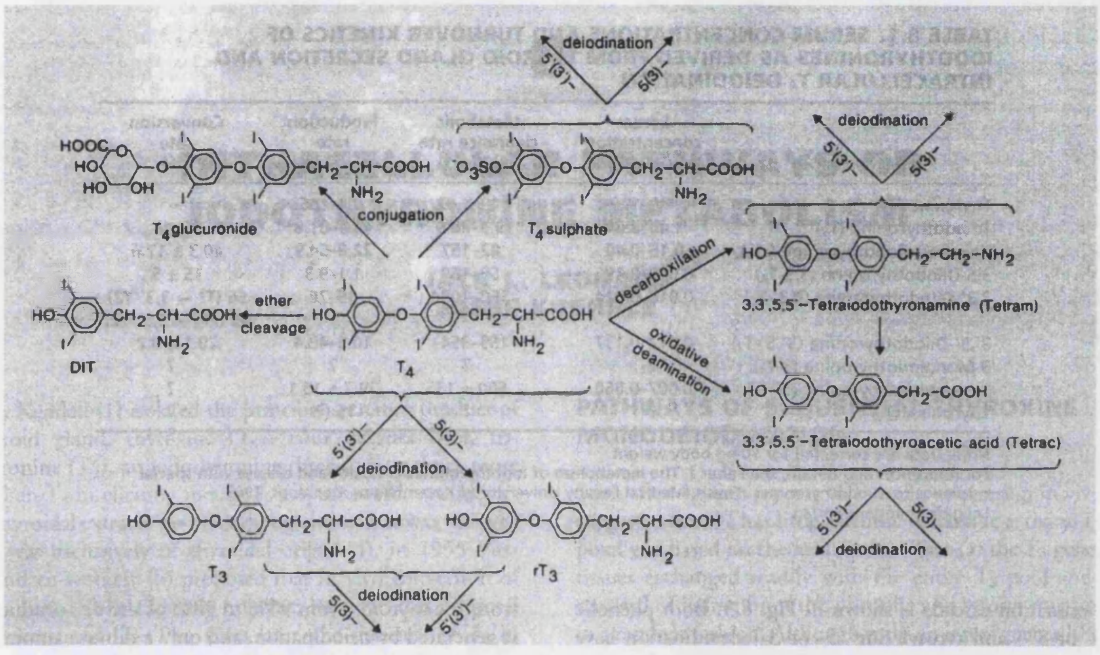


Figure 1.6 **The pathway of thyroxine (T_4) metabolism.** The metabolism of thyroxine is performed by two types of deiodinase enzymes: 5′-deiodinase and 5-deiodinase. They are responsible for the metabolism of ~80% of T_4. The remaining ~20% of T_4 is metabolised as iodothyronine glucuronide and sulphate conjugates, formed in the liver, by the deamination and decarboxylation. (Köhrle J, Brabant G & Hesch RD. Metabolism of thyroid hormones. Hormone Res; 1987, 26:58)

Type I 5′-deiodinase is distinguished from type II, not only by its tissue distribution, but also its substrate specificity. This isoenzyme deiodinized T_4 to T_3 as well as rT_3 to 3,3′-T_2. Although type I 5′-deiodinase has a high affinity for T_4 and plays a significant role in the production of the bioactive thyroid hormone T_3, rT_3 has been suggested to be its preferred substrate and it is therefore important for in the degradation of rT_3. It is inhibited by propylthiouracil (PTU) and aurothioglucoase (ATG) (Hesch RD *et. al.*, 1975; Visser TJ *et. al.*, 1975; Köhrle J, 1994). In contrast, type II has a higher affinity for T_4 than rT_3. As a result, type II 5′-deiodinase plays a more important role in the generation of local T_3 at the target sites in a tissue such as the pituitary.

Another deiodinase, 5-deiodinase, is located in the liver and the kidneys. It converts T_4 to the alternative triiodothyronine rT_3 which results from the removal of an iodine atom from the inner (tyrosyl) ring. It has no known biological activity and is rapidly metabolised. Hence, this appears to be a metabolic clearance pathway for thyroxine. As shown in Figure 1.6, both T_3 and rT_3 are then further metabolised via stepwise deiodination into iodine-free thyronines.

All of the above three enzymes are responsible for the metabolism of ~80% of T_4 in the circulation. As a result of the substrate specificities of these isoenzymes, a higher percentage of rT_3 (55%) is normally generated than T_3 (45%). Nevertheless, there are various factors that can affect this balance. These include systemic illnesses, starvation, medication and even age. The remaining 20% of T_4 is metabolised via an alternative pathway. This involves the formation of iodothyronine glucuronide and sulphate conjugates in the liver. These are deaminated and decarboxylated and final removal of their ether groups results in metabolically inert compounds. Two

marginally biologically active metabolites, tetrac and triac, are formed by deamination of T_4 and T_3 respectively. These are further subjected to deiodination to eliminate all activities. The liver, kidneys and skeletal muscles are the major degradation sites. The metabolically inert compounds are then excreted in the bile, and the iodide reused by the thyroid.

The two iodothyronines synthesised and secreted by the thyroid gland are the major iodinated products in serum. However, both inorganic iodide and other iodinated materials are also present. These include iodotyrosines and iodoproteins such as Tg and iodoalbumin. They are the result of direct thyroid secretions and/or are byproducts of peripheral thyroid hormone metabolism. Their deiodination and metabolism follows the processes described for T_3 and T_4 and the I^- is once more scavenged for recycling.

1.3.6 Actions of Thyroid Hormones

The diverse actions of the thyroid hormones are exerted via a nuclear pathway (Figure 1.7). It involves the interaction of T_3 with specific nuclear receptors, which then, acting essentially as transcription factors, modify the expression of specific target genes. Thyroid hormones have numerous target genes, which include those coding for growth hormone, lipogenic enzymes, TSH, myosin heavy chain expressed in cardiac and skeletal muscles and specific brain proteins such as the myelin basic protein. It has long been recognised that intracellular thyroid hormone action depends upon T_3 and as discussed above, it appears that T_4 only acts as a prohormone (Surks MI &

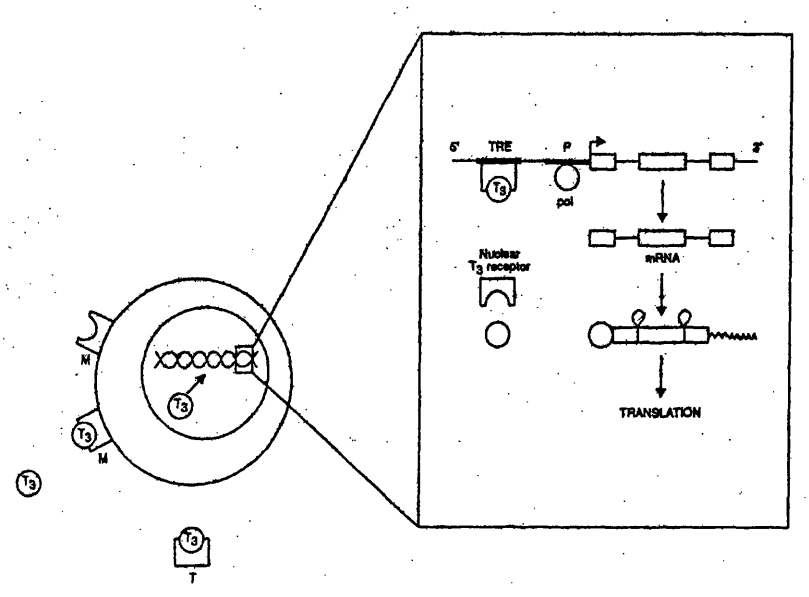


Figure 1.7 **The molecular action of T_3 via the nuclear pathway.** The more potent thyroid hormone, free T_3, binds to membrane thyroid receptors (M) to initiate the gene transcription cascade. Once bound, the hormone is the internalised and transported into the nucleus. It then binds to nuclear receptor proteins and, as a complex, acting as a transcription factor, interacts with thyroid hormone response elements (TRE) within the promoter (P) of specific target genes to regulate gene transcription. (Franklyn JA. Thyroid Physiology (2b) thyroid hormone action. In Wheeler MH & Lazarus JH, eds. Disease of the Thyroid – Pathophysiology and Management. Chapman & Hall Medical; 1994, p30)

Oppenheimer JH, 1977). Both iodothyronines enter the cells via a cell-mediated, energy-dependent mechanism and then within the cell, the majority of T_4 is converted to the more potent T_3. Although, all the iodothyronines including rT_3 are found intracellularly, T_3 has the highest affinity for the thyroid hormone receptors (TR). Thus, T_3 is the predominant ligand which binds to TR and initiates the responsive gene transcription cascade.

Thyroid hormone receptors

There are two isoforms of the human TR, namely the α and β receptors. These are encoded by two separate genes located on chromosomes 3 (Weinberger C *et. al.*, 1986) and 17 (Dayton AI *et. al.*, 1984) respectively. The structures of the TRs and the variants described here are shown in Figure 1.8. β receptors are the foetal form of the TR and two isoforms, TR-β1 and TR-β2 arise due to alternate splicing. Similarly, there are also two isoforms of the α receptors, termed TR-α1 and TR-α2, which are again the products of alternate processing. All of the TRs have molecular weights of 50 - 55kd. The TR-β1 isoform is identical to TR-β2 except for a deletion in the amino terminal region (Hodin RA *et. al.*, 1989). Similarly, the α receptor isoforms are identical for the first 370 amino acids after which TR-α2 differs from TR-α1 at the carboxyl terminus. The protein TR-α2 contains a 122 amino acid sequence which replaces a 40 amino acid segment essential for ligand binding present in the TR-α1 isoform (Dayton AI *et. al.*, 1984; Benbrook D & Pfahl M, 1987; Thompson CC & Evans RM, 1989). This short amino acid sequence responsible for ligand binding is also present in both the β receptor isoforms. Thus TR-α1, -β1 and -β2 bind to T_3 with high affinity leading to the transcription of target genes. Furthermore, there are two

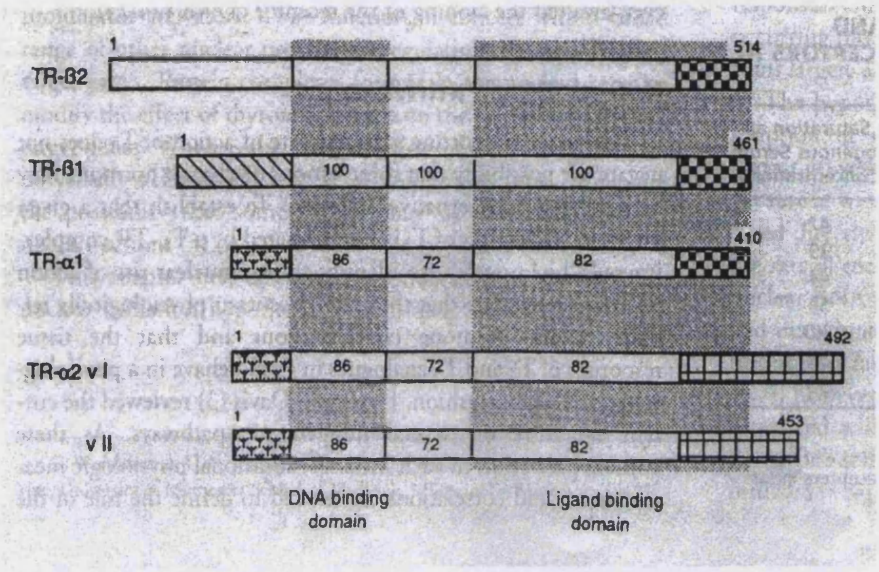


Figure 1.8 **The structures of the thyroid hormone receptor variants**. There are two forms of the human thyroid hormone receptor (TR): α and β receptors. There are two isoforms of the β receptor, β1 and β2, and similarly, also two isoform of the α receptor, α1 and α2. These isoforms are the result of alternate splicing. However, despite their difference in amino acid sequences, they all have an identical receptor structure which contains three distinctive domains: the ligand binding domain, a transmembrane domain and the DNA binding domain which is responsible for the interaction with thyroid hormone response elements on specific genes. (Schwartz HL, Strait KA & Oppenheimer JH. Molecular mechanisms of thyroid hormone action: a physiological perspective. In Klee G, ed. Clinics in laboratory medicine: pathophysiology of thyroid disease. Philadelphia: WB Saunders; 1993, p543)

variants of the TR-α2 receptors, namely TR-α2vI and TR-α2vII (Mitsuhashi T *et. al.*, 1988; Izumo S & Mahdavi V, 1988). However, as TR-α2 receptors lack the sequence essential for ligand binding, neither of the TR-α2 variants can bind to T_3 and cause transcription of the target genes. Although, the function of TR-α2 is still unknown, there has been a suggestion that it may inhibit the action of the other TRs (Koenig RJ *et. al.*, 1989; Lazar MA *et. al.*, 1989).

TRs are members of the steroid hormone receptor superfamily, as evident from the substantial amino acid homology found between TR and steroid hormone receptors (Evans RM, 1988; O'Malley B, 1990). Furthermore, TRs belong to a family of receptors that share a common structural configuration including receptors for retinoic acid and vitamin D. Receptors of the superfamily contain three distinct domains (Kumar V *et. al.*, 1987). The two functional domains are the DNA-binding and the ligand-binding domains located at the amino and carboxyl terminal respectively. Situated between the above two domains is a central element, termed the "hinge" region, whose function is unclear. In the DNA-binding domain, located at the amino terminus, there are 70 amino acids that form two conserved zinc finger motifs (Evans RM & Hollenberg SM, 1988). The zinc finger motifs interact with specific DNA segments called hormone response elements (HRE) found in the promoter regions of target genes. Furthermore, there are specific sequences located at the base of the zinc fingers, designated the P and D boxes, that recognise different but specific HREs to generate heterogeneity (Danielson M *et. al.*, 1989; Umesono K & Evans RM, 1989). The carboxyl domain is fundamental for ligand binding and the activation of intranuclear activities. Within this terminal there are specific sequences, such as a leucine zipper, that allow dimerization with other receptors or associated nuclear

proteins (Forman BA & Samuels HH, 1990). Dimerization of TRs with other receptors is important for initiation of the transcription cascade (O'Donnell AL & Koenig RJ, 1990; O'Donnell *et. al.*, 1991).

The distribution of these TR isoforms is widespread with varying concentrations and tissue specificities (Chin WW, 1991). They can be found in the liver, kidneys and heart and also within the brain (Carlson DE *et. al.*, 1994). As immunohistochemical studies have shown, TRs are notably localised on the nuclei of all the target cells (Strait KA *et. al.*, 1991; Schwartz HL *et. al.*, 1994). In addition, TRs have been located on intracellular organelles including ribosomes, mitochondria and the plasma membrane. These TR may be involved in the posttranscriptional and pretranslational modifications of mRNA.

Thyroid hormone response elements

A mentioned above, HREs are specific DNA sequences that are able to exert regulatory influences on gene expression. There are positive and negative HREs which stimulate or inhibit gene transcription respectively (Mitchell PJ & Tijan R, 1989; Diamond MI *et. al.*, 1990). These regulatory sequences are located within the promoter region of target genes downstream of the 5′ transcription start site. However, sometimes they can also be found within the 3′-untranslated regions. These sequences act as recognition sites for the binding of the ligand / steroid-thyroid hormone receptor complexes. Binding then results in triggering the recruitment of various other specific gene transcription factors (Schlief R, 1989). These factors form a complex near the transcription site to either initiate or inhibit DNA transcription. There are two

proposed mechanisms by which protein-DNA interactions influence the rate of transcription (Magasanik B, 1989; Ptashne M & Gan AAF, 1990). Firstly, with the favoured mechanism, the receptor binds directly to the transcription initiation site. The ligand/receptor complex either binds near to or a small distance away from the start site allowing direct receptor-initiation site interaction. Secondly, an alternative mechanism is indirect exertion on transcription via the mediation of an intermediate protein.

The HRE regulatory sequences that specifically bind the ligand-TR complex are termed the thyroid hormone response element (TRE). As with the other regulatory elements, there are positive and negative TREs that activate and inhibit gene transcription respectively. TR bind to all these sequences with high affinity and confer T_3 activity. Gel-shift (Garner MM & Revzin A, 1981) and avidin-biotin complex assays (Glass CK *et. al.*, 1987) were used to identify the binding affinity of TR to TREs, whereas footprinting and methylation interference analyses determined the specific sequences of the TREs. Using reporter gene transient transfection assays, the function of the identified TREs was analysed. As a result, a hexamer core consensus sequence, (A/G)GGT(C/A)A, has been identified as the minimal motif required for TR binding (Brent GA *et. al.*, 1989). Furthermore, the optimal sequence is believed to be an octamer which, in addition to the core consensus sequence, are the two nucleotides (TA) located at the 5′ end (Katz RW & Koenig RJ, 1993). Yet, despite the identification of the core motif, all the positive TREs identified to date contain a binding sequence with some degree of divergence from the above hexamer (Williams GR & Brent GA, 1995). Although, a single core sequence is sufficient to bind TR as a monomer, it is insufficient to activate gene transcription. The majority of the positive

TREs comprise of a pair of the hexameric sequences (Koenig RJ *et. al.*, 1987; Samuels HH *et. al.*, 1988) and as a result a single core hexamer has been designated as a "half site". Typically, the two "half sites" in the target genes are arranged as a palindrome (Glass CK *et. al.*, 1987), direct repeat with a 4-base spacing (Umesono K *et. al.*, 1991) or an inverted repeat with a 6-base separation (Farsetti A *et. al.*, 1991). These TRE can form homodimers or heterodimers with other nuclear proteins, termed thyroid hormone receptor auxiliary proteins (TRAPs), which act to stabilize the binding of TR to TREs (Murray MB & Towle HC, 1989; Darling DS *et. al.*, 1991). Dimerization with TRAPs and the specific spacing between the "half sites" convey specificity of binding to the TREs and the capacity to initiate gene transcription.

Only a few negative TREs (nTREs) have been identified and they do not differ significantly from the positive TREs. They loosely preserve the core consensus sequence as described above. However, the most important difference is the requirement of only one "half site" to initiate a response. Many of the identified nTREs contain only one binding site (Brent GA *et. al.*, 1991; Carr FE *et. al.*, 1992). Furthermore, there is evidence which suggests that nTREs may form dimers with other nuclear proteins. The mechanism by which nTREs suppress gene transcription is not known. It has been suggested that such TRE may be located close to the transcription start site and thereby interfere with the formation of the initiation complex or obstruct the transcription enzyme. Furthermore, the ability for nTREs to exert their negative effect is independent of their position so that they can be equally effective when placed upstream of the start site (Brent GA *et. al.*, 1991; Carr FE *et. al.*, 1992). Consequently, nTREs may have a more complex mechanism than hindrance of gene transcription.

Extranuclear Actions

In essence, thyroid hormones control the basal rate of oxygen consumption and heat production. The mRNA generated within the nucleus stimulates and exerts a diverse range of extranuclear activities, which include (Figure 1.9):

i) the synthesis of new proteins for growth and maturation,

ii) increases in both the biogenesis of mitochondria and the activities of their respiratory enzymes such as NADPH cytochrome C reductase and cytochrome oxidase,

iii) intracellular Na^+ increases due to heightened activity of the Na^+ pump. This results in the generation of an excess of ADP which increases the utilisation of oxygen by the enzyme K^+-ATPase in the mitochondria, and

iv) activation of other enzymes concerned with glucose oxidation and glucogenesis.

All these actions eventually result in the increase in oxygen consumption followed by the increase in the metabolic rate. This overall effect affects the metabolism of the whole body (Figure 1.9). As a consequence there is:

i) an increase in carbon dioxide production due to increase in oxygen consumption,

ii) increased fatty acid and glucose oxidation,

iii) increased thermogenesis which, however, does not increase body temperature as this is accompanied by an increase in blood flow, sweating and ventilation. These annul each other,

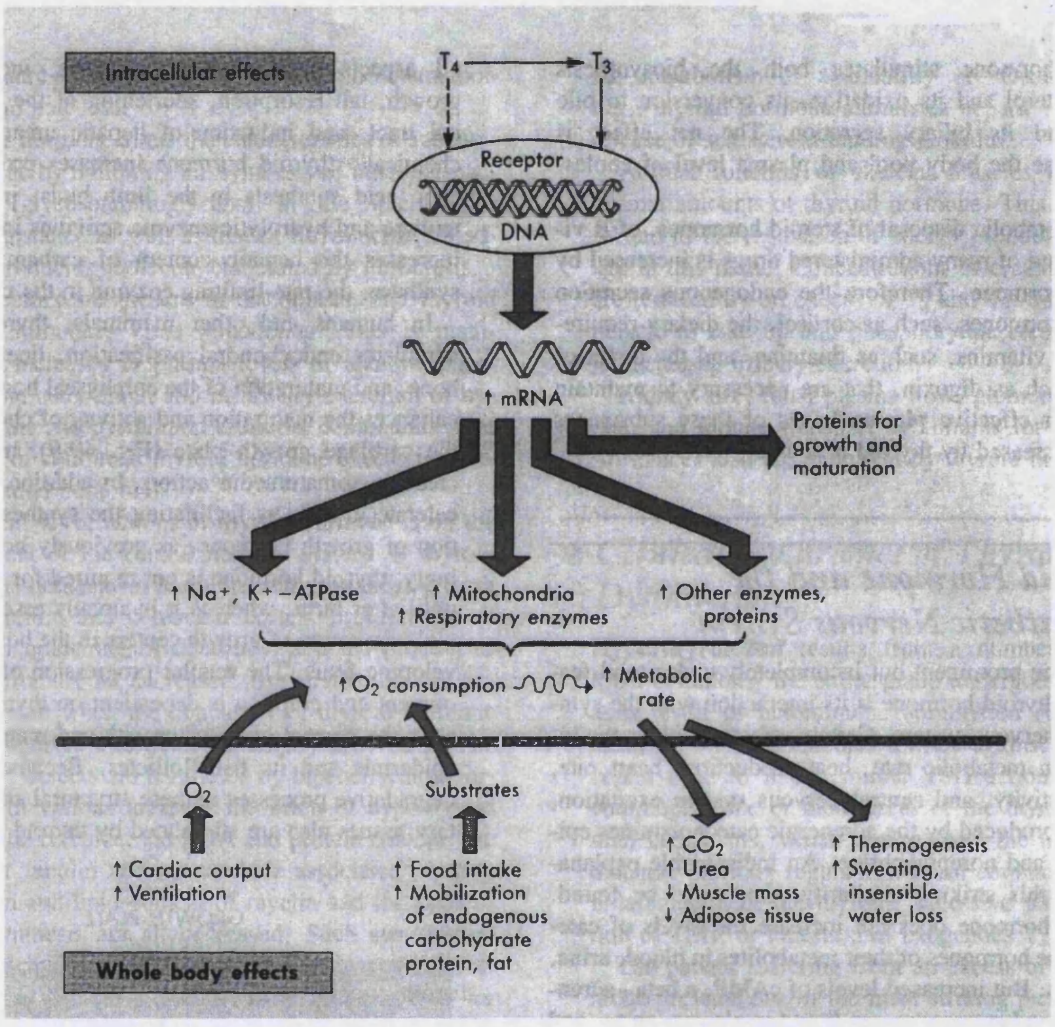


Figure 1.9 **The extranuclear effects of the thyroid hormones**. In addition to the regulation of the expression of specific genes, a diverse range of extranuclear activities are exerted as illustrated in the lower portion of the above scheme. These actions result in the increase in oxygen consumption and metabolic rate. The overall effect then affects the metabolism of the whole body. (Genuth SM. The Endocrine System – The Thyroid Gland. In Berne RM & Levy MN, eds. Physiology, 3rd Ed. St. Louis: Mosby – Year Book; 1993, p943).

iv) an enhanced oxygen supply through increases in ventilation and cardiac output in which heart rate and stroke volume is augmented, and

v) an increase in substrates required for oxidation such as food intake and mobilisation of storage carbohydrate, protein and fat.

Section 2

Graves' Disease

Graves' Disease (GD) is the commonest cause of hyperthyroidism (~50%) and results in clinical thyrotoxicosis (Table 2.1). GD patients have increased levels of thyroid hormones in their circulation. This is the consequence of enhanced hormone synthesis and secretion. Elevated levels of iodothyronines suppress TSH release from the anterior pituitary via the negative feedback mechanism. In this section we will discuss GD in detail.

2.1 Aetiology of Graves' disease

In an autoimmune disease such as GD, antibodies recognize self antigens (autoantigens) and initiate an inappropriate immune response. As reviewed by McLachlan & Rapoport (McLachlan SM & Rapoport B, 1993) in autoimmune thyroid disease, B- and T-lymphocytes of the immune system can potentially recognize three distinct thyroid autoantigens. These are the thyroid stimulating hormone receptor (TSHR), TPO and Tg. Recognition of these autoantigens by T-cells results in the production of autoantibodies by B-cells. TSHR is the primary autoantigen associated with GD whereas anti-TPO and anti-Tg autoantibodies are largely found associated with hypothyroidism, in particular with Hashimoto's thyroiditis. However in some GD patients, anti-TPO and anti-Tg autoantibodies may also be found, but at levels that are incidental to the anti-TSHR antibodies and play no part in thyroid stimulation.

Common Causes

THYROTOXICOSIS ASSOCIATED WITH HYPERTHYROIDISM

Graves' disease

Intrinsic thyroid autonomy
- Toxic adenoma
- Toxic multinodular goitre

THYROTOXICOSIS NOT ASSOCIATED WITH HYPERTHYROIDISM

Inflammatory disease
- Silent thyroiditis
- Subacute thyroditis

Extrathyroidal source of thyroid hormone
- Exogenous hormone

Uncommon Causes

THYROTOXICOSIS ASSOCIATED WITH HYPERTHYROIDISM

Production of thyroid stimulators
- TSH hypersecretion
- Trophoblastic tumor
- Hyperemesis gravidarum

Intrinsic thyroid autonomy
- Thyroid carcinoma
- Nonautoimmune autosomal dominant hyperthyroidism
- Struma ovarii

Drug-induced hyperthyroidism
- Iodine and iodine-containing drugs and radiographic contrast agents
- Lithium

THYROTOXICOSIS NOT ASSOCIATED WITH HYPERTHYROIDISM

Inflammatory disease
- Drug induced thyroiditis (amiodarone, interferon-α)
- Infarction of thyroid adenoma
- Radiation thyroiditis

Table 2.1 **The common and uncommon causes of thyrotoxicosis which are either associated with or not associated with hyperthyroidisim.** (Braverman LE & Utiger RD. Introduction to Thyrotoxicosis. In Braverman LE & Utiger RD, eds. Werner& Ingbar's The Thyroid – A Fundamental and Clinical Text, 7th Ed. Lippincott Raven Publishers; 1996, p523)

2.1.1 Autoantibodies against the thyroid stimulating hormone receptor

The TSHR, found on the basal membrane of the thyroid follicular cells, is discussed in detail in Section 3. The TSHR has epitopes that are recognised by both T- and B-lymphocytes. Although different regions (epitopes) of the TSHR antigen are recognized by these lymphocytes (Livingstone AM & Fathmann CG, 1987), the initial interaction is dependent on T-cells (Figure 2.1). T-cells recognize short, linear TSHR peptide fragments of 9 – 17 amino acids that are produced by proteolysis. These fragments bind to major histocompatability complex (MHC) class I and II molecules expressed on the membranes of antigen-presenting cells. Their interactions form peptide-MHC complexes which anti-TSHR T-lymphocytes are able to distinguish. Furthermore, there are subsets of anti-TSHR T-cells that each only recognize a specific combination of peptide and MHC molecule. The interaction between T-cells and MHC complexes initiates T-cell proliferation. This response is dependent on the presence of a number of stimulatory cytokines such as interleukines (IL) and adhesion molecules. IL-1 is secreted to stimulate T-cell expression of IL-2 receptors. Activated T-cells further release IL-2 and IL-4. They stimulate other T- and B-lymphocytes to proliferate. Thus both the increase in the number of T-cells and the generation of a range of cytokines are responsible for the activation of B-cells. Subsequently, activated B-cells then produce numerous heterogeneous autoantibodies against TSHR epitopes.

These autoantibodies, primarily of the immunoglobulin G (IgG) subclass, could be receptor stimulating or inhibiting. Both recognize and bind to multiple non-linear and linear epitopes in the extracellular domain of the receptor which has been

Stage 1:
T-cell epitope presentation to the MHC molecule on the APC. The peptide-MHC complex then activates T-cell.

Stage 2:
Activated T-cell then stimulate and activate B-cells via the release of IL-2 and IL-4. Subsequently, B-cell then produces specific antibodies against the T-dependent antigen.

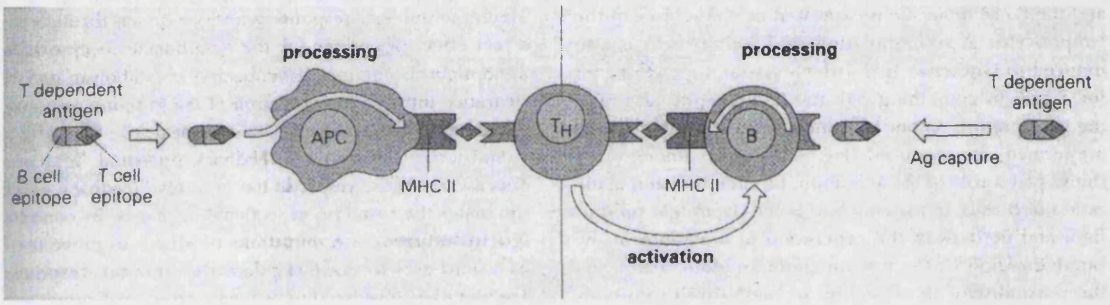


Figure 2.1 **The mechanism of B-cell activation by a T-cell to produce specific antibodies against a T-dependent antigen**. A diagram illustrating the immunological mechanism in which T-cells stimulate B-cells to produce antibodies against a specific antigen. B-cell activation is also aided by the release of interleukins such as IL-2 and IL-4. MHC, major-histocompatibility complex; APC, antigen-presenting cell; IL, interleukin. (adapted from Mims CA, Playfair JHL, Roitt IM, Wakelin D & Williams R. Medical Microbiology. Mosby – Year Book European Limited; 1993, p7.5)

acknowledged to be the major immunogenic region. As their name suggests, thyroid stimulating antibodies (TSAb) not only bind to TSHR but also activate intracellular response pathways, thereby acting as mimics of TSH. In contrast, TSHR-blocking antibodies inhibit receptor activation by blocking TSH binding. Both classes of autoantibodies can be found simultaneously in GD patients' serum and the net result will then depend upon their relative concentrations and potencies as stimulators. In GD patients, TSAbs prevail and activate the TSHR. It has been suggested that autoantibodies receptor activation is persistent, since receptor desensitization only occurs at very high concentrations of antibodies and via a decrease in the coupling of TSHR with G protein (Damante G *et. al.*, 1987; Kraiem Z *et. al.*, 1988). Kraiem and colleagues reported that significant desensitization of the receptors in human thyroid cells only occurred with TSAb doses ≥4mU/ml and also only after a prolonged stimulation of 48hr (Kraiem Z *et. al.*, 1988). This may explain the mechanism by which prolonged exposure to TSAb in a patient can cause persistent activation of TSHR without receptor desensitization. This then results in hyperthyroidism, due to increased thyroid hormone synthesis and secretion from the thyroid gland. Increased levels of T_3 and T_4 induce a decrease in circulating TSH, via negative feedback. Consequently, without any negative feedback mechanism inhibiting autoantibodies production and persistent receptor activation, the disease persists indefinitely.

It has been suggested that there are numerous binding sites for both stimulatory and blocking TSHR autoantibodies in the extracellular domain of TSHR (Nagayama Y *et. al.*, 1991b; Tahara K *et. al.*, 1991). Although none of their epitopes are identical to the TSH binding site (Nagayama Y *et. al.*, 1991b), the epitopes in the carboxyl region of the extracellular domain for both classes of autoantibodies largely overlap with the

TSH binding site (reviewed by Rapoport B *et. al.*, 1998). In contrast, epitopes in the amino terminus shared little overlap with the binding sites for TSH. The binding of TSHR autoantibodies to TSHR is further discussed in Section 3 of the Introduction. The numerous attempts to identify the TSHR T-cell epitopes have, unlike the B-cell epitopes, met with little success. Furthermore, no consensus can be made with regard to the major immunogenic site of the receptor (Tandon N *et. al.*, 1992; Fan J-L *et. al.*, 1993a; Soliman M *et. al.*, 1995). However, as reviewed by Graves and Davies (Graves PN & Davies TF, 2000), four TSHR epitopes (amino acids 51-71, 142-161, 202-221 & 247-266) were identified which resulted in a significant increase in T-cell proliferation in patients with GD compared to normal individuals (Martin A *et. al.*, 1997). Furthermore, transfection of TSHR into lymphoblastoid cells, acting as antigen-presenting cells, caused a T-cell response (Mullins RJ *et. al.*, 1994). These results suggest that T-cells may play a role in GD autoimmunity.

2.1.2 Autoantibodies against thyroid peroxidase

TPO is the membrane bound enzyme involved in the iodination of Tg and the coupling of iodotyrosines (see Section 1.3.1). Again, autoantibodies directed at TPO are produced by B-cells via the mechanism described. Using synthetic and recombinant peptides in primary lymphocyte cultures and T-cell lines, numerous TPO peptide fragments have been investigated for their ability to induce T-cell responses and proliferation. The consensus TPO epitopes, as well as intact TPO, recognized by T-cells have been identified and named NP-7 (amino acids 535-551) and B6 (amino acids 632-645, Dayan CM *et. al.*, 1991; Feldmann M *et. al.*, 1992). Similar to the anti-TSHR antibodies, numerous heterogeneous autoantibodies against TPO epitopes have been

produced (McLachlan SM & Rapoport B, 1992). Each autoantibody recognized a specific TPO epitope which was highly conformational and only expressed on native TPO (Finke R *et. al.*, 1990; Portolano S *et. al.*, 1992). Furthermore, the majority of anti-TPO autoantibodies have also been found to interact with conformational intact TPO (McLachlan SM & Rapoport B, 1992). In addition, as TPO is a membrane bound protein, autoantibodies were also found to recognise the extracellular domain of the enzyme. Within this region, two consensus linear epitopes located between amino acids 590-622 (Libert F *et. al.*, 1991) and 713-721 (Finke R *et. al.*, 1991; Libert F *et. al.*, 1991) have been identified. As mentioned previously, although anti-TPO antibodies, may be detected in the sera of patients with GD, they do not play a role in the pathogenesis of GD.

2.1.3 Autoantibodies against thyroglobulin

Tg, a glycoprotein, is an essential intermediary during the synthesis of thyroid hormones (see Section 1.3.1). It appears to be only a minor antigen in GD. Autoantibodies directed against this protein are likewise generated by the interactions of T- and B-cells to epitopes located on Tg. B-cells recognize larger conformational epitopes generating antibodies primarily of the IgG class. However T-cells interact with peptide fragments and further help to promote autoantibody production and secretion. The majority of the B-cell epitopes identified are located between amino acids 1097 – 1560 in which an epitope between amino acids 1149-1250 is particularly significant (Bouanani M *et. al.*, 1989; Henry M *et. al.*, 1992). There is evidence of cross-reaction of TPO autoantibodies to Tg epitopes (Martin A & Davies RF, 1992)

and *vice versa* (Ruf J *et. al.*, 1993) suggesting that common epitopes may exist between Tg and TPO.

2.2 Causes of Graves' disease

Despite many years of investigation, the fundamental lesion which results in GD is still unknown. The immune system, especially the B- and T-cells, plays a major part as described above. Intrathyroidal lymphocytic infiltration consists of mostly CD4+ helper T-cells. There appears to be a lack of CD8+ cytotoxic T-cells. This may be responsible for a diminished tolerance in GD. As reviewed by Davies, there are several possibilities as to how these B- and T-cells become autoimmune lymphocytes (Davies TF, 1996):

- a viral infection may expose hidden antigens to lymphocytes or cause the virus to develop into an endogenous antigen,
- antibody specificity crossover may occur, when some thyroid proteins may be similar to foreign antigens,
- potent T-cell stimulators called superantigens, which form complexes with MHC class II molecules and are then recognized by T-cells, may be present,
- auto-anti-idiotypic antibodies may be generated as part of the normal primary immune response to a foreign antigen in order to regulate the immune response (Jerne NK, 1974) . However, these auto-idiotypic antibodies may then develop imprints of the epitopes of the initial exogenous antigen, thus causing other antibodies to react to them and result in an autoimmune reaction (Tomer Y & Shoenfeld Y, 1993). In support of this, there was a report that TSH immunized animals developed anti-idiotypic antibodies that recognized and stimulated TSHR (Islam MN *et. al.*, 1983),

- immunogenic heat shock proteins are induced by heat shock or other stressful stimuli such as an infection. During a bacterial infection, the antibodies and T-cells responding against microbial heat shock proteins, may then cross-react against the conserved epitopes on host heat shock proteins, thus initiating an autoimmune response (Lamb JR & Young DB, 1994). This is supported by the detection of the expression of heat shock protein 72 in GD patients which was not found in normal subjects (Bahn RS *et. al.*, 1991),
- there may be induction of MHC molecules on thyroid follicular cells by γ-interferon and cytokines, whose production was stimulated by a local viral infection. The expression of such MHC molecules could then result in the presentation of autoantigens by thyroid cells acting as antigen presenting cells and this may play a role in the pathogenesis of GD.

Several other precipitating factors, including infections, stress and psychiatric traumas have also been suggested to play a role in the development of GD. While infections themselves would not be expected to be a direct cause of GD (Toivanen P & Tovianen A, 1994), they could be involved via the mechanisms described above. However, whilst viral infections have been shown to induce autoimmune thyroid disease in experimental animals, there is no evidence demonstrating such a role in GD in humans (Carter JK & Simth RE, 1983; Tomer Y & Davies TF, 1993). As mentioned above stress and psychic traumas have also been associated with the onset of GD. Several studies have reported a higher incidence of a history of stress and trauma amongst GD patients compared to normal subjects (Leclere J *et. al.*, 1991; Winsa B *et. al.*, 1991; Sonino N *et. al.*, 1993). Acute stress has been thought to induce immune suppression and it is possible that hyperactivity of the immune system may then follow. This may

subsequently promote autoimmunity. Finally, since GD is more common in women, estrogens may also play a role in its pathogenesis.

All the above are speculations based upon limited and somewhat anecdotal evidence. In addition, it is well recognized that hereditary factors influence one's predisposition to GD. The genetic contribution to GD is notable from both family and twin studies. As reviewed by Gough (Gough SCL, 2000), the concordance rate for monozygotic (identical) twins when developing GD is 30 - 50%, which is far greater than that found for dizygotic (non-identical) twins, which is only 5% (Brix TH *et. al.*, 1998). Furthermore, the chance of the sister or brother of a sibling with GD in developing the disease themselves has been reported to be 5.4 - 12.6 and 1.2 - 7.4 times higher than normal respectively (Brix TH *et. al.*, 1998). These enhanced probabilities strongly indicate an association between genetic factors and GD. Using human DNA samples and techniques such as classic linkage analysis and allelic association, two susceptible loci have been identified. GD has been found to associate with a human MHC region known as the human leukocyte antigen (HLA) system located on chromosome 6. The MHC-HLA system consists of three main gene clusters in which class II genes, including the HLA-D genes, are strongly linked with the autoimmune process, as they encode proteins expressed on antigen-presenting cells. A particularly consistent association has been reported between the gene DR3 and gene polymorphisms. The association with this HLA-DR3 factor increases the relative risk factor for GD 2.5 – 3.7-fold (Mangklabruks A *et. al.*, 1991; Badenhoop K *et. al.*, 1992; Yanagawa T *et. al.*, 1993; Barlow AB *et. al.*, 1996; Cuddihy RM & Bahn RS, 1996). For example, one report observed that DR3 was found in 56% of Graves' patients (n = 65) compared to 26% in the general population and that DR3 association increases the risk factor by

3.4-fold in the population (Mangklabruks A *et. al.*, 1991). Linkage disequilibrium within other regions of the HLA system, for example DQB1*02 (Mangklabruks A *et. al.*, 1991), DRB1*03 (Heward JM *et. al.*, 1998) and DAQ1*05, (Barlow AB *et. al.*, 1996) has also been reported to be linked to GD. Furthermore, there is evidence to suggest that distinct HLA linkages are associated with specific ethic groups. For example, Caucasians were reported to be linked to HLA-DR3 (Farid NR *et. al.*, 1980), whereas Japanese were linked to HLA-DR5 (Uno H *et. al.*, 1981) and Chinese with HLA-Bw46 and DR9 (Yeo PPB *et. al.*, 1989). Although there is no definitive conclusion, results from association and linkage studies have provided strong evidence that the HLA region is involved in the development of GD.

Recently, the cytotoxic T-lymphocyte-associated-4 gene (CTLA-4) region has also been reported to be involved in the development of GD. Genes CD28 and CTLA-4, closely located on chromosome 2, are part of the complementary component of the immune response. They regulate the normal primary immune response from B- and T-cells. CD28 is expressed on T-cells and, when bound to its ligand CD86 (B7.1) expressed on antigen-presenting cells, plays a role in T-cell proliferation and cytokine production (Krummel MF & Allison JP, 1996; Walunas TL *et. al.*, 1996). In contrast, CTLA-4 which is also expressed on T-lymphocytes, modulates T-cells by decreasing T-cell receptors once stimulated by its ligand CD80 or B7.2 (Marengere LEM *et. al.*, 1996; Lee KM *et. al.*, 1998). Gene polymorphism of a single nucleotide in exon 1 of the CTLA-4 gene, in which an A allele was replaced by a G allele, was found to be in linkage disequilibrium and thus associated with GD (Yanagawa T *et. al.*, 1995). This A-G polymorphism has also been reported in other studies with GD patients (Donner H *et. al.*, 1997; Kotsa K *et. al.*, 1997; Heward JM *et. al.*, 1999) and is associated with an

increase relative risk for GD from 1.6 to 2.6 (Vaidya B *et. al.*, 1999; Heward JM & Gough SCL, unpublished data, 1999). Although the mechanism by which CTLA-4 polymorphism results in susceptibility for GD is not known, there is speculation that the resulting imbalance in the regulation of the immune response may contribute to autoimmunity. Furthermore, in addition to the HLA region and CTLA-4 gene, Davies and colleagues have recently reported three chromosomal regions, designated *GD-1*, *GD-2* and *GD-3*, which are also linked to GD (Tomer Y *et. al.*, 1997; Tomer Y *et. al.*, 1998a; Tomer Y *et. al.*, 1998b). Clearly further investigation into the role of genetic linkages is necessary.

2.3 Pathology of Graves' disease

Although GD affects both males and females, the prevalence in women (0.5 - 2%) is 10 times higher than for men (Tunbridge WMG *et. al.*, 1977; Konno N *et. al.*, 1993; Vanderpump MPJ & Tunbridge WMG, 1996; Chiovato L *et. al.*, 1997). GD can occur at any age, but the highest frequency of initial presentation occurs between the years 40 - 60. Thyrotoxic patients exhibit a range of signs and symptoms, the majority of which are common to all hyperthyroid cases. However, there are several GD specific clinical features. Both these common and specific signs and symptoms are discussed below. The diagnosis of hyperthyroidism must be confirmed biochemically despite the presentation of these clinical features (Vanderpump MPJ *et. al.*, 1996). This is accomplished with thyroid function tests. Either total thyroid hormones or free T_3 and T_4 together with TSH concentrations are measured. In most GD cases, TSH levels will be below the normal range and at times even undetectable. Using the highly sensitive third generation TSH immunoassays, which can detect as little as 0.01 - 0.02mU

TSH/L (Spencer CA *et. al.*, 1995), a suppressed serum TSH level provides the initial biochemical confirmation of the diagnosis of hyperthyroidism following clinical presentation. Confirmation may be obtained by measuring the concentrations of thyroid hormones, which will be raised. Occasionally the level of free T_3, and not free T_4, is enhanced; this condition is named T_3-thyrotoxicosis. Additional tests such as thyroid scans and assays for thyroid antibodies should ideally also be performed, but are often restricted to selected patients (Vanderpump MPJ *et. al.*, 1996). A definitive diagnosis of GD is usually achieved by the finding of a diffuse goitre together with detectable TSAb.

2.3.1 Common signs and symptoms of thyrotoxicosis

All thyrotoxic patients, irrespective of the cause of hyperthyroidism, exhibit the signs and symptoms of thyrotoxicosis. However, there are a number of features specifically associated with GD (see Section 2.3.2). The common clinical features are listed in Table 2.2 (Braverman LE & Utiger RD, 1996). The severity and frequency of these signs and symptoms varies from patient to patient and may be age related (Nordyke RA *et. al.*, 1988). For example, elderly patients may exhibit more symptoms of cardiovascular dysfunction whereas the sympathetic activation giving rise to anxiety may be more prominent in younger patients. Furthermore, the extent of these clinical manifestations on a given patient may be independent of the biochemical severity (Trzepacz PT *et. al.*, 1989).

Common symptoms:	**Common signs:**
Nervousness	Tachycardia or atrial arrhythmia
Fatigue	Systolic hypertension
Weakness	Warm, moist, smooth skin
↑ perspiration	Stare & eyelid retraction
Heat intolerance	Tremor
Hyperactivity	Hyperreflexia
Palpitation	Muscle weakness
↑ appetite	Goitre (maybe)
Weight loss	
Menstrual disturbances	

Table 2.2 **The common clinical manifestations of thyrotoxicosis.** (Braverman LE & Utiger RD, Introduction to Thyrotoxicosis. In Braverman LE & Utiger RD, eds. Werner& Ingbar's The Thyroid – A Fundamental and Clinical Text, 7th Ed. Lippincott Raven Publishers; 1996, p524)

As shown in Table 2.2, a goitre may be observed in a thyrotoxic patient, the increase in the size of the thyroid gland being a consequence of both hyperplasia and hypertrophy. There are several types of goitres and GD is usually associated with a diffuse goitre. Several features e.g. palpitations and tachycardia, indicate that the cardiovascular system is affected, particularly in elderly patients. Cardiac output is increased as a result of an increased demand for oxygen and blood flow to organs such as muscles, the brain and kidneys. This is accompanied by a decrease in the systemic vascular resistance (Klemperer JD *et. al.*, 1995) and increased in blood volume, blood pressure and heart rate (Anthonisen P *et. al.*, 1960). Subsequently, >90% of patients exhibit signs of tachycardia with heart rates of >120 beats/min (Delit C *et. al.*, 1961; Roti E *et. al.*, 1988; van-Olshausen KV *et. al.*, 1989). Moreover, atrial fibrillation and ectopic beats are also commonly observed. Patients also complain of muscle weakness, fatigue and tremors. The extent of muscle weakness is variable and linked to the severity and duration of thyrotoxicosis. In addition, mild muscle atrophy may also occur as well as hyperreflexia. In the latter, there is hyperactivity in both the contraction and relaxation of the tendons and skeletal tissue. The skin of thyrotoxic patients is often warm and moist as a result of increased heat production and perspiration. The nails and hair are also affected in which nails become shiny and maybe soft and friable while there is increased hair loss. Patients may also develop a stare in conjunction with retracted eyelids. This results from a non-infiltrative eye disease in which the extrocular muscles are restricted in their movements. This condition is not the result of an immune response with B- and T-lymphocytes, as in Graves' ophthalmopathy (see below). Finally, in addition to the neuropsychiatric symptoms mentioned above, in extreme cases depression, mania and even schizophrenia have been reported to occur in

association with GD (Lazarus A & Jaffe R, 1986). All these symptoms are reversible upon treatment and the achievement of euthyroid status.

2.3.2 Graves' specific features

As mentioned above, there are several features that are unique to GD. These are Graves' ophthalmopathy, pretibial myxoedema and thyroid acropachy. While Graves' ophthalmopathy has been extensively investigated and reviewed by Burch and colleagues (Burch HB *et. al.*, 1996), pretibial myxoedema and thyroid acropachy have been studied to a lesser extent (Fatourechi V, 1996; Bayliss RIS & Tunbridge WMG, 1998).

2.3.2.1 Graves' ophthalmopathy

It is well recognised that patients with hyperthyroidism *per se* are liable to develop eye changes. The main signs are retracted eye lids and a staring appearance. This is the result of noninfiltrating eye disease, i.e. without the infiltration of B- and T-cells into the orbit, and causes restriction in the movement of the eye muscles. Graves' ophthalmopathy however, is more complex and unique to GD, and not found with the other forms of hyperthyroidism (Figure 2.2). These patients present a range of clinical features listed below (Bayliss RIS & Tunbridge WMG, 1998):

- The eyes often feel gritty and sore as they are poorly protected by the retracted eyelids. They are also vulnerable to irritations and infections.
- The eyes and eyelids are swollen as the result the increase in pressure, which leads to impaired drainage of fluid from the eye.

Figure 2.2 **Graves' ophthalamopathy, the eye signs specifically associated with Graves' disease, in a patient**. (Wise PH. Colour Guild Endocrinology. Churchill Livingstone; 1994, p10)

- Lid retraction is present due to restriction in the movement of the inflamed eyelid muscles.
- Ophthalmoplegia, which is restriction in directional movements of the eyeball, may occur. The oculamotor muscles are principally affected, so that the upward movement is most severely influenced.
- Proptosis, which is the protrusion of the eye due to the anterior displacement of the globe, may be present. This is due to increased pressure, swelling and enlargement of the muscles and retro-orbital tissues.
- The patient may complain of diplopia (double vision) which results from the enlarged extraocular muscles.
- There may be a loss of visual acuity, which is the consequence of the reduction in the movements of the muscles responsible for the focusing of the lens.
- Optic nerve neuropathy which results from compression of the optic nerve by the enlarged posterior muscles may, in the most severe cases, result in the loss of sight.

Graves' ophthalmopathy occurs in both females and males. Although it is more common in women, men (Perros P & Kendall-Talyor P, 1992) and smokers are the more severely affected (Hägg E & Asplund K, 1987; Bartalena L *et. al.*, 1989; Shine B *et. al.*, 1990; Prummel MF & Wiersinga WM, 1993). Mild ophthalmopathy is generally left untreated. Generally, improvements in ophthalmopathy are concurrent with treatment of GD. However, specific features may be treated separately (Bayliss RIS & Tunbridge WMG, 1998). For example, lid retraction can be reduced by a β-blocker, whereas eye drops and lubrications provide relieve of discomfort in swollen and gritty eyes. Diuretics may be used to increase drainage of fluids and thus decrease oedema.

However, in more severe cases, surgery may be required to relieve symptoms. These, however, are secondary treatments which do not treat the underlying cause of GD.

Despite intense investigations the aetiology of Graves' ophthalmopathy is still unknown. In general, the eye changes are thought to result from a secondary autoimmune reaction (Bahn RS & Heufelder AE, 1993). One hypothesis is that autoantigens common to the thyroid and the orbit coexist. Infiltration of GD specific T- and B-lymphocytes into the retroorbital space then initiates an inappropriate immune response. Epitope recognition then stimulates the secretion of secondary signals such as interferons, interleukines and tumor necrosis factors (Heufeld AE & Bahn RS, 1993). B-cells within the orbit then proliferate in response to these stimulators and secrete autoantibodies. In addition, the cytokines can also stimulate proliferation of orbital fibroblasts (Heufelder AE & Bahn RS, 1994) and their production of glycosaminoglycans (Smith TJ *et. al.*, 1991; Korducki JM *et. al.*, 1992). Subsequently, there is an accumulation of glycosaminoglycans within connective tissues and between muscle fibres in retroorbital tissues. Furthermore, this immune response could result in inflammation and oedema of the extraocular muscles and retro-ocular fibrous tissues (Riley FC, 1972). Inflammation and the deposits of glycosaminoglycans eventually cause the muscles and tissues to enlarge and restrict the movement of the muscles. Moreover, localised oedema causes increased pressure within the orbit.

Graves' ophthalmopathy generally affects both eyes although the degree of severity may differ between each eye. On rare occasions, only one eye may be affected, which is difficult to explain by the mechanisms proposed above. Furthermore, the timing of its occurrence is not totally predictable, although its appearance generally coincides

with the onset of GD. However, it can arise shortly before obvious presentation with GD or even after treatment and the achievement of a euthyroid state. Furthermore, it may exist, although only rarely, in the total absence of any other biochemical or clinical signs of GD, when the condition is named ophthalmic GD.

2.3.2.2 Pretibial myxoedema

This condition is so named because of the thickening of skin occurring in the pretibial region (Figure 2.3). Occasionally the feet and toes are also affected (Kriss JP, 1987; Fatourechi V *et. al.*, 1994). The characteristic lesions are caused by the accumulation of glycosaminoglycans in the subcutaneous connective tissues and the dermis (Watson EM & Pearce RH, 1947; Sisson JC, 1968; Hanke CW *et. al.*, 1983; Smith TJ *et. al.*, 1989). The reason for the increase in glycosaminoglycan production by dermal fibroblasts is not clear. The lesions can appear in a number of forms: diffuse, nonpitting oedema; raised plaque lesions above nonpitting oedema; distinctive tubular or nodular lesions and a rare elephantiasic form. The nonpitting oedema lesions are most common (Fatourechi V *et. al.*, 1994); they are round and highly pigmented, with colours ranging from pink to brown.

Pretibial myxoedema occurs in ~ 4% of Graves' patients and is commoner in women who are 3.5 times more likely to develop the disorder than men (Fatourechi V *et. al.*, 1994). This condition is nearly always associated with Graves' ophthalmopathy (Beierwaltes WH, 1954; Kriss JP, 1987; Fatourechi V *et. al.*, 1994) and has been reported to occur in 4% (Bartley GB *et. al.*, 1996) and 12 – 15% (Kriss JP, 1987;

Figure 2.3 **Pretibial myxeodema, the thickening of the skin occurring in the pretibial region, associated with Graves' disease**. (Wise PH. Colour Guild Endocrinology. Churchill Livingstone; 1994, p 12)

Fatourechi V *et. al.*, 1993) of patients with notable and severe Graves' ophthalmopathy respectively. Furthermore, a subclinical form of the condition i.e. without obvious changes, and only identified by skin biopsies, is believed to be more common (Wortsman J *et. al.*, 1981; Salvi M *et. al.*, 1994). In most cases, the lesions are mild and tend to regress with time and hence do not require treatment (Fatourechi V *et. al.*, 1994). However, medication may become necessary both to ease discomfort and for cosmetic reasons. The routine regimen is the topical administration of a corticosteroid ointment (Benoit FL & Greenspan FS, 1967; Kriss JP *et. al.*, 1967). The cream is applied to the affected area and covered with polythene film overnight which is then removed in the morning (Kriss JP *et. al.*, 1967). The goal of therapy is to improve both appearance and function, and to avoid tissue breakdown and any further complications. Treatment may span several months with a gradual reduction in its frequency with partial remission being observed after several weeks.

2.3.2.3 Thyroid acropachy

Thyroid acropachy is rare, occurring in only ~ 1% of GD patients. It is invariably associated with Graves' opthalmopathy and pretibial myxoedema (Goette DK, 1980). It affects both males and females equally (Winkler A & Wilson D, 1985). The distinctive feature of thyroid acropachy is the clubbing of the distal phalanges of the fingers and toes (Figure 2.4). There are also swellings in the soft tissues surrounding the digits. In addition, new bone formation is observed in the proximal and middle phalanges of the fingers and in the metatarsal and proximal phalanges of the toes. The skin is likely to be pigmented as well. This condition is asymmetric and can even occur in only one digit (Chapman ME *et. al.*, 1993). Acropachy is usually not painful,

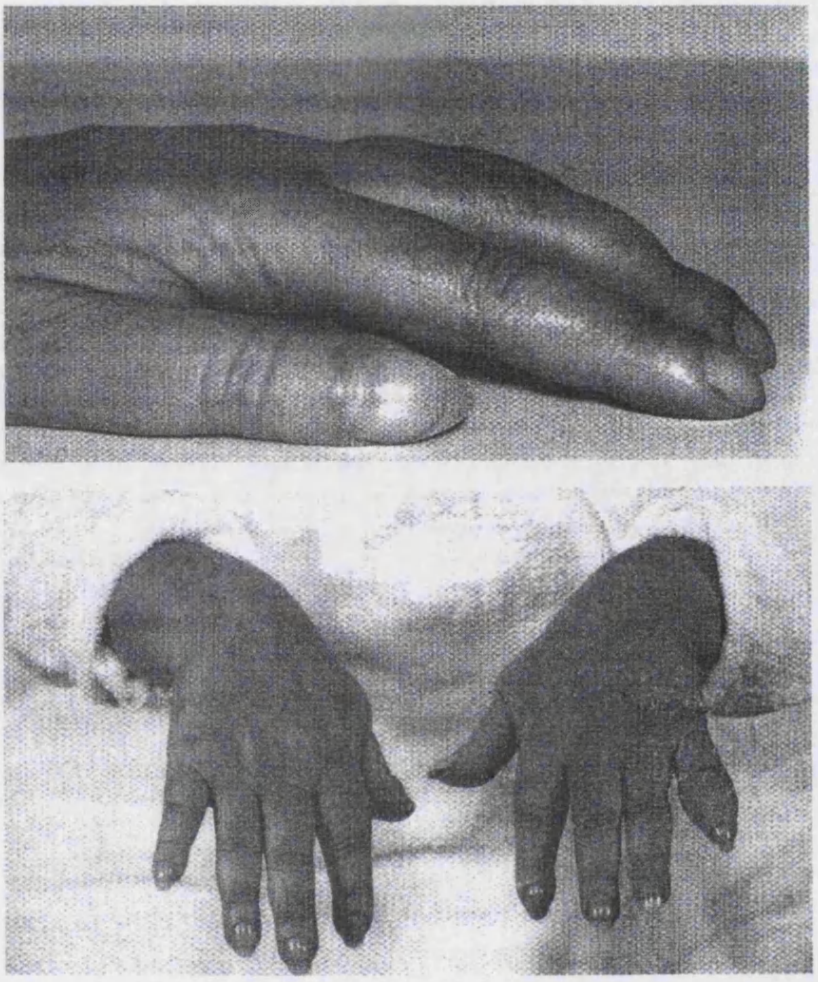


Figure 2.4 **The distinctive feature of thyroid acropachy, the clubbing of the distal phalanges of the fingers and toes**. (Fatourechi V. Localized myxedema and thyroid acropachy. In Braverman LE & Utiger RD, Eds. Werner & Ingbar's The Thyroid – A Fundamental and Clinical Text, 7th Ed. Lippincott Raven Publishers; 1996, p 556)

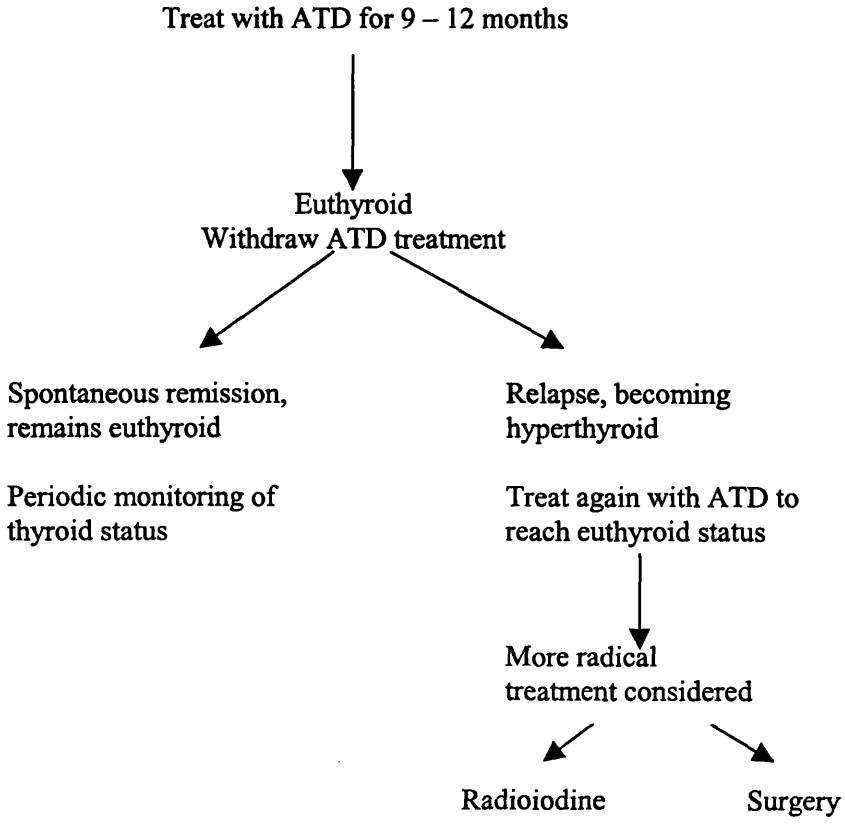
but it has been reported that some patients experience extreme swellings associated with lymphatic blockage, loss of function of the fingers, discomfort and some degree of pain (Rothschild BM & Yoon BH, 1982). There is currently no effective treatment available although local applications of corticosteroid therapy have been reported to benefit some patients (Parker LN *et. al.*, 1982).

2.4 Treatments to control Graves' hyperthyroidism

Once the patient has been diagnosed as suffering from GD, treatment to control the hyperthyroidism is essential. Unfortunately, there is no absolute cure for GD in terms of the therapeutic elimination of the TSAb responsible for the condition. Thus, any therapy only treats the hyperthyroidism. There is no single definitive therapy and the contrasting treatments available may not be totally successful. There are three main forms of treatment: antithyroid drugs (ATD), radioactive iodine and surgery. As none of these may be totally effective, they are not exclusive of each other. However, as GD is an autoimmune disease, patients may undergo spontaneous remission. The aim therefore, in the first instance, is to render the patient euthyroid and hope that he or she might enter remission spontaneously. This is tested for by withdrawal of preliminary ATD treatment.

A treatment strategy adopted for GD, as opposed to non-Graves' hyperthyroidism, is shown in Figure 2.5. Diagnosis of GD is dependent on the presentation symptoms, thyroid function tests together with, ideally, isotope uptake studies which should reveal a diffuse uptake and thyroid antibody assays. Once diagnosed, GD patients are, initially, treated with ATD for 9 – 12 months after which time the drug is withdrawn to

GRAVES' HYPERTHYROID PATIENT



NON-GRAVES' HYPERTHYROID PATIENT E.G. TOXIC NODULE

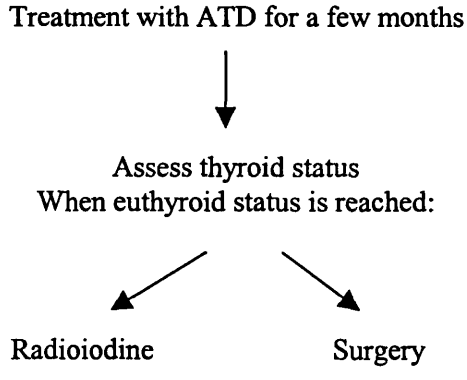


Figure 2.5 **Comparing the different treatment protocols for Graves' and non-Graves' (e.g. toxic nodule) hyperthyroid patients.** ATD = antithyroid drugs

test for a spontaneous remission. Leech and Dayan reported a 50% remission rate in GD patients after a 6 – 24 month of ATD treatment (Leech NJ & Dayan CM, 1998). If the patient does enter remission, no further treatment is required but thyroid status must be periodically monitored. If, however, the patient relapses, further and more radical treatment with radioiodine or surgery is required.

Treatment for a particular patient is determined by consultation between the clinician and the patients. Important personal factors to consider (Bayliss & Tunbridge, 1998) include:

- the age and sex of the patient,
- the position and size of the thyroid gland,
- whether it is convenient for the patient to be maintained under long term medical supervision or a more rapid approach is more suitable,
- whether or not there is Graves' ophthalmology,
- previous treatments if relapses occur after remission and,
- financial considerations.

A particular course of treatment is only selected after careful consideration of patient's personal circumstances.

2.4.1 Antithyroid medication

ATD is the initial treatment for hyperthyroidism. The drugs used may be divided into five groups:

- By far the most commonly used are those that interfere with thyroid hormone synthesis at the cellular level so that the synthesis of thyroid hormones is suspended. These are the thionamides such as propylthiouracil (PTU), carbimazole (CBZ) and methimazole. These drugs reduce the iodination of tyrosine by inhibiting with the enzyme TPO. Subsequently, the coupling of iodotyrosines is also inhibited. As a result, there is a reduction in the synthesis and release of T_4 and T_3.
- Those which inhibit the deiodination of T_4 to T_3 in the circulation. Propanolol and also PTU, can be used this way.
- β-blockers such as propranolol that inhibit the effect of the thyroid hormones on the peripheral tissues. Obviously cardiac side-effects must be considered.
- Medications that have immunosuppressive effects. These include methimazole and PTU (Weetman AP *et. al.*, 1984).
- Those which interfere with the release of the thyroid hormones. Inorganic iodine and lithium are able to decrease thyroid hormone secretion.

The thionamides are the most commonly used drugs in the "first-line" medical treatment of GD. PTU and CBZ are both effective as described above. Initially, high doses are used, which may be adjusted accordingly to the severity of the hyperthyroidism and the age of the patient. The doses are gradually reduced concurrently with improvements in the condition until the patient is rendered euthyroid. The patient is then maintained on the lowest dosage to sustain euthyroidism. Although these drugs can occasionally have serious side effects such as agranulocytosis, hepatitis and lupus-like syndromes, such problems are rarely encountered, and only minor reactions such as skin rashes are usually encountered.

Numerous studies have attempted to determine the influence of thionamides on remission rates and to devise a definitive treatment protocol for GD (e.g. Braverman LE, 1996). For example, one trait reported has been that remission rates improve with longer treatments with ATD (Allanic H *et. al.*, 1990) and higher doses (Romaldini J *et. al.*, 1983). Unfortunately, this was not confirmed in a later trial conducted by Reinwein *et. al.* (1993). Wilson and colleagues reported that a constant higher daily dosage of CBZ (60mg), as oppose to 20mg, for 12 months, resulted in a higher remission rate of 64% compared to only 43% with the lower dose (Wilson R *et. al.*, 1996) but, these differences were not statistically significant. Another study reported no difference in remission rates, between patients on a high (40mg) or low (10mg) dose (Benker G *et. al.*, 1998). Since side-effects are more frequent in patients receiving the higher doses of ATD (Myer-Gefner M *et. al.*, 1994), if no benefit may be obtained in terms of remission rates with the higher doses, lower doses may be preferable.

Hashizume and colleagues reported that a therapy, sometimes referred to as "block-replacement" treatment, which requires T_4 to be prescribed concurrently with a high dose of thionamide, decreased TSAb levels and improved remission rates (Hashizume K *et. al.* 1991). However, several subsequent studies investigating the effectiveness of this regimen have produced conflicting conclusions as reviewed in Hershman (1995). In a recent study by Pujol and colleagues, they have reported that CBZ combined with T_3 or Triac (3,5,3'-triiodothyroacetic acid) has no significant effect on the rate of remission and relapse (Pujol P *et. al.*, 1998). McIver and colleagues also concluded that the administration of T_4 with CBZ neither delayed nor reduced relapses (McIver B *et. al.*, 1996). In general, it is accepted that ~50% of patients relapse after initial ATD

treatment (e.g. Leech NJ & Dayan CM, 1998), and that only ~20% of all patients remain in remission after 20 years (Sugrue D *et. al.*, 1980).

2.3.2 Radioiodine Therapy

^{131}I is the radioactive isotope of iodine used in radioiodine therapy for thyrotoxicosis. The radioiodine is trapped by the thyroid, incorporated into Tg and stored within the lumen. β-particles and high energy γ-rays emitted by the ^{131}I irradiates surrounding follicular cells. Since the uptake of radioactive iodine is homogenous in the thyroid of a Graves' patient, the irradiation and destruction of cells is uniform throughout the gland. Consequently, the synthesis and release of the thyroid hormones is diminished and patients can achieve euthyroidism. The dose of radioiodine given should aim to restore the patient to an euthyroid status within 2 - 3 months. After administration of a dose of radioiodine, thyroid function declines gradually. 50 – 70% of patients achieve a euthyroid status in 6 – 8 weeks (Holm LE *et. al.*, 1981). Furthermore, 80 – 90% of patients regain normal thyroid function after only one dose of radioiodine. The others will require a second dose and, rarely, a third dose may be necessary (Holm LE *et. al.*, 1981).

No serious adverse effects have been attributed to thyroid ablation with radioactive iodine. Radioiodine is therefore recommended for most GD patients except for children, pregnant and breast feeding women. Caution should be taken with patients with Graves' ophthalmopathy since this can be exacerbated by this treatment. Radioiodine therapy has a number of advantages. These are summarised below:

- no admission into the hospital required,

- no complications associated with surgery,
- can be administrated orally,
- short term treatment - usually only one dose is necessary and,
- comparatively inexpensive.

However, there are two major disadvantages. Firstly, the relatively long period of time required to achieve total benefit. Secondly, the likelihood of the development of hypothyroidism in subsequent years. As >90% of GD patients become hypothyroid within the first year after treatment (Cunnien AJ *et. al.*, 1982), hypothyroidism is considered as an inevitable consequence of radioiodine treatment rather than as a side-effect (Graham GD & Burman KD, 1986). However, hypothyroidism can be easily remedied with thyroxine replacement therapy. GD patients should be warned of this as a probable final outcome and be prepared for the need of long term replacement therapy. For this reason, long term monitoring of thyroid function is essential after radioiodine treatment (Mcdougall IR, 1992; Vanderpump MJP *et. al.*, 1996; Bayliss RIS & Tunbridge WMG, 1998).

2.4.3 Surgery

Subtotal thyroidectomy is an effective treatment of GD especially when performed by experienced surgeons. This procedure removes the majority of the thyroid gland, leaving only a remnant of <10g (Klementschitsch P *et. al.*, 1979). The need for surgery depends upon patient's personal circumstances and should be determined after discussion with both the physician and surgeon. Surgical treatment is preferred for patients with large goitres which may be unsightly or causing breathing difficulties. It

is also recommended when other forms of treatments have failed or are unsuitable. This is likewise an option for pregnant GD patients when drugs may be unacceptable.

Patients undergoing surgery must be rendered euthyroid before surgery. This is generally achieved with a short course of ATD. There are a number of factors that could influence the outcome of surgery including the age of the patient and the size of any goitre. It has been reported that hypothyroidism is more likely to occur with smaller glands (Cusick EL *et. al.*, 1987) and high iodine intake is associated with relapses (Thjodleifsson B *et. al.*, 1977). As with any other surgical procedures, there may be risks and complications. These are listed below:

- Haemorrhage.
- Wound infections.
- Damage to the recurrent laryngeal nerves on either side of the neck. These nerves activate the vocal cord and hence patients may experience a temporary hoarse voice for a few days after surgery. Permanent hoarseness would ensue if the nerves were to be cut.
- Transient (≤20%) and permanent (2%) hypocalcaemia may result due to damage to the parathyroid glands during the operation (Neis C *et. al.*, 1994).
- Hypothyroidism may develop. 80% of patients are euthyroid one year after surgery, but subsequently 5 - 40% of patients develop permanent hypothyroidism (Franklyn JA *et. al.*, 1991). The incidence increases with time. Thyroxine replacement treatment is obviously then required.
- Recurrent hyperthyroidism is also a minor risk. Hyperthyroidism reoccurs in about 1 - 3% of patients within the year of surgery and 1% in the subsequent years

(Sugrue D *et. al.*, 1980; Cusick EL *et. al.*, 1987). Radioiodine is the most suitable treatment for recurrent hyperthyroidism.

2.4.4 The use of TSAb to monitor patients with Graves' disease

From the above review of the current treatment protocols, it is clear that a test which could be used to predict whether a patient might enter a spontaneous remission at the end of the initial 9 – 12 months of treatment of ATD would improved patient management. It would not then be necessary to withdrawal the ATD and wait for a relapse to occur, an event which must cause anxiety to the patient and a process which must be stressful. Theoretically, monitoring TSAb levels might be expected to provide just such a prognostic indicator. One might anticipate that a low TSAb level at the end of ATD would indicate that remission was likely, whereas a persistently high TSAb levels would indicate relapse and so point to the immediate used of a more radical therapy such as radioactive iodine or surgical ablation. To date, as is discussed in the final Discussion, no such clear picture has emerged. However, since we do not yet have an ideal detection and measurement system for TSAb, this may be the confounding factor. It is our aspiration that a TSAb bioassay, which is sensitive, precise, robust and technically amenable will help to resolve this issue. This is the main reason for our attempts to devise just such a TSAb bioassay.

Section 3

The TSH Receptor: interactions with TSH and autoantibodies

As previously mentioned TSH is the dominant regulator of thyroid function. It binds to a cell-surface receptor (TSHR) on the surface of the follicular cell and initiates a cAMP mediated intracellular signalling cascade. Ultimately, this increases the synthesis and release of thyroid hormones. The TSHR also plays a fundamental role in autoimmune hyperthyroidism, namely GD, since the receptor is acknowledged to be the primary autoantigen. Stimulating autoantibodies interact with the TSHR and thereby mimic TSH. Consequently, inappropriately high levels of thyroid hormones are persistently released into the circulation, causing Graves' hyperthyroidism. These actions have been discussed in greater detail in previous sections.

Clearly, an improved understanding of the structural and functional relationship between the receptor, TSH and autoantibodies should reveal significant findings relating to the underlying pathology of GD. This may lead to improvements in the diagnosis and treatment of the disease. The molecular cloning of the human TSHR in 1989 (Libert F *et. al.*, 1989; Nagyama Y *et. al.*, 1989) together with other technical advances have led to significant progress in this area. This will be summarised in the current section. In addition, particular attention will be paid to the application of this new understanding to the design and relative merits of diagnostic assays for GD.

3.1 Thyroid Stimulating Hormone Receptor

Cloning of the human TSHR allowed the sequencing of the receptor (Libert F *et. al.*, 1989; Nagayama Y *et. al.*, 1989). The full length human TSHR cDNA is ~4kb. It contains a single open reading frame of 2292bp which encodes a protein of 764 amino acids. The TSHR amino acid structure is shown in Figure 3.1. The TSHR is a glycoprotein hormone receptor with a molecular mass of ~ 84.5kd. It is closely related to the other glycoprotein receptors for the pituitary hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), and for placental human chorionic gonadotropin (hCG). The molecular and genomic structures of human TSHR are reviewed below (Nagataki S & Nagayama Y, 1997; Rapoport B *et. al.*, 1998).

3.1.1 Molecular Structure

All the glycoprotein hormone receptors belong to the GTP-binding protein (G-protein) coupled receptor family. G-protein coupled receptors have distinctive characteristics. These receptors have three differentiated domains: the amino (N-) terminal extracellular region (also named the ectodomain), the seven pass transmembrane-spanning domain and the carboxyl (C-) terminal intracellular cytoplasmic region (Figure 3.1). The ectodomain and the cytoplasmic region are the ligand binding and catalytic domains respectively. The transmembrane domain consists of seven transmembrane segments which are linked by three extracellular and three cytoplasmic loops. Stimulation of these receptors activates the receptor linked heterotrimeric G-proteins. They in turn trigger the intracellular secondary phosphorylation cascades, predominantly for TSH the cAMP cascade, which mediates a diverse range of cellular effects (see Section 3.1.1.2).

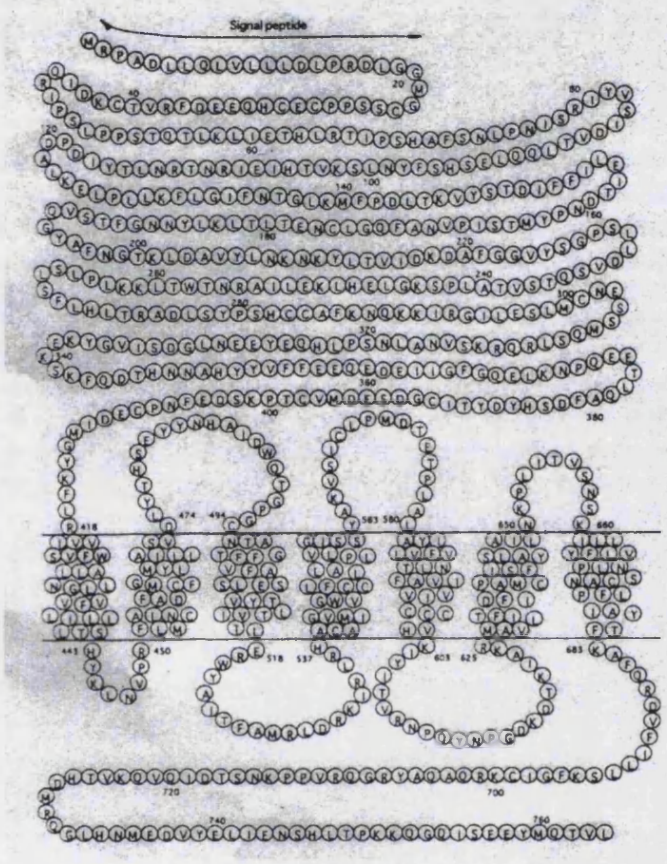


Figure 3.1 **The amino acid sequence and structure of the human TSHR**. (Nagataki S & Nagayama Y. Molecular Biology of the Thyroid Stimulating Hormone Receptor. In Falk SA, ed. Thyroid Disease: Endocrinology, Surgery, Nuclear Medicine, and Radiotherapy, 2nd Ed. Philadelphia: Lippincott Raven Publishers; 1997, p210)

3.1.1.1 Extracellular Domain

Primary structure

The ectodomain of the TSHR is relatively large, comprising 418 amino acids in which a 21 amino acid signal peptide is present at the extreme N-terminal (Figure 3.1; Libert F *et. al.*, 1989; Nagayama Y *et. al.*, 1989). The extracellular domains of the TSHRs derived from human, dog and rat thyroids have very high amino acid homology (85 - 90%). In contrast, the homology is low (35 - 45%) between the ectodomains of the receptors for the other human glycoprotein hormones (LH, FSH and hCG). The differences are concentrated at both ends of the extracellular domain (amino acids 1 - 57 and 287 - 404). Within these unconserved regions, the TSHR has two additional insertions at residues 38 - 45 (8 amino acids) and 317 - 366 (50 amino acids). The former is thought to be involved in both TSH binding and signalling (Wadsworth HL *et. al.*, 1990), and its deletion results in the loss of TSH binding. Furthermore, mutational analysis revealed residue Cys41 within this segment to be critical for TSHR function (Wadsworth HL *et. al.*, 1992). The function of the 50 amino acid insert remains unknown. There is a high degree of homology in the mid region of the ectodomains of the receptors for the glycoprotein hormones. In this conserved section there are nine leucine-rich repeats (LRRs) between residues 58 – 277 (Nagayama Y & Rapoport B, 1992a; Nagayama Y & Nagataki S, 1994). These LRRs are important for protein-protein interactions (Takahashi N *et. al.*, 1985). They contain numerous aliphatic amino acids and have a consensus sequence as follows: x-Leu-x-x-Thr-x-x-Leu-Thr-x-Leu-Pro-x-x-Ala-Phe-x-x-Leu-x-x-Leu-x-x-x-Leu. There are also six potential N-linked glycosylation sites, Asn-x-Ser/Thr (x being any amino acid except Pro), and eleven cysteine residues (Cys) which form disulfide bridges.

Subunit Structure

Prior to the cloning of the TSHR, a variety of subunit structures within the receptor had been reported. It is now recognised that there are two subunit structures, A and B, which link via disulfide bonds to form the holoreceptor. These subunits are ~ 35 and 42kd in size respectively and are produced by intramolecular cleavage of a single polypeptide chain which is encoded by a single mRNA. In addition to the two-subunit form, the existence of the single chain form of the receptor has also been reported (Russo D *et. al.*, 1991a). The latter was found on the surface of intact cultured cells in monolayers. While both of these forms of the receptor bind TSH with similarly high affinities, there is speculation as to which is the physiological form. Rapoport and colleagues proposed that both are present on the surface of the thyroid follicular cells (Rapoport B *et. al.*, 1998).

Intramolecular cleavage of the TSHR occurs simultaneously at two distinct sites within the ectodomain (Figure 3.2; Chazenbalk GD *et. al.*, 1997b). This results in the two-subunit form of the TSHR being derived from the single-chain form. The two sites, 1 and 2, are upstream and downstream in the extracellular region respectively. Site 1 has been speculated to locate downstream of residue Asn302, between residues 303 – 317 (Wadsworth HL *et. al.*, 1990; Russo D *et. al.*, 1991a). Light trypsinization of the TSHR releases a small polypeptide fragment containing an N-linked glycan moiety from the C-terminal of the A subunit (Tanaka K *et. al.*, 1998). The Asn302 N-linked residue is the last and furthest downstream within the ectodomain (see below). Cleavage downstream of this residue produces an A subunit of the anticipated size. Surprisingly mutation of all the residues between 302 and 317 does not abolish cleavage at site 1 (Tanaka K *et. al.*, 1998). This has lead to the speculation that a non

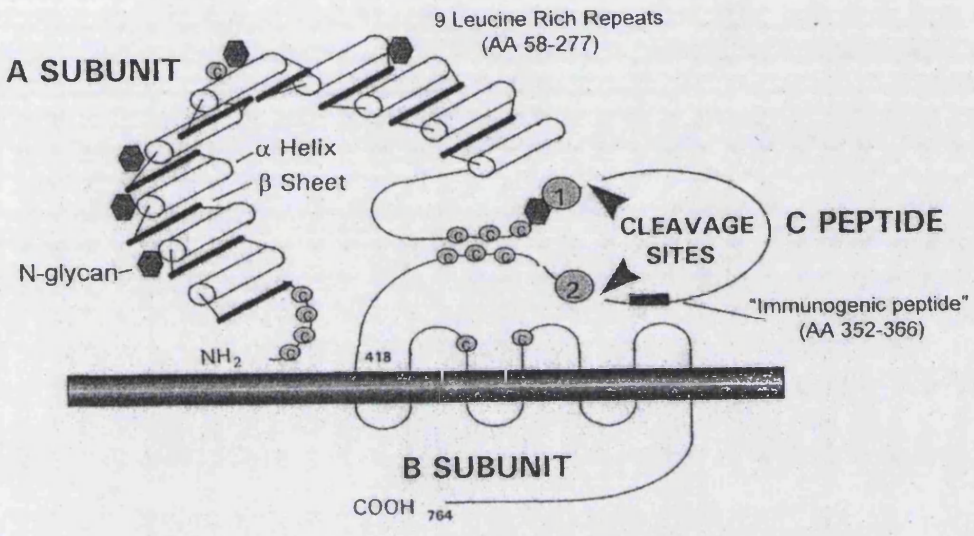


Figure 3.2 **An illustration showing the subunit structures, A and B, of the human TSHR**. The subunits are produced as a result of an intramolecular cleavage of the single polypeptide chain in the extracellular domain (ectodomain). This cleavage also results in the release of the C-peptide. (Rapoport B *et. al.*. The thyrotropin (TSH)-releasing hormone receptor: interaction with TSH and autoantibodies. Endo Rev; 1998, 19:673)

specific amino acid motif is involved. Furthermore, the 50 amino acid "insertion" (residues 317 - 366) is also required for cleavage at site 1, and deletion of this sequence eliminates cleavage of the receptor. Site 2 has been identified to be at the three residues Gly367, Gln368 and Glu369 ($GQE_{367-369}$). Mutagenesis studies using chimeric TSH-LH/CGRs have lead to the discovery that these residues are critical for cleavage at site 2. Simultaneous substitution of all three residues prevents cleavage. Specifically, the introduction of an N-linked glycosylation site within this region, N-X-S/T (where X is any amino acid with variable glycosylation efficiency), will abolish site 2 (Shakin-Eshleman SH *et. al.*, 1996). However, in contrast to site 1, the 50 amino acid "insertion" is not necessary for cleavage at this site.

The mechanism of TSHR cleavage is not clear. There have been a number of hypothesises. The most recent proposal involves enzymes named matrix metalloproteinases (MMPs). These enzymes are able to release membrane-anchored growth factors, receptors, adhesion molecules and proinflammatory cytokines from the cell surface (reviewed by Arribas J *et. al.*, 1996). An MMP inhibitor, BB-2116, has been reported to inhibit cleavage of the TSHR into its subunits, thus suggesting the involvement of the MMPs (Couet J *et. al.*, 1996). Unfortunately, since MMPs do not interact with any specific amino acid sequences, the residues involved at the cleavage site cannot be identified. Yet, this further supported the theory that MMPs are responsible due to the absence of specific amino acid motifs at the two cleavage sites (Kakinuma A *et. al.*, 1997; Tanaka K *et. al.*, 1998). However, further studies are necessary for confirmation. The simultaneous cleavage at both sites produces not only the TSHR subunits, but also releases a small peptide chain named the C-peptide (Figure 3.2). At present it is not possible to detect this peptide even in the culture

medium of cell lines expressing the TSHR. After cleavage of the receptor, A and B subunits are linked together only by disulfide bonds formed between Cys residues (see below for details).

Amongst the receptors for the glycoprotein hormones, the 50 amino acid "insertion" (residues 317 - 366) is unique to the TSHR. The precise boundaries of the insertion are, however, unclear. This "insertion" is located between the two cleavage sites 1 and 2 as described above, and is the C-peptide released after cleavage of the TSHR. However, the role of this fragment is unclear. As discussed previously, this "insertion" is required for the cleavage at site 1 but not site 2. Furthermore, it is not necessary for both TSH binding and consequent signal transduction (Wadsworth HL *et. al.*, 1990). Nevertheless since it is released into the circulation, it may play a role in the pathogenesis of GD, and a synthetic peptide, containing the corresponding C-terminal residues of the C-peptide (residues 352-367), has been reported to be recognized by TSHR autoantibodies in Graves' sera (Kosugi S *et. al.*, 1991a & 1991b).

As TSH binds to both cleaved and uncleaved forms of the receptor with similar affinities, the question remains as to what is the functional importance of receptor cleavage (Russo D *et. al.*, 1991a). Furthermore, a cleaved receptor is not necessary to mediate TSH actions. This has lead to the suggestion that cleavage of the receptor may affect the basal level of TSHR activity. The recognition that the basal, ligand-independent activity of a TSHR is higher than that of other noncleaving glycoprotein receptors supports this theory (Kosugi S & Mori T, 1995; Parma J *et. al.*, 1995; Van Sande J *et. al.*, 1995). Light trypsinization of cells expressing the receptors converts the monomeric form of the TSHR into the heteromeric form (Tanaka K *et. al.*, 1998)

as well as activating the receptors which have lost an epitope at residues 354 - 359 (Van Sande J *et. al.*, 1996). Increases in TSHR activity was also reported after deletion of the residues 339 - 367 at the C-terminal of the C-peptide (Zhang M-L *et. al.*, 1995). However, further studies are required to support this speculation.

Carbohydrate Moieties

The mature TSHR is glycosylated with complex carbohydrates moieties transformed from the high-mannose variety in the rER. This transformation is essential for the trafficking of the receptor to the cell surface. There are six potential N-linked glycosylation sites within the A subunit of a cleaved TSHR, these being asparagine (Asn) residues located at residues 77, 99, 113, 117, 198 and 302 (Figure 3.3). However, despite the cloning of the receptor, it remains difficult to determine the extent of glycosylation in the extracellular domain. Expression of the human TSHR in mouse L cells (Misrahi M *et. al.*, 1994) and Chinese hamster ovarian (CHO) cells (Chazenbalk GD *et. al.*, 1997b) have yielded an estimation of 25 - 27kDa of N-linked glycan. This is more than previous estimates of 10 - 14kDa (Kohn LD *et. al.*, 1995; Graves PN *et. al.*, 1996), yet consistent with the hypothesis that all six sites are glycosylated (Kohn LD *et. al.*, 1995). To date, only Asn302 has been directly shown to be glycosylated. Individual elimination of Asn99, 177, 198 and 302 by mutation studies had no effect on receptor trafficking to the cell surface (Russo D *et. al.*, 1991b). Mutation of Asn77 and Asn113 abolished receptor expression and decreased TSH binding affinity respectively (Russo D *et. al.*, 1991b). However, it is not certain

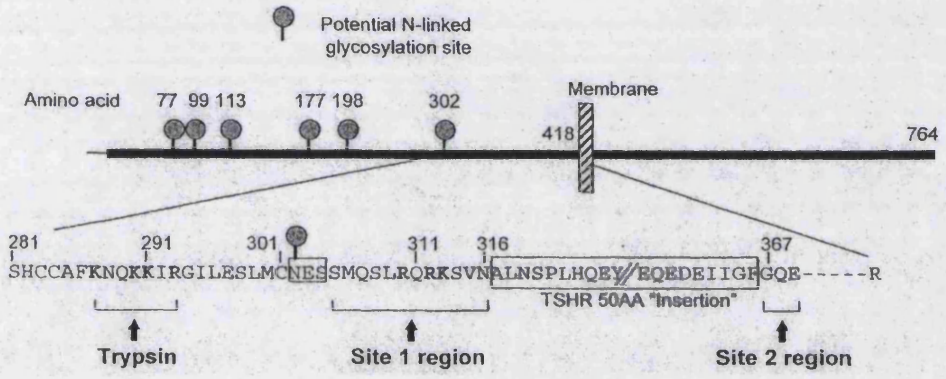


Figure 3.3 **A diagram showing the six potential N-linked glycosylation sites in the human TSHR ectodomain**. (Tanaka K *et. al.*. Thyrotropin receptor cleavage at site 1 does not involve a specific amino acid motif but instead depends on the presence of the unique, 50 amino acid insertion. J Biol Chem; 1998, 273:1959)

if these effects are primarily due to the loss of glycan, or secondary conformation changes as a result of the amino acid substitutions.

Disulfide Bonds

Disulfide bonds are essential for the correct folding of any protein. They are also vital for retaining its tertiary conformation. In the TSHR, they are also involved in the quarternary structure of subunits A and B (Buckland PR *et. al.*, 1982; Kajita Y *et. al.*, 1985). As mentioned above, there are eleven Cys residues in the ectodomain of the TSHR. In addition to these, there are two Cys residues located in the first and second extracellular loop of the transmembrane domain (see Figures 3.1 & 3.4). These two residues are highly conserved amongst other G-protein receptors and are likely to form a disulfide bond. The eleven Cys residues in the ectodomain can be divided into four groups (Figure 3.4):

- Group I consists of a cluster of four Cys residues located between residues 24 - 41 at the N-terminal of the extracellular domain. Furthermore, this cluster is found at the N-terminus of the first of the nine conserved LRRs in the ectodomain. This is a consistent characteristic of glycoproteins with LRRs (Mikol DD *et. al.*, 1990).
- Group II is another characteristic cluster of Cys residues found on the C-terminal end of the LRRs. In the human TSHR, this group contains three Cys residues found within residues 281 - 301.
- Group III is also a cluster of three Cys residues. They are situated between residues 390 - 408 at the extreme C-terminal of the ectodomain. Group III Cys

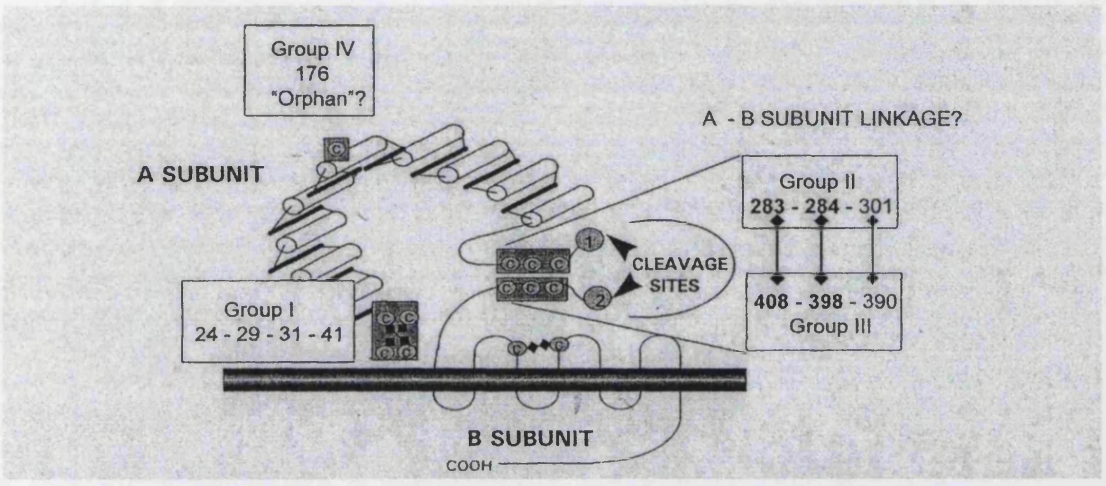


Figure 3.4 **The hypothetical model of the disulfide bonds involved in the quaternary structure of the human TSHR ectodomain**. There are 11 cysteine (C) residues in the ectodomain and they are divided into 4 groups. The diagram shows the hypothetical model, proposed by Rapoport and colleagues, of the possible pairing of cysteine residues to form disulfide bonds. (Rapoport B *et. al.*. The thyrotropin (TSH) - releasing hormone receptor: interaction with TSH and autoantibodies. Endo Rev; 1998, 19:673)

residues are the only ones within the B subunit, other than the two located in the extracellular loops.

- Group IV is an orphan Cys at residue 176 which is within a LRR region (residues 58-277).

Currently, it is not known which of these Cys residues are paired to form bonds. However, indirect data have lead to the postulation that these Cys form 5 disulfide bonds with Cys176 being an orphan. Rapoport and colleagues proposed the following hypothetical model (Figure 3.4; Rapopart B *et. al.*, 1998):

- As groups II and III are located at the approximate cleavage sites of the TSHR, they are likely to be the essential links between subunits A and B after cleavage of the C peptide. In particular, Cys residues 283 and 284 in group II form bonds with residues 408 and 398 in group III respectively. All four of these residues are essential for TSH binding (Kosugi S *et. al.*, 1992a; Nagayama Y & Rapoport B, 1992b).
- In contrast, Cys390 and Cys301 are not vital for TSH binding, but substitution of either reduces receptor affinity (Kosugi S *et. al.*, 1992a; Nagayama Y & Rapoport B, 1992b). This indicates that Cys390 is probably paired with Cys301 in group II.
- Cys residues 24, 29, 31 and 41 in group I are inclined to form bonds with themselves. They are unlikely to pair with residues within the B subunit due to the anticipated three dimension model of the LRR region. Within group I, only Cys41 is essential for the high affinity binding of TSH (Kosugi S *et. al.*, 1992a; Wadsworth HL *et. al.*, 1992). One would therefore expect that its paired Cys residue should be similarly significant. However, mutations of the other three

group I residues have no effect on high affinity TSH binding (Kosugi S *et. al.*, 1992a). As a result, Cys41 remains a paradox.

3.1.1.2 Transmembrane & Cytoplasmic Regions

The transmembrane and cytoplasmic domains of the TSHR are characteristic of any G-protein coupled receptor (Figure 3.1). As mentioned before, the transmembrane domain contains seven segments which span the membrane and which are linked by three extracellular and three cytoplasmic loops. The cytoplasmic domain ends with a C-terminal cytoplasmic tail. There is high homology (85 - 90%) of these two regions to the TSHRs from other species and also to receptors for other human glycoprotein hormones (70 - 75%). However, there is only ~20% homology between the TSHR and non-glycoprotein hormone G-protein coupled receptors. There are conserved proline residues within the transmembrane segments. These are important for the correct insertion of the receptor into the plasma membrane (Brandl CJ & Deber CM, 1986). Although lowest homology occurs within the cytoplasmic tail and the third intracellular loop, even here there are a number of consensus sequences for phosphorylation sites by intracellular regulators such as protein kinase C (PKC).

Stimulation of G-protein coupled receptors, including the TSHR, results in the activation of intracellular signalling cascades initiated by the second messengers cAMP (Figure 3.5) or inositol 1,4,5-triphosphate (IP_3) / diacylglycerol (DAG). The G-proteins involved in both systems are a heterotrimer consisting of three polypeptide chains: α, β and γ. The α chain binds and hydrolyses guanine 5'-triphosphate (GTP) whilst the β and γ chains form a tight complex which anchors the G-protein to the

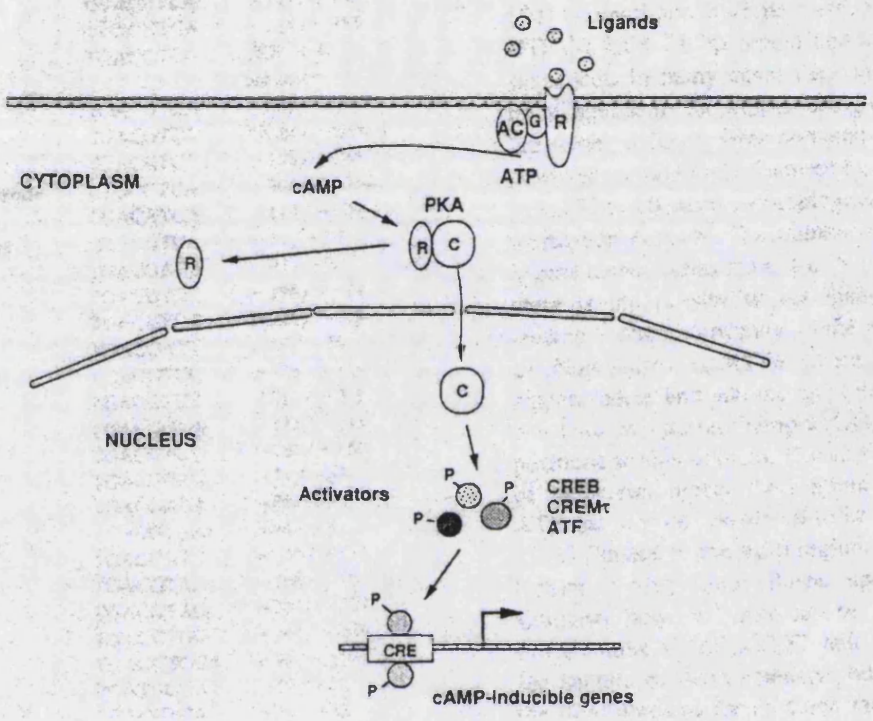


Figure 3.5 T**he intracellular second messenger cAMP and its signal transduction cascade**. The activation of the G-protein (G) coupled receptor (R) by ligands stimulates adenylate cyclase (AC) and results in the increase in intracellular cAMP. cAMP then in turn activates, with the dissociation of the regulatory subunits (R), the catalytic subunit (C) of protein kinase A (PKA). The catalytic subunit then migrates into the nucleus and, phosphorylates (P) and activates transcription factors such as CREB and CREMτ within the nucleus. These activated transcription factors then bind to cAMP-response element (CRE) in the promoter region of the target cAMP-inducible gene to initiate gene transcription. (Foulkes NS & Sassone-Corsi P. Transcription factors coupled to the cAMP-signalling pathway. Biochim Biophys Acta; 1996, 1288:F101)

cytoplasmic side of the plasma membrane. Although the α-subunit interacts with a catalytic subunit such as AC, the βγ complexes also play a role in signal transduction. They regulate a large number of enzymes involved in cellular signalling transduction pathways including AC, phospholipase Cβ (PLCβ) and several kinases (Birnbaumer L, 1992; Clapham DE & Neer EJ, 1993; Sternweis PC, 1994). An inactive G-protein exists as the heterotrimer with a guanine 5′-diphosphate (GDP) bound to the α-subunit. The following outlines the mechanism by which TSH stimulates the activation of AC resulting in an increase of intracellular cAMP (Figure 3.6):

i) TSH binding to the TSHR induces a slight conformational change within the receptor which exposes the G-protein binding site.

ii) This allows the association of the G-protein with the TSHR and the displacement of GDP by GTP.

iii) Once GTP is bound, the α-subunit dissociates from the βγ complex and activates AC. This then dephosphorylates numerous ATP molecules into cAMP, the intracellular second messenger.

iv) Finally, hydrolysis of the GTP by the α-subunit itself returns the G protein to its inactive GDP-bound conformation. The α-subunit then dissociates from AC and re-associates with the βγ complex, and returns to the G-protein pool in the plasma membrane.

v) The cycle then repeats until the dissociation of TSH from the receptor.

Numerous receptors belong to the G-protein coupled receptor family, but the hormonally specific cellular effects are exerted by the recruitment of different G-proteins. The latter are divided into five main G-proteins families: G_s, G_i, G_q, G_o and G_{12} (Simon MI *et. al.*, 1991; Hepler JR & Gilman AG, 1992). These have been

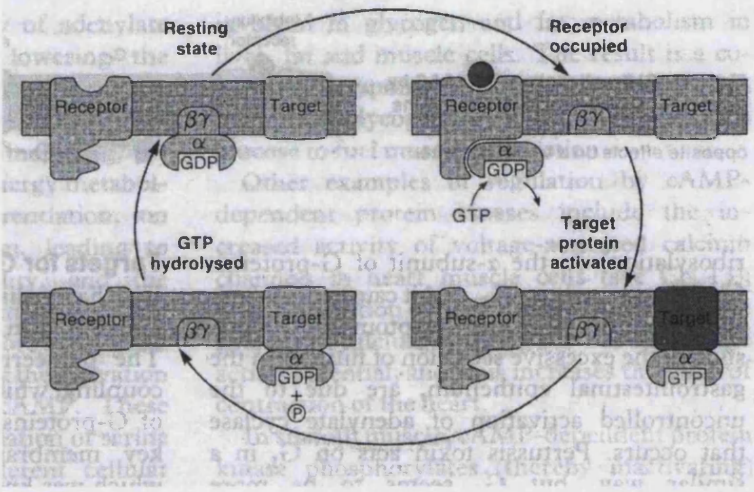


Figure 3.6 **The activation and deactivation mechanisms of the G-protein**. The G-protein consists of 3 subunits: α, β and γ. When resting, the βγ complex anchors the G-protein to the cytoplasmic side of the membrane and the catalytic α-subunit is bound to GDP. When activated, by the binding of ligands to the receptor, GDP is displaced with GTP which then allows the dissociation of the α-subunit from the βγ complex. The GTP-bound α-subunit then activates adenylate cyclase, the Target shown here. Deactivation occurs with the hydrolysis of the GTP to GDP (+ phosphate) by the α-subunit itself and its re-association with the βγ complex. (Rang HP & Dale MM. Pharmacology, 2nd Ed. Churchill Livingstone; 1991, p37)

defined by homologies of the α chains, which are structurally and functionally distinctive, and their preferred effector targets such as AC and phospholipase C (PLC; Simon MI *et. al.*, 1991). Some receptors, including the human TSHR, have been found to associate with several G-proteins and thereby elicit an array of cellular effects. It has long been recognised that TSH binding to the TSHR stimulates both G_s- and G_q-proteins (Allgeier A *et. al.*, 1994) resulting in the activation of AC and PLC respectively (Van Sande J *et. al.*, 1990). These effects are, however, TSH-dose dependent with G_q mediated activation of PLC only occurring with the higher TSH doses. Laugwitz and colleagues have however reported that the human TSHR is capable of interacting with all five G-protein families and identified α subunits of G-proteins $G_{s\ short}$, $G_{s\ long}$, G_{i1}, G_{i2}, G_{i3}, G_o, G_q, G_{11}, G_{12} and G_{13} in the membrane of the human thyroid gland (Laugwitz K-L *et. al.*, 1996). Thus, it appears that the activated TSHR can couple to not only G_s and $G_{q/11}$, but also G_i, G_{12} and several other subtypes. Furthermore, similar results were obtained when TSAbs from Graves' patients sera were substituted for TSH. Although all five families of G-proteins have been found capable of associating with the TSHR, the G_s-mediated signalling cascade is recognised to be the dominant pathway, leading to the increase in intracellular cAMP (Vassart G & Dumont JE, 1992). The coupling of activated TSHR to G_i-proteins was an unexpected finding, since G_i-proteins inhibit AC activity and thus it opposes the effect of G_s-proteins. However, it has been speculated that the inhibitory effects of the G_i families may be masked by dominant stimulatory effects. A similar situation was described for the receptors for the parathyroid hormone (Akiko IK *et. al.*, 1995). G_o-proteins, which are usually associated with inhibition of the voltage-gated Ca^{++} channels within the neuronal system (Kleuss C *et. al.*, 1991; Hescheler J & Schultz G, 1993), have also been suggested to play a role in the secretory processes of the

thyrocytes (Laugwitz K-L *et. al.*, 1996). Finally, although not much is known about the more recently discovered G-proteins G_{12} and G_{13}, Laugwitz and colleagues suggested that their activation may elicit growth and/or differentiation of thyrocytes (Laugwitz K-L *et. al.*, 1996).

As mentioned above, cAMP initiated signal transduction is by far the most important intracellular signalling cascade which is activated by the TSHR. Mutagenesis studies of the receptor have identified several key regions within the transmembrane and cytoplasmic domain for this. Amino acids residues 528-537 in the second cytoplasmic loop (Chazenbalk GD *et. al.*, 1990a, Kosugi S *et. al.*, 1994), residues 617-625 in the carboxyl terminus of the third cytoplasmic loop and residues 683-708 in the terminal of the cytoplasmic tail (Chazenbalk GD *et. al.*, 1990a) are especially important. Furthermore, alanine 623 appeared to be essential for cAMP initiated signal transduction but not the pathway initiated by IP_3/DAG (Kosugi S *et. al.*, 1992c).

The cAMP initiated signalling cascade results in the regulation of gene transcription (Figure 3.5). The increase in intracellular cAMP activates a specific protein kinase, namely protein kinase A (PKA). In its resting state PKA contains 2 catalytic subunits and one homodimeric regulatory subunit. cAMP binding leads to dissociation of the regulatory subunit thus activating the enzyme and enabling it to initiate a phosphorylation cascade. The substrates may be other protein kinases, glucose and ultimately transcription factors. Phosphorylated transcription factors such as the cAMP-response element binding protein (CREB) are translocated to the nucleus where they bind to short palindromic sequences in promoter regions of target genes

referred to as the cAMP-response element (CRE). They thereby activate or inhibit gene transcription.

3.1.2 Genomic Structure

The human TSHR gene is located on chromosome 14q31 (Libert F *et. al.*, 1990; Rousseau-Merck MF *et. al.*, 1990). Its translated region is encoded by ten exons which span more than 60kb (Gross B *et. al.*, 1991). The extracellular domain of the receptor is encoded by the first nine and a fraction of the larger tenth exon. The rest of the tenth exon encodes the remaining receptor, namely the transmembrane and cytoplasmic regions. This tenth exon is relatively conserved amongst the G-protein receptor family. Exons two to eight each correspond to one of the nine LRRs mentioned previously. This concurs with the molecular structure of the extracellular domain of the TSHR described above. The unconserved regions at the extreme ends of the TSHR ectodomain are coded by exons one and nine. These have low homology to the other glycoprotein hormone receptors, whereas the mid region of the ectodomain, corresponding to the LRRs, is highly conserved.

3.1.2.1 TSHR gene expression

Gene transcription takes place within the cell nucleus and is outlined below:

i) Firstly, DNA containing the relevant genes is transcribed into RNA by the enzyme RNA polymerase.

ii) This is followed by the maturation of RNA into mRNA. This procedure includes the addition of the 5′ cap and the poly(A) tail to the 5′ and 3′ ends respectively.

iii) The final stage is gene splicing in which introns are removed from the transcribed mRNA. This is performed by ribonucleoproteins referred to as spliceosomes.

iv) The mRNA is then transported to the rER in the cell cytoplasm to be synthesised into proteins.

3.1.2.2 Alternative splicing and tissue distribution

TSHR mRNA expression has been studied using techniques such as Northern blot analysis, immunohistochemistry, reverse transcription (or RT-PCR) and polymerase chain reaction (PCR). Using Northern blot analysis, multiple TSHR mRNA transcripts have been identified of which the majority are 4.6 and 3.9kb in length. These are likely to encode the full length receptor. However, smaller transcripts of 1.8 and 1.2kb or shorter have also been detected. At least 5 shorter TSHR cDNAs have been isolated and they are liable to be the result of alternate splicing (e.g. Graves PN *et. al.*, 1992; Takeshita A *et. al.*, 1994). These variants encode a variety of smaller TSHR extracellular domains without the transmembrane and cytoplasmic regions. This suggests the possibility of soluble and functional autoantigens that can be secreted into the periphery.

The tissue distribution of TSHR mRNA has also been studied using the above techniques. The TSHR gene is expressed in both thyroid tissue and several other extrathyroidal tissues (Endo T *et. al.*, 1993). For example, it is also expressed in adipocytes (Roselli-Rehfuss L *et. al.*, 1992; Endo T *et. al.*, 1993), which is to be expected since TSH stimulates lipolysis in isolated fat cells. Other extrathyroidal tissues found to express TSHR mRNA include skeletal muscle, cardiac muscle,

lymphocytes, fibroblasts and retroorbital tissues. However, caution must be exercised regarding the significance of these findings since conflicting results have been reported by different research groups. The detection of human TSHR mRNA in retroorbital tissues has generated particular interest since it may provide an explanation for the Graves' ophthalmopathy described in Section 2.3.2. Numerous research groups have reported supporting observations. These include Feliciello A *et. al.* (1993) who reported the presence of full length human TSHR mRNA in both healthy and Graves' retroorbital tissue using PCR, Burch HB *et. al.* (1994) and Heufelder AE (1995) who both detected human TSHR protein in retroorbital fibroblasts by immunofluorescent staining with a polyclonal antibody and Perros and Kendall-Taylor (1994) who demonstrated radiolabelled TSH binding to retroocular connective tissues. Furthermore, mRNA of TSHR gene splice variants were also encountered in extrathyroidal sites including peripheral blood and skeletal and extraocular muscles (Nakashima M *et. al.*, 1994; Paschke R *et. al.*, 1994). The existence of TSHR gene variant mRNAs raises the possibility of a soluble form of the TSHR ectodomain being secreted. The detection of this within orbital tissues may provide new insights into the aetiology of Graves' ophthalmopathy. However, despite demonstration of the presence of these variant mRNAs in extrathyroidal tissues, whether any of them are translated into functional receptors remains questionable.

3.1.2.3 Regulation of gene expression

The transcription of DNA into mRNA is highly controlled to ensure that genes are not activated and transcribed randomly. The transcription of the TSHR gene is regulated by basic promoter elements and their corresponding transcription factors. The

promoter region, also named the 5'-flanking region, is located upstream from the transcription initiation site towards the 5' end (Figure 3.7). The promoter region of both the human and rat TSHR genes has been isolated and studied (Gross B *et. al.*, 1991; Ikuyama S *et. al.*, 1992a). Within these segments are multiple transcription start sites, promoter sequences, a CRE and binding sites for several transcription factors. These include AP1, AP2 and thyroid-specific transcription factor-1 (TTF-1). However, they lack other common features such as a TATA box, CCAAT site or the GC box motif.

FRTL-5 cells have been used extensively to study the functional properties of the promoter region regulating the TSHR gene. A minimum promoter region consisting of the first 200bp is required for basal transcriptional activity, tissue specificity and autoregulation by TSH and insulin (Ikuyama S *et. al.*, 1992a & b). Within the minimum promoter region is one CRE (-193 to -131bp) and two transcription factor TTF-1 binding sites (-180 to -175, -134 to -116bp). The CRE acts as an essential enhancer, a positive regulatory sequence located a distant away from the transcription start site, to increase transcription. Yet, the flanking sequences of the CRE act to suppress its activity (Ikuyama S *et. al.*, 1992a & b; Shimura H *et. al.*, 1993). The CRE, however, is the most important binding site involved in the cAMP signal cascade. A CREB which is activated by the phosphorylation of a serine residue at position 133 by PKA following the increase in cAMP (see Section 3.1.1.2), becomes a powerful transcription factor which binds to the CRE (Gonzalez GA & Montminy MR, 1989; De Groot RP *et. al.*, 1993). Binding of both TTF-1 and CREB to the TTF-1 and CRE sites respectively is required for the full expression of the receptor gene in

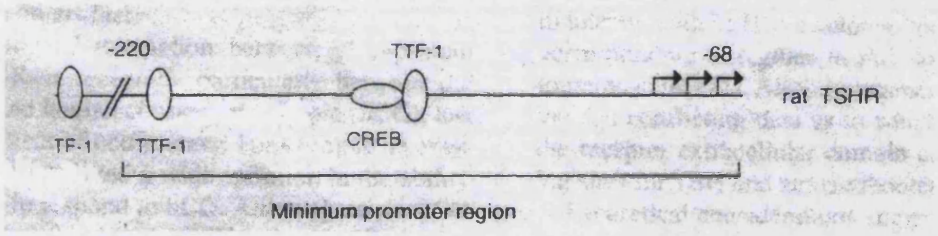


Figure 3.7 **A schematic representation of the promoter region of the rat TSHR**. Although the rat TSHR promoter region is shown, the human TSHR promoter has similar structures including a cAMP-response element (CRE) and binding sites for certain transcription factors such as AP1 and thyroid-specific factor-1 (TTF-1). (Nagataki S & Nagayama Y. Molecular Biology of the Thyroid Stimulating Hormone Receptor. In Falk SA, ed. Thyroid Disease: Endocrinology, Surgery, Nuclear Medicine, and Radiotherapy, 2nd Ed. Philadelphia: Lippincott Raven Publishers; 1997, p213)

thyroid cells (Civitareale D *et. al.*, 1993; Shimura H *et. al.*, 1994). However, binding of TTF-1 to the upstream TTF-1 binding site at -180 to -175 and another located upstream of the minimum promoter region (at -881 to -866bp) determines thyroid specificity and TSH/cAMP-mediated regulation of the receptor (Civitareale D *et. al.*, 1993; Shimura H *et. al.*, 1994; Ohmori M *et. al.*, 1995). The TTF-1 binding site located at -143 to -116bp is not directly involved in TSHR promoter activities, but it is required for the synergistic action of CREB and TTF-1 (Saiardi A *et. al.*, 1995).

TSH plays a role in the regulation of TSHR mRNA production. It stimulates the phosphorylation and binding of TTF-1 to its binding site thus increasing TSHR mRNA levels transiently. However, TSH is also able to down regulate TSHR gene expression. TSH induces the activation of the inducible cAMP early repressor (ICER), an isoform of the CRE modulator, which binds to the CRE to repress its expression (Lalli E & Sassone-Corsi P, 1995). Furthermore, it has been reported that thyroid hormones also have an inhibitory effect on TSHR promoter activity (Saiardi A *et. al.*, 1994). It should, however, be noted that as the above observations were generated from *in vitro* studies using FRTL-5 cells, they may not represent the *in vivo* situation in humans.

3.2 Thyroid Stimulating Hormone

TSH is a member of the glycoprotein hormone family which includes LH, FSH and hCG. TSH is one of two hormones involved in thyroid hormone regulation. The mechanism by which TSH and TRH regulate triiodothyronine secretion was discussed in Section 1.3.3.

3.2.1 Molecular and Genomic Structures

TSH is a glycoprotein with a molecular weight of ~ 30kD. It is synthesised and secreted by thyrotrophs located in the anterior pituitary. Like all other glycoprotein hormones in the family (LH, FSH and hCG), TSH consists of two non-covalently linked subunits, referred to as the α and β subunits respectively (Pierce JG & Parsons TF, 1981; Pierce JG, 1986). The α-chain is common between the four glycoprotein hormones within a specific species, hence it is also named the human glycoprotein hormone α-subunit (hGPHα) gene. However, their respective β-subunits are hormonally specific. The β-subunit chains are associated with specificity, receptor binding and function for each individual hormone. Nonetheless, both subunits are required for bioactivity, the β-subunit on its own being non-functional.

3.2.1.1 The α-subunit

The α-subunit of human TSH contains an apoprotein core of 92 amino acid residues. This subunit also contains 10 cystine residues which are linked into pairs, by disulfide bonds, with the corresponding cystine residues in the β-chain. In addition, covalently linked carbohydrate chains are also found. These sugar chains are linked to asparagine residues (N-linked) and they consist of a variety of sugar residues including mannose, fucose, N-acetylglucoasmine, galactose, N-acetylgalactose and sialic acid. Furthermore, sulphate groups, which terminate sugar chains, are found on the TSH subunits. They are similarly abundant on the subunits of LH but less so on FSH and are absent from hCG subunits (Green ED *et. al.*, 1984; Green ED & Baenziger JU, 1988a & b).

The genes coding for the α subunit of various species have been identified. They are a similar size and contain four exons and three introns. The gene encoding the human α-subuit common to all the glycoprotein hormones is located on chromosome 6. The gene is ~ 9.4kb in size. Intron 1 is 6.4kb and located between the 5′ untranslated region of exons 1 and 2. Intron 2 is 1.7kb in length and found to interrupt codon 6 within the α-subunit coding regions. Lastly, intron 3 is only 0.4kb in size and situated between codons 67 and 68.

Upstream of the transcription start site lies the promoter region of the gene (see Result Section 7 Figure 7.4; Delegean AM *et. al.*, 1987; Deutsch PJ *et. al.*, 1987; Silver BJ *et. al.*, 1987; Jameson JL *et. al.*, 1988). It has been shown that the CRE of the hGPHα gene lies within the initial 180 base pairs of the 5′ flanking promoter region. Within this region, there are three separate domains important for the modulation of basal and cAMP stimulated expression. These are: an upstream regulatory element (URE), CRE and a downstream α promoter region that includes a CCAAT motif. The URE is a unique motif located at -178 to -156 upstream of CRE. It is a distinct element. It has no structural similarity to any other elements and regulates the expression of basal activity. A deletion of this region causes ~80% decrease in transcription activity but preserves cAMP responsiveness. Activation of this element increases basal activity 5-fold and stablizes the interaction of binding factors to these elements and motifs. CRE is located at -146 to -112 and consists of two repeated 18bp elements each with the 8bp palindromic core. It is tissue specific in expression but non-specific in response. This CRE showed the characteristics properties of the others, namely:

i) its function is orientation and relatively distance independent,

ii) it is a non-essential promoter, and

iii) it confers responsiveness for heterologous promoters.

However, it also has a number of unique characteristics:

i) it is a duplicated structure where one copy is sufficient for activation but two copies gives full activity, increasing basal expression by another 5-fold,

ii) not only is the 8bp palindromic core important but the entire 18bp are required for function,

iii) it is bifunctional as it is required for tissue specific activity of URE but also independently contributes to tissue specific expression.

Finally, the α-promoter region is located at -99 to -72 and has a consensus CCAAT motif on both strands. It has no fully defined function as yet but may respond to a family of different factors that interacts with the CCAAT motif. This region on its own is not enough to activate transcription but allows CRE to function more efficiently. In conclusion, all three promoter regions are required for transcription activation. Basal expression is regulated by URE where activation increases activity 5-fold, however, when synergising with the two copies of the CRE, the activity increases by another 5-fold. And finally when stimulated by cAMP, there is another 10-fold increase showing that these domains function co-operatively in response to cAMP and in the activation of gene transcription.

3.2.1.2 The β-subunit

While the α-subunit is common to all the glycoprotein hormones, the β-chains are hormonally specific. Although they are distinctive, they all have a similar three dimensional structure as they need to combine with a common α-chain to form a functional hormone. As with the α-subunit, the human TSH β-subunit contains a 118 amino acid residue apoprotein core and 12 cystine residues which are in disulfide linkages. There are also covalently linked carbohydrate chains like those described in the α-subunit (see Section 3.2.1.1).

Whereas the common α-chain is encoded by a single gene on chromosome 6, in contrast, the respective glycoprotein hormone β-subunits are encoded by different genes located on different chromosomes (Dracopoli NC *et. al.*, 1986). The β-subunit gene of a number of species has been identified and they share a similar genomic organisation. The human TSH β-chain is found on chromosome 1 and contains three exons. The first exon is 37bp in size but it is untranslated. The second exon encodes the leader peptide and the first 34 amino acids of the mature β-chain. The remainder of the subunit is encoded by the third exon.

3.2.2 Regulation of TSH Synthesis

The process of gene expression, in which proteins are synthesised from DNA, has been outlined above in Section 3.1.2.1. The transcription and translation of both the common hGPHα- and TSH-β-subunit genes are required for the synthesis of bioactive TSH. TSH synthesis is regulated pretranslationally by a number of factors. These are described below and summarised in Figure 3.8.

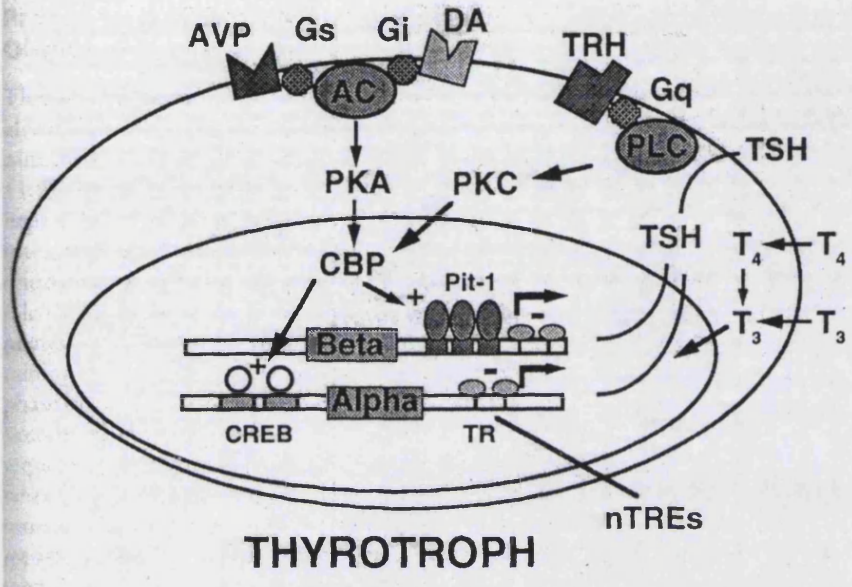


Figure 3.8 **The pretranslational regulation of TSH synthesis**. A diagrammatic presentation of the factors regulating pretranslationally the synthesis of the TSH subunits in thyrotrophs. AC, adenylate cyclase; AVP, arginine vasopressin; DA, dopamine; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; CBP; cAMP binding protein; CREB, cAMP-response element binding protein; TRH, thyrotropin-releasing hormone; TR, thyroid receptor; nTRE, negative thyroid hormone response element. (Cohen RN, Weintraub BD & Wondisford FE. Thyrotropin – chemistry and biosynthesis of thyrotropin. In Braverman LE & Utiger RD. Werner & Ingbar's The Thyroid – A Fundamental and Clinical Text, 8th Ed. Philadelphia: Lippincott Williams & Wilkins; 2000, p209)

Thyrotropin-releasing hormone

TRH stimulates the release of TSH from the anterior pituitary which then in turn stimulates thyroid hormone release from the thyroid gland (see Section 1.3.3). Consequently, TRH is the dominant positive regulator of TSH synthesis, increasing the transcription of TSH-subunit genes via the PLC signalling cascade. TRH activates PLC, via a G-protein coupled receptor, which then hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to DAG and IP_3. DAG in turns activates PKC which phosphorylates enzymes leading to the activation and binding of transcription factors responsible for gene transcription. In the TSH-β-subunit gene, there are two cis-elements which mediate the TRH-induced transcription and they are located between -128 to -91bp and -28 to +8bp (Weintraub BD *et. al.*, 1989). The pituitary-specific transcription factor 1 (Pit-1) binds to the upstream cis-element (Nelson C *et. al.*, 1988) and may be involved by enhancing TRH responsiveness of the TSH-β gene (Steinfelder HJ *et. al.*, 1992b). The other product of the hydrolyses of PIP_2 is Ca^{++}. Currently, the role of the Ca^{++} in the regulation of TSH-subunit gene expression is not clear.

cAMP

Intracellular cAMP also increases the expression of both TSH α- and β-subunit genes. Arginine vasopressin (AVP), from the hypothalamus / posterior pituitary has been reported to stimulate TSH release from thyrotrophs (Lumpkin MD *et. al.*, 1987) via a cAMP signalling cascade such as that described in Section 3.1.1.2 (Figure 3.5). For the common α-gene, transcription is via two CREs located in the 5′ promoter region (Section 3.2.1.1). Although cAMP mediation of gene expression of the β-subunit gene

is located predominantly between -128 to -28bp of the 5′ flanking region (Weintraub BD *et. al.*, 1989), there is no recognisable CRE within this region (Hoeffler JP *et. al.*, 1988). Moreover, Pit-1, once phosphorylated by PKC, also mediates cAMP induction of TSH-subunit gene expression (Steinfelder HJ *et. al.*, 1992a & b).

Thyroid hormones

As described in Section 1.3.3, thyroid hormone synthesis and release is regulated by both TSH and TRH. In addition, T_3 and T_4 in the circulation then form a negative feedback system acting on both the hypothalamus and the anterior pituitary. Thus, thyroid hormones are major regulators of TSH synthesis. They directly inhibit the synthesis of TSH at the anterior pituitary (Gurr JA & Kourides IA, 1985; Shupnik MA *et. al.*, 1985) and indirectly via a decrease in TRH secretion (Segerson TP *et. al.*, 1987; Taylor T *et. al.*, 1990). Shupnik and colleagues (1985) have shown the thyroid hormones to negatively regulate the transcription of the common α- and also the TSH-β-subunit genes, resulting in a decrease in their respective mRNA levels. The rates and magnitudes of decrease, however, differed between the two subunits. The β-subunit was suppressed more rapidly than the α-subunit. A 61% decrease after 30min and a maximum of >95% after 4hr was observed for the β-subunit mRNA synthesis, whereas, only a reduction of 28% and a maximal of 70% was obtained for the common α-subunit. The inhibitory nuclear action of the thyroid hormones, via nuclear receptors and nTREs, has been previously described in Section 1.3.6 and in this case, the inhibition of TSH-subunit genes transcription, adheres to the same process. It is via nuclear thyroid hormone receptor, termed c-erbA, and cis-acting elements (Sap J *et. al.*, 1986; Weinberger C *et. al.*, 1986). The thyroid hormone-receptor complex is

internalised and then binds to cis-acting elements located near the transcription start site of the subunit genes (Burnside J *et. al.*, 1989; Chatterjee VKK *et. al.*, 1989; Wondisford FE *et. al.*, 1989; Wood WM *et. al.*, 1989). Located within cis-acting elements are nTREs, named thyroid hormone inhibitory elements. In the hGPHα gene, the thyroid hormone inhibitory element is found in the region between -100 to +4bp (Chatterjee VKK *et. al.*, 1989). However, recently another site, located further downstream of the 5′ flanking region, has been implicated in the inhibition of the common α-subunit gene by thyroid hormones (Pennathur S *et. al.*, 1993). The cis-acting element of the β-subunit gene has been localised to between +3 to +37bp within exon 1 (Wondisford FE *et. al.*, 1989). This cis-acting element contains two thyroid hormone inhibitory sites, both of which are necessary for inhibition. The binding of the hormone-receptor complex to cis-acting elements is thought to interrupt transcription by interfering with the transcription initiation complex. As a result, there is an inhibition of gene expression.

Steroid hormones and Dopamine

Regulation of TSH secretion and gene expression by steroid hormones, such as estradiol and testosterone has also been studied. The consensus is that although they are not major regulators of TSH secretion, they may play a repressive role under pathological conditions. In euthyroid animals and men, estrogen administration did not have any effect on the level of TSH induced by TRH (Spitz IM *et. al.*, 1983; Ahlquist JAO *et. al.*, 1987). However, in hypothyroid rats, a pharmacological dose of estrogen enhanced the suppression of TSH-gene expression by replacement thyroid

hormones (Ahlquist JAO *et. al.*, 1987). Furthermore, in animals, testosterone appeared to have a similar effect to estrogen (Glass CK *et. al.*, 1988).

Finally, dopamine may also be a minor negative regulator of TSH secretion and synthesis. It had been reported that dopamine rapidly decreased basal and TRH-induced TSH release in man by ~50% (Cooper DS *et. al.*, 1983). It may inhibit the cAMP initiated signalling cascade.

3.3 TSHR Autoantibody

In 1956, Adam and Purves (1956) discovered a thyroid stimulating activity distinct from TSH, in the sera of Graves' patients, using a bioassay for TSH. They termed this stimulator long-acting thyroid stimulator (LATS). Extensive further investigation determined that LATS was a unique, thyroid stimulating, autoantibody which is able to mimic the effects of TSH (e.g. Dorrington KJ & Munro DS, 1966; McKenzie JM, 1968; Rees Smith B, 1981). TSAbs compete with TSH for binding to the TSHR and subsequently activating the cAMP initiated signalling cascade. This was demonstrated by the inhibition of ^{125}I-labelled TSH binding to TSHRs by TSAb in a dose-dependent manner (Manley SW *et. al.*, 1974; Smith BR & Hall R, 1974; Mehdi SQ & Nussey SS, 1975). As mentioned in section 2.1, in addition to the TSAb in a Graves' patient, TSHR blocking antibodies (TBAb) can occur. These will also compete with TSH for its binding to TSHRs, but they do not subsequently stimulate. TSAb and TBAb may also occur simultaneously and the net result will then depend upon their relative concentrations and potencies. In GD patients, TSAbs are reasoned to be the

predominant antibodies and thus prevail. The specific trigger resulting in the emergence of either of these TSHR autoantibodies is uncertain.

3.3.1 Molecular structure

The production of TSAb by B- and T-cells has been discussed in Section 2.1. All antibodies have a common structure containing two identical light chains and two identical heavy chains. The light chains are always ~ 24kd, however the heavy chains are either ~ 55 or 70kd in size depending on the specific antibody isotype (see below). Each light chain is attached to one heavy chain and the heavy chains are then linked by disulfide bonds to form a characteristic "Y" form (Figure 3.9). There are five classes (or isotypes) of antibodies: IgA, IgD, IgE, IgG and IgM. Isotypes IgA and IgG can be further subdivided into subclasses e.g. IgA1, IgA2 and IgG1-4. Each isotype and its subtypes are identified by the homology of their heavy chains which differ between the classes (reviewed by Abbas AK *et. al.*, Chapter 3, 1997).

Light chain structure

All light chains belong to two classes, these being either κ or λ. A light chain has the conformation of a straight chain with two projecting loops termed immunoglobulin (Ig) domains. The two Ig domains, formed by the folding of the polypeptide chain, consist of a variable (V) and a constant (C) domain located at the amino and carboxyl terminal respectively (Figure 3.9). These domains are ~ 110 amino acids long and labelled V_L and C_L. The C domains of the κ and λ light chains differ in their amino

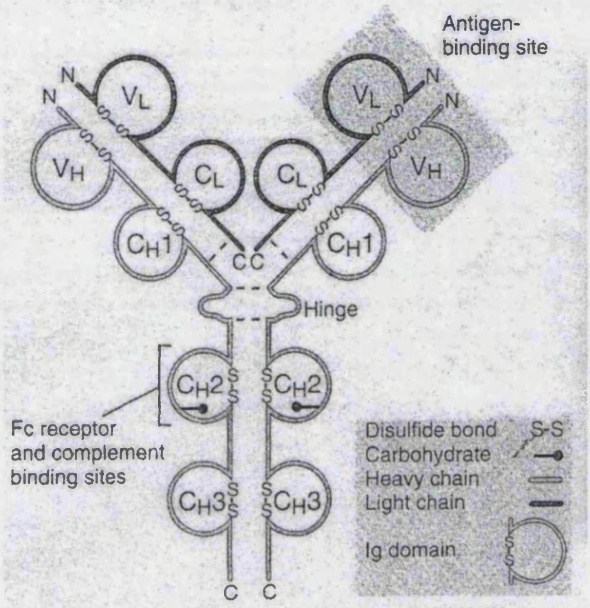


Figure 3.9 **A schematic diagram of the basic structure of an antibody molecule**. The basic "Y" form of an antibody is formed by the association of two light (L) and two heavy (H) chains. Each light chain is attached to one heavy chain and the heavy chains are linked by disulfide bonds. (Abbas AK, Lichtman AH & Pober JS. Cellular and Molecular Immunology, 4th Ed. W.B. Saunders Company; 2000, p 43)

acid sequences. However, the amino acid sequence of each member of the same light chain class is identical. Yet, despite the difference in amino acid sequence between C_κ and C_λ, they are structurally homologous to each other. In the light chain, the V_L domain has the most variable amino acid sequence, particularly since, within this domain, there are three hypervariable regions. These are also termed complementarity-determining regions (CDRs) and each is ~10 amino acids long. These hypervariable regions are named CDR1-3 of which CDR3 is the most variable. The sequences adjacent to the CDRs, termed framework regions, determine the folding of the V region. These framework regions also differ between the two light chain classes. However, within these framework regions, there are also conserved segments such as an internal disulfide loop, of ~90 amino acid residues, which is found in all V_L domains. When the V domain of either light chain class is folded into an Ig domain, the CDRs are located at the surface of the projecting loop.

Heavy chain structure

As for the light chains, the heavy chains also contain V and C domains, each being ~100 amino acids long. The amino terminal of the heavy chain contains a variable domain (V_H) and the remaining chain forms the C domain (C_H, Figure 3.9). Again, the V_H region shows the greatest sequence variability including three CDRs, 1-3, of which CDR3 is the most diverse. As mentioned, the rest of the heavy chain is the C region. However, unlike the light chains, this domain differs between antibody isotypes but is consistent amongst antibodies of same isotype. In IgM and IgE C regions fold to form 4 Ig domains whereas in IgG, IgA and IgD, which have shorter C regions, there are only 3 Ig domains. These Ig domains are labelled C_H1-3 or 1-4 from the N- to C-

terminal. There is also a hinge region of 10 - 60 amino acid residues within the heavy chains. The hinge, located between domains C_H1 and C_H2, allows the antibody to conform to the 'Y' shape. Heavy chains of each isotype may be expressed in two forms: secreted or cell membrane bound. The difference between these two is found at the carboxyl terminal end of the last C_H domain. The cell membrane form is only found on the cell membrane of B-lymphocytes. At its carboxyl terminal, there is a sequence of amino acids similar to a transmembrane protein. This includes ~26 uncharged hydrophobic side chains followed by a variable number of charged amino acid residues. In contrast, in the secreted form of heavy chains, the carboxyl terminal ends with charged and hydrophilic amino acid residues. Furthermore, all heavy chains are N-glycosylated at asparagine residues. However, the locations for the attachment of these N-linked oliogosaccharide groups, vary between each antibody isotype.

Association of light and heavy chains

The basic "Y" shape of an antibody is formed by specific associations between the light and heavy chains. As mentioned, an antibody consists of two light and heavy chains each and each light chain is linked to the N-terminal angled branches of the heavy chains whereas the C-terminus of the 2 heavy chains are paired (Figure 3.9). These associations are the result of both covalent disulfide bonds and non-covalent bonds. A disulfide bond between cysteine residues, located at the carboxyl terminus of the light chain and the C_H1 domain, links each light chain to a heavy chain. Consequently, the V domains (V_L and V_H) and C_L and C_H1 are paired. The pairing of the V_L and V_H domains forms an antigen binding site; this is termed the antigen-binding fragment, which is referred to as Fab. Thus each antibody contains 2 antigen

binding sites. The hinge region in each heavy chain provides flexibility to allow the antibody to orient its antigen binding sites so that two antigens maybe bound at the same time. In addition, there are also non-covalent hydrophobic interactions between these paired Ig domains. Similarly, the pairing of the heavy chains also involves both disulfide bonds and non-covalent interactions. Using IgG as an example, two disulfide bonds are found at the region carboxyl to the hinge and there are extensive non-covalent associations between the two C_H3 domains. Consequently, the C_H2 and C_H3 domains on each heavy chain are paired. Furthermore, the N- linked carbohydrates found on each heavy chain may also interact with each other, thus enhancing interchain associations.

3.3.2 Genomic Structure

Antibodies are produced as the result of immune responses against specific antigens. It is estimated that there are 10^7 – 10^9 structurally specific antibodies in every individual. Each antibody has a unique amino acid sequence in its antigen binding site as a result of the hypervariable regions in the V domains. Consequently, every antibody is specific to only one antigen. Diversity and specificity is achieved by alternative splicing of the locus of the heavy and both κ and λ light chains. The human heavy chain locus is located on chromosome 14 whereas the κ and λ light chain loci are found on chromosome 2 and 22 respectively (Figure 3.10; Abbas AK *et. al.*, Chapter 4, 1997).

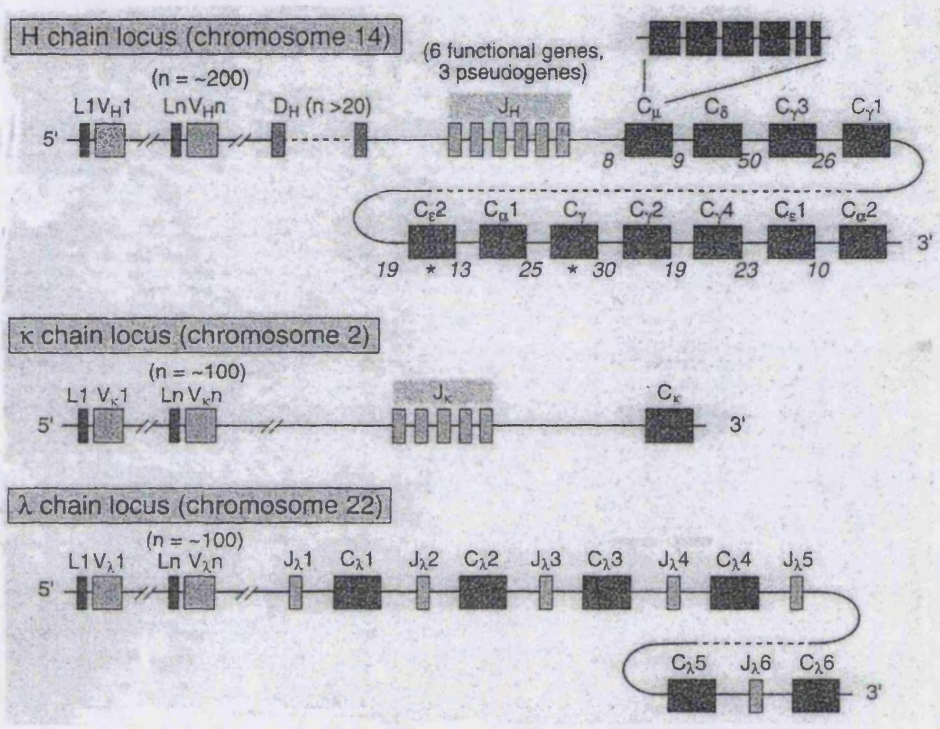


Figure 3.10 **The germline organization of the human heavy (H) and light (κ and λ) chains loci**. Each chain locus consists of numerous gene segments such as the variable (V), constant (C), diversity (D) and joining (J) exons. (Abbas AK, Lichtman AH & Pober JS. Cellular and Molecular Immunology, 4th Ed. W.B. Saunders Company; 2000, p 132)

Each locus contains multiple genes which encode the V and C domains which are separated by non-coding DNA. The V region exons are located at the 5′ end of the locus. Each of these is ~ 300bp long and are disjoined by introns of varying lengths. Furthermore, at ~ 90bp 5′ of each V region exon there is another smaller exon, 60 - 90bp long, that encodes the translation initiation signal and is responsible for the 20 - 30 terminal residues of the translated protein. These residues are hydrophobic in nature and function as a signal sequence important in the synthesis and maturation of the antibodies. In the human Ig genes, there are 100 - 200 V region exons in each heavy or light chain locus. The C region exons are located far downstream of the V exons, at the 3′ end of the locus. The human κ and λ light chain loci contain 1 and 3 – 6 C exons respectively. On the heavy chain locus, the different C region genes for the different isotypes of antibodies are arranged in tandem. Furthermore, there are 3 - 4 exons of similar size to a V region exon within each C gene and even smaller exons that encode the carboxyl terminal ends. Between the exons for the V and C regions, which are separated by introns, there are additional coding sequences. They are the joining (J) segments, of 30 - 50bp, found on all three loci, and diversity (D) segments present only in the heavy chain locus. There are less than 20 D and 6 functional (plus 3 pseudogenes) J segments. These individual exons are also separated by introns of variable size. These J and D genes encode for the carboxyl terminal ends of the V region including the third CDR and thus provide additional specificity for the antibodies. In both κ and λ light chains, the V domain is encoded by both the V and J exons and the C domain by the C gene. In the heavy chain protein, the variable region is encoded by all V, J and D exons while the C region is derived from multiple C genes.

For each individual antibody, there is a specific combination of V and C regions. Before the translation and transcription of the light and/or heavy chains from DNA to proteins, specific exons are selected from the locus and rearranged with the deletion of intervening DNA. For a heavy chain, using the synthesis of the mouse μ heavy chain as an example, firstly the selected D and J segments (i.e. D2 & J1) are joined together with the deletion of all the other D and J segments (i.e. D3-12 but no J exon) in between (Figure 3.11). However, the exons 5′ and 3′ of the rearranged D and J exons respectively (i.e. D1 & J2-4) are not removed. This is followed by the selection of one V exon (V1) which is then attached to the DJ complex, thus resulting in a rearranged and selected VDJ gene (V1D2J1). At this point, the intervening V exons (i.e. V2-n) and the remaining D segments (i.e. D1) are also deleted. The C region gene (Cμ) is only rearranged to join the VDJ complex after transcription and RNA processing (splicing). A similar process occurs in the synthesis of the light chain proteins (Figure 3.11). By this method, individuals are able to generate copious numbers of specific antibodies.

3.3.3 Classification of TSHR Autoantibodies

Different TSAb bind to specific epitopes on the receptor ectodomain. The different names used can be confusing, but often usefully reflect the particular assay used for their detection. As mentioned previously, fundamentally there are two major types of thyroid receptor antibodies (TRAbs). The first are the autoantibodies that stimulate the TSHR, causing hyperthyroidism, which are known as the TSAb or thyroid stimulating immunoglobulins (TSI). The second are autoantibodies, termed TBAb, which block the binding of TSH to its receptor and do not themselves activate the

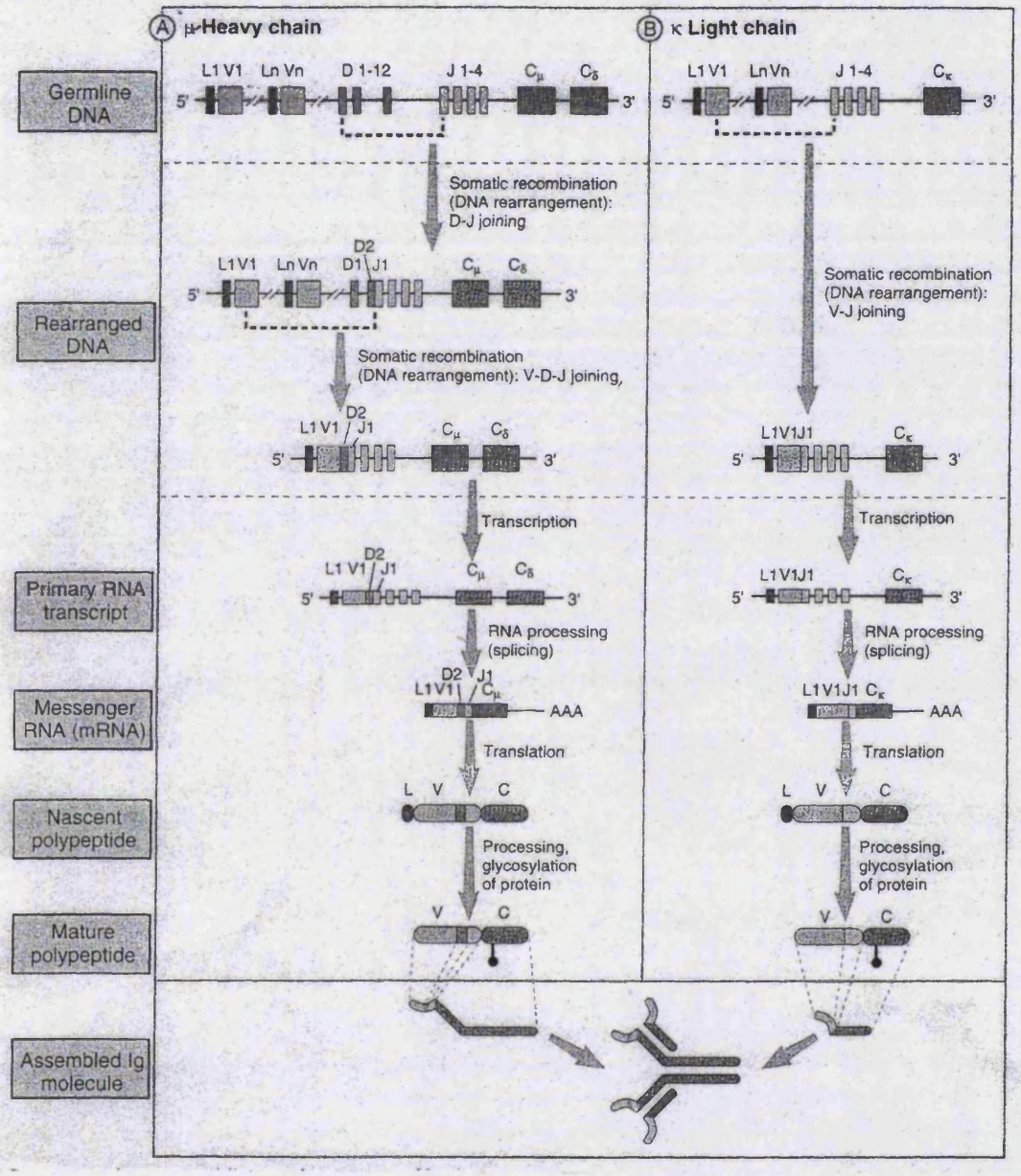


Figure 3.11 **A diagrammatic illustration of the Ig heavy and light chain gene recombination and expression to produce a specific antibody**. Using the examples of the μ heavy chain and κ light chain to demonstrate the sequence of events in which these chains are synthesised. In particular, the focus on the recombination of the selected gene segments from each class of exon, for example in the μ heavy chain, V1, D2, J1 and Cμ gene segments. (Abbas AK, Lichtman AH & Pober JS. Cellular and Molecular Immunology, 4th Ed. W.B. Saunders Company; 2000, p 136)

receptor. The inhibition of TSH action can result in hypothyroidism, but this is very rare. In addition to these two forms of TRAbs, a number of others, which are claimed to have diverse functions, have also been reported, as reviewed by Rapoport and colleagues (1998). These include:

- Thyroid growth autoantibodies, which were reported to induce growth, but not function, of the thyroid gland (e.g. Prabhakar BS *et. al.*, 1997). These are hypothesised to cause some goitres.
- Thyroid blocking autoantibodies that do not interact with the TSHR. As yet, these IgG have been reported to inhibit thyroid function without blocking TSHR (e.g. Desai RK *et. al.*, 1993; Dallas JS *et. al.*, 1996).
- Thyroid blocking antibodies that can convert to stimulators (Amino N *et. al.*, 1987; Watanabe Y *et. al.*, 1997). These antibodies are essentially blocking IgG and their conversion was reported to be achieved by the binding of divalent anti-IgG molecules.
- Neutral TSHR autoantibodies which do neither activate nor block the TSHR (Tonacchera M *et. al.*, 1996).

However, there is only limited and sometimes conflicting evidence for the existence of the TSHR autoantibodies listed above.

The ability to identify specific TSHR epitopes has resulted in an additional classification scheme of TRAb according to the epitopes with which they interact (e.g. Jaume JC *et. al.*, 1993; Kim WB *et. al.*, 1996). However, epitope interaction does not, at present, define a function. In summary, it is likely that multiple types of TRAb may

be present in a given patient serum at any point. This together with their low circulating concentration, complicates their detection and potency determinations.

3.4 TSH and Autoantibody binding to the TSHR

The extracellular domain of the TSHR is the obvious primary binding site of both TSAb and TBAb in addition to TSH (Figure 3.12). Many studies have suggested that TSH and TSHR autoantibodies have overlapping but different binding sites within the ectodomain. A summary of the main conclusions is reviewed below.

3.4.1 TSH binding

Direct evidence that TSH bound to the ectodomain was provided when covalent cross-linking of TSH to recombinant receptors on the surface of intact cells was observed (Furmaniak J *et. al.*, 1987; Russo D *et. al.*, 1991a). "Domain-exchange experiments", employing chimeric receptors, have yielded particularly significant results in this respect. For example, an LH/hCG chimeric receptor with a TSHR ectodomain was generated and high affinity TSH binding was observed (Nagayama Y *et. al.*, 1994). In a reverse experiment, where the LH/hCGR ectodomain was spliced to the transmembrane/intracellular domains of the TSHR, LH was likewise observed to bind specifically and activate this chimeric receptor (Nagayama Y *et. al.*, 1990). The question of whether TSH binds to the isolated TSHR ectodomain with the same affinity as to the full receptor remains uncertain. Some studies have reported a high (Shi Y *et. al.*, 1993) to moderate binding affinity ($K_D = 10^{-9}$M, Seetharamaiah GS *et.*

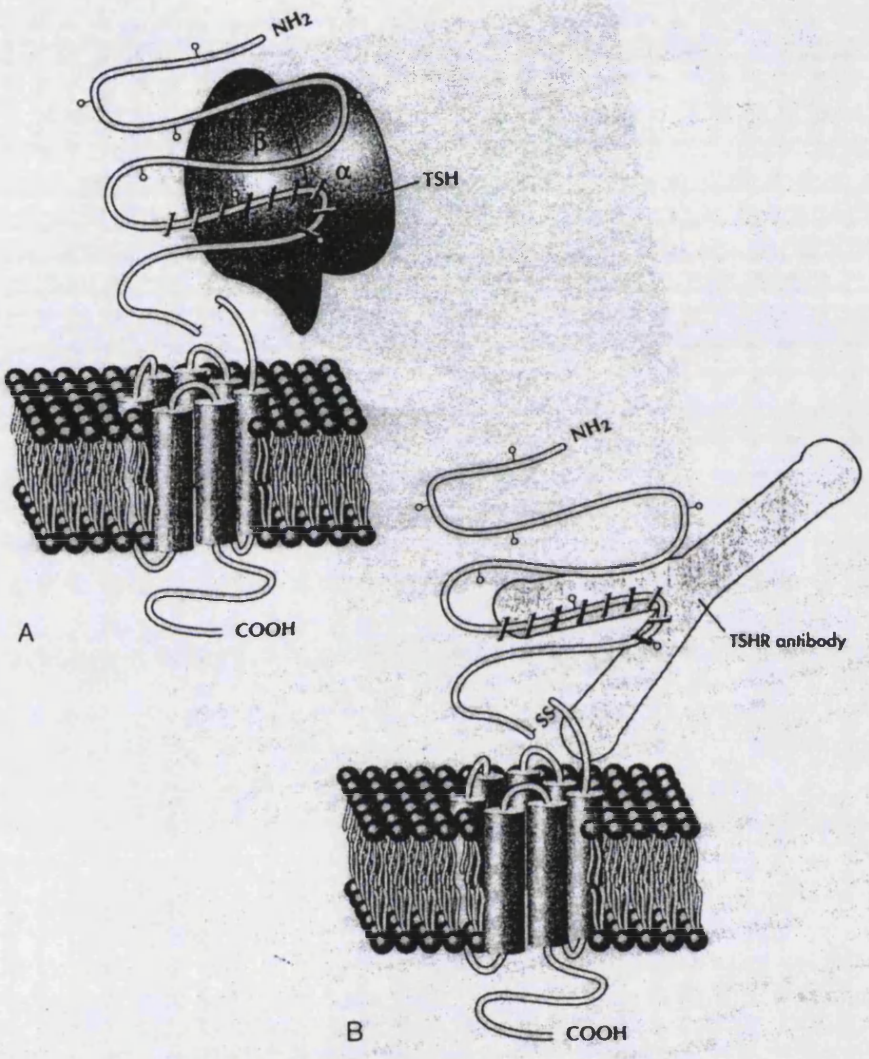


Figure 3.12 **A postulated scheme for the binding of (A) TSH and (B) TSHR autoantibodies to the ectodomain of the TSHR**. (Graves PN & Davies TF. New insights into the thyroid-stimulating hormone receptor – the major antigen of Graves' disease. Endocrinol Metab Clin North American; 2000, 29:267)

al., 1994), whereas others have obtained only low affinity binding (Chazenbalk GD & Rapoport B, 1995; Vlase H *et. al.*, 1997). In an extreme case, one investigation was unable to detect any specific binding (Osuga Y *et. al.*, 1997). Although it is generally considered that the ectodomain is the major TSH binding site, it does not preclude a role for the other regions of the receptor. For example, Rapport and colleagues (1998) reported that mutations introduced to all three exo-loops abolished TSH binding to stably transfected cells, although at present detailed results remain to be published. Compromised binding was also obtained in mutation studies of the first two loops (Haraguchi K *et. al.*, 1994; Kaneshige M *et. al.*, 1995). Furthermore, mutations in the third loop retained TSH binding but resulted in a reduced signal transduction (Kosugi S & Mori T, 1994). Interestingly, studies in which the TSHR ectodomain was divided into 5 arbitrary segments, and each segment was then substituted in turn with the corresponding region of the LH/hCGR, did not reduce the high affinity of TSH binding (Nagayama Y *et. al.*, 1990 & 1991c). This suggested that multiple interrupted segments contribute to TSH binding.

Investigations to determine the amino acid sequence in the TSHR responsible for TSH binding have taken two different approaches. Either synthetic peptides or TSHR mutagenesis studies have been used. The former implicated the involvement of more than 300 out of the 397 amino acid residues, in the interaction of TSH with its receptor (e.g. Ohmori M *et. al.*, 1991a & b; Morris JC *et. al.*, 1993; Hidaka Y *et. al.*, 1995). It seems improbable that TSH would interact with such a large number of residues, and the significance of some of the results has been questioned. For example, the binding affinities of TSH to these individual linear peptides are generally low and may even be difficult to distinguish from low-affinity binding of the hormone

to plastic culture dishes and cells which do not express the TSHR (Yamamoto M & Rapoport B, 1978; Chazenbalk GD *et. al.*, 1990b). Mutagenesis studies involve either the substitution or deletion of individual or a cluster of amino acid residues in specific regions of the TSHR ectodomain, which are suspected to be part of the TSH binding site. This technique also includes the replacement of TSHR residues with the corresponding residues from a closely related receptor such as the LH/hCGR (homologous substitutions). The deletion or substitution of residues has resulted in modification in the nature of TSH binding to the TSHR (e.g. Nagayama Y *et. al.*, 1990, 1991c & 1992b; Wadsworth HL *et. al.*, 1992; Kosugi S *et. al.*, 1993; Akamizu T *et. al.*, 1994). However, caution must be taken when interpreting these results, since such mutagenesis may have altered the overall conformation of the TSHR. Thus, in these studies, it is essential to establish conformational integrity, and in this respect the use of homologous substitutions may provide more reliable data. Homologous substitutions have localised residues between 201 - 211 and 222 - 230 within the mid-region of the receptor as significant TSH contact points (Nagayama Y *et. al.*, 1991a). In addition, non-homologous substitutions or deletions have indicated that residues between 295 - 302 and 387 - 395, and especially Tyr385, within the carboxyl terminal of the TSHR, contain significant TSH contact points (Kosugi S *et. al.*, 1991b).

3.4.2 The binding of Autoantibodies to the TSHR

The binding of an antibody to an antigen is dependent on the recognition of linear and/or conformational determinants within the antigen. Linear determinants are epitopes formed by adjacent amino acid residues linked in a covalent sequence. They are usually about six amino acid residues in size and form a direct contact with a

specific antibody. Conformational determinants, in contrast, are formed by several separate linear determinants that have been folded into a precise conformation. Commonly, antibodies against a large antigen such as the TSHR are likely to recognise conformational epitopes (Davies DR *et. al.*, 1990). However, a particular antibody may recognise both determinants of an epitope. Chimeric receptor studies using the TSH-LH/hCGR have concluded that autoantibodies bind to multiple segments of the ectodomain (Nagayama Y *et. al.*, 1991b; Tahara K *et. al.*, 1991). These results favour discontinuous conformational epitopes. The interaction of autoantibodies with the extracellular domain of the TSHR was further confirmed by the demonstration that they bound to isolated ectodomains (e.g. Harfst E *et. al.* 1992; Chazenbalk GD *et. al.*, 1997a; Seetharamaiah GS *et. al.*, 1997). Furthermore, carbohydrate moieties on the TSHR have also been implicated in antibody binding. However, at present, conflicting reports have been published (e.g. Fan J-L *et. al.*, 1993b; Chazenbalk GD & Rapoport B, 1995; Vlase H *et. al.*, 1995; Seetharamaiah GS *et. al.*, 1997). Both nonglycosylated and glycosylated ectodomains were recognized by autoantibodies in patient's sera but nonglycosylated recognition was reported to be dominant (Vlase H *et. al.*, 1995). In contrast, another study suggested that glycosylation may be more important (Seetharamaiah GS *et. al.*, 1997). However, there is still no direct evidence of antibody interaction with TSHR glycan (Prabhakar BS *et. al.*, 1997; Seetharamaiah GS *et. al.*, 1997). Hence it remains questionable as to whether these carbohydrate moieties are part of the epitope.

The identification of TRAb epitopes has been complicated by the existence of the two major forms of the TRAb, namely stimulating and blocking autoantibodies. As with the TSH studies the twin approach of investigation, namely using synthetic peptides

and investigating the effect of TSHR mutagenesis, has been utilized. Studies with synthetic peptides implied that more than 180 and 330 amino acids of the TSHR were involved in stimulating (e.g. Kosugi S *et. al.*, 1991b; Mori T *et. al.*, 1991; Fan J-L *et. al.*, 1993b; Ikeda M *et. al.*, 1993; Ueda Y *et. al.*, 1995) and blocking (e.g. Morris JC *et. al.*, 1994; Bryant WP *et. al.*, 1995) autoantibodies respectively. It has been reported that non-specific hexapeptides, rich in the highly charged amino acids glutamate, aspartate and histidine, are particularly effective in the neutralization of TSHR autoantibodies (Park JY *et. al.*, 1997). This suggests these amino acids may play a role in influencing TRAb binding. However, the most conclusive evidence has been gained from mutagenesis studies, and in particular, those using TSH-LH/hCG chimeric receptors. TSAbs were found to interact with the N-terminal region (amino acids 22 – 160) of the ectodomain of the TSHR (Nagayama Y *et. al.*, 1991b). Moreover, to a lesser extent, they were also found to bind to discontinuous segments within the C-terminal end (Nagayama Y *et. al.*, 1991b). Furthermore, certain amino acid residues at the N-terminal, namely Ser25 - Glu30 and Thr40, have been shown to be particularly important for TSAb binding (Kosugi S *et. al.*, 1992). Although both TSAb and TBAb were found to have overlapping binding sites with TSH (Nagayama Y *et. al.*, 1991b; Tahara K *et. al.*, 1991), the blocking antibodies shared a particularly large number of binding sites with TSH. TBAb have been reported to interact strongly with the C-terminal (Nagayama Y *et. al.*, 1991b), in particularly with amino acid residues 295 - 302, 385 and 387 – 395 (Kosugi S *et. al.*, 1992b). In addition, they also bound to the N-terminus or the mid-region of the receptor ectodomain (Nagayama Y *et. al.*, 1991b). Furthermore, the presence of an "immunogenic peptide" (YYVFFEEQEDEIIGF), which corresponds to TSHR residues 352–366 (Takai O *et. al.*, 1991) or 352–367 (Kosugi S *et. al.*, 1991b) has generated great interest. This peptide, when it was

administered, was highly immunogenic in both rabbits and mice. Moreover, sera from GD patients, compared to normal subjects, reacted particularly strongly to this peptide (Kosugi S *et. al.*, 1991a; Takai O *et. al.*, 1991). Since this peptide is located within the cleaved C-peptide (Figure 3.3) and has been predicted to be hydrophilic, it has led to speculation about a potential GD epitope being readily accessible within the circulation.

3.5 Assays for Autoantibodies against the TSHR

The identification of autoantibodies as the cause of GD has stimulated the development of assays for diagnostic purposes. Assays based upon binding to the TSHR have been devised together with *in vivo* and *in vitro* bioassays. Attempts have been made to adapt some of them to measure both TSAb and TBAb.

3.5.1 TSH Binding-Inhibitory assays

Currently, the most widely used *in vitro* assay employed for the detection of anti-TSHR autoantibodies and to aid the clinical diagnosis of GD is a TSH-binding inhibitory (TBI) assay. This assay is sometimes named the thyroid receptor assay (TRAK assay). It is based upon the competitive binding principle similar to that used in radioimmunoassays. Autoantibodies in a patient serum sample and TSH compete for binding to TSHRs. A constant amount of [^{125}I]TSH is incubated with a fixed amount of soluble receptors, and TRAb compete with the labelled hormone for the limited number of soluble receptors. Increased TRAb leads to a decrease in the bound radioactivity. This assay has the merit that it is precise and reproducible. It is also

convenient, technically easy to perform and inexpensive, facilitating high sample throughputs.

Although this is the most commonly encountered *in vitro* diagnostic assay for TRAbs, it has severe limitations. The main problem is that it is a non-functional assay. It cannot distinguish between the stimulating and blocking autoantibodies. In addition, it cannot indicate the biological potency of a given TSAb but merely its binding efficiency to the receptors. Interpretation of the results is further complicated by the fact that the latter is a function of both the concentration of a given autoantibody and its affinity for the receptor. Consequently, results generated by this assay would not be expected to correlate well with bioassays which measure thyroid stimulation. Indeed, there are reports of clinically hyperthyroid patients with negative TBII activity (Vitti P *et. al.*, 1993; Morgenthaler NG *et. al.*, 1998). Hence, clinical status of the patients does not necessarily correlate with the result provided by a TBI assay. Another major disadvantage of this assay might be due to species specificity of autoantibodies. The assay frequently uses porcine TSHR and bovine TSH to detect human autoantibodies to the human TSHR. Recently, Costagliola and colleagues have developed a second generation TBI assay (Costagliola S *et. al.*, 1999). In this assay, the problem of species specificity was eliminated by the use of the full length recombinant human TSHR expressed in the K562 leukaemia cell line. This cell line, expressing the human TSHR, was grown in suspension at a high density and it was thus able to provide large quantities of extracted human TSHR for use in the binding assay. Other later assays used either radioactive or luminescent labelled human TSH, and was developed in coated tube formats. Costagliola *et. al.* compared their assays to a commercially available conventional TBI assay. They tested 328 Graves' sera, from untreated and

treated patients and those in remission, together with 520 controls, which included euthyroid and non-thyroid disease patients. Both forms of the new assay were equally specific (99.6%) and significantly more sensitive (98.8% to 80.2%, $p<0.001$) than the conventional TBI assay. Their new assay, based upon human specific reagents require further use by different laboratories for a full evaluation. However the fundamental problem that these assays are non-functional, and thus cannot distinguish between stimulating and blocking antibodies, remains.

3.5.2 *In vivo* and *in vitro* bioassays

Despite repeated attempts there is at present no ideal *in vitro* clinical diagnostic bioassay for TSAb available. A reliable bioassay is needed for both the initial diagnosis of GD and subsequent long-term patient management. This includes monitoring during the initial period of ATD treatment (see Section 2.4.1), and attempting to predict remission or relapse upon cessation of treatment. It may also be useful as an indicator of neonatal GD. As technology has advanced, the bioassays have been developed and improved as is summarised below.

3.5.2.1 Thyroid Stimulating Antibodies

In vivo bioassays

Currently, no animal *in vivo* bioassays are routinely used for clinical purposes. The first *in vivo* bioassay was described by Adams and Purves in 1956 (Adams and Purves, 1956). Their assay employed guinea pigs in which they measured the release of radioiodine from pre-labelled thyroids in response to an intravenous injection of

Graves' patient sera. This resulted in the release in radioiodine after a delay of 9hr which contrasted with the 3hr time course obtained for the corresponding response to TSH. Because of the delayed response to the TSAb, which has never been adequately explained, they referred to the antibodies as "long acting thyroid stimulators" (LATS). The term LATS is now linked only to this assay and the antibodies that were measured with it are not considered to be different from TSAbs (Kriss JP *et. al.*, 1964; Meek JC *et. al.*, 1964). This *in vivo* method was adapted and used extensively by McKenzie and colleagues using mice ("The McKenzie bioassay", McKenzie JM, 1958a & b). Although, because of their expensive cumbersome nature and poor performance characteristics, *in vivo* bioassays are no longer used for the routine detection of TSAb, the system is still used in attempts to develop animal models of GD.

***In vitro* bioassays**

Over the last 30 years, numerous attempts have been made to develop *in vitro* bioassays for TSAb, which are suited for clinical use. These bioassays have largely been based upon the activation by autoantibodies to the TSHR of the intracellular second messenger signalling system AC, leading to the generation of cAMP. In the majority of these bioassays, the assay end point has therefore been the measurement of the increased intracellular cAMP. However, other assays have also been devised which utilised [^{3}H]-thymidine incorporation (e.g. Chiovato L *et. al.*, 1983), the increase in inorganic iodide uptake (e.g. Bidey SP *et. al.*, 1984) and naphthylamidase activity (e.g. McMullan NM & Smyth PPA, 1984) as end points.

Bioassays based upon plasma membrane preparations

This system utilised a sub-cellular fraction of thyroid tissue enriched with plasma membranes. With the latter, the AC embedded within the plasma membrane responds to TSH and TSAb, the bioassay response then being the increase in cAMP in the incubation medium. Though there was consistent activation by TSH in a dose- and time-dependent manner, contrasting results regarding the potencies, time courses and synergistic effects with TSAb were obtained (e.g. Orgiazzi J *et. al.*, 1976; Holmes SD *et. al.*, 1978). This method was insensitive, since it required supraphysiological doses of TSH e.g. 500mU/L to provoke a response, and moreover that magnitude of response to even such high doses was relatively small. Compared with later systems developed on intact thyroid tissue and cultured cells, the errors in response were, however, advantageously small and this enhanced the precision of the system. This characteristic was largely due to the homogenous nature of the target tissue preparation i.e. the membrane suspension. This system provided reasonable between assay reproducibility and could be described as a rugged, if insensitive, bioassay.

Human thyroid slices

This system used thyroid tissue obtained from patients undergoing laryngectomy or partial thyroidectomy. Slices were prepared and incubated in culture medium together with the stimulators. TSH and TSAb increased cAMP concentrations in both slices and culture medium (e.g. Holmes SD *et. al.*, 1978; Bidey SP *et. al.*, 1980). This technique proved to be more sensitive than that based upon plasma membrane preparations, and yielded larger magnitudes of response. However, the assays were ultimately less precise, since the response system, namely the slices of thyroid tissue,

were inherently heterogeneous. This inevitably resulted in large within assay errors (Bidey SP *et. al.*, 1981a). This problem seriously detracted from the ruggedness of this bioassay system. In addition, frustration was encountered due to the unreliable supply of thyroids as the starting tissue.

Primary monolayer cultures of normal human thyroid cells

This system was based upon cultures of normal human thyroid cells from thyroid tissue obtained from patients undergoing partial thyroidectomy for non-thyroidal diseases e.g. laryngectomies. Responses to TSH (Bidey SP *et. al.*, 1981b & 1982) and TSAb (Kasagi K *et. al.*, 1982; Bidey SP *et. al.*, 1983) varied in a dose- and time-dependent manner. Bidey and colleagues described a dose-dependent increase in the level of intracellular cAMP after both 1hr and a prolonged (16hr) exposure (Bidey SP *et. al.*, 1982). However, there was no difference in the magnitude of responses between the two incubation times. Whether incubated for 1 or 16hr, there was only a 2-fold increase in the intracellular levels of cAMP in response to 5mU TSH/ml. Similar observations were also observed for TSAb (Bidey SP *et. al.*, 1983). Dose-dependent increases in intracellular cAMP were observed, with the greatest occurring after incubation for 16hr. However, in contrast to TSH, there was an approximately 4-fold difference in the magnitude of responses between the incubation times of 1 and 16hr. Furthermore, after such prolonged exposure (16hr), a significant ($p<0.01$) increase in cAMP was observed at a dose of 0.95mU LATS-B/ml, LATS-B being the international reference preparation. More importantly, all the IgG preparations from sera from 28 newly-diagnosed GD patients significantly ($p<0.05$) increased the level of cAMP in the thyroid cell monolayers.

In attempts to improve the sensitivity of this bioassay system, Kasagi and colleagues discovered that the magnitudes of the increase in cAMP accumulation were influenced by the concentration of sodium chloride in the assay medium (Kasagi K *et. al.*, 1982). Bidey and colleagues had already demonstrated that, in response to TSH, the accumulation of cAMP in the medium was significantly greater than the intracellular concentration, particularly after a prolonged exposure e.g. 16hr (Bidey SP *et. al.*, 1982). Thus, a prolonged incubation time and assay of medium cAMP generally resulted in lower TSH detection limits and improved the bioassay. This effect was enhanced by the use of Kasagi's hypotonic medium (Kasagi K *et. al.*, 1982). It increased the efflux of cAMP into the medium due to an apparent increase in the permeability of the cells to this cyclic nucleotide; this improved the magnitude of response (Rapoport B *et. al.*, 1982). By measuring cAMP concentrations in the medium, this bioassay was simplified and became technically more straightforward. It also resulted in improved sensitivity and precision since several steps which were prone to errors were eliminated. However, despite the potential of this bioassay system, there was still the problem of availability and variability of the starting material. This can be partially solved by cryopreserving the thyroid cells and using aliquots for successive bioassays. A more serious problem however was due to the finding that the monolayers de-differentiated into fibroblasts when passaged for longer than two weeks. This greatly inconvenienced the system.

FRTL-5 cell cultures

As just mentioned although the problem of tissue availability and variability with primary cultures can be partially solved by cryopreservation, one particularly serious

limitation was the limited time that the cells retained their thyroid characteristics in culture; after about 2 weeks they were de-differentiated so that they were no longer responsive to TSH. Obviously a system which would provide a continuous culture would make the basis for a more stable, consistent and reproducible bioassay. The development of FRTL-5 cells, a rat thyroid follicular cell line which may be cultured over many passages, provided the stability required. FRTL-5 cells responded to TSH (Bidey SP *et. al.*, 1984), LATS-B (Bidey SP *et. al.*, 1985) and forskolin (Ealey PA *et. al.*, 1987) in a dose-dependent manner. Forskolin at a concentration that on its own did not provoke a response, could however potentiate cAMP accumulation in synergy with TSH or TSAb (Bidey SP *et. al.*, 1985). The following were some of the many advantages of this system:

i) it was a stable continuous culture thus the problem of tissue variability and short life span of the primary cultures was eliminated,

ii) it proved to be even more sensitive to TSH than the monolayers of human thyroid cells since larger magnitude of responses could be reliably obtained. It also had the advantage of the cell culture systems that each microculture was highly reproducible, leading to only small between-replicate errors,

iii) FRTL-5 cells could be sensitized to TSH to further improve sensitivity. This was achieved by a brief culture period in medium lacking foetal calf serum, prior to use in the bioassay,

iv) several parallel bioassays could be performed which have different end points on parallel microcultures. For example enhancement of iodide uptake, [^{3}H]-thymidine uptake, cell number and metaphase number have been followed in parallel (Marshall NJ & Ealey PA 1986).

Even with all of the above advantages, there remained one major disadvantage - namely species specificity (Vitti P *et. al.*, 1983). Since there has long been evidence that some TSAb are human specific, rat thyroid cells such as the FRTL-5 line were thought not to be the most appropriate for measuring human TSAb. It was appreciated that there was a need to develop a microculture bioassay, which whilst possessing all the advantages discussed above, could be based upon the stimulation of cells which express the human TSHR.

Cytochemical bioassays

Cytochemical bioassays, using quantitative cytochemistry, were also developed to assess responses to hormone stimulation. One based upon the stimulation of leucylnaphthylamidase utilizing thyroid tissue segments or sections for the detection of both TSH and TSAbs was developed in the early 1970s (Bitensky L *et. al.*, 1974; Ealey PA *et. al.*, 1981). This bioassay was exceptionally sensitive, responding to sub-physiological doses of TSH, but suffer from poor precision. The bioassay was technically demanding and laborious, and thus, only had a very limited sample throughput. It could be used in a research context, but not as a clinically useful routine assay.

With the development of immortalized cells such as the FRTL-5 line, which were responsive to specific hormones, cytochemical bioassays were also adapted to utilise microcultures rather than tissue sections. With the FRTL-5 cells, cAMP is a positive regulator of cell metabolism and proliferation. This facilitated the development of a microculture tetrazolium salt assay, which was a straightforward colorimetric assay

for thyroid stimulators (Ealey PA *et. al.*, 1988). With this system, no separate and error-prone procedure to measure cAMP concentrations was required. Instead a colorimetric reaction responding to enhanced cell number and metabolism was employed, so that quantification was achieved by measuring optical density (O.D.) of the medium above the microcultures. The colour change was due to the intracellular reduction of an added tetrazolium salt to its a non-substantive formazan. Dose-response curves were obtained in response to TSH, TSAb and forskolin (Ealey PA *et. al.*, 1988). This system, also known as an Eluted Stain Assay (ESTA) was much more technically straightforward than the previously described assays, which required the separate measurement of cAMP. Because minimal manual manipulation was involved, it was capable of enhanced precision.

Chinese hamster ovarian cell lines

The molecular cloning and expression of the functional human TSHR made possible the development of more appropriate microculture TSAb bioassays, which should possess the appropriate species specificity. The first research group to exploit this system was that of Dumont and colleagues in Brussels (Perret J *et. al.*, 1990). They stably transfected the human TSHR into CHO cells with a pSVL expression vector. They cotransfected the cells with a pSV_2Neo Neomycin Resistance Gene vector. This conferred resistance to Geneticin and so selection of successfully transfected cells could be achieved by the addition of G418 to the culture medium. A total of 24 successful clones were generated and amplified. Their responses to TSH (10mU/ml), forskolin (10^{-5}M) and TSAb (1.5mg/ml) were characterized. Of the 24 clones, 21 showed responses to all of these stimulators. However, the relative accumulation of

cAMP varied from clone to clone and only those that responded equally well to TSH and TSAb were further characterized. This included the cell line JP_{26}. Though there were variations in the maximal response to TSH, 0.4mU/ml TSH gave half maximal responses (in three clones). The differences were attributed to differing numbers of receptors per cell in each clone. It was estimated that JP_{26} had ~1750 receptor molecules/cell whilst another, JP09, had ~90,000 receptor molecules/cell (Costagliola S *et. al.*, 1992). The investigators also indicated that the responses could be potentiated by forskolin ($<10^{-7}$M) although those results were not shown.

CHO cell lines transfected with the hTSHR were tested for use in a TSAb bioassay based upon increase cAMP by two groups (Ludgate M *et. al.*, 1990; Vitti P *et. al.*, 1993). This bioassay was compared to other assays, namely the TRAK binding assay and the bioassay based upon increased cAMP in FRTL-5 cells. Relatively good correlations were found between these two assays and the CHO cell lines when sera from Graves' patients were tested. A correlation of $r = 0.83$, $p<0.0001$ was obtained when compared to the TRAK assay when sera from 55 patients were tested (Costagliola S *et. al.*, 1992), and when compared to the FRTL-5 cell system, a correlation of $r = 0.60$, $p<0.0001$ was attained with 74% of sera from active GD patients testing positive in both assays (Vitti P *et. al.*, 1993). They also reported that by using hypotonic conditions and measuring cAMP in the medium, the sensitivity to TSAb improved (Ludgate M *et. al.*, 1990; Vitti P *et. al.*, 1993). They concluded that these CHO cell lines provided a stable and convenient basis for bioassays for TSAb which could be exploited clinically in the future.

Since those early reports described above, the JP09, as opposed to the JP_{26}, CHO cell line has been adopted by several other groups for the development of TSAb bioassays based upon increases in cAMP. One such group is Morgenthaler and colleagues in Germany who used the JP09 CHO cell line to develop a bioassay for the routine detection of both TSAb and TBAb (Morgenthaler NG *et. al.*, 1998). They reported the presence of TSAb in 85% of Graves' patients in contrast to 2% in the euthyroid controls. Bioassay specificity was validated by testing different patients with different, non-thyroid, autoimmune diseases such as insulin dependent diabetes mellitus and rheumatoid arthritis. Only 1 out of 64 patients with non-thyroid autoimmune disease was positive. Furthermore, a good correlation ($r = 0.76$, $p<0.001$) was also obtained between unfractionated sera (84%) and IgG preparations (87%) prepared using the polyethylene glycol (PEG) precipitation technique. Morgenthaler and colleagues concluded that it was feasible to use the JP09 CHO cell line as a routine bioassay for TSAb. However, in an analogues study (Wallaschofski H & Paschke R, 1999) it was claimed that the JP_{26} cells, with their lower TSH receptor number, were superior to the JP09 cells.

3.5.2.2 Thyroid Blocking Antibodies

The heterogeneity of TRAbs in the serum of a GD patient has long been recognised although the majority of bioassays, such as those described above, have focused upon the stimulatory effects of these autoantibodies. Blocking antibodies have been detected by their ability to block TSH stimulation. They have been demonstrated in the sera of GD patients, by adapting the same bioassay systems (e.g. Kuzuya N *et. al.*, 1979; Madsen SN & Beck K, 1979; Macchia E *et. al.*, 1981 & 1988). However, their

presence has chiefly been demonstrated in patients with autoimmune atrophic thyroiditis (primary myxoedema) and autoimmune goitrous (hypothyroid Hashimoto's) thyroiditis and appear to be particularly prevalent amongst hypothyroid patients of Japanese and Chinese origin (e.g. Takasu N *et. al.*, 1987; Tokuda Y *et. al.*, 1988; Cho BY *et. al.*, 1990).

Takasu and colleagues studied the effects of TBAbs in both forms of autoimmune thyroiditis on the inhibition of TSH binding (TBII), TSH-stimulated thyroid cAMP response (TSII) and TSH-stimulated cell growth (TGII; Takasu N *et. al.*, 1987). They observed that IgGs (1mg/ml) from a number of patients with either atrophic or Hashimoto's thyroiditis inhibited the cAMP response to TSH (10mU/ml) in both the FRTL-5 and porcine cell assay systems. Furthermore, cell growth in response to TSH stimulation (10mU/ml) was also inhibited by the same IgGs when thymidine incorporation was measured in FRTL-5 cells. They concluded that multiple forms of TBAbs (TBII, TSII and / or TGII) could be found in some patients with either forms of autoimmune thyroiditis, their frequency being higher in atrophic thyroiditis. Similar findings were reported by Tokuda and colleagues in which they studied the effect of crude IgG from patients with either forms of autoimmune thyroiditis on ^{125}I uptake in FRTL-5 cells (Tokuda Y *et. al.*, 1988). They found that TSH-stimulated ^{125}I uptake was inhibited by IgGs from atrophic patients, whether they were TBII positive or negative, and also those from goitrous hypothyroid patients. Moreover, the mean inhibition by these three groups of IgGs correlated with the inhibition of TSH-stimulated cAMP accumulation ($r = 0.929$, $p<0.001$). Thus, Tokuda and colleagues further established the heterogeneous nature of TBAbs in autoimmune thyroiditis patients.

As mentioned above, TBAb have also been detected in the sera of patients with GD. Formerly, the bioassay system based upon the stimulation of AC in thyroid plasma membranes was utilized (e.g. Madsen SN & Beck K, 1979; Macchia E *et. al.*, 1988). Macchia and colleagues tested 135 hyperthyroid GD patients for both TSAb and TBAb activity. They reported that 54.1% of sera were positive for both TSAb and TBAb and that 12.6% were TBAb positive only.

More recently, with the development of the CHO cell lines expressing the hTSHR (see above), Morgenthaler and colleagues attempted to detect TBAbs using the JP09 CHO cell bioassay mentioned above (Morgenthaler NG *et. al.*, 1998). TBAbs were detected in 4 out of 24 (17%) TSAb negative Graves' patients who were highly positive for TBII. They reported up to 75% inhibition of TSH induced cAMP production by the unfractionated patient sera.

In another study, Wallaschofski and Paschke compared the detection of TSAb and TBAb in GD patients using cAMP production by two CHO cell lines expressing the hTSHR (Wallaschofski H & Paschke R, 1999). These 2 cell lines, JP09 and JP_{26}, differ only in the number of TSHR expressed per cell (~90,000 and ~1,750 receptors respectively; Perret J *et. al.*, 1990). The authors reported that under optimal assay conditions ($4x10^4$ cells/ml, 1:10 serum dilution, pH 7.6 at room temperature), the JP_{26} cell line had a higher stimulation index and was more sensitive, particularly for the detection of TBAb. Using these cells, 50 and 34 out of 84, sera from GD patients treated with antithyroid drugs, were positive for TSAb and TBAb respectively.

Section 4

Luciferase Reporter Gene Bioassays

The use of bio- and chemiluminescence to generate signals for analytical purposes is now widespread. One important example is in gene transfer studies. This technique uses reporter genes to monitor and assess the function and/or activity of specific genes. There are a growing number of bio- and chemiluminescent reporter gene assays. Bio- and chemiluminescence emissions are the result of biochemical and chemical reactions respectively. Both result in the emission of photons due to the deactivation of excited molecules as they return to their resting state. Of the two, bioluminescence reporter genes are the more widespread. These include aequorin, alkaline phosphatase, β-galactosidase and a family of luciferase reporter genes. Within the latter, luciferase from the firefly has been the most extensively exploited.

4.1 Reporter Gene Vectors

The study of the regulation of mammalian genes has been greatly aided by reporter genes. Reporter genes code for an enzyme or protein that is distinguishable from all naturally expressed intra- and extracellular proteins. They are used to examine the transcriptional activity of a given regulatory sequence. The regulatory DNA can be either a promoter or an enhancer element. Test promoter or enhancer sequences are ligated upstream and downstream of the reporter gene respectively to generate a chimeric gene. This gene thus contains a test regulatory element that will control the transcription of the reporter gene. The chimeric gene is then transfected into cultured

cells. The transcriptional activity of the putative regulatory element is then quantified by measurement of the production of the protein encoded by the reporter gene.

The development of reporter gene vectors has aided the widespread exploitation of the luciferase reporter gene. Basic reporter gene vectors have a common structure. They usually comprise of the following, on a vector backbone (Alam J & Cook JL, 1990):

1. A procaryotic origin of replication which will allow replication of the vector in *Escherichia coli.*
2. An antibiotic resistance gene that is necessary for the selection of the vector in *Echerichia coli.*
3. Cloning sites, containing multiple restriction enzyme sites, upstream and downstream of the reporter gene.
4. A eucaryotic poly(A) addition signal and an intron both of which are necessary for efficient RNA production and processing (Sambrook J *et. al.*, 1989; Carswell S & Alwine JC, 1989).

The number and specificity of restriction enzyme cloning sites upstream and downstream of the reporter gene determines the effectiveness of a given vector. Multiple distinct sites provide flexibility and options regarding the insertion of the DNA. They also allow the accommodation of a broad range of different test sequences. For any given reporter gene, a family of reporter gene vectors are usually designed. They include (Alam J & Cook JL, 1990):

- a basic vector which is promoter- and enhancer-less and will allow the inserted test sequence to regulate reporter gene transcription,
- a promoter vector which has an in-built promoter upstream of the reporter gene,

- an enhancer vector containing an enhancer downstream of the reporter gene and,
- a control vector which has both the promoter and enhancer elements.

Promoter and enhancer vectors have intrinsic transcriptional activity and, thus, can act as positive controls in an experiment. Basic vectors can serve as negative controls and control vectors are useful in monitoring transfection efficiencies.

4.2 Firefly Luciferase

De Wet and colleagues first cloned the firefly luciferase gene from the North American firefly *Photinus pyralis* (De Wet JR *et. al.*, 1985 & 1987). The gene encodes the monomeric enzyme, luciferase (Luc), which has a molecular weight of 62kd (Wood KV *et. al.*, 1984). This protein requires no post-translational modification and catalyses the following reaction (Sutherland CA & Johnson RA, 1974; DeLuca M, 1976):

$$\text{Luc} + \text{D-luciferin} + \text{ATP} + Mg^{++} \rightleftharpoons \text{Luc-luciferyl-AMP} + PP_i$$

$$\text{Luc-luciferyl-AMP} + O_2 \rightarrow \text{Luc} + \text{oxyluciferin} + \text{AMP} + CO_2 + \text{LIGHT}$$

This reaction utilises adenosine 5′-triphosphate (ATP) and O_2 as co-substrates and results in the emission of a flash of light (Seliger HH & McElroy WD, 1960). At pH 8.0, the light emits at the wavelength 560nm and is yellow-green in colour. Luciferase derived from other firefly species will also catalyse the above reaction, but the colours of the light emitted are different. Since the substrates are the same, these differences must be due to variations in the structures of the species specific luciferase enzymes (Seliger HH & McElroy MD, 1964). Optimum conditions for the luminescence

reaction are critical. For example, changes in the pH alter the wavelength of the light emitted, with a shift to longer wavelengths occurring at lower, more acidic, pHs which results in redder emissions. In addition, the presence of several divalent metal ions such as Zn^{2+} also changes the wavelength of the emitted light (Seliger HH & McElroy MD, 1964).

This reaction, catalyzed by luciferase, is ATP specific, and other nucleotide triphosphates can act as inhibitors (Lee RJ *et. al.*, 1970). Using ATP analogues, it was determined that the 6-amino group of the adenine is important for the binding at the ATP-Mg binding site (Rhodes WC & McElroy WD, 1958). The reaction product, adenosine 5'-monophosphate (AMP), is a competitive inhibitor of luciferase (Lee RJ & McElroy WD, 1971). Hence this forms a negative feedback loop. Mg^{2+} is the most potent divalent cation but Mn^{2+}, Co^{2+} and Zn^{2+} can be substituted. However, as mentioned above, this results in a shift in the emission wavelength.

4.3 Luciferase Reporter Gene Assays

Since the cloning of the firefly luciferase reporter gene and the development of its vectors, luciferase has become one of the most widely used reporter genes. When luciferase catalyses the reaction discussed above (see Section 4.2), it oxidises the substrate, D-luciferin, to oxyluciferin. The reaction, in the presence of Mg^{2+}, utilises ATP and O_2 to produce light which can then be quantified by a luminometer. The availability of the substrates is the limiting factor. Under optimal conditions, light production is highly efficient with a quantum yield of >0.88 (Selinger HH & McElroy WD, 1960). The quantity of light produced is linearly proportional to the amount of

enzyme transcribed. This linearity is maintained over a range of 8 orders of magnitude of enzyme concentration. Because of the exceptionally high quantum yield, subattomole quantities of enzyme can be detected (Bronstein I *et. al.*, 1994), but ultimately the sensitivity is limited by the efficiency and background noise of the luminometer.

Luciferase reporter gene assays are based upon the same principles as other reporter gene assays. They were mentioned above (see Section 4.1) but are outlined here (Champiat D *et. al.*, 1994):

1. The regulatory DNA sequence to be tested, which can be either a promoter or enhancer, is ligated into the desired cloning site of the appropriate luciferase vector.
2. The resultant chimeric construct is transfected into suitable immortalised cells, such as CHO, NIH3T3 or HeLa cells.
3. Luciferase is synthesised under the regulation and according to the functionality of the test element.
4. Most protocol have required the cells to then be harvested and lysed to obtain cell lysates.
5. The assay is performed, by the addition of optimised concentrations of substrates and co-substrates, to determine the amount of luciferase produced.
6. Light emitted is measured by a luminometer.

The vectors which are currently used contain a luciferase gene which has undergone deliberate specific modifications from its native form. These have generated vectors with increased compatibility for a larger range of hosts. For example, the Promega

pGL3 Luciferase Reporter Gene vector family contains a modified firefly luciferase cDNA designated *luc+* (Sherf BA & Wood KV, 1994). Modifications to the native firefly luciferase gene have been engineered to achieve the following:

- eliminate peroxisome targeting of the transcribed enzyme
- increase expression in both plant and animal cells.
- remove any potential binding sites for regulatory and transcription proteins within the native cDNA.
- remove existing restriction enzyme sites within the native luciferase gene.
- disrupt native palindromes.

These changes were made without altering the encoding amino acids. They minimize any potential interference by the reporter gene, which could lead to misleading signals. Furthermore, the modifications have also enhanced the reliability and convenience of luciferase as a reporter gene. The resulting *luc+* is "a biologically neutral form" of the native luciferase gene (Sherf BA & Wood KV, 1994). The vector backbone was also altered to enhance gene expression (Doyle K ed., 1996). The modifications achieve the following:

- maximise efficient RNA processing.
- increase reporter gene expression.
- increase luciferase translation initiation efficiency.
- eliminate any transcription originated by the vector backbone.

The resultant family of pGL3 Luciferase Reporter Gene vectors has greater flexibility and sensitivity, minimal background noise and increased luciferase expression (Doyle K ed., 1996). Furthermore, they are suitable for a wide range of cell types, enhancing their ease and convenience of use.

In the original assay, D-luciferin was added to a mixture of expressed luciferase, ATP and Mg^{2+} in a buffered aqueous solution. The light was emitted as a flash, which peaked within 1sec and subsequently rapidly decayed (DeLuca M & McElroy WD, 1978). There were two major disadvantages to this basic system. Firstly, the light output had to be measured immediately and, secondly, the product, oxyluciferin, acted as an inhibitor of the luciferase. Consequently, the assay was redesigned to eliminate enzyme inhibition and also yield sustained luminescence. This "glow", as opposed to "flash" luminescence would obviously ease quantification. The addition of coenzyme A in micromolar concentrations was found to both increase the intensity of the light produced and generated a more sustained light output (Airth RL *et. al.*, 1958; Wood KV, 1991). Other additions which helped produce a glow-type luminescence included inorganic pyrophosphate (McElroy WD *et. al.*, 1953; Lundin A., 1993), triphosphate and a number of cytidine nucleotides (Leach FR *et. al.*, 1988). In addition, detergents, such as Triton X-100, were added, at above their critical micelle concentration, to further improve both the intensity and duration of light emission (Kricka LJ & DeLuca M, 1982; Williams TW *et. al.*, 1989). The micelles were thought to act as a barrier between oxyluciferin and luciferase, hence minimizing product inhibition (Williams TW *et. al.*, 1989). It was also determined that optimal results were obtained when the assay was performed at room temperature (20 - 25°C). Consequently, during an assay, it is important to equilibrate all reagents to room temperature before use. These modifications have led to striking improvements in the performance of luciferase reporter gene assays. Such major changes to both the assay and the reporter gene itself, did not interfere with the generation of the rapid response, which is a useful characteristic of the luciferase system. Thus stable and intense response signals can be measured within minutes of the initiation of the reaction.

The discovery of firefly luciferin esters, such as the 2-hydroxyethyl ester, which are water soluble, has resulted in the development of a direct, one-step luciferase assay (Craig FF *et. al.*, 1992), so that luciferase expression can now be determined in intact cells. The uncharged soluble esters readily cross the cell membrane into the cytosol. However, they are not the immediate substrates for firefly luciferase. It is necessary for intracellular esterases to hydrolyse these soluble esters into luciferin. Luciferase assay systems developed using this strategy include the Steady-Glo and, more recently, the Bright-Glo Luciferase Assay System from Promega. They are both single-step assays which are designed to be used for high-throughput screening. In these protocols, the addition of the assay reagent mixture in fact induces both cell lysis and light emission. This eliminates the need to separately lyse cells and then determine light emission from aliquots of the cell lysates as was necessary in the prototype of this system. This inevitably, as we demonstrate later, incurs smaller manipulative errors and thereby contributes to greater assay precision in the final reporter gene assay. The notable advantages of this system include glow-type luminescence, assay flexibility, tolerance to different assay compositions, rapid responses and reproducibility (Hawkins *et. al.*, 1999; Hawkins *et. al.*, 2000).

4.4 The Development of a Firefly Luciferase Reporter Gene Bioassay for Thyroid Stimulating Antibodies

In Section 3.5.2, we reviewed the numerous different *in vitro* bioassays for TSAb, which have been developed in the past. None of these have been ideal for clinical exploitation. In particular they are multiple-step techniques which are error-prone, cumbersome and only capable of small sample throughputs. However, it was anticipated that luciferase reporter gene technology could be advantageously

exploited to develop an improved TSAb bioassay. This system, like most of those previously developed, would fundamentally be based upon the increase in cAMP which result from TSHR activation. It would however differ from the previous systems, since luminescence could be measured as the end point. This would avoid the cumbersome steps involved in the extraction of, and separate measurement of, intracellular cAMP. Albanese and colleagues demonstrated the successful use of a firefly luciferase reporter gene with a CRE promoter, in a prototype bioassay for FSH (Albanese C *et. al.*, 1994). However, subsequent to this early work, there have now been many improvements in luminescence technology, as discussed in the preceding section. Our aim is to exploit these recent advances for a luciferase reporter gene bioassay for TSAb. We discuss the current status regarding the key components for this as follows.

cAMP and its actions as a nuclear response element

cAMP has long been established as one of the most important intracellular signalling second messengers for hormones which act at the cell surface. Numerous cellular effects, including gene transcription, are the result of an increase in intracellular cAMP. The activation of PKA and a subsequent intracellular phosphorylation cascade, by cAMP, results in the activation of gene transcription via CRE promoters. For example, the role of cAMP in the transcription of the genes of both the α- and β-subunits of TSH, via CRE in the promoter region, has already been detailed (see Sections 3.2.1 & 3.2.2). Briefly, the rise in intracellular cAMP activates the tetrameric PKA by the removal of its two regulatory subunits. Exposure of its catalytic subunits leads to phosphorylation of crucial serine and threonine residues in specific DNA binding proteins. These then bind to specific DNA sequences in the nucleus to

promote gene transcription. There are a large number of DNA binding proteins such as CREB and CREM (CRE modulator), which, when phosphorylated, bind to selected CREs. All CREs contain a conserved core consensus sequence, TGACGTCA. This 8 base pair palindromic sequence is important in regulating gene transcription. The binding of the DNA binding proteins promotes the subsequent attachment of other transcription factors and enzymes leading to fully activated gene transcription (Foulkes NS & Sassone-Corsi P, 1996).

Stable cell lines expressing G-protein linked glycoprotein hormone receptors

The activation of G-protein linked glycoprotein hormone receptors, such as FSHR, TSHR and LHR, results in an increase in intracellular cAMP (see Section 3.1.1.2). These receptors have been cloned and can therefore be transfected into continuous cell lines and stably expressed. Such cell lines have proved useful tools for study of the structures, functions and actions of the receptors. However, they are also useful target tissues of the development of diagnostic assays for receptor activators such as TSAb, such as those described in Section 3.5.2.

The exploitation of CRE in the human glycoprotein hormone α-subunit gene

The human glycoprotein hormone α-subunit (hGPHα) gene, common to all the glycoprotein hormones, has been extensively studied (e.g. Fiddes JC & Goodman HM, 1981; Fiddes JC & Talmadge K, 1984). The promoter region of the gene has been of special interest. The details have already been described (see Section 3.2.1.1). Clearly, the presence of cAMP responsive CREs within the promoter can be exploited

as a key component in a cAMP detection system, such as the proposed cAMP-responsive luciferase reporter gene assay.

In the first attempt to exploit this system, Albanese and colleagues utilized the cAMP responsive hGPHα promoter to create a luciferase construct in which luciferase transcription was under the control of CRE (Albanese C *et. al.*, 1994). This vector was cotransfected into a CHO cell line expressing the human FSHR. Stimulation by FSH led to the accumulation of intracellular cAMP and, subsequently, the activation of the cAMP signalling transduction pathway. The intracellular phosphorylation cascade resulted in the transcription of luciferase via the activated CRE promoters. Good correlation between increases in luciferase activity and cAMP concentrations was observed. Thus, Albanese and coworkers reported the generation of a cell line which measured the activation of FSHR via the light generated by luciferase. This cell line provided a stable target tissue for FSH which could be maintained in continuous culture. They thereby obtained a luciferase reporter gene bioassay for FSH.

Given the stable expression of the hTSHR in CHO cells (Perret J *et. al.*, 1990), it therefore become possible to develop a luciferase reporter gene bioassay for TSAb, in GD patient sera, based upon the same principles. This reporter gene system is particularly attractive since any increase in cAMP would initiate a cascade of responses which could culminate in enzymatic amplification by luciferase. Such magnification of a response might enhance the sensitivity of the assay. In addition, by using the newly developed Steady-Glo, single step direct away system (see Section 4.3), which does not require extraction of luciferase from the target cell prior to its measurement by light output, it is anticipated that the bioassay will suffer minimal

transfer errors. This should yield a bioassay which is technically straightforward and adaptable to 96-well plate technology. It is anticipated that these advantages will enhance both the bioassay precision and sample throughput, both of which would be key development for obtaining a TSAb bioassay suitable for routine clinical exploitation.

CHAPTER 2

COMMON MATERIALS & METHODS

Section 5

Cell Culture

During the course of this project, a number of different transfected CHO cells, which expressed the human TSHR, were used. This brief section details the common methods and materials used in the handling of the CHO cells. More specific materials and methods, relating to particular investigations, are detailed, as appropriate, in each section in the Results (Chapter 3). For all the CHO cells, standard protocols for routine cell culture and cryopreservation were used and these are described below.

5.1 Chinese Hamster Ovarian cell lines

5.1.1 JP_{26} and $JP_{26/26}$ cells

These cells were a kind gift from Dr. J. Van Sande (University of Brussels, Belgium). JP_{26} and its subclone $JP_{26/26}$, are CHO cells which had been stably transfected with the wild type human TSHR. They have been co-transfected with a hTSHR-pSVL expression shuttle and a pSV2 NEO neomycin resistance gene vector. A modified calcium phosphate precipitate method of transfection was used (Perret J *et. al.*, 1990).

They were routinely subcultured in $25cm^2$ tissue culture flasks. The culture medium was Ham's F-12 nutrient medium supplemented with 10% foetal calf serum (FCS), 100U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml amphotericin-B (Fungizone) and 400μg/ml Geneticin G418 for resistance selection.

5.1.2 YSW cells and subclones

The JP_{26} cells were stably co-transfected in our laboratory with the pGL3hαCRE plasmid and the pWZL hygromycin resistance vector (for details see Results Section 8). This generated stable YSW2 and YSW3 cells. After the characterization of these two cells lines, YSW3 cells were further cloned by limiting dilution. This yielded 54 YSW3 subclones (for details see Results Section 8).

These cells were routinely cultured in a Ham's F12 selection culture medium containing 10% FCS, 100U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml amphotericin-B (Fungizone), 400μg/ml Geneticin G418 and 500μg/ml hygromycin. The latter three antibiotics were for resistance selection.

5.1.3 CHO25LUC cells

The CHO25LUC cells were a kind gift from Dr. L. D. Kohn at the National Institute of Health (N.I.H.), USA. CHO25LUC cells are CHO cells stably transfected with the wild type human TSHR and a firefly luciferase reporter gene. The luciferase plasmid contained the promoter region of the human glycoprotein hormone α-subunit (α-Gly) gene. The promoter contained two CRE promoters and was placed upstream of the luciferase reporter gene. CHO cells expressing the human TSHR were transfected with the α-Gly luciferase plasmid in Dr. Kohn's laboratory by electroporation. Briefly, 40μg of the plasmid was mixed with 4μg of puromycin, which was used as the selection marker. The mixture was transfected into 10^6 cells. After electroporation, the cells were expanded for 2 weeks in Ham's F12 medium containing 10% FCS and 10mg/ml puromycin. Surviving cells were cloned by limiting dilution. Clones were

selected by analysing their response to acute stimulation with 10^{-8} mol/L TSH or a standard Graves' IgG preparation.

The CHO25LUC cells were routinely cultured in Ham's F12 nutrient medium supplemented with 10% FCS, 100U/ml penicillin and 100μg/ml streptomycin. For every 4th passage, either or both selection antibiotics were added to the culture medium. These antibiotics were puromycin at 10μg/ml and geneticin G418 at 1mg/ml.

5.2 Materials

Ham's F-12 medium (supplemented with 1mM sodium pyruvate, L-glutamine and sodium bicarbonate), penicillin/streptomycin, amphotericin (fungizone) and G418 Geneticin were obtained from Gibco, Paisley, Scotland, UK. FCS was either obtained from Gibco, Paisley, Scotland, UK, TSC Biological Ltd, Buckingham, UK or Insight Biotechnology Ltd, Wembley, Middlesex, UK. Hygromycin and puromycin were obtained from Calbiochem, Ja Jolla, CA, USA. 2.5% Trypsin in Hank's Balanced Salt Solution (modified) was ordered from ICN Pharmaceutical Inc., CA, USA.

5.3 Routine procedures

5.3.1 Cryopreservation

Cells were first harvested by trypsinisation, in which culture medium was removed and a 2.5% trypsin solution (3ml) was added. The cells were then allowed to detach for 10min at 37°C. Finally, the cell suspension was centrifuged for 5min at 1500rpm. The cells were then suspended in their respective culture media supplemented with

10% dimethylsulphoxide (DMSO) at the density of 10^7cells/ml. The suspension was then aliquoted into cryotubes (1ml/tube). The aliquots were frozen slowly overnight at -70°C within a polystyrene box. Finally, all the aliquots were transferred for long-term storage in liquid nitrogen.

To start a routine cell culture

One aliquot of cells was rapidly thawed in a 37°C waterbath. The cells were then washed twice in culture medium. They were finally resuspended in 20ml of fresh culture medium and cultured in two 25cm^2 tissue culture flasks (10ml/flask). Cells such as these could be used for experiments after the first passage.

5.3.2 Routine cell culture

CHO cells were cultured as attached monolayers. All cells were grown in their respective culture media described above. Cells were routinely cultured in 25cm^2 tissue culture flasks containing 10ml of culture medium. Subculture was necessary once a week, and required two subsequent medium changes during a given week. The cells were trypsinized as described above, washed and resuspended in 10ml of culture medium. They were plated into the tissue culture flasks at an initial density of 4 – 6x10^3 cells/ml. All cultures were routinely maintained in a 5% CO_2 / 95% air, water saturated incubator at 37°C.

CHAPTER 3

RESULTS

Section 6

The characterization of the CHO JP_{26} and $JP_{26/26}$ cell lines

6.1 INTRODUCTION

Two groups first cloned the human TSHR simultaneously (Libert F *et. al.*, 1989; Nagayama Y *et. al.*, 1989). This led to the transfection of the human TSHR into CHO cells (Perret J *et. al.*, 1990). The selection and characterization of the successfully transfected stable clones was described in the Section 3.5.2.1.

Several groups have used CHO JP cell lines to develop TSAb bioassays based upon the conventional measurement of cAMP (Lugate M *et. al.*, 1990; Costagliola S *et. al.*, 1992; Vitti P *et. al.*, 1993; Morgenthaler NG *et. al.*, 1998). They concluded that these CHO cell lines provided a stable and convenient continuous culture system that was suitable as the target tissue for a clinical bioassay for TSAb.

The aim of this project was to develop a cAMP responsive luciferase reporter gene bioassay for thyroid stimulators using a CHO cell line stably expressing the human TSHR. Two of these cell lines, JP_{26} and its subclone $JP_{26/26}$, were obtained with the intention of transfecting them so as to express luciferase under the regulation of a cAMP-responsive promoter. Prior to using either of the cell lines, they were subjected to a number of preliminary investigations to confirm their characteristics previously described by Perret and colleagues (Perret J *et. al.*, 1990). In addition, we tested their suitability for use in a microculture tetrazolium salt colorimetric assay.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Horse serum was obtained from Gibco, Paisley, Scotland, UK. 3-isobutyl-1-methylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, used at the final concentration of 2mM and PMS (Phenazine methosulphate) were obtained from Sigma Chemical Co, Poole, UK. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) was obtained from Promega, Madison, Wisconsin, USA. Forskolin, an AC activator was obtained from Calbiochem, La Jolla, CA, USA. It was dissolved in culture medium (see Section 5.1.1) supplemented with DMSO giving a stock concentration of 1mM in 10% DMSO. The hTSH used was the Second International Reference Preparation of TSH, human, for immunoassays, coded 80/558 with an assigned biological potency of 37mU/ampoule. The preparation was reconstituted in hypotonic medium i.e. Kasagi's hypotonic medium (see Section 3.5.2.1), pH 7.4 with 0.4% BSA to yield an initial stock concentration of 1U/L. All such stocks were aliquoted and stored in liquid nitrogen. Long Acting Thyroid Stimulator (LATS-B), a human TSAb coded 65/122, was an MRC standard for thyroid stimulating antibodies, prepared by lyophilizing a patient serum preparation; it was obtained from NIBSC. It has an assigned biological potency of 15mU/ampoule. For use, the preparation was reconstituted in hypotonic medium (see above) with IBMX and 0.4% BSA to give a stock concentration of 10U/L. In addition, a Thyroid Stimulating Antibody (TSAb) standard from NIBSC was used; this was the First International Standard coded 90/672 which has an assigned biological potency of 100mU/ampoule. For use, the preparation was reconstituted in hypotonic medium (see above) with IBMX and 0.4% BSA to give a

stock concentration of 50U/L. The cAMP Enzymeimmunoassay (EIA) system (RPN 225, 96-wells) was obtained from Amersham, Buckinghamshire, UK. The two CHO cell lines JP_{26} and $JP_{26/26}$ were gifts from Dr. J. Dumont, Brussels, Belgium.

6.2.2 METHODS

6.2.2.1 cAMP Bioassays

The following protocol was based upon that kindly provided by Dr. J Van Sande, Brussels, Belgium.

Cells were transported to our laboratory as a monolayer in a $25cm^2$ tissue culture flask. After several passages, they were harvested by trypsinization and plated into 96-well plates at $5x10^4$ cells/well containing 200µl of culture medium/well (see Section 5.1.1). The plates were then incubated for 24hr in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. For the assay, the culture medium was removed and the cells were rinsed once with Krebs Ringer Hepes Buffer which did not contain NaCl (i.e. Kasagi's hypotonic medium, see Section 3.5.2.1). Stimulators or controls in this medium, supplemented with 2mM IBMX (200µl), were added to the wells and incubated for 4hr at 37°C. After 4hr, 150µl of medium was removed and stored at -20°C. The cAMP concentration in the medium was determined using the EIA system according to manufacturer's instructions. The EIA assay was a competitive binding assay in which unlabelled cAMP (in standards and test samples) competed against a fixed quantity of peroxidase-labelled cAMP for a limited number of binding sites on a cAMP specific rabbit antibody. As a result, the amount of peroxidase-labelled cAMP bound to the rabbit antibody was inversely related to the concentration of the

unlabelled samples. The anti-cAMP rabbit antibody was in turn bound to the microtiter plate wells by a precoated second antibody, an anti-rabbit IgG antibody raised in a donkey. Any unbound ligand was easily removed by a washing step. The amount of peroxidase-labelled cAMP bound was then determined by the addition of a tetramethylbenzidine (TMB)/hydrogen peroxide substrate. Finally, an acid solution was added to stop the colorimetric reaction. In brief, for the non-acetylation assay protocol: 10 standard concentrations of cAMP from 0 – 32.0 pmol/ml were generated by serial dilution. 100µl of the standards or samples were plated into appropriate wells followed by 100µl of anti-cAMP rabbit antibody. The plate was then incubated for 2hr at 3 - 5°C. After the addition of 50µl of a cAMP peroxidase conjugate, the plate was further incubated for 60min at 3 - 5°C. The plate was aspirated and washed four times. Then TMB/peroxidase substrate (150µl) was added to all wells. The plate was then shaken on a plate shaker for another 60min. Finally, to stop the colorimetric reaction, 100µl of sulphuric acid (1.0M) was added to all wells. Optical densities (O.Ds.) were read at the wavelength of 450nm with a reference wavelength of 655nm, within 30min, using a microtiter plate reader (Bio-Rad, Model 3550, Richmond, CA).

6.2.2.2 Tetrazolium Salt Colorimetric Assays

Cells were harvested by trypsiniztion and seeded into 96-well plates. Each well contained 100µl of cells in culture medium (see Section 5.1.1) at a constant cell density, which ranged in different experiments from 10^4 – 10^5cells/ml. Cells were stimulated with hTSH or forskolin (10µl) and incubated in a humidified incubator as before, for 1 – 4 days. After the initial incubation, 20µl of the tetrazolium salt colorimetric system [MTS (4.1mM) / PMS (0.15mM)] was added to each well. The plates were then further incubated in a dry 5% CO_2 / 95% air incubator at 37°C, for 1

– 3hr. The colorimetric reaction shifted the colour from yellow to orange/red and this was measured using a microtiter plate reader (Bio-Rad, Model 3550, Richmond, CA). O.Ds. were measured at a wavelength of 490nm using a reference wavelength of 655nm (Goodwin CJ *et. al.*, 1995). Although successive readings were routinely made every 30min, the results read at 180min were usually used for final response assessments.

6.2.3 Quantitative assessment of assay performance characteristics

The results for both the cAMP bioassays and the tetrazolium salt colorimetric assays are expressed as means ± standard deviations (S.D.) of triplicate cultures, unless stated otherwise. For both assays, the initial results were in the form of O.D.. However in the cAMP bioassays, O.Ds. were interpolated from the standard curve so as to be expressed as cAMP concentrations (pmol/ml).

6.3 RESULTS

6.3.1 cAMP Bioassays

RESPONSE TO hTSH 80/558

Both JP_{26} and $JP_{26/26}$ cell lines responded to TSH in terms of a dose-related rise in intracellular cAMP (Figure 6.1). With the highest dose tested (500mU/l), the JP_{26} cells gave the greatest cAMP accumulation of 100.0 ± 13.9 pmol/ml, which was a 9-fold increase above the zero control response (11.5 ± 0.4 pmol/ml). In contrast, with the $JP_{26/26}$ cells, the maximal response (79.3 ± 7.6 pmol/ml) was achieved by a 10-fold

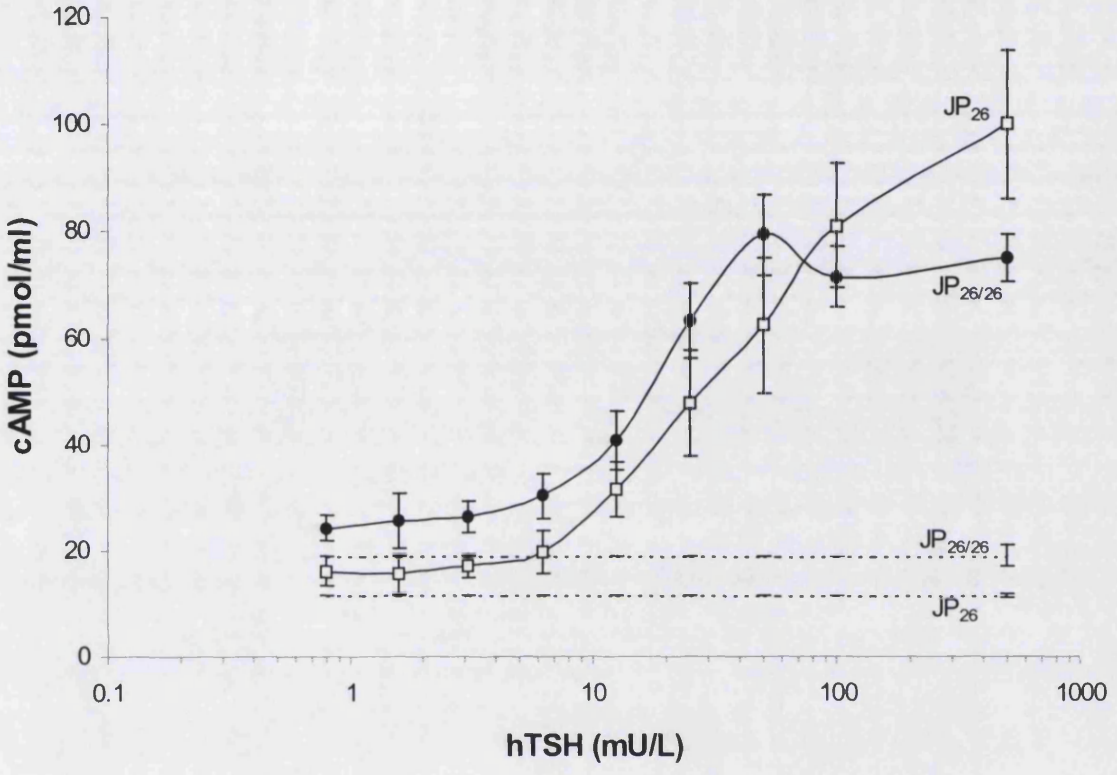


Figure 6.1 **The accumulation of cAMP in CHO cell lines JP_{26} (□) and $JP_{26/26}$ (●) in response to increasing concentrations of the Second International Standard Preparation of hTSH (80/558).** --- represents the unstimulated control microcultures. Results are the mean ± S.D. for each set of triplicate microcultures exposed to each dose.

lower stimulator dose (50mU TSH/L). However, this was only a 4-fold increase above the zero control response of 19.0 ± 2.1 pmol/ml.

RESPONSE TO FORSKOLIN

Forskolin, an AC activator, stimulates the increase in intracellular cAMP by directly activating the catalytic subunit. This mechanism is independent of both the hTSH receptor and the G-proteins. The dose-response curves of the two JP_{26} cell lines in response to forskolin stimulation are shown in Figure 6.2. For both JP_{26} and $JP_{26/26}$ cells the greatest rise in cAMP was obtained with the highest dose of forskolin tested (100μM). This represented a 73-fold increase above the control response (2.8 ± 0.5 pmol/ml) for the JP_{26} cells.

RESPONSE TO THYROID STIMULATING ANTIBODIES

TSAb preparations were tested on JP_{26} cells. Responses to the two alternative International Reference preparations, LATS-B 65/122 and TSAb 90/672, were compared. These are both well characterized preparations of autoantibodies that stimulate the human TSHR resulting in an increase in intracellular cAMP. The dose-response curves for both are shown (Figure 6.3a & b). The greatest responses observed were 240.0 ± 16.7 pmol/ml at the concentration of 5.0mU/ml and 303.0 ± 15.5 pmol/ml at the concentration of 12.5mU/ml for LATS-B 65/122 and TSAb 90/672 respectively. However, the curves showed no evidence of approaching a plateau hence implying that even higher responses could be obtained at higher concentrations of these antibodies. These responses represented a 34- and 95-fold

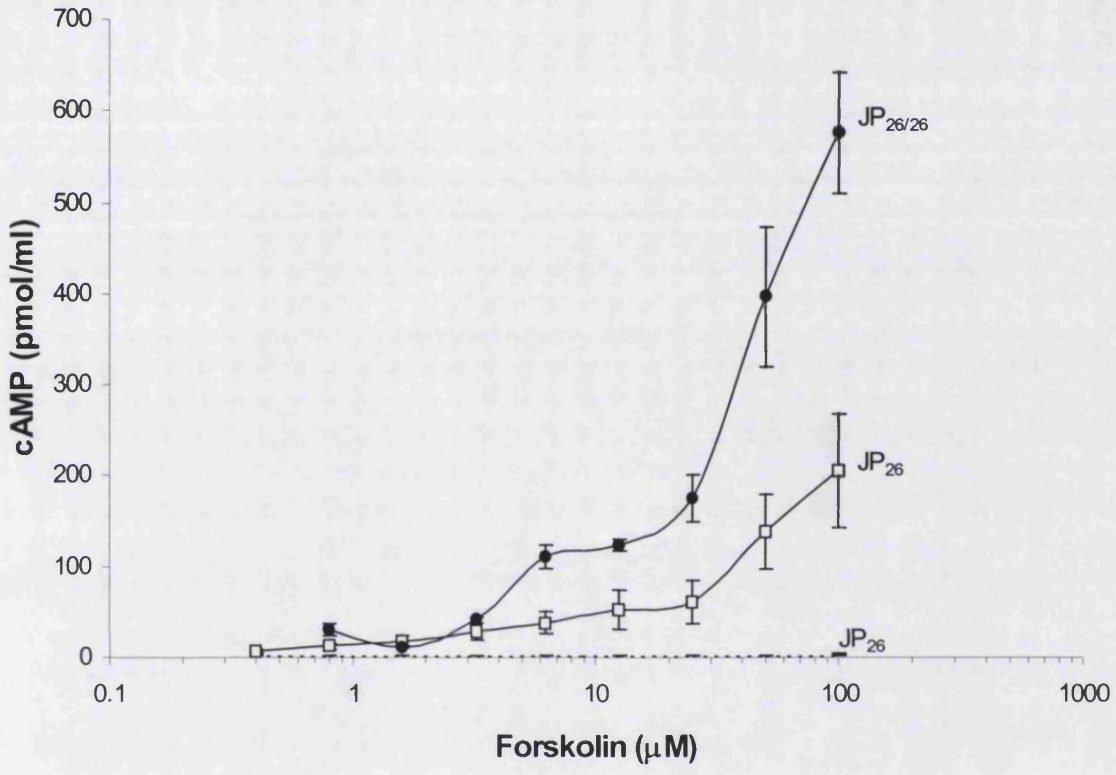


Figure 6.2 **The accumulation of cAMP in CHO cell lines JP_{26} (□) and $JP_{26/26}$ (●) in response to increasing concentrations of forskolin**. --- represents the unstimulated control microcultures for the cell line JP_{26} only. An unstimulated control was not obtained for the cell line $JP_{26/26}$. Results are the mean ± S.D. for each set of triplicate microcultures exposed to each dose.

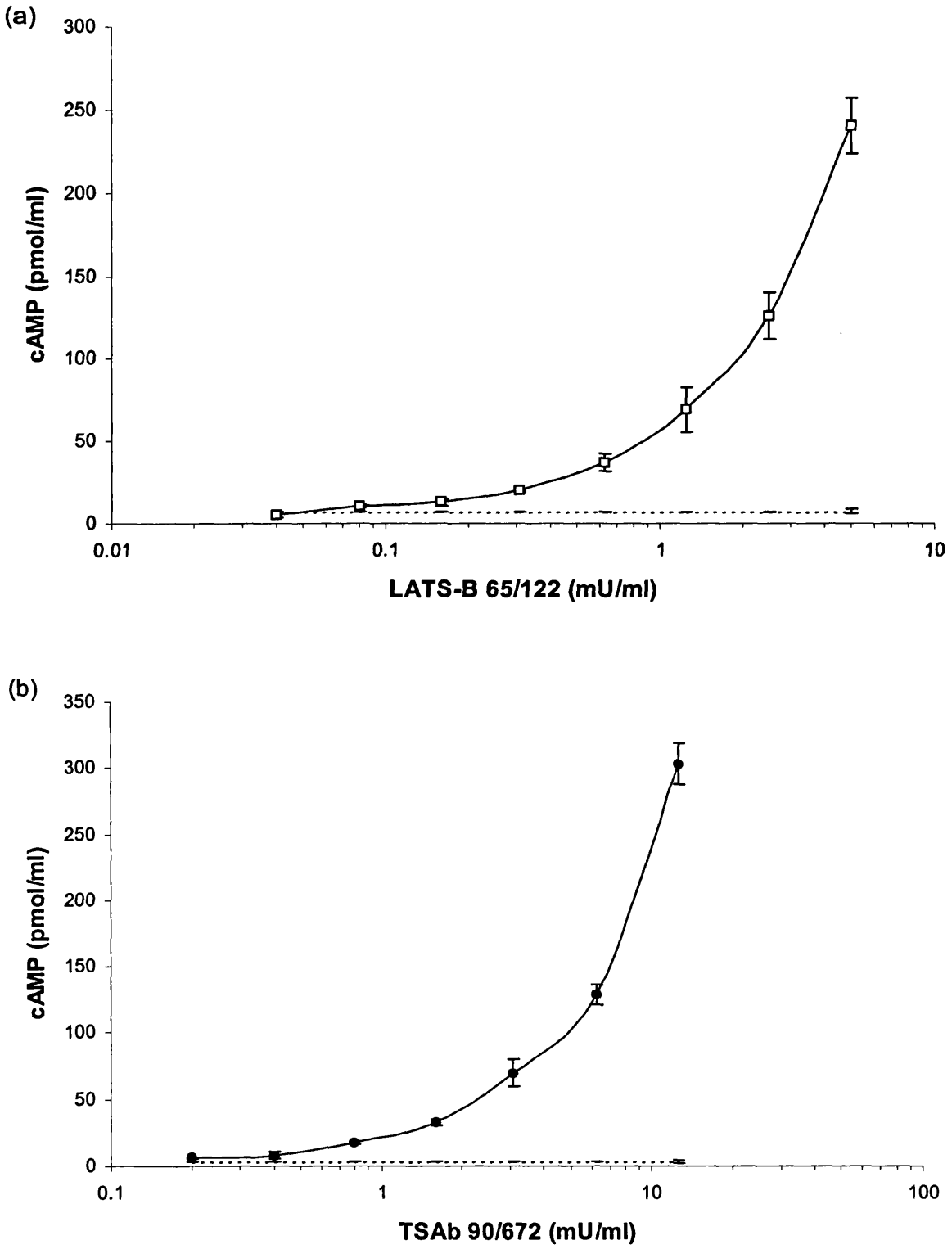


Figure 6.3 **The accumulation of cAMP in CHO cell line JP_{26} in response to increasing concentrations of thyroid stimulating antibodies: (a) MRC LATS-B Standard coded 65/122 (□) and (b) First International Standard for TSAb coded 90/672 (●).** --- represents the unstimulated control microcultures. Results are the mean ± S.D. for each set of triplicate microcultures exposed to each dose.

increase above the zero control responses (7.1 ± 1.3 and 3.2 ± 0.8 pmol/ml) of LATS-B 65/122 and TSAb 90/672 respectively.

Possible synergy between forskolin and TSAb was tested by the co-incubation of a low dose of forskolin (0.8μM), together with increasing doses of the TSAb standard 90/672 (Figure 6.4). No significant enhancement of the responses to lower doses of TSAb 90/672 (0.2 – 1.6mU/ml) was obtained in the presence of the low dose of forskolin. There was a slight decrease, rather then increase, in cAMP accumulation at the higher doses of TSAb 90/672 (3.1 – 12.5mU/ml). Without forskolin, the greatest response to 12.5mU/ml TSAb 90/672 was 303.3 ± 15.5 pmol/ml and with forskolin, the response was only 245.0 ± 4.2 pmol/ml. Thus this low dose of forskolin did not synergise with TSAb.

6.3.2 Tetrazolium Salt Colorimetric Assays

RESPONSE OF THE CELL LINE $JP_{26/26}$ TO hTSH 80/558 AND FORSKOLIN

Increasing concentrations of hTSH 80/558 did not induce in a rise in formazan production by $JP_{26/26}$ cells, after an incubation of 48hr (Figure 6.5a). On the contrary, there was a 13.7% decrease from the zero control O.D.x100 of 205 ± 2.5 to 177 ± 2.5 at the concentration of 100.0mU/L. A similar lack of response to forskolin was obtained after 48h incubation (Figure 6.5b). At the highest forskolin concentration (100μM), there was a 15.3% decrease in O.D.x100 from 202 ± 1.1, the zero control response, to 171 ± 10.9.

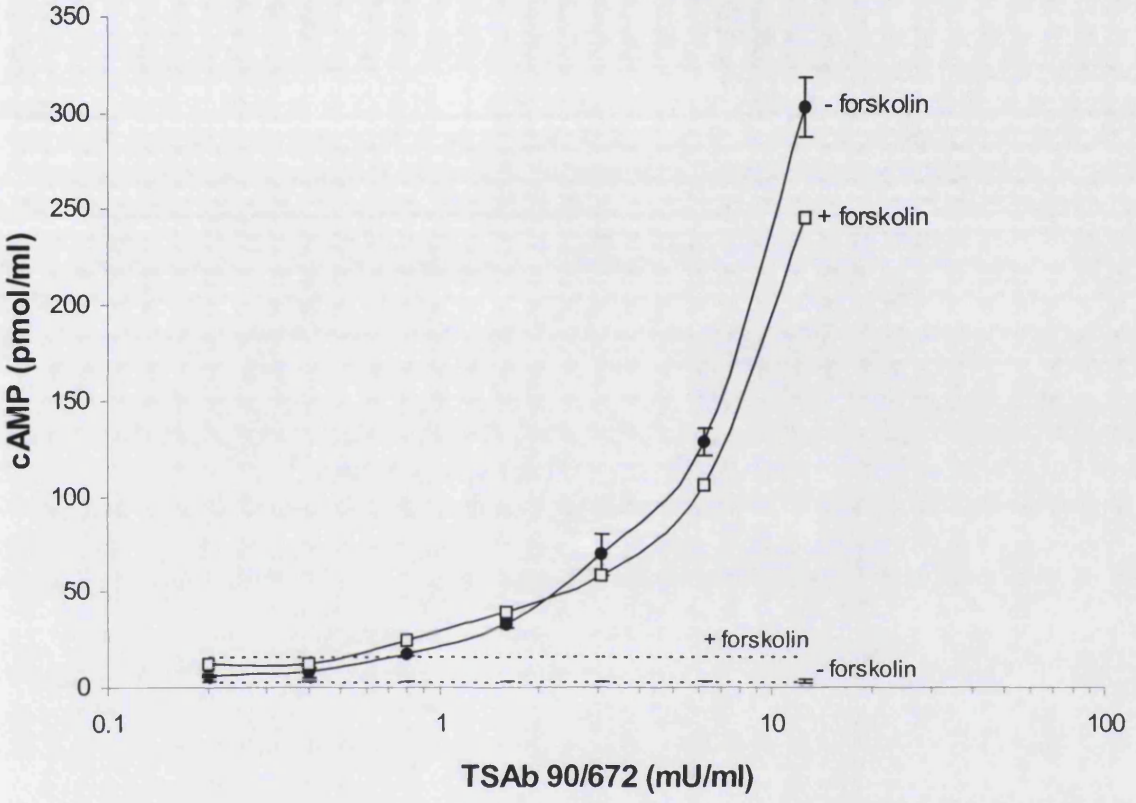


Figure 6.4 **The accumulation of cAMP in CHO cell line JP_{26} in response to increasing concentrations of the First International Standard Preparation TSAb (90/672) in the presence (□) and absence (●) of 0.8μM forskolin**. --- represents the unstimulated control microcultures. In the absence of forskolin, results are the mean ± S.D. for each set of triplicate microcultures exposed to each dose whereas the results are only the mean for each set of duplicate microcultures in the presence of forskolin.

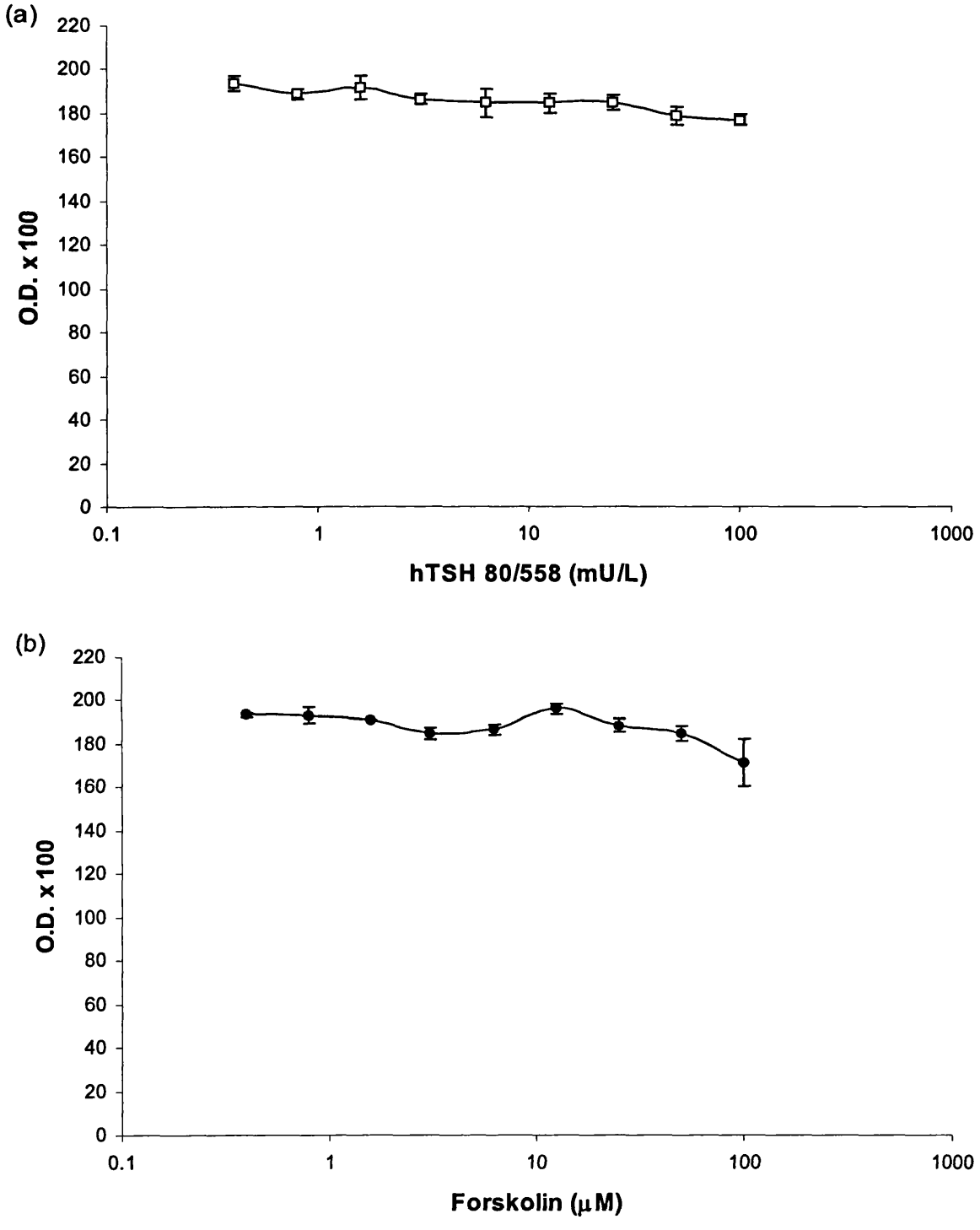


Figure 6.5 **MTS-formazan production by the CHO cell line $JP_{26/26}$ in response to increasing concentrations of (a) hTSH (80/558; □) and (b) forskolin in a 1% DMSO vehicle (●).** The optical densities (O.D.x100) are the mean ± S.D. determined from three replicate microcultures used for each dose of stimulator. Cells were incubated with the stimulators for 48hr in a culture medium (see Section 5.1.2) supplemented with 5% of horse serum instead of 10% FCS.

Extended investigations were made with the MTS assay, but the results are not detailed here. In brief, selected combinations of FCS and horse serum were tested in the culture medium to determine whether conditions could be devised which could support a positive response to TSH and forskolin in terms of MTS-formazan production. We concluded that this was not possible and that intracellular cAMP is, in fact, a negative regulator for formazan production in CHO cells.

6.4 CONCLUSIONS

In this chapter, we have summarized our preliminary work performed on CHO cell line JP_{26} and its sub-clone $JP_{26/26}$. Several previous studies have already characterized these and other CHO cell lines transfected with the hTSHR (Perret J *et. al.*, 1990; Ludgate M *et. al.*, 1990; Costagliola S *et. al.*, 1992; Vitti P *et. al.*, 1993). We have confirmed that these CHO cell lines responded positively to TSH, TSAb and forskolin stimulation with increases in intracellular cAMP. We also investigated the application of the MTS assay system to these CHO cell lines.

The results from the cAMP bioassays clearly showed positive responses to all three stimulators (Figures 6.1, 6.2, & 6.3). The results thus supported the rationale of using these CHO cell lines for the development of a new *in vitro* bioassay for TSAb. Although both cell lines responded to stimulation, the magnitudes of response were different. In preliminary experiments (not shown), we found that a first batch of $JP_{26/26}$ cells responded well to forskolin but not TSH. This suggested that although the AC system was present, these cells had lost their TSHR. The results shown here were all obtained with second batches of cells, freshly obtained from Brussels, which responded both to the hormone and the diterpene. We could not confirm a synergistic

effect with a low dose of forskolin (Figure 6.4), which had been reported by Perret and colleagues (Perret J *et. al.*, 1990). They claimed, though data was not shown, that the presence of forskolin at concentrations ≤100nM potentiated responses to TSH in these cell lines. The potentiation of responses by forskolin has also been reported in other systems. For example in rat FRTL-5 thyroid cells (Ealey PA *et. al.*, 1987) and dog thyroid slices (Van Sande J *et. al.*, 1983). Such potentiation would prove beneficial in the development of any bioassay for thyroid stimulators, since it might lower the detection limit.

Experiments with the MTS assay system gave unexpected results. Our investigations showed that, unlike the FRTL-5 cells (Ealey PA *et. al.*, 1988), no positive response could be obtained with the MTS assay applied to CHO cells in response to TSH or forskolin. In the FRTL-5 cells, increased intracellular cAMP acts as a positive regulator of mitogenesis leading to increased cell metabolism and division. This induced increased formazan production and hence the assoicated increase in O.D. could be used as a bioassay end point (Marshall NJ & Ealey PA, 1986; Ealey PA *et. al.*, 1988). In contrast, in CHO cell lines, cAMP appears to be an anti-mitogen, since both TSH and forskolin, when added at concentrations which we have demonstrated increase cAMP (Figure 6.1 & 6.2), resulted in a slight decrease in formazan production (Figure 6.5a & b). This confirmed an observation by Roger PP *et. al.* (1995) that cAMP was a negative regulator of mitogenesis in CHO cells.

Extensive work in which we manipulated the serum conditions failed to yield a formulation which resulted in TSH or forskolin stimulating cell proliferation, as reflected by increased MTS-formazan production. This supported our conclusion that

cAMP was a weak anti-mitogen for CHO cells. Elimination of all serum from the medium resulted in cell death. Increased serum concentrations in the medium merely resulted in greater baseline MTS-formazan production by the reducing components present in the medium itself, even in the absence of the cells. We concluded that it was not possible to establish a microculture tetrazolium salt assay (MTA) for thyroid stimulators using these transfected CHO cells. However, our results did confirm one attractive feature of the MTA system, which was its precision. This is evident in a comparison between Figures 6.1 – 6.3, which rely upon the cAMP immunoassay for their end point and Figure 6.5 which is obtained with the MTA. The errors of response are considerable for the former but consistently reduced for the MTA. Finally, we concluded that because of the larger fold-stimulation in response to TSH (Figure 6.1), the JP_{26} cell line was preferable for the future development of bioassays for thyroid stimulators.

Section 7

The construction of the pGL3hαCRE, cAMP responsive, Luciferase Reporter Gene Plasmid

7.1 INTRODUCTION

The aim of this project is to develop a clinical diagnostic bioassay for TSAb in Graves' disease patients. This bioassay will use a cell line transfected with the human TSHR and a cAMP responsive luciferase reporter plasmid. Albanese and colleagues have established the principle of the use of the luciferase reporter gene for a bioassay (Albanese C *et. al.*, 1994), when they exploited the firefly luciferase reporter gene in a bioassay for FSH (see Section 4.4).

The generation of the stable CHO cell line JP_{26} expressing the human TSHR (Perret J *et. al.*, 1990) has led to the prospect of a TSAb luciferase luminescence bioassay. The characterization of the JP_{26} cell line was reported in Results (see Section 6). The cells were found to respond to hTSH, forskolin and TSAb stimulation with dose-dependent increases in intracellular cAMP.

As mentioned above, the TSAb bioassay will require the JP_{26} cell line to be transfected to express a cAMP responsive luciferase reporter plasmid in addition to the human TSHR. A 224bp fragment of the promoter region of the hGPHα gene (-180 to +44) was used in the FSH bioassay developed by Albanese and colleagues (Albanese C *et. al.*, 1994). In view of the success of this luminescence bioassay, a similar region was selected for the pGL3hαCRE plasmid. In this section, we describe

the detailed construction of the pGL3hαCRE cAMP responsive luciferase reporter plasmid which will then be stably transfected into the JP_{26} cells.

7.2 MATERIALS AND METHOD

7.2.1 Materials

The plasmids pGL3 Basic, pGL3 Promoter and pGEM-T Easy vector and also Bioexact taq polymerase were obtained from Promega, Madison, Wisconsin, USA. All the restriction enzymes and their buffers were obtained from either Promega or New England Biolabs, Beverly, MA, USA. The primers PR1-3 were custom made by Cruchem, Glasgow, UK. IBMX, a cyclic nucleotide phosphodiesterase inhibitor, was obtained from Sigma Chemical Co., Poole, UK. Forskolin, an AC activator was obtained from Calbiochem, La Jolla, CA, USA. It was dissolved in culture medium (see Section 5.1.1) supplemented with DMSO giving a stock concentration of 1mM of forskolin in 10% DMSO. The hTSH used was the 2nd International Reference Preparation of TSH, human, for immunoassays, coded 80/558 which has an assigned biological potency of 37mU/ampoule. For use, the preparation was reconstituted in hypotonic medium (see Section 6.2.1), pH 7.4 with 0.4% BSA to yield an initial concentration of 1U/L. Wizard PCR Clean Up Kit, Wizard Plus Maxiprep DNA Purification System and the Luciferase Assay System with Reporter Lysis Buffer were obtained from Promega, Madison, Wisconsin, USA. The Galacto-Light™ chemiluminescence reporter assay for the detection of β-galactosidase was obtained from Tropix, Bedford, UK.

7.2.2 Methods

7.2.2.1 Designing primers

Primers are short DNA fragments complementary to the target molecule. One primer is needed for each strand of the double helix. These primers mark the region to be amplified. Therefore, the sequence of the targeted fragment must be known in order to design the primers. Primers PR1, 2 & 3 were used in the construction of the luciferase construct pGL3hαCRE. Primers or oligonucleotides (oligos) are required for the Polymerase Chain Reaction (PCR), which is used to amplify specific DNA fragments with DNA polymerase. Hybridization of primers to the DNA molecule is essential for DNA synthesis.

A PCR protocol follows a repeated cycle. Each cycle consists of three stages. First, denaturation of the double stranded DNA template occurs at 94°C for ~1min. This separates and releases the two single strands. This is followed by the second, annealing stage. Once the strands have been dissociated, the primers hybridize to the templates at a predetermined annealing temperature. This stage, again, takes ~1min. Once they have bound, DNA synthesis occurs. This is referred to as the extension stage, and relies upon the added DNA-polymerase. This stage is usually set for ~2min. However, the duration depends on the length of the fragment to be synthesised. The cycle is repeated ~30 times. This results in amplification of the target DNA molecule.

A successful PCR depends on correctly designed primers. The sequences of the primers are not difficult to determine, as they have to be complementary to their target templates. However, the primer has to satisfy other specifications. These include:

1. A primer should be between 17 – 24 bases long. A minimum of 17 bases (17-mer) is required for DNA specificity. This is based upon the calculated statistic that the expected frequency of a 17-mer occurs only once in the human genomic DNA. Hence there is one hybridization site for the 17-mer thus ensuring specificity. The upper limit of 24 bases is not definitive. However, the rate of hybridization decreases with increasing length, so that if the primers are too long, the rate of hybridization and consequently PCR efficiency is reduced.
2. Primers should have ~50% G and C bases. This affects the annealing temperature (see below).
3. There should be no internal complements, since this would cause the primer to "fold" and hybridize to itself.
4. The ends of the primers should not be complementary either, since this could result in primers annealing to each other.
5. The melting temperature (Tm) of the primers selected should ideally be identical, or not more than 2°C different. The normal range is between 55 – 72°C. The upper limit, 72°C, is the optimum temperature for Bioexact *Taq* polymerase which was used for all our PCR protocols. Unlike *Taq* polymerase, Bioexact *Taq* polymerase has the ability to proofread and correct mistakes.
6. Tm is highly significant as it determines the annealing temperature. The annealing temperature is usually 2 – 5°C lower than the Tm. If the annealing temperature is too high, it will obstruct primer hybridization, while too low a temperature causes

stable mismatched hybrids to form. Both will result in the failure of the PCR. The Tm is determined using the formula -

$$Tm = (4 \times [G + C]) + (2 \times [A + T])^{\circ}C$$

All the primers PR1, 2 & 3 were designed according to these principles. They were complementary to the targeted regions of the hGPHα gene (Figure 7.1). PR1 & 2 were used in the amplification of the CRE promoter. Primer PR2 has a complementary copy of the *Bam*HI (GGATCC) restriction enzyme site (+44). Primer PR1 has a synthetic *Kpn*I restriction site (-192) added on to the 5′ end of the primer fragment. This restriction site was amplified as part of the primer. Primer PR3 is an internal primer immediately inward to PR1. This primer was used in another PCR to check successful recombinant colonies. Details of the primers are summarised in the Table 7.1.

7.2.2.2 Construction of the pGL3hαCRE, cAMP responsive, luciferase reporter gene plasmid

The detailed construction of the pGL3hαCRE plasmid is described below. A flow-chart illustrates the multi-step process (Figure 7.2).

(I) Cloning of the promoter region of the human glycoprotein hormone α gene

The 230bp promoter region of the hGPHα gene (-186 to +44) contains the URE, two repeated 18bp CRE and a downstream region with a CCAAT motif (Delgeane AM *et. al.*, 1987; Deutsch PJ *et. al.*, 1987; Silver BJ *et. al.*, 1987; Jameson JL *et. al.*, 1988). It was cloned and amplified by PCR using human genomic DNA together with primers PR1 and 2. Bioexact *taq* polymerase was used for this reaction. Primer PR2 has a

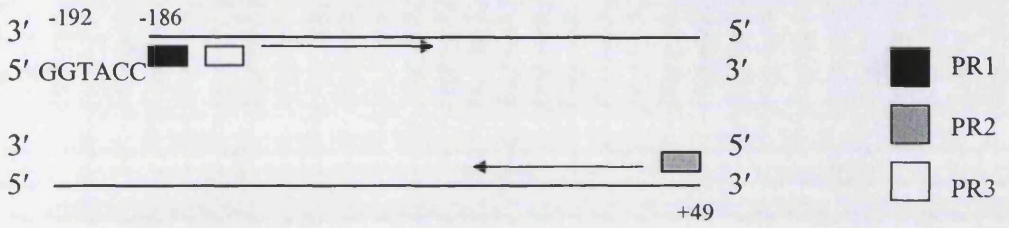


Figure 7.1 **A schematic representation of the targeted positions of the primers PR 1 – 3 in reference to the promoter region of the hGPHα gene**. They are complementary to the targeted regions of the gene.

Primer	No. of bases	Sequence (5′ → 3′)	Position in reference to target DNA	% of GC content	Tm (°C)
PR1	22	GGTACCCCAAACAAAAAT GACC	-192 to -171	45.5	64
PR2	22	GGATCCGAAGAGGGATTT TAGC	+28 to +49	50	66
PR3	24	TAAGGGTTGAAACAAGAT AAGATC	-170 to -147	33.3	64

Table 7.1 **A detail description of the three primers designed for the construction of the pGL3hαCRE reporter plasmid**. The primers PR1 – 3 are targeted to a specific promoter region of the hGPHα gene. They were designed in accordance with the principles outlined in the text.

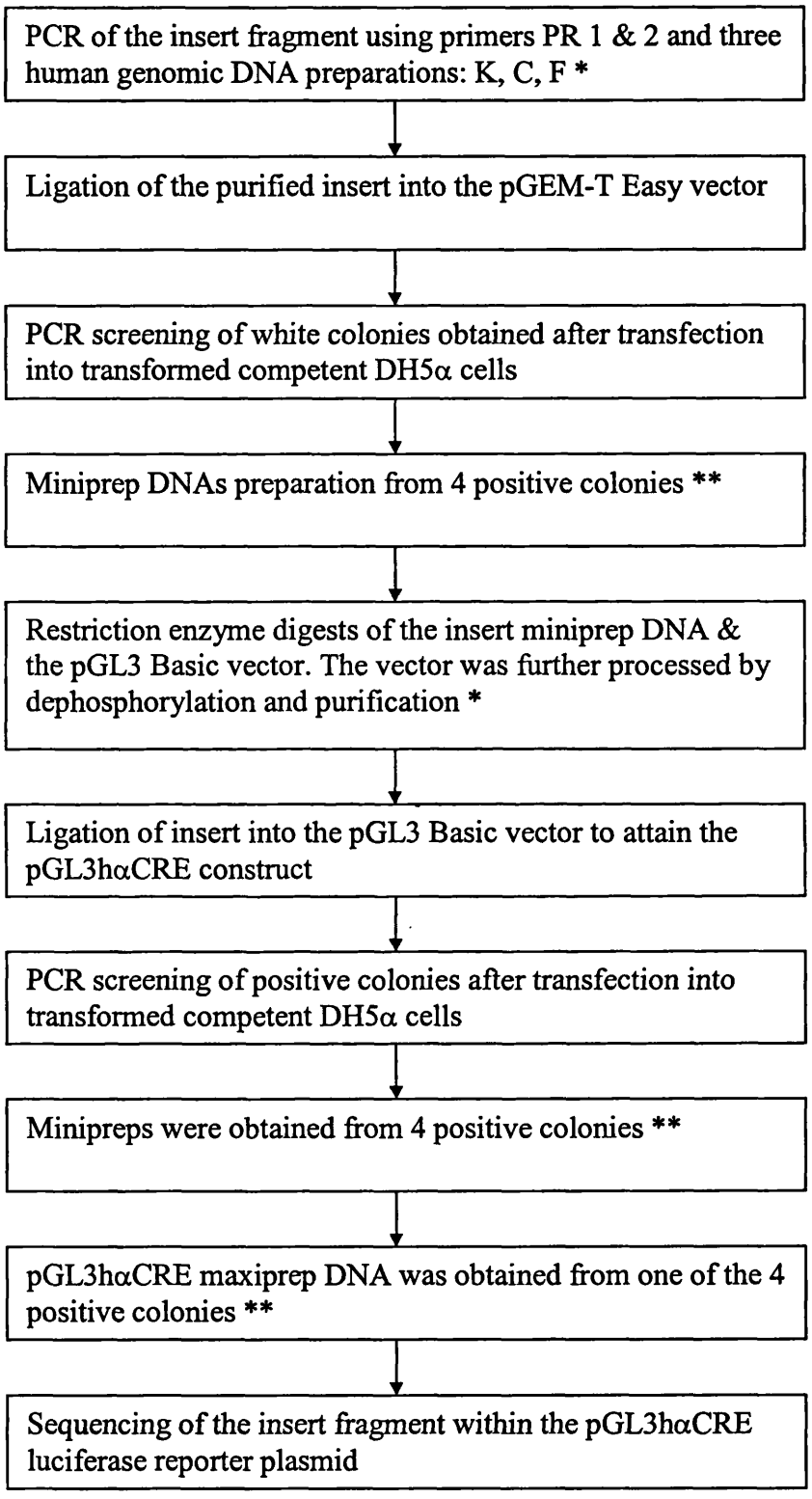


Figure 7.2 **Outline of the procedure used to construct the pGL3hαCRE luciferase reporter gene plasmid.** * denotes that both the restriction enzyme diagnostic digest and the purification of DNA were performed following the procedures detailed in the text whereas ** denotes that only the restriction enzyme diagnostic digest was performed.

complementary copy of the *Bam*HI site (+44) within the promoter region. Primer PR1 has a synthetic *Kpn*I site added on the 5′ end of the promoter region giving the insert a total of 241bp. The addition of the *Kpn*I site would allow the insert to be ligated into the *Kpn*I and *Bgl*II (compatible with *Bam*HI) restriction enzyme sites within the pGL3 basic vector. The PCR product was purified (Wizard PCR Clean-up kit) and ligated into pGEM-T Easy vector. This was followed by the transfection of the ligation product into transformed competent DH5α cells. Transfected cells were plated on an Ampicillin/IPTG/X-Gal agar plate. Recombinant clones were identified directly by the screening of blue/white colonies on the plate. PCR screening was performed to check the recombinant colonies (white) using the primer PR3, the internal oligo of the hGPHα insert, and the T7 oligo of the vector. Miniprep DNA was obtained from successful PCR screened colonies.

(II) Construction of a cAMP Responsive Element - Luciferase Reporter Gene

The reporter plasmid, pGL3hαCRE (Figures 7.3 & 7.4), was generated by the ligation of the cloned insert fragment (241bp) into the promoterless firefly luciferase pGL3 Basic vector. The fragment was inserted into the *Kpn*I (+5) and *Bgl*II (+36) restriction enzyme sites located upstream of the *luc*+ gene in the vector. Both the fragment and vector were prepared prior to the ligation by restriction enzyme digests. Restriction enzymes *Kpn*I and *Bam*HI were used to digest the insert miniprep DNA obtained (see above) to extract the insert fragment. The DNA of the pGL3 Basic vector was, firstly, digested by the restriction enzymes *Kpn*I and *Bgl*II. This was possible as *Bam*HI and *Bgl*II generated compatible restriction ends and would allow their ligation. The cut vector was then dephosphorylated and purified. After the ligation, the constructed plasmid pGL3hαCRE was again transfected into transformed competent DH5α cells.

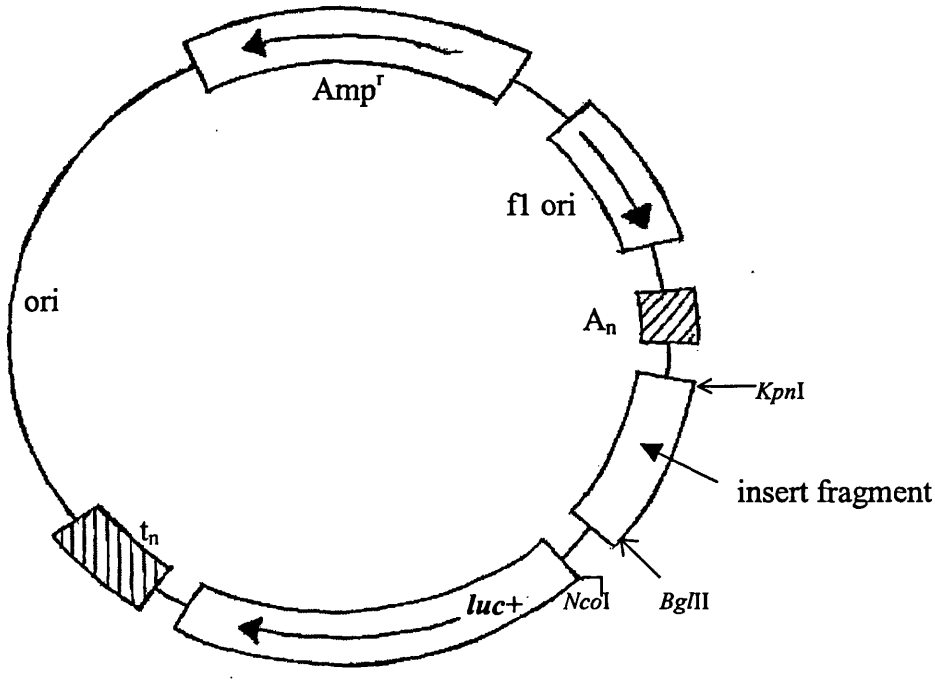
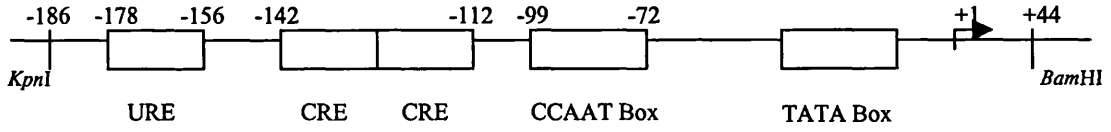


Figure 7.3 **The pGL3hαCRE reporter plasmid construct with a luciferase gene (*luc+*) and and an inserted promoter and enhancer sequence.** The insert fragment (see Figure 7.4) was ligated into restriction enzyme sites *Kpn*I and *Bgl*II. A_n and t_n represent a synthetic poly(A) signal / transcription pause site and an SV40 late poly(A) signal respectively. Amp^r is a gene conferring ampicillin resistance in *E.coli.*; f1 ori is the origin of replication derived from filamentous phage; ori is the origin of plasmid replication in *E.coli.*. Arrows within *luc+* and Amp^r gene indicate the direction of transcription, while in f1 ori, it indicates the direction of ssDNA synthesis.

(a)



(b)

*Kpn*I -186 -178 -156 -142

GGTACC CCAAACAA AAATGACCTA AGGGTTGAAA CAAGATAAAGA TCAAATTGAC GTCATGGTAA

-124 -106

AAATTGACGT CATGGTAATT ACACCAAGTA CCCTTCAATC ATTGGATGGA ATTTCCTGTT

TATA Box

GATCCCAGGG CTTAGATGCA GGTGGAAACA CTCTGCTGGT ATAAAAGCAG GTGAGGACTT

+1

CATTAACTGC AGTTACTGAG AACTCATAAG ACGAAGCTAA AATCCCTCTT CGGATCC

*Bam*HI

Figure 7.4 **(a) A detailed diagram showing the insert fragment from the 5′ promoter region of the human glycoprotein hormone α-subunit (hGPHα) gene (-186 to +44).** The insert contains a synthetic restriction enzyme site *Kpn*I and an original *Bam*HI site. It also has an upstream regulatory element (URE), two repeated 18bp cAMP response elements (CRE) and a downstream promoter region that contains a CCAAT motif. +1 is the transcription start site. This insert was ligated into restriction enzyme sites *Kpn*I and *Bgl*II of the pGL3 basic vector. NB. The *Bam*HI site is compatible with the *Bgl*II site. (b) The complete sequence of the insert fragment.

Colonised cells were grown on an ampicillin agar plate. PCR screening was performed on the colonies using the internal primer PR3 and the GL2 primer to the vector. Minipreps and maxipreps of pGL3hαCRE DNA were obtained from positive colonies (Wizard Plus Maxiprep DNA Purification System). Finally, pGL3hαCRE was sequenced to ensured base to base accuracy of the fragment which has been ligated into the pGL3 basic vector. Both the miniprep and maxiprep DNA were stored at -20°C until required for transfection into JP_{26} CHO cells.

7.2.2.3 Transient transfection studies into JP_{26} cells

Transient transfections were performed using the standard calcium phosphate technique. JP_{26} cells were plated into 6cm tissue culture dishes containing 5ml of culture medium (see Section 5.1.1). They were seeded to attain 60 – 70% confluency after 24hr. Several different DNAs, in addition to pGL3hαCRE, were included in each experiment. The pGL3 Basic and pGL3 Promoter vectors were transfected separately to function as negative and positive controls respectively. Transfections of the CMV β-galactosidase plasmid were also performed to monitor transfection efficiency. For each transfection experiment, 5μg of DNA/dish was used. The cells were incubated with the DNA-calcium phosphate precipitate in a 5% CO_2 / 95% air, water saturated, 37°C incubator. After 7 – 8hr (unless stated otherwise), the dishes were washed and replaced with fresh culture medium. 48hr after transfection, the cells were tested for their responses to forskolin and hTSH, with and without IBMX (2mM). The cells were incubated with the stimulants for 4hr (unless otherwise stated). Luminescence was detected using the Promega Luciferase Assay System with Reporter Lysis Buffer according to manufacturer's instructions. Briefly, assay medium was removed from the dish and the cells were washed twice with PBS buffer. 400μl of Reporter Lysis

buffer was added to the dish. Cell lysate and cell debris were collected into microcentrifuge tubes and placed on ice. The tubes were vortexed for 10 – 15sec and centrifuged at 12,000xg for 15sec at room temperature. The supernatants were transferred to a fresh microcentrifuge tube and stored at -70°C. To test for luminescence, 20µl of cell extract and 100µl of luciferase assay reagent (both at room temperature) were mixed in a luminescence cuvette. Light emission was measured by a luminometer for 10sec, after a 2sec delay.

7.2.3 Quantitative analysis

The results were measured as relative light units (R.L.U.). They are shown as R.L.U. for each singlicate culture and also as means ± standard deviations (S.D.) of triplicate cultures.

7.3 RESULTS

The aim was to create a firefly luciferase reporter plasmid with the specific 5′ promoter region of the hGPHα subunit gene (-186 to +44). This included the two 18bp repeats of CRE which retained cAMP responsive gene expression (Delegeane AM *et. al.*, 1987; Silver BJ *et. al.*, 1987; Jameson JL *et. al.*, 1988; Albanese C *et. al.*, 1994). Considering the success of the hGPHα subunit CRE gene promoter in the previous work, which established a bioassay for FSH, we used the same promoter region and ligated it into the pGL3 Basic vector at the *Kpn*I and *Bgl*II restriction sites. The procedure was described in Methods (see Sections 7.2.2.1 & 7.2.2.2).

7.3.1 Cloning of the promoter region of the hGPHα gene

The successful cloning and amplification of the hGPHα CRE promoter from three different human genomic DNA preparations (K, C & F), by PCR, is shown in Figure 7.5. The distinctive bands clearly showed that all three PCRs were successful but to different degrees, with K and C being more successful than F. A clear control lane (no band) indicated that there was no contamination in these reactions. The 1kb ladder further confirmed that the PCR products were of the right size, being ~ 250bp long. The PCR product from K was purified and ligated into pGEM-T Easy vector. The ligated product was then transfected into transformed competent DH5α cells. 95 white colonies on the ampicillin/IPTG/X-Gal agar plate were selected for PCR screening. The reaction was performed in a 96-well plate and the gel analysis is shown in Figure 7.6. Each lane represented a single colony cultured in separate wells. A bright distinctive band in the lanes indicated a successful recombinant colony. Miniprep DNA was prepared from 4 individual wells (2A, 3D, 5B & 6B) since each of these was a successful recombinant colony. The minipreps were examined with restriction enzyme diagnostic digests and shown to contain the recombinant pGEM-T Easy vector ligated with the insert fragment. Restriction enzymes *Kpn*I and *Bam*HI were used for the digests. These cut at their specific restriction enzyme sites in the ligated vector and the gel analysis (Figure 7.7) showed two bands: a larger vector band (~3kb) and the insert band (~250bp). This figure also clearly showed the cut miniprep DNA compared to the uncut. These restriction enzyme digests verified that successful ligation had occurred.

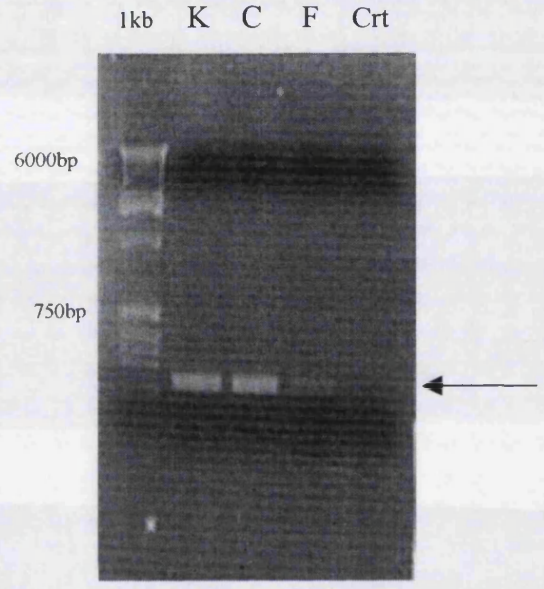


Figure 7.5 **Analysis of the cloning of the 5′ promoter region of the hGPHα gene (-186 to +44) using three different human cDNA preparations: K, C & F by PCR**. The end results were compared to a control reaction (Crt). The 1kb ladder marker shows that the bands are of the right size, i.e. ~250bp long.

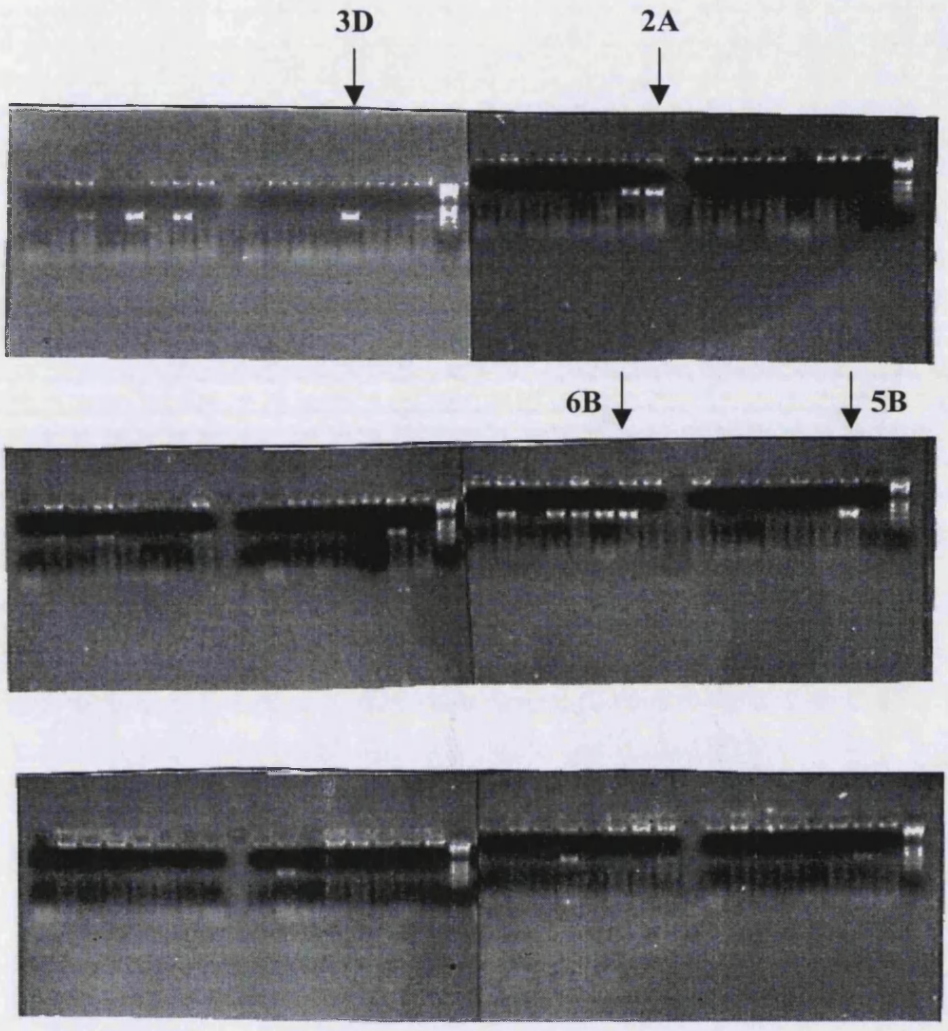


Figure 7.6 **Analysis of the PCR screening of 95 ligated pGEM-T easy vectors**. The pGEM-T easy vector was ligated with the insert and the product was transfected into transformed competent DH5α cells. Each lane represents a single colony cultured in a 96-wells plate. The highly visible and distinctive bands in a number of lanes are the PCR products indicating a positive recombinant colony. Four successful wells: 2A, 3D, 5B & 6B were further cultured to obtain miniprep DNA.

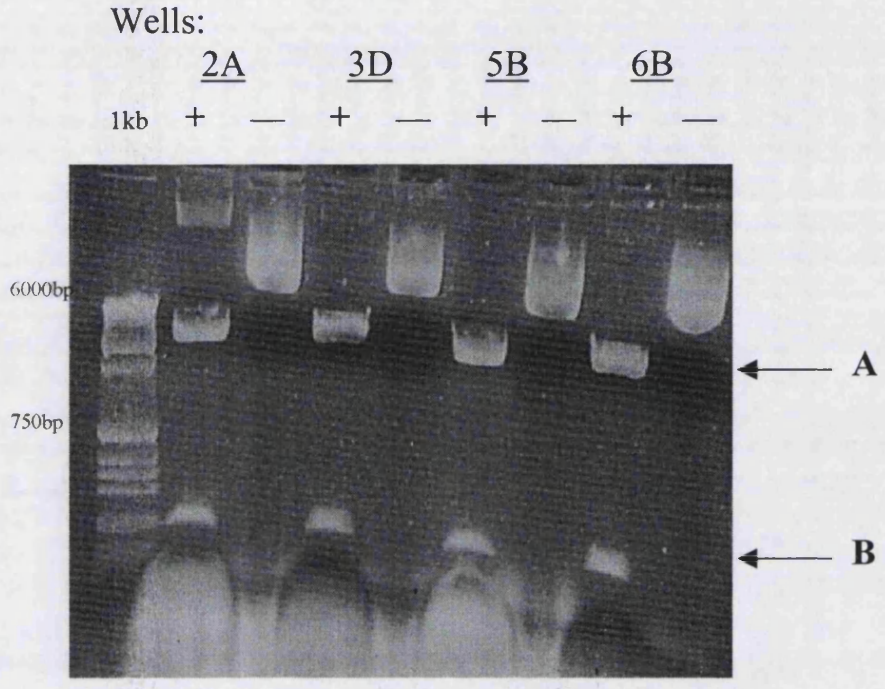


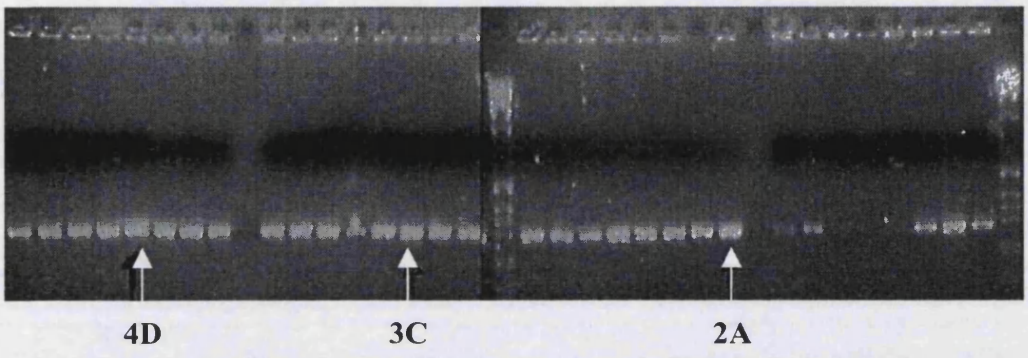
Figure 7.7 **Minipreps checked by a restriction enzyme diagnostic digest with restriction enzymes *Kpn*I and *Bam*HI**. The + and – refer to cut and uncut miniprep DNA respectively. These enzymes were expected to produce two bands. They are the vector band of ~ 3kb and the insert band of ~ 250bp; these are indicated by arrows A & B respectively. The 1kb ladder marker record the size of the two bands.

7.3.2 Construction of a cAMP Responsive Element - Luciferase Reporter Gene

Following the preparation described in the Methods (see Section 7.2.2.2), the cut pGL3 Basic vector and insert (miniprep DNA from well 2A) were ligated to form the pGL3hαCRE reporter plasmid (Figure 7.3). The 47 recombinant colonies which arose on the ampicillin agar plate were screened by PCR to ensure ligation had been achieved. Analysis of the PCR products is shown in Figure 7.8. The presence of a bright band indicated a successful recombinant colony. Minipreps were obtained from four individual wells (wells 2A, 3C, 4D & 5B). The restriction enzyme diagnostic digests of the 4 miniprep DNAs are shown in Figure 7.9. *Kpn*I and *Nco*I restriction enzymes, the latter being another restriction enzyme site within the pGL3 Basic vector, were used. The vector bands (~6kb) and the insert bands (~250bp) were clearly visible in the lanes of the digested DNA compared to the uncut preparation. This verified the positive ligation of the insert into the vector in these four colonies. Maxiprep DNA was then prepared from well 4D and the same restriction enzyme diagnostic digest check (*Kpn*I and *Nco*I) showed the two bands of the anticipated size (Figure 7.10). Finally, the resultant sequence of the insert within the pGL3hαCRE plasmid was determined by reading the consecutive distinct bands as they appeared on the film (data not shown). The sequence was verified, being 100% correct and we therefore concluded that the selected 241bp insert had been correctly ligated into the pGL3 Basic vector.

7.3.3 Transient transfection Studies

Prior to the stable transfection of the JP_{26} cells with the pGL3hαCRE construct, the plasmid was tested for its response to forskolin and hTSH stimulation. Transient



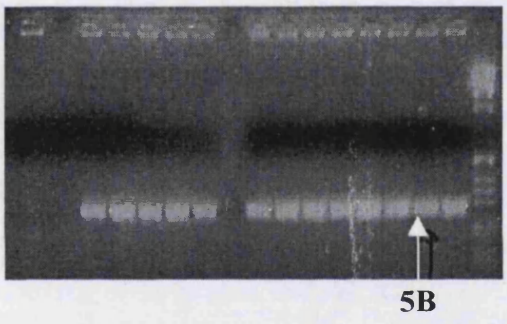


Figure 7.8 **Analysis of the PCR screening of 47 possible colonies of pGL3hαCRE.** The insert was ligated into the pGL3 Basic vector followed by transfection into transformed competent DH5α cells. Each lane represents a single colony cultured in a 96-well plate. They were compared to a control well which had not been seeded. A bright and distinctive band indicates a successful ligation of the particular colony in the well. Successful colony cultures in wells 2A, 3C, 4D & 5B were used to obtain miniprep DNA.

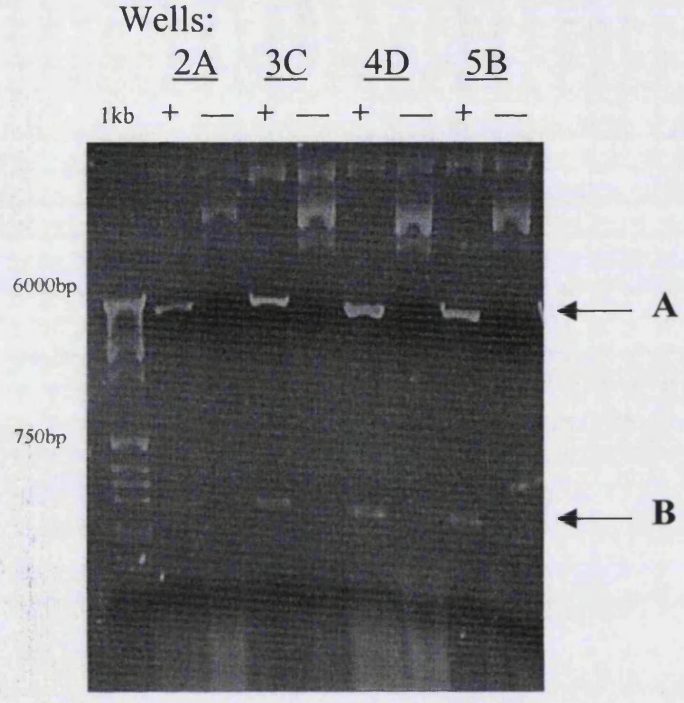


Figure 7.9 **Minipreps from wells 2A, 3C, 4D and 5B checked by a restriction enzyme diagnostic digest with restriction enzymes *Kpn*I and *Nco*I**. The + and – refer to cut and uncut miniprep DNA respectively. These enzymes were expected to produce two bands. They are the vector band of ~ 6kb and the insert band of ~ 250bp indicated by arrows A & B respectively. The 1kb ladder marker record the size of the two bands.

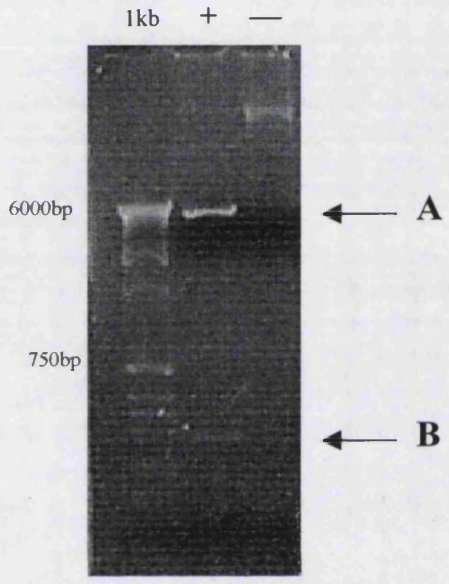
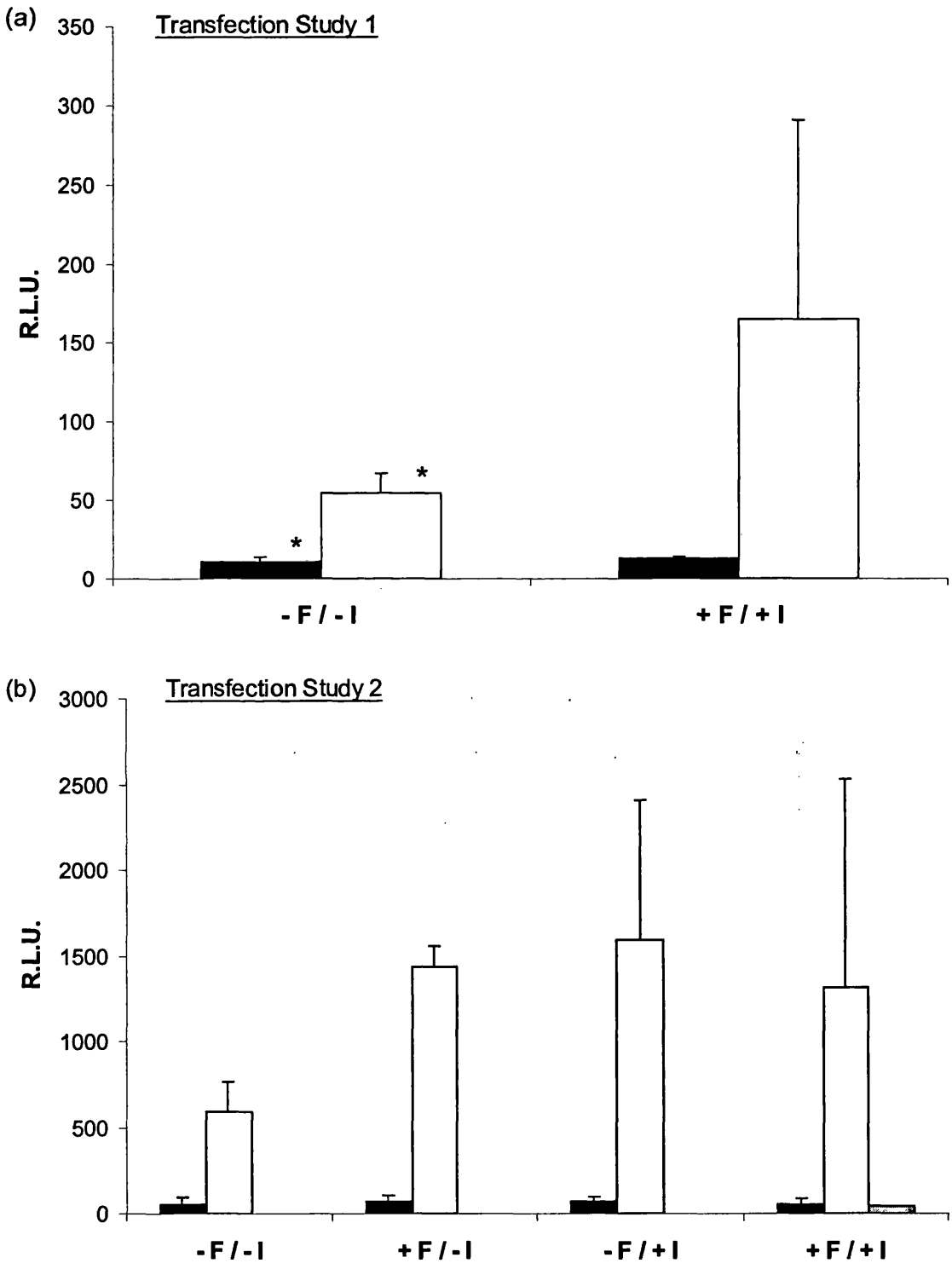
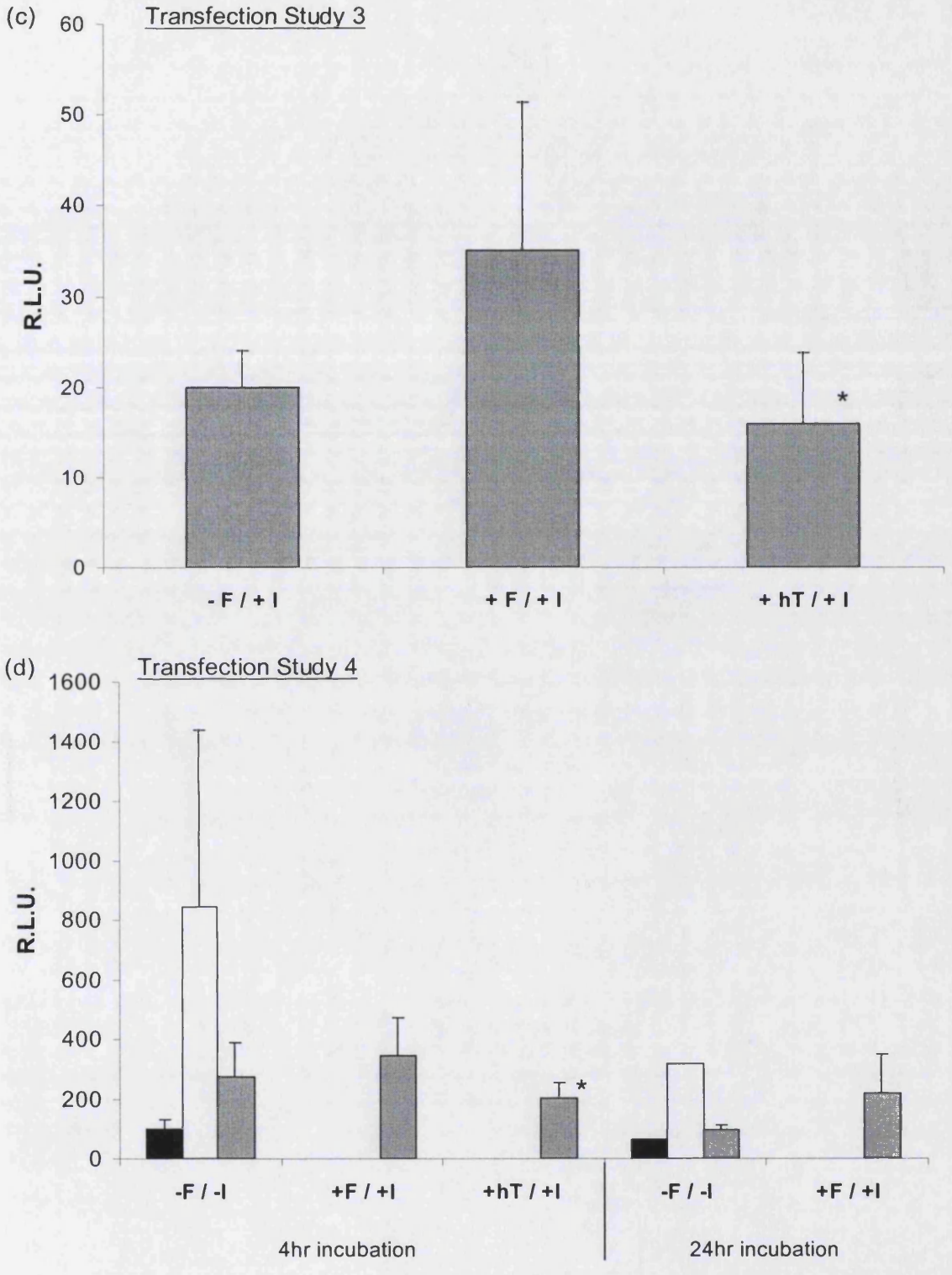


Figure 7.10 **Analysis of the restriction enzyme diagnostic digest of pGL3hαCRE maxiprep DNA**. The + and – refer to cut and uncut DNA respectively. pGL3hαCRE was cut by restriction enzymes *Kpn*I and *Nco*I. This produced two bands, the vector band of ~6kb (A) and the insert band of ~250bp (B).

transfection studies were performed and transfected JP_{26} cells were stimulated with forskolin (100μM) and hTSH (250mU/L), 48hr after transfection. The results are shown in Figures 7.11a - e. In all the experiments, a trend was observed in which the level of luminescence produced by the 3 different vectors was as follows: pGL3 Promoter > pGL3hαCRE > pGL3 Basic. In Transfection Study 1 and 2, forskolin, with and without IBMX, did not increase luminescence in cells transfected with the basic plasmid. However, a 2 to 3-fold increase above the unstimulated controls was observed from cells transfected with the promoter construct when stimulated with forskolin in the presence and absence of IBMX (Figures 7.11a & b). Slight increases in luminescence, these being 1.8- and 1.3-fold, were observed in response to forskolin (+IBMX) in Transfection Studies 3 and 4a respectively (Figures 7.11c & d). However in the same studies, hTSH stimulation, in the presence of IBMX, resulted in a decrease rather than an increase in light output. In addition, a 2.3-fold increase in R.L.U was observed in the cells transfected with the pGL3hαCRE when the incubation with forskolin (+IBMX) was prolonged to 24hr (Figure 7.11d). In Transfection Study 5, we examined whether the duration in which the cells had been in culture would affect transfection and the subsequent responses to stimulation. In Study 5(i) and 5(ii), the JP_{26} cells had been routinely passaged for 3 and 22 times respectively (see Chapter 2 Section 5.5.2). Forskolin (+IBMX) stimulation resulted in a mixed response. The light outputs obtained were 1.3- and 0.7-fold of the control, i.e. they were increased and decreased, in Studies 5(i) and 5(ii) respectively. Light outputs to IBMX alone (-hT/+I) were unexpected only ~12 – 29% of the unstimulated control responses (-F/-I). Consequently, a 9.5- and 3.2-fold increase above these control respones (-F/-I) was observed in response to hTSH (+IBMX) in Transfection Studies 5(i) and 5(ii) respectively (Figure 7.11e). However, none of the results obtained were



Figures 7.11 **Increases in light output in (a) Transfection Study 1 and (b) Transfection Study 2.** Cells were transiently transfected with pGL3 Basic (■), pGL3 Promoter (□) and pGL3hαCRE (▣) DNA. Transfected cells were exposed to forskolin (100μM) in the presence and absence of IBMX (2mM) for 4hr. Duplicate* and triplicate cultures were used. The mean ± S.E.M. and mean ± S.D. are plotted for duplicate and triplicate cultures respectively. Key: Forskolin (F), IBMX (I), + and – indicated the presence and absence of the stimulator respectively.



Figures 7.11 **Increases in light output in (c) Transfection Study 3 and (d) Transfection Study 4**. Cells were transiently transfected with pGL3 Basic (■), pGL3 Promoter (□) and pGL3hαCRE (■) DNA. Transfected cells were exposed to forskolin (100μM) or hTSH (80/558; 250mU/L) in the presence and absence of IBMX (2mM) for 4hr or 24hr as indicated. Duplicate* and triplicate cultures were used. The mean ± S.E.M. and mean ± S.D. are plotted for duplicate and triplicate cultures respectively. If not indicated, the error bar was within the symbol. Key: Forskolin (F), IBMX (I), hTSH (hT), + and – indicated the presence and absence of the stimulator respectively.

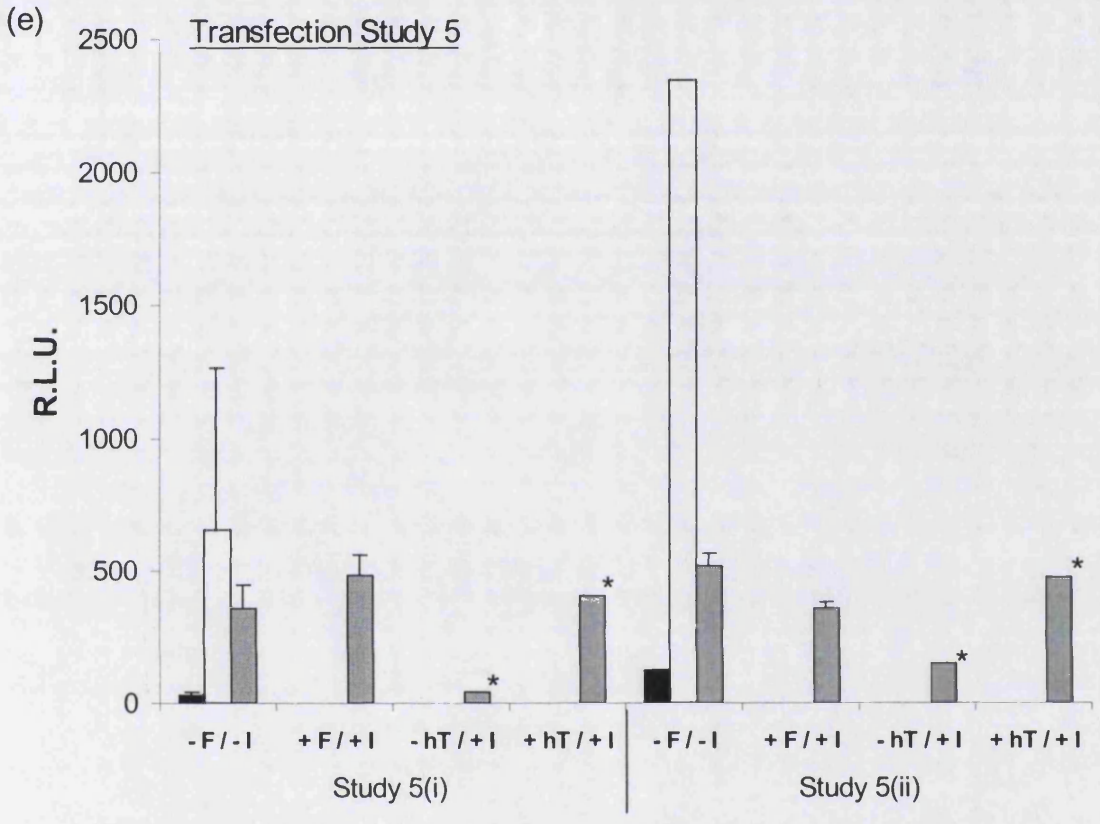


Figure 7.11e **Increases in light output in Transfection Studies 5 (i) & (ii)**. Cells were transiently transfected with pGL3 Basic (■), pGL3 Promoter (□) and pGL3hαCRE (■) DNA. Transfected cells were exposed to forskolin (100μM) or hTSH (80/558; 250mU/L) in the presence and absence of IBMX (2mM) for 4hr. Duplicate and single* cultures were used. The mean ± S.E.M. are plotted for duplicate cultures. * A singlicate culture was used for all the hTSH stimulation investigations. Key: Forskolin (F), IBMX (I), hTSH (hT), + and – indicated the presence and absence of the stimulator respectively.

considered to be significantly different due to the large errors incurred with the assay system.

7.4 CONCLUSIONS

We have outlined the construction of the pGL3hαCRE luciferase reporter plasmid. The procedure and the critical checks performed to ensure that the desired promoter region of the hGPHα subunit gene (-186 to +44) was inserted have been described in detail. We demonstrated that the final pGL3hαCRE maxiprep DNA had the insert ligated into the two specific restriction enzyme sites, *Kpn*I and *Bgl*II, within the pGL3 basic vector (Figures 7.7, 7.9 & 7.10). The final sequencing of the insert revealed that there was the correct sequence within the designated positions of the plasmid, using two internal oligos of the pGL3 Basic vector (data not shown). The next step was the stable transfection of the CHO cell line JP_{26} with the new construct.

The pGL3hαCRE construct was assessed by transient transfection luciferase assays. The reporter gene plasmid was transfected into JP_{26} cells and tested for responses to forskolin and hTSH in the presence and absence of IBMX (2mM). In addition, two contrasting control plasmids, pGL3 Basic and pGL3 Promoter, were also included in these experiments. Since the pGL3 Basic vector lacked a eukaryotic promoter, low levels of luminescence would be anticipated, whist in contrast, the pGL3 Promoter plasmid contained an SV40 promoter upstream of the luciferase gene, and therefore, higher levels of luminescence would be expected. It was therefore notable that light emitted by the positive pGL3 Promoter control vector was generally the greatest whereas the negative pGL3 Basic plasmid yielded the lowest levels of luminescence. Since there was no cAMP responsive promoter in both of these vectors, stimulation

by forskolin and hTSH should have no effect upon the responses. Therefore, stimulation of these vectors in Transfection Studies 1 and 2 was inappropriate.

Despite several attempts, an increase in luminescence following hTSH stimulation was only observed in transient Transfection Study 5. In Study 5(i), there was a 9.5-fold (401 R.L.U.) increase above the control light output in the presence of IBMX (42 R.L.U.) in response to the hTSH. Whereas, a 3.2-fold increase (474.3 R.L.U.) above the control response in the presence of IBMX (148.8 R.L.U.) was observed in Study 5(ii) when stimulated with hTSH (250mU/L). These increases in light output were only observed when cells stimulated with IBMX (2mM) alone were used as the controls. It may be significant that improved responses were observed with the cells in Study 5(i), which were in culture for only a short duration (3 passages), compared to Study 5(ii) which had been passaged for 22 times. No increases in light production were observed when compared to cells incubated in the absence of IBMX. We could not explain why the addition of IBMX would result in decreased luminescence. Furthermore, since in this experiment only single cultures were used, no estimate of replicate error could be made. However increases in light production in response to forskolin stimulation were observed in Transfection Studies 3 and 4, but large replicate errors were experienced. Despite the evidence to support that the correct promoter region had been inserted into the pGL3hαCRE plasmid, these results with the transient transfections did not give a strong indication that subsequent stable transfection of the JP_{26} cells might yield cAMP responsive cells for the development of a TSAb bioassay.

Section 8

Stable transfection and limiting dilution cloning of the YSW cell line

8.1 INTRODUCTION

To develop a luminescence bioassay for TSAb, a cell line stably expressing the human TSHR and a cAMP responsive luciferase reporter gene plasmid is required. The CHO JP_{26} cell line stably expressing the human TSHR was characterised and found to respond to hTSH, forskolin and TSAbs (see Results Section 6). The construction of pGL3hαCRE, the cAMP responsive luciferase reporter plasmid, was described in Results (see Section 7). The stable transfection of pGL3hαCRE into JP_{26} cells should generate a cell line which can then be used in the development of a luminescence bioassay for thyroid stimulators.

Hygromycin B (hmB) is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* (Pettinger RC *et. al.*, 1953). It inhibits protein synthesis in eukaryotic cells by interfering with ribosomal translocation and aminoacyl-tRNA recognition (Cabañas MJ *et. al.*, 1978; Gonzalas A *et. al.*, 1978). No cell line, including the CHO cell line, has been found to be naturally resistant to hmB. The hygromycin-B-phosphotransferase (*hph*) gene has been identified and sequenced (Gritz L & Davies J, 1983). The coding sequence of the *hph* gene was inserted into the Moloney sarcoma virus vector and transfected into eukaryotic cell lines LTK^- and CCL64 (Blochlinger K & Diggelmann H, 1984). Successfully transfected cells were found to be hmB resistant. Thus, hmB was considered to be a suitable selection marker for transfection studies in eukaryotic cells. In addition, Blochlinger and Diggelmann reported that

CHO cells transfected with the *neo* gene, resistant to the antibiotic G418, were found to remain sensitive to hmB. This raises the possibility of using both these selection markers in transfection studies. Consequently, hmB was chosen to be the selection marker for the stable transfection of the pGL3hαCRE plasmid into the JP_{26} cells.

A population of successfully transfected cells, named YSW3, was established to respond to hTSH. The cells also responded to 8-bromo-cAMP stimulation in a dose dependent manner. The YSW3 cells were subsequently cloned by limiting dilution to produce individual clones. The 54 clones generated were characterized in an attempt to obtain a highly responsive clone, which could then be used in the luminescence TSAb bioassay.

8.2 MATERIALS AND METHODS

8.2.1 Materials

Wizard Plus Maxiprep DNA Purification System and MTS were obtained from Promega, Madison, Wisconsin, USA. All the restriction enzymes and their buffers were obtained from either Promega, Madison, Wisconsin, USA or New England Biolabs, Beverly, MA, USA. HmB and forskolin, an AC activator, were obtained from Calbiochem, La Jolla, CA, USA. Forskolin was dissolved in culture medium (see Section 5.1.2) supplemented with DMSO to give a stock concentration of 1mM of forskolin in 10% DMSO. The hTSH used was the 2nd International Reference Preparation of TSH, human for immunoassays, coded 80/558 with an assigned biological potency of 37mU/ampoule. The preparation was reconstituted in a hypotonic medium (see Section 6.2.1), pH 7.4 with 0.4% BSA to yield an initial

concentration of 1U/L. The two dyes used, trypan blue and erythrocin B, were both obtained from Sigma Chemical Co., St. Louis, MO, USA IBMX, 8-bromo-cAMP (sodium salt) and PMS were also obtained from Sigma Chemical Co., St. Louis, MO, USA. IBMX, a cyclic nucleotide phosphodiesterase inhibitor, was made up in DMSO to give a stock concentration of 400mM. 8-bromo-cAMP was reconstituted in dH_2O to give a stock concentration of 50mM.

8.2.2 Methods

8.2.2.1 pWZL, a hygromycin resistance plasmid

The hygromycin resistance plasmid, pWZL, was co-transfected with the pGL3hαCRE plasmid into CHO JP_{26} cells. This plasmid confers resistance to hmB. Prior to the transfection, pWZL DNA was obtained by miniprep and maxiprep procedures using the Wizard Plus Maxiprep DNA Purification System. Restriction enzyme diagnostic digests were performed to verify both the miniprep and maxiprep DNA. Three separate restriction enzymes were used to generate a diagnostic pattern. These were *Bam*HI, to give a linear fragment, and *Pst*I and *Kpn*I to give four and three distinct fragments of definitive sizes respectively. Both miniprep and maxiprep DNA preparations were stored at -20°C until required for stable transfection.

8.2.2.2 Hygromycin B toxicity test

HmB was chosen to select stably transfected JP_{26} cells and the optimum concentration of hmB was required. To investigate the sensitivity of JP_{26} cells to hmB, a dose-response curve was performed. JP_{26} cells were harvested by trypsinization and plated

into 96-well plates at $5x10^4$ cells/ml. The cells were seeded into wells containing 100µl of culture medium (see Section 5.1.1) and supplemented with a series of hmB concentrations (0 – 500µg/ml). The plates were incubated in a 37°C, water saturated, 5% CO_2 / 95% air incubator for 24 – 72hr. The % of cell vitality was obtained using the MTS/PMS tetrazolium salt colorimetric system. For the assay, 20µl of MTS (4.1mM) / PMS (0.15mM) reagent mixture was added to each well. The plates were then further incubated in a 5% CO_2 / 95% air dry incubator at 37°C for 1 – 3hr. Formazan production was measured by a microtiter plate reader (Bio-Rad, Model 3550, Richmond, CA). Before the plates were read, they were shaken on a plate shaker. O.Ds. were measured after 150min at a wavelength of 490nm using a reference wavelength of 655nm (Goodwin CJ *et. al.*, 1995).

8.2.2.3 Stable transfection of JP_{26} cells

In an attempt to obtain a cAMP responsive luciferase cell line, JP_{26} cells were stably co-transfected with the pGL3hαCRE plasmid and pWZL hygromycin resistance vector. The standard calcium phosphate transfection technique with glycerol shock was used. JP_{26} cells were harvested with trypsin and seeded into 9cm tissue culture dishes containing 10ml of culture medium (see Section 5.1.1). The cells were plated at $2.2x10^5$ cells/ml to yield 60% confluency after 24hr. The cells were co-transfected with 10µg of pGL3hαCRE and pWZL hygromycin DNA each per dish. "Mock" transfections, which used no DNA, were also prepared as negative controls. The dishes were incubated in a 5% CO_2, water saturated, 37°C incubator for 3.5 – 4hr. The DNA precipitates were removed followed by the addition of a 15% glycerol-HBS solution (3ml/dish) for 1.5min at 37°C. Fresh culture medium (10ml/dish) was added following the 'glycerol shock' treatment. After 24hr, selection medium (i.e. culture

medium as described in Section 5.1.1, supplemented with 500μg/ml hmB; see Section 5.1.2) replaced culture medium in the dishes to start selection of positively transfected cells. Selection medium was changed every 4 – 5 days. Colonies were harvested, characterized (see Section 8.2.2.6) and cyropreserved (see Section 5.3.1).

8.2.2.4 Limiting dilution cloning of YSW3 cells

The stable transfection JP_{26} cells described above yielded a population of cells, which we named YSW3 cells, which responded to 8-bromo-cAMP and hTSH stimulation (see Section 8.3.3). The YSW3 cells were subsequently subcloned by limiting dilution to produce individual clonal cell lines. This technique applies the Poisson distribution statistical theory. The procedure involves culturing cells at decreasing cell densities until the cells occur at a frequency of less than 1 cell/well. Briefly, doubling dilutions of YSW3 cells were performed in selection medium (see Sections 8.2.2.3 & 5.1.2), to yield densities ranging from 0.4 – 100 cells/ml. The cells were then seeded into 96-well plates at 200μl/well. The plates were incubated in a 5% CO_2 / 95% air, water saturated, 37°C incubator for 10 days to allow colonies to form. After 10 days, the plates were screened microscopically for negative i.e. empty wells. Plates with >37% negative wells were selected and the wells located in these plates were then further cultured until confluency. In these wells, the colonies were likely to have arisen from one or two cells. Once confluent, they were trypsinized and amplified by re-seeding first into 24-well plates and finally 25cm^2 flasks. The individual clones were harvested and cryopreserved as soon as these flasks were confluent.

8.2.2.5 Luciferase Bioassays

Two distinctive hmB resistant populations of cells, named YSW2 and YSW3 cells, were established following the stable transfection of JP_{26} cells (see Section 8.2.2.3). In addition, individual YS clones were obtained from the limiting dilution of YSW3 cells (see Section 8.2.2.4). These cells were characterized for their response to forskolin (+2mM IBMX), 8-bromo-cAMP and hTSH (+0.5mM IBMX) stimulation. The cells were harvested by trypsinization and seeded at $5x10^5$ cells/ml into 6cm dishes containing 5ml of selection medium (see Section 8.2.2.3 & 5.1.2). The plates were cultured for 72hr (unless stated otherwise) before assay. The appropriate stimulators were added directly into the medium to reach the desired final concentrations. The cells were then incubated for 4hr in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. Luminescence was determined using the Promega Luciferase Assay System with Reporter Lysis Buffer according to manufacturer's instructions as described in Section 7.2.2.3.

8.2.2.6 The stability of the luciferase reporter gene in YSW3 cells

The YSW3 cells were subjected to 10 consecutive luciferase bioassays (once/week) to determine the stability of the transfected luciferase reporter gene. The cells were routinely cultured and passaged as described in Section 5.3.2. Each week, the cells were trypsinized and seeded at $5x10^5$ cells/ml into 6cm tissue culture dishes containing 5ml of selection medium (see Section 8.2.2.3 & 5.1.2). They were incubated for 72hr to reach confluency prior to assay. For the assay, triplicate cultures were exposed to ±1mM 8-bromo-cAMP for 4hr in the 37°C incubator. Luminescence

was determined using the Promega Luciferase Assay System with Reporter Lysis Buffer as described in Section 7.2.2.3.

8.2.3 Quantitative analysis

The results for luminescence are expressed either as raw data (single culture) or means ± standard deviations (S.D.) of triplicate cultures, unless stated otherwise. The results are in the form of relative light units (R.L.U.). The results for the tetrazolium salt colorimetric assays are expressed as means ± standard deviations (S.D.) calculated from six replicate cultures. The raw data is in the form of optical densities (O.D.s).

8.3 RESULTS

8.3.1 pWZL Hygromycin resistance plasmid

The pWZL hygromycin maxiprep DNA obtained was subjected to a restriction enzyme diagnostic digest check (Figure 8.1). Three restriction enzymes were used to generate a specific band pattern. Restriction enzyme *Bam*HI gave a single band of ~6kb (linear DNA) while *Pst*I gave four separate bands of the following sizes - 175, 895, 1185 and 3600bp. Finally, *Kpn*I resulted in three bands whose sizes were 1435, 1680 and 2800bp. All the anticipated bands were observed with their correct sizes. This was indicative of the integrity of the hygromycin maxiprep DNA preparation.

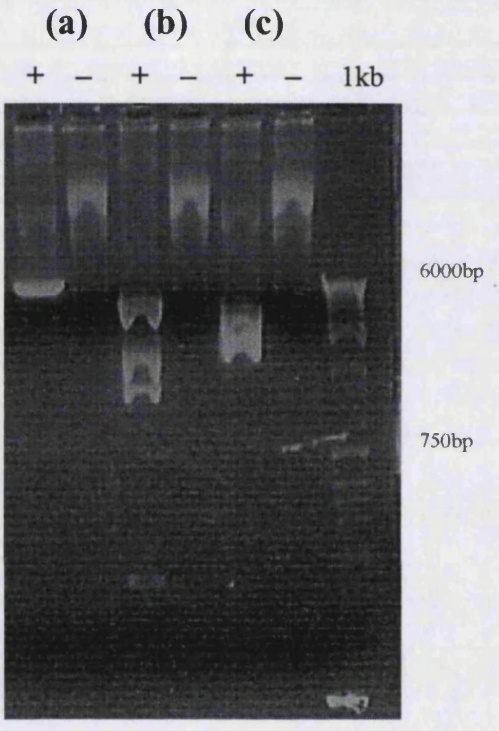


Figure 8.1 **The restriction enzyme diagnostic digest check on the pWZL hygromycin maxiprep DNA**. Three restriction enzymes were used to generate a specific band pattern: a) *Bam*HI to give a single band of ~6kb (linear DNA), b) *Pst*I to generate four separate bands of the following sizes – 175, 895, 1185 & 3600bp and, c) *Kpn*I to produce three bands whose sizes were 1435, 1680 & 2800bp. The + and - refer to cut compared to uncut DNA. The 1kb marker is to record the size of the bands.

8.3.2 Hygromycin toxicity test

The effect of increasing concentrations of hmB on JP_{26} cells is shown in Figure 8.2. The cells were found to be sensitive to hmB in a dose dependent manner. These dose-response curves were used to determine the optimal concentration of hmB to be included in the selection medium. The control (i.e. no hmB added) O.D.s increased with increasing incubation time. Cells cultured for 24hr had the lowest control O.D. of 57.5 ± 1.1 compared to cells after 48 and 72hr of culture which produced O.D.s of 92.7 ± 1.2 and 169.6 ± 1.8 respectively. All three plates showed a decrease in O.D.s when the cells were grown in culture medium (see Section 5.1.1) supplemented with increasing concentrations of hmB. The rate of decline was dependent on the incubation duration. Cells incubated for 72hr showed the fastest rate of decline between the concentration range of 4.0 and 125.0μg/ml hmB. The curves intercept at the concentration of 125.0μg/ml hmB at which point the O.D. readings were approximately 50. At concentrations >125.0μg/ml, the rate of decline reached a plateau in all 3 plates. The % of cell viability as a function of hmB concentration is shown (Figure 8.3). Viability of the controls was defined as 100%, since these cells had not been exposed to hmB. The % of viable cells decreased with increasing concentrations of hmB. The baseline (O.D.s of wells containing culture medium alone) for plates incubated for 48 and 72hr were 37.1 ± 0.8 (40.0% viability) and 35.9 ± 1.0 (21.2% viability) respectively. These results demonstrated that JP_{26} cells were unable to resist hmB selection in the presence of 1000μg/ml (40.1% viability) and 500μg/ml (20.3% viability) after 48 and 72hr respectively. At these concentrations, the baseline O.D.s were reached. However, this did not occur in the plate incubated for 24hr. The baseline O.D. (67.1% viability) was not reached at the concentration of 500μg/ml hmB despite obtaining a lowest % of viability of 74.8%. It

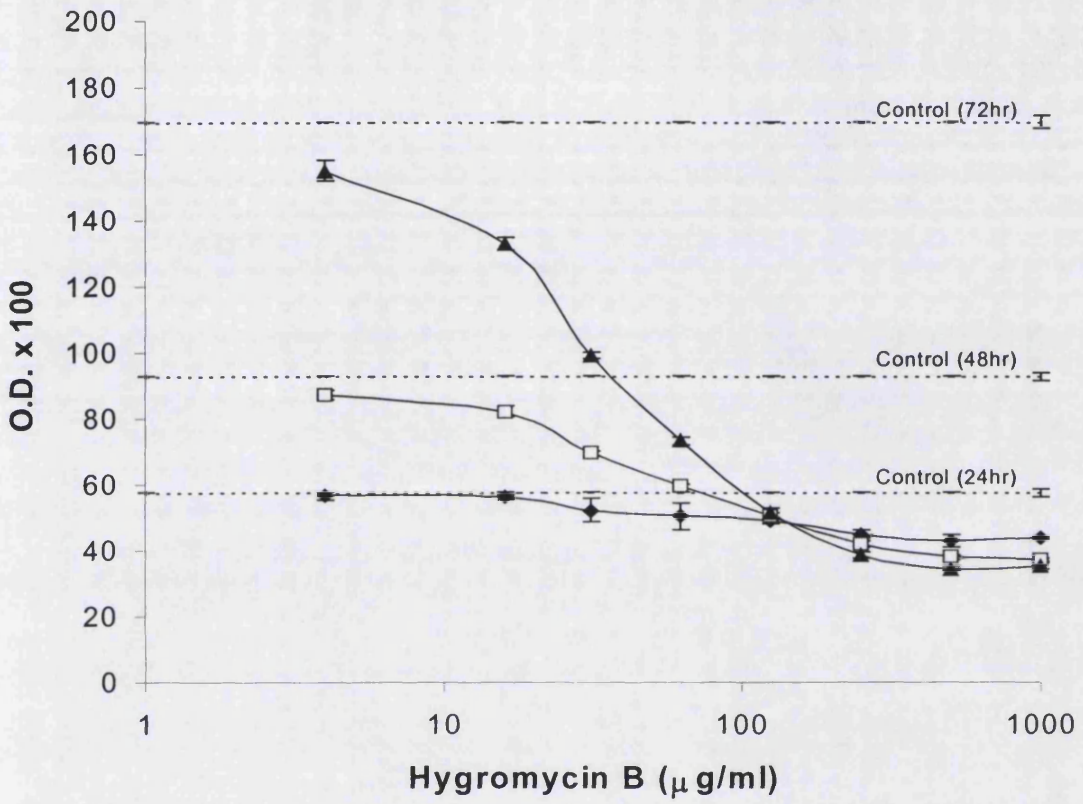


Figure 8.2 **The effect of hygromycin B on JP_{26} cells**. The cells were cultured in three 96-well plates for 24hr (◆), 48hr (□) and 72hr (▲) in culture medium (see Section 5.1.1) supplemented with increasing concentrations of hygromycin B. The hygromycin B concentration ranged from 0 – 1000μg/ml. The means ± S.D. are plotted (n = 6). If not indicated, the error bar was within the symbol.

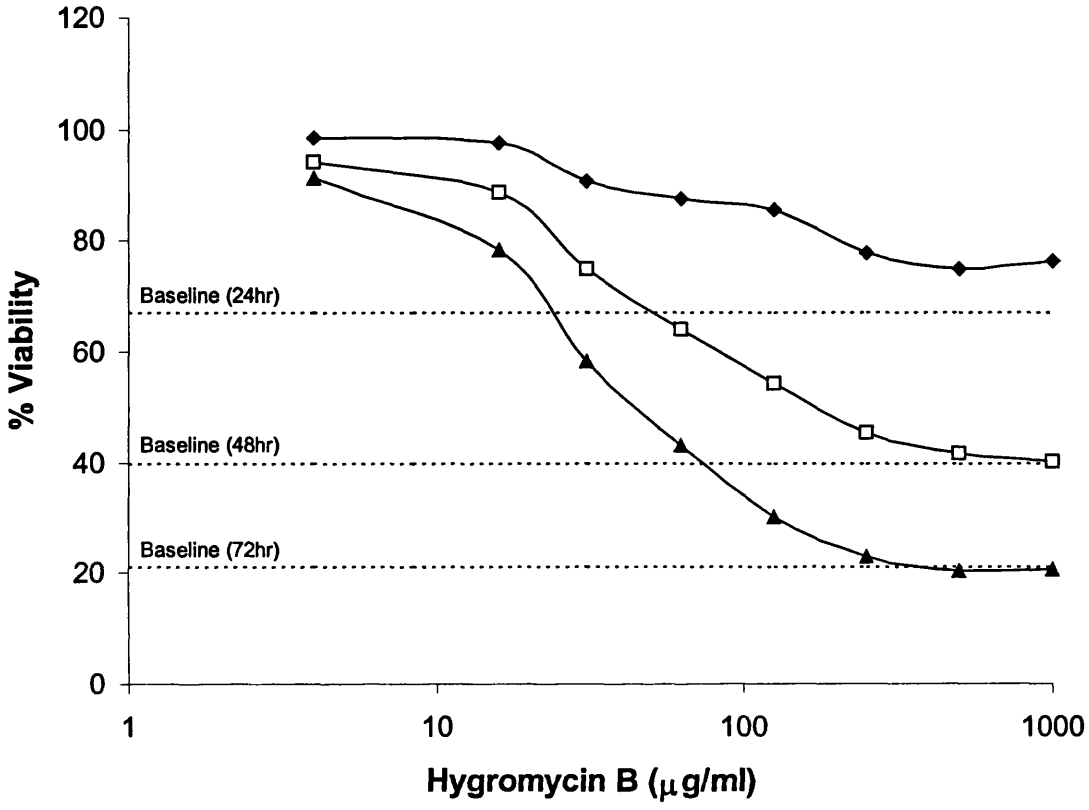


Figure 8.3 **The effect of hygromycin B on the viability of JP_{26} cells.** The cells were incubated with increasing concentrations of hygromycin B (0 – 1000μg/ml) for 24hr (◆), 48hr (□) and 72hr (▲). The calculated % of viability is plotted.

was concluded that culture medium supplemented with 500μg/ml hmB was optimal for the selection of stably transfected JP_{26} cells.

8.3.3 Stable transfection of JP_{26} cells

Triplicate cultures of JP_{26} cells seeded in 9cm dishes were stably transfected as described in Section 8.2.2.3. The dishes were labelled: Plate 1 – 3. In addition, three 'mock' transfection dishes were also prepared as negative controls. With the introduction of the selection medium i.e. culture medium supplemented with 500μg/ml hmB, cells numbers within the controls gradually declined, so that by Day 18, none were detected. In contrast, multiple colonies could be observed in the three transfected plates The first small colonies, each with 5 – 10 cells, were observed 7 days after transfection in all three plates. The colonies were allowed to grow to confluence. Unfortunately, due to an infection, Plate 1 was discarded. The colonies in Plates 2 and 3 were however further amplified by re-seeding into $25cm^2$ tissue culture flasks. When the latter were confluent, they were harvested and cryopreserved in liquid N_2. These two stably transfected cell populations were named YSW2 and YSW3.

8.3.4 Characterization of YSW cells

The YSW2 and YSW3 cells were initially tested for their responses to both forskolin and 8-bromo-cAMP stimulation. For details of the assay protocol, see Section 8.2.2.5. The results are shown in Figure 8.4a & b. YSW2 and YSW3 cells, cultured for either 24 and 72hr, were stimulated with 100μM forskolin in the presence of 2mM IBMX (Fig 8.4a). Nil light outputs were obtained with both the stimulated and the

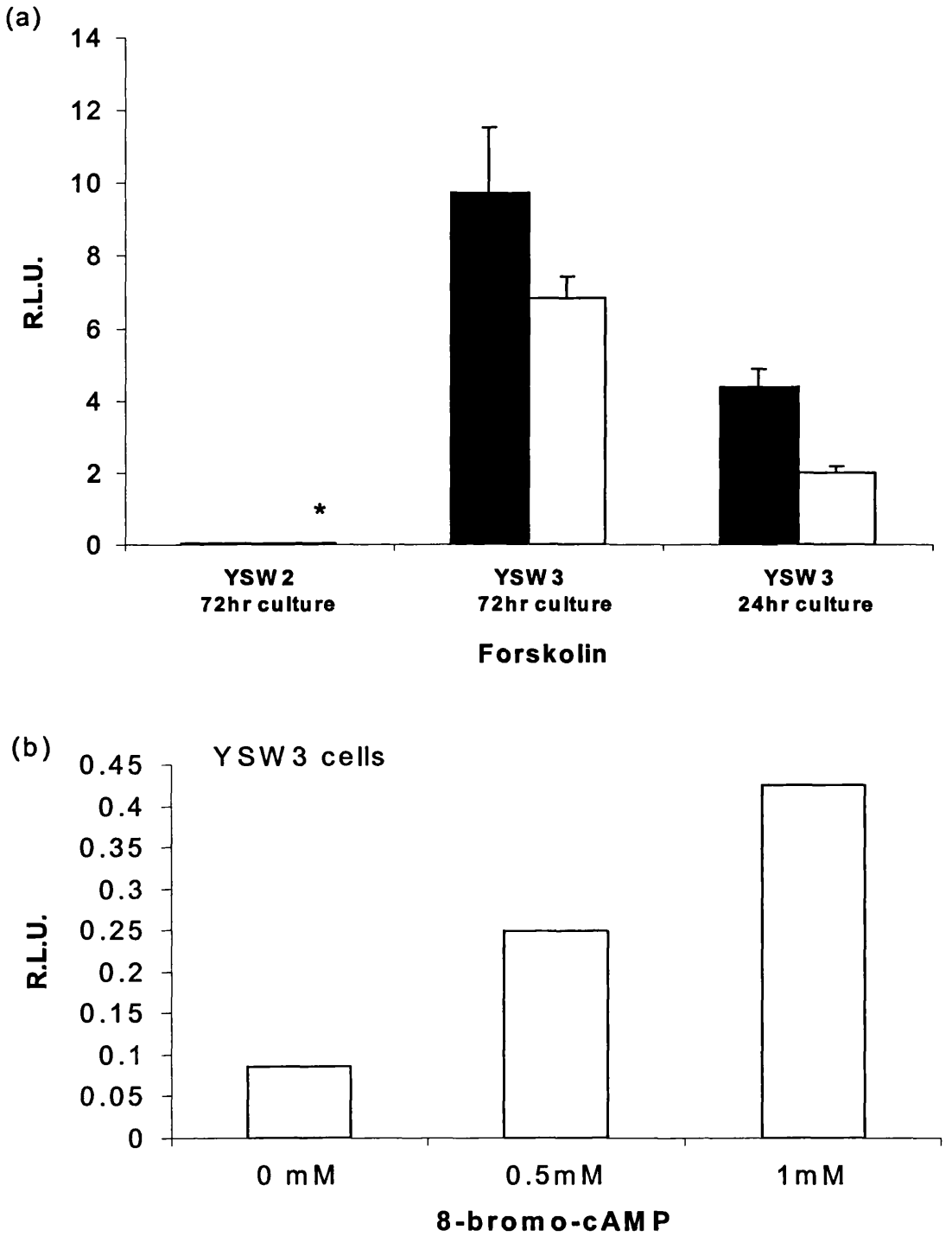


Figure 8.4 **The increase in luminescence in response to (a) forskolin and (b) 8-bromo-cAMP stimulation by YSW2 and YSW3 cells.** a) Cells, cultured for 24 or 72hr, were incubated in the presence (□) and absence (■) of 100μM forskolin +IBMX (2mM). Duplicate* and triplicate microcultures were used for YSW2 and YSW3 cells respectively. The means ± S.D. are plotted for the YSW3 cells. b) YSW3 cells were incubated with 2 concentrations of 8-bromo-cAMP. Singlicate cultures were used.

unstimulated YSW2 cells. Luminescence was detectable from the YSW3 cells cultured for 24 and 72hr prior to assay. Non-stimulated control light outputs of 9.73 ± 1.8 and 4.39 ± 0.5 R.L.U. were produced by YSW3 cells cultured for 72 and 24hr respectively. Unfortunately, forskolin stimulation did not result in an increase in luminescence from basal unstimulated controls. Furthermore, decreases in luminescence were observed in stimulated cells. Light outputs of 6.82 ± 0.6 and 1.98 ± 0.2 R.L.U. were obtained for those cultured for 72 and 24hr respectively. In contrast, YSW3 cells responded to 8-bromo-cAMP stimulation. A dose-dependent increase in R.L.U. was observed in response to 0.5 and 1mM 8-bromo-cAMP (Fig 8.4b). There was a 2.9- and 5-fold increase above the control response to 0.5 and 1mM 8-bromo-cAMP stimulation respectively.

The responses of YSW3 cells to hTSH were also investigated (Figure 8.5). In a preliminary experiment, incubation with a relatively high dose of TSH (200mU/L) for 4hr, stimulated light output by 244% above the unstimulated control. When IBMX (0.5mM) was added, light output from the control itself increased by 170%, as might be expected. However, in the presence of the IBMX, although TSH stimulation (200mU/L) was reduced to 172% (5.06 ± 0.1 *v* 8.72 ± 0.5; control *v* stimulated; $p<0.001$), as opposed to the higher value of 244% observed in its absence, it was statistically a highly significant increase.

8.3.5 The stability of the luciferase reporter gene in YSW3 cells

Light outputs from cultures of unstimulated YSW3 cells were noted to vary widely from one subculture to another. This led us to question the stability of the transfected

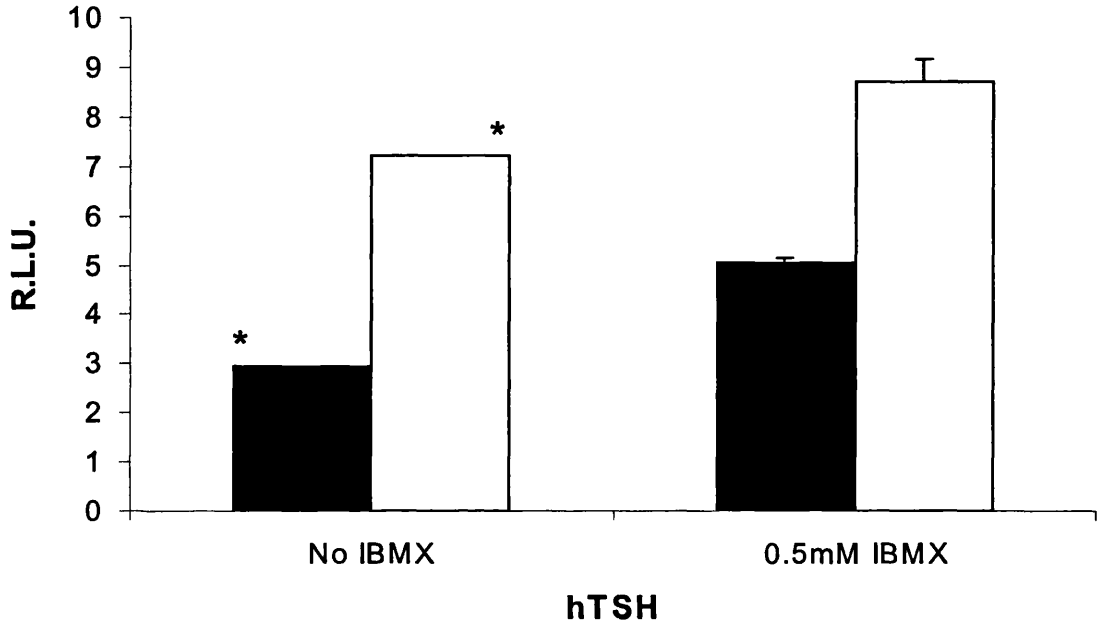


Figure 8.5 **The increase in luminescence in response to 200mU/L hTSH stimulation by YSW3 cells.** The cells were incubated in the presence (□) and absence (■) of hTSH with and without IBMX (0.5mM) as indicated. Singlicate* culture and triplicate cultures were used. Raw data are plotted for the singlicate* cultures. The means ± S.D. are plotted for triplicate cultures.

luciferase reporter gene. There was the possibility that upon repeated subculture, the cells would gradually reject the reporter gene or the gene would lose its activity. Thus, over a period of 10 weeks, with 10 weekly passages, light outputs from each subculture were tested in the presence of 8-bromo-cAMP (1mM). The absolute light outputs and the % increases are shown (Figure 8.6a & b). Light outputs from the unstimulated controls ranged from 0.25 – 3.78 R.L.U.. We noted that the outputs from subcultures 1 – 3 were the highest. However, all subcultures responded to added 8-bromo-cAMP but, the % increase ranged from 126% to 652%. Despite the decline in light outputs for the controls, over the 10 week period, a response to 8-bromo-cAMP of >300% was maintained, except for passage 3. Thus we concluded that responsiveness to 8-bromo-cAMP was relatively stable.

8.3.6 Limiting dilution cloning of YSW3 cells

Having established that the YSW3 cell population responded in terms of light outputs to 8-bromo-cAMP and also hTSH stimulation, this population was subjected to limiting dilution cloning to obtain individual clonal cell lines. The inner 60 wells of nine 96-well plates were plated with YSW3 cells at decreasing cell densities (Table 8.1). After 10 days, the plates were inspected microscopically and the number of wells seeded with and without cells (positive and negative wells respectively) were recorded for each plate. The % of negative wells in each plate was then calculated. The results are summarised in Table 8.1. Plates 6 – 9 were found to have >37% of negative wells. These four plates were further incubated for 14 days to allow the cells to grow to confluence. This yielded 54 individual clones. These clones were then

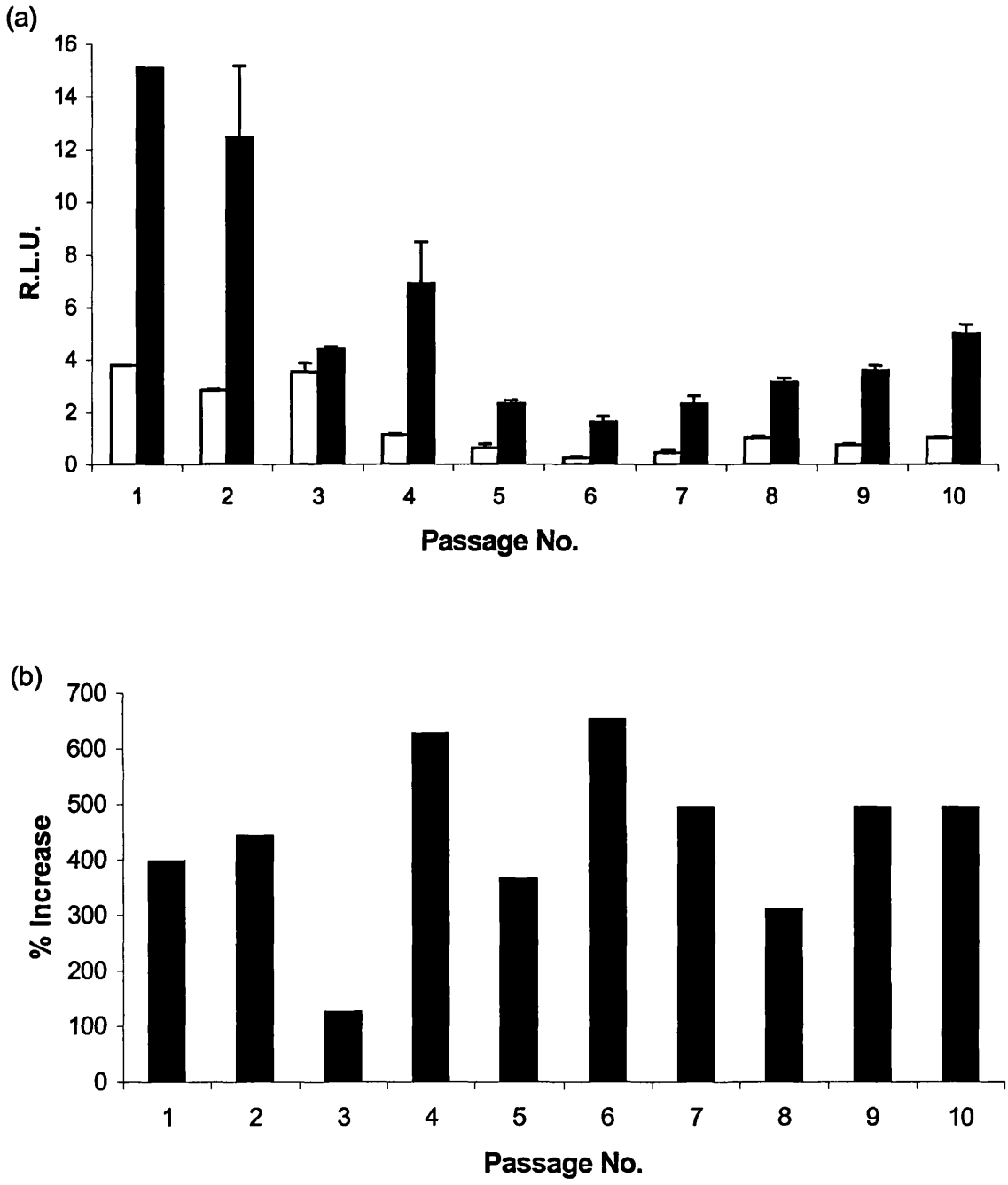


Figure 8.6 **Luminscence assays on 10 consecutive subcultures of YSW3 cells, set up to investigate the stability of the expression of the pGL3hαCRE plasmid over a period of 10 weeks.** (a) The increase in luminescence in response to 8-bromo-cAMP stimulation is plotted. Each subculture was incubated in the presence (■) and absence (□) of 8-bromo-cAMP (1mM) for 4hr before being assayed for luciferase activity. Triplicate cultures were used and the means ± S.D. have been plotted. (b) Results from (a) expressed as the % increase in luminescence in response to 8-bromo-cAMP.

Plate No.	Plating cell density (cells/ml)	No. of wells plated	No. of +ve wells	No. of –ve wells	% of –ve wells
1	100	60	60	0	0
2	50	60	60	0	0
3	25	60	59	1	1.67
4	12.5	60	51	9	15.0
5	6.25	60	38	22	36.67
6	3.1	60	27	33	55.0
7	1.6	60	13	47	78.33
8	0.8	60	9	51	85.0
9	0.4	60	5	55	91.67

Table 8.1 **The results obtained from limiting dilution cloning of YSW3 cells.** Nine 96-well plates were plated at the cell densities indicated. The outerwells were not used and hence only 60 wells/plate were seeded. After 10 days incubation, the wells were inspected microscopically. The number of positive (+ve) and negative (-ve) wells was recorded. The % of negative wells for each plate was calculated and shown.

expanded. They were harvested by trypsinization and re-seeded, first into 24-well plates, followed by a further re-seeding into 25cm^2 flasks. When the flasks were confluent, the clones were harvested and cryopreserved in liquid N_2 (see Chapter 2 Section 5.3.1).

8.3.7 Screening of YS Clones

The 54 clones resulting from the limiting dilution of YSW3 cells were named YS clones 01 – 54. 22 clones (YS01 – YS22) were tested for their luciferase response to 8-bromo-cAMP (1mM). For details of the assay system see Section 8.2.2.6. The results are summarised in Table 8.2. Unfortunately, none showed any significant increase in light outputs in response to 8-bromo-cAMP stimulation. Light outputs were in fact consistently low in both the stimulated and unstimulated cells.

8.4 CONCLUSIONS

We were successful in achieving stable transfection of our pGL3hαCRE construct into the JP_{26} CHO cell line. Using luminescence assays we demonstrated that YSW3 cells increased light output after incubation with 8-bromo-cAMP, and that the 3-fold increase was stable over 10 weekly passages. However, paradoxically, forskolin decreased light output. Using a relatively high dose of hTSH (200mU/L), we observed an anticipated increase in light output, after a 4hr incubation in the presence of the thyroid stimulator. However, the response, was only of the order of 200%. Nevertheless, encouraged by this response, we undertook a cloning of YSW3 cells by limiting dilution.

YS Clones No.	Unstimulated Controls (R.L.U.)	+ 1mM 8-bromo-cAMP (R.L.U.)
01	0.034	0.017
02	0.040	0.015
03	0.028	0.016
04	0.025	0.017
05	0.062	0.067
06	0.057	0.035
07	0.025	0.018
08	0.051	0.036
09	0.022	0.015
10	0.023	0.013
11	0.049	0.030
12	0.047	0.034
13	0.018	0.014
14	0.048	0.034
15	0.020	0.010
16	0.018	0.009
17	0.043	0.027
18	0.039	0.029
19	0.035	0.023
20	0.031	0.021
21	0.034	0.026
22	0.028	0.024

Table 8.2 **Showing the light outputs from the YS clones, YS01 – YS22, in response to 8-bromo-cAMP**. Singlicate cultures of each clone were tested with and without 8-bromo-cAMP.

Transfection and subsequent cloning was facilitated by hygromycin selection, since we had co-transfected the gene which confers hygromycin resistance, namely the *hph* gene. A total of 54 individual clonal cell lines were obtained. These were harvested and cryopreserved. However, when we screened 22 of these for responsiveness to 8-bromo-cAMP, we found that (a) light outputs were consistently low and (b) there was no increased output in response to 8-bromo-cAMP. 3 of these clones (YS05, 11 and 22) were also tested later for responsiveness to forskolin and hTSH, using an improved luminescence system (see Section 9.2.2.1.4, Bioassay D). Unfortunately, nil responses were observed with these as well.

During the course of this labour-intensive cloning and screening, we were offered the use of a newly developed cell line which preliminary tests had suggested responded well to thyroid stimulators in terms of light output. These, developed at the N.I.H., Bethesda, Maryland, appeared well suited for further testing with the aim of developing a luminescence bioassay for TSAb. We therefore concluded that rather than spend further considerable effort on our transfected clones, which were at best only poorly responsive, it would be more suitable to exploit newly emerging luminescence technology using the cells from N.I.H.. We describe this work in the succeeding sections.

Section 9

The development of a Luminescence Bioassay for Thyroid Stimulating Antibodies using the Chinese Hamster Ovarian cell line CHO25LUC

9.1 INTRODUCTION

We previously reported the construction of the cAMP responsive luciferase reporter plasmid, pGL3hαCRE, and its transfection into the CHO cell line JP_{26}. As described in Section 8, after stable transfection and limiting dilution we were unable to obtain any functional clones which responded to thyroid stimulators.

An alternative CHO cell line which stably expressed both the human TSHR and a cAMP responsive promoter region upstream of a luciferase reporter gene was obtained. This cell line, named CHO25LUC, was kindly given to us by Dr. L.D. Kohn at the N.I.H., USA. These cells had been stably transfected with the wild type human TSHR. They also contained the promoter region of the human glycoprotein hormone α-subunit (α-Gly) upstream of the luciferase reporter gene. This promoter region has the same two CRE elements as were described in the pGL3hαCRE plasmid (see Section 7.2.2.2). A puromycin resistance gene vector (pPUR), had been co-transfected into the CHO hTSHR cell line as a selection marker. Transfections had been performed by electroporation and surviving cells had been cloned by limiting dilution. Clones were selected by their responses to TSH and thyroid stimulating antibodies. This section summarizes our work on the development and optimization of a luminescence bioassay for thyroid stimulators using the CHO25LUC cell line.

9.2 MATERIALS AND METHODS

9.2.1 Materials

Both the initial Luciferase Assay System with Reporter Lysis Buffer (E4030) and also the later Steady-Glo™ Luciferase Assay System (E2510) were obtained from Promega, Madison, Wisconsin, USA. These will be referred to in this report as Promega Systems I and II respectively. Several International Reference Preparations for thyroid stimulators were obtained from NIBSC as follows. The main hTSH preparation used was the 2nd International Reference Preparation of TSH, human for immunoassays, coded 80/558. This had an assigned biological activity of 37mU/ampoule. The preparation was reconstituted in assay medium (see Section 9.2.2.2) to yield a starting concentration of 1U/L. A secondary hTSH preparation, coded 84/703, was also used. This preparation was reconstituted in assay medium to yield a stock concentration of 2U/L. The Long Acting Thyroid Stimulator (LATS-B), a human TSAb, coded 65/122, was an MRC standard for thyroid stimulating antibodies. It had been prepared by lyophilizing a patient serum preparation. LATS-B has an assigned biological activity of 15mU/ampoule. For use, the preparation was reconstituted in 1ml of assay medium to give a stock concentration of 15U/L. In addition a more recently established Thyroid Stimulating Antibody (TSAb) standard was also used; this was the 1st International Standard coded 90/672 which had an assigned biological activity of 100mU/ampoule. For use, the preparation was reconstituted in 1ml of assay medium to give a stock concentration of 100U/L. It should be noted that there was no equivalence between the units of bioactivity for TSH and TSAb. Horse serum was obtained from Gibco, Paisley, Scotland, UK. Hepes buffer (1M) and IBMX, a cyclic nucleotide phosphodiesterase inhibitor, were obtained

from Sigma Chemical Co., St. Louis, MO, USA. A stock of IBMX at 100mM was prepared in DMSO. Forskolin, a ubiquitous AC activator, was obtained from Calbiochem, La Jolla, CA, USA. It was dissolved in culture medium supplemented with DMSO (10%) giving a stock concentration of 1mM. The Regenerated Cellulose Tubular Membrane, T2 dialysis membrane, used for dialysis of the TSAb preparation had a molecular weight cut-off between 6,000 – 8,000. It was obtained from Cellu.Sep Membrane Filtration Products Inc., San Antonio, Texas, USA. Starvation medium and AM3 (see Section 9.2.2.2) were obtained from Diagnostic Hybrids, Inc. (DHI), Athens, OH, USA. They were kindly donated by Drs. L.D. Kohn and J. Brown.

9.2.2 Methods

During the development of the luciferase bioassay, numerous systematic changes were made to a starting assay protocol suggested by our colleagues at N.I.H.. These changes varied from using contrasting assay media and microculture incubation conditions, to the selection of alternative Promega luciferase assay systems. The changes were systematically selected so that they resulted in progressive improvement to the original bioassay. To ease the organisation of our report we will subdivide the assay protocol into three separate phases. These are as follows:

- **Phase 1 – preparation of the monolayer of CHO25LUC cells.** The cells were harvested, seeded into 96-well microtiter plates and incubated to attain confluency.
- **Phase 2 – exposure of the cells to stimulators.** The confluent monolayers were exposed to selected stimulators (TSH, TSAb, forskolin) for a selected time.

- **Phase 3 – signal development.** Luminescence from the activated microcultures was determined using one of two Promega luciferase assay systems and light output was measured using a luminometer.

In addition, to further aid the clarity of our report, we have grouped the variations tested into four different bioassay designs (A – D). These are described below.

9.2.2.1 Outline protocols for Bioassays A – D

9.2.2.1.1 Bioassay A

Major features: Cells were exposed to stimulators in AM1 and Promega System I was used.

For Phase 1, CHO25LUC cells were harvested by trypsinization and seeded into 96-well microtiter plates (clear or white). The cells were seeded into the inner wells at $4x10^5$ cells/ml (100µl/well). The plates were incubated for 48hr in a humidified atmosphere of 5% CO_2 / 95% air at 37°C prior to assay. For Phase 2, the culture medium (see Section 9.2.2.2) was aspirated and the appropriate stimulator then added to the wells (100µl/well). Test stimulators were diluted in AM1 (see Section 9.2.2.2). The cells were exposed to the stimulators for 4hr (unless stated otherwise) in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. The wells were then washed twice with AM2 (200µl/well, see Section 9.2.2.2). For Phase 3, luminescence was determined using Promega System I (see Section 9.2.2.3) and light outputs were measured with the Anthos Lucy 1 microplate luminometer.

9.2.2.1.2 Bioassay B

Major feature: Promega System II used.

This bioassay used an identical protocol to Bioassay A with two exceptions. First, at the end of Phase 2, the cells were not washed after exposure to thyroid stimulators. Secondly, for Phase 3, luminescence was determined using Promega System II (see Section 9.2.2.3).

9.2.2.1.3 Bioassay C

Major feature: Cells exposed to stimulators in culture medium.

This is a development of Bioassay B. Thus cells were prepared for the bioassay as described for Bioassays A and B. However, for Bioassay C, culture medium (see Section 9.2.2.2) was used for both the growth of the monolayer (Phase 1) and also for the exposure of the cells to the thyroid stimulators (Phase 2). At the end of the 48hr standard incubation prior to Phase 2, just 50µl of the 100µl of the culture medium in each well was removed i.e. the wells were not fully aspirated. Subsequent stimulators were then added to each well, as appropriate, in 50µl of fresh culture medium, restoring the volume to 100µl/well for Phase 2. As for Bioassays A and B, the cells were exposed to the stimulators for 4hr, prior to the addition of the luciferase reagent (20µl/well). As for Bioassay B, Promega System II was used for Phase 3.

9.2.2.1.4 Bioassay D

Major feature: Cells exposed to stimulators in AM3.

For this protocol the monolayers were exposed to the thyroid stimulators in AM3 (see Section 9.2.2.2). This was a proprietary solution provided by DHI. The confluent microcultures were prepared as for the other 3 protocols except that all 96-wells were seeded; in the previous bioassays, because of possible "edge" effects, the outer wells had not been used. After the 48hr incubation i.e. at the end of Phase 1, the culture medium (100µl) was completely removed and replaced with thyroid stimulators in AM3 (110µl). As before the bioassay incubation for Phase 2 was for 4hr prior to Phase 3, when Promega System II was used (50µl/well).

9.2.2.2 Media used for Phase 2

Several different media were used for Phase 2 of the bioassay, i.e. the exposure for the cells to the stimulators. They were as follows:

Culture Medium This medium was the same as that used in the routine culture of CHO25LUC cells. It comprised of Ham's F12 nutrient medium supplemented with 10% FCS and antibiotics penicillin and streptomycin (see Section 5.1.3).

Assay Medium 1 (AM1) This medium was identical to the above culture medium, except that 10% FCS was replaced with 10% horse serum.

Assay Medium 2 (AM2) This was Hank's Balanced Salt Solution (HBSS) without Ca^{++} and Mg^{++} ions but supplemented with 20mM Hepes and 1% BSA.

Assay Medium 3 (AM3) This was a proprietary solution supplied by DHI.

9.2.2.3 Luciferase Assay Systems

Promega System I

This was described by the manufacturer as the "Promega Luciferase Assay System with Reporter Lysis Buffer" (Promega Code E4030). Crucially, this protocol required an extraction step, in which the cells were first lysed to release the intracellular luciferase, prior to the separate addition of the luciferase substrate. After Phase 2, i.e. the exposure to the stimulators, assay medium was completely removed from the wells and the cells were washed twice with PBS buffer (150µl/well). 50µl of the Reporter Lysis Buffer, provided by Promega, was then added to each well. The monolayers were then scraped, using a pipette tip, to extract the luciferase from the lysed cells. To quantify the luciferase in each extract, 20µl of the latter were pipetted into the wells of a white 96-well plate. Using the auto-injection function of the luminometer, 100µl of luciferase assay reagent (equilibrated to room temperature) was then added to a given well. This provided the substrate (luciferin) for the luciferase. After a pre-set 2sec delay, light emission from this well was measured for 10sec. This system was used for Bioassay A.

This system was initially designed for use with larger cultures growing in 6 – 10cm dishes. After preliminary experiments on this larger scale, we scaled it down for use with microcultures in 96-well plates.

Promega System II

This single step system used the Promega "Steady-Glo" luciferase assay system (Promega Code E2510). In contrast to Promega System I, described above, the cell lysis reagent and the luciferase substrate were added simultaneously, as the "luciferase reagent". This system was used for Bioassays B, C and D. However for Bioassays B and C, only 20µl luciferase reagent was added to each well whereas for Bioassay D, 50µl/well was added, unless stated otherwise. After the addition of the pre-warmed luciferase reagent, the plate was then mixed and left for 10min (Bioassays B & C) or 40 – 60min (Bioassay D), unless stated otherwise, before being placed in the luminometer to quantify light emission. Thus the auto-injection function of the luminometer was not used. This major alteration in the mode of luminometer use was because the luciferase substrate had been formulated so that Promega System II yielded a much more sustained and stable light output than System I. Hence, with this "Steady-Glo" system, it was not necessary to add the reagent with the auto-injection function of the luminometer.

Table 9.1 summarises the different assay conditions and variations used for the Bioassays A – D.

	BIOASSAY	A	B	C	D
Phase (1)	96-well plate	clear then white	white	white	white
	Plating cell density (cells/ml)	4×10^5	4×10^5	4×10^5	4×10^5
	Volume in wells (µl/well)	100	100	100	100
	Time to form monolayer (hr)	48	48	48	48
Phase (2)	Assay media	AM1	AM1	CM	AM3
	Volume aspirated / added to wells (µl/well)	100/100	100/100	50/50	100/110
	Incubation duration (hr)	4	4	4	4
	Wash	AM2	none	none	none
Phase (3)	Promega system	I	II	II	II
	Volume of luciferase reagent used (µl/well)	100	20	20	50
	Incubation time with the reagent before measurement of light emission	2secs (auto-injection function)	10mins	10mins	40 - 60mins
	Reading time (sec/well)	10	10	10	1

Table 9.1 **A summary of the conditions used for Bioassays A – D.** Each bioassay protocol has been subdivided into 3 discrete *Phases*: *(1)* preparation of the monolayer of CHO25LUC cells; *(2)* exposure of the cells to the thyroid stimulators; and *(3)* signal development (see Section 9.2.2). The standard conditions for each protocol are listed above. More detailed descriptions of the protocols for Bioassays A – D are described in Section 9.2.2.1 and when the results from each Bioassay are separately reported (see Sections 9.3.1 – 9.3.4). The media and luciferase systems used in the Bioassays are detailed in Sections 9.2.2.2 and 9.2.2.3 respectively.

9.2.3 Quantitative assessment of bioassay performance characteristics

The succession of bioassay protocols (Bioassays A – D) were each ultimately tested with dose-response curves, in response to TSH (Figure 9.1a). These curves were designed so that they could be critically examined by the use of a number of quantitative criteria, as described below.

Response and Response Errors

Relative Light Units

Light outputs from individual microcultures were measured as Relative Light Units (R.L.U.) in which luminescence was measured by the luminometer on an arbitrary scale. The manufacturer also described this reading as representing kilocounts/second, and claimed that this device was adopted to accommodate a broad dynamic range. The R.L.U. readings were the fundamental bioassay ***Response Metameter*, R** (Figure 9.1a).

Apart from the earliest bioassays, triplicate cultures were used when assessing dose-response curves. The light outputs could then be expressed as the ***Means ± S.D.*** of these triplicates. The S.D. at a given point on the dose-response curve is a statistical assessment of the ***Response Error*** incurred at that point and is conventionally referred to as **ΔR** (Figure 9.1a). This could be transformed to a ***% Error*** by expressing ΔR as a % of the mean R i.e. as a ***Coefficient of Variation*** (CV).

Zero-dose, unstimulated controls, were included in all dose-response curves. This enabled us to estimate the ***Detection Limit*** of a given assay. This was defined as the stimulator concentration interpolated from the dose-response curve, when the response was 3 S.D.s above the mean of the zero-dose controls (Figure 9.1a). In the event that this point fell below the lowest stimulator concentration tested (X), then the result was reported as <X. The detection limit of an assay may also be referred to as its "sensitivity".

The ***Magnitude of Response*** was defined as **the light output at a particular point on the dose-response curve – the light output from the zero-dose controls** (Figure 9.1a). This, when combined with the response error, determines the imprecision (ΔH) of an assay, as explained later with Figure 9.1c.

At times, the magnitude of response was transformed to a ***Fold-response***. This was, **the light output at a particular point on a dose-response curve / the light output of the zero-dose control**. Sometimes this has been expressed as a ***% Fold-response***. This is the conventional method for comparing the relative potencies of different TSAbs when they are expressed as a Thyroid Stimulation Index (TSI) in Section 10.2.3.

Systematic changes across a dose-response curve

Both of the two determinants of assay precision, namely ΔR and the magnitude of response, change in a systematic manner across any dose-response curve (Figure 9.1b). When our emphasis has been on minimizing ΔR, it has at times been useful to

compare the mean $\Delta R \pm$ S.D. obtained across complete dose-response curves. Clearly, to do this the absolute ΔR must first be transformed to a % error (CV) and the mean % error across a dose-response curve has then been referred to as the ϕCV (Figure 9.1b).

The Imprecision Profile

Assay optimization is usually directed at achieving maximum assay precision or minimal assay imprecision. Conventionally, for a given stimulator concentration H, the imprecision is represented by ΔH (Figure 9.1c). ΔH is seen to be a function of both ΔR and the slope of the response curve at H, since Slope = ΔR / ΔH. Should significant curviture occur over the range $R \pm 2\Delta R$, then ΔH_1 and ΔH_2 will be slightly different. ΔH is then estimated as $(\Delta H_1 + \Delta H_2) / 2$. Very often it is useful to express ΔH as a % of H. The imprecision of the bioassay can then be described by constructing an imprecision profile (Figure 9.1d). This is usually a plot of $\%\Delta H$ against H, estimated at selected points on the dose-response curve (Figure 9.1d). A clinically useful assay would be expected to have an imprecision of <10% across its target concentration range.

In practice, when constructing an imprecision profile, it is best to attempt to estimate the response-error relationship (Figure 9.1e). To do this, ΔR is plotted against R across the dose-response curve. A smoothed relationship can then be estimated, which reveals the systematic changes in ΔR despite the inevitable variation caused by random fluctuations. The imprecision profile can then be calculated as $\Delta R_{smoothed}$ / Slope at selected points on the dose-response curve.

Figure 9.1

Diagrammatic representation of a typical sigmoidal dose-response curve and the quantitative criteria used to critically assess the bioassay performance.

(a) A typical sigmoidal dose-response curve plotted semi-logarithmically. Triplicate microcultures have been used so that the response errors (ΔR) could be estimated as S.D.s. The detection limit is estimated as shown using $R_0 + 3x\Delta R_0$, where R_0 is the response obtained from zero-dose controls and ΔR_0 is its associated response error.

(b) A typical dose-response curve, established with 8 different stimulator concentrations, showing the systematic change in ΔR as the curve is ascended, and the derivation of ϕCV (see text).

(c) Illustrating how assay imprecision (ΔH) is a function of the ΔR and the slope of the dose-response curve. See text for details.

(d) An imprecision profile: $\%\Delta H$ is plotted against H for selected points in the dose-response curve.

(e) The response-error relationship estimated for the result (hTSH 80/558) obtained with Bioassay C (Figure 9.29). ΔR has been plotted against R for all the stimulator doses tested.

Figure 9.1a

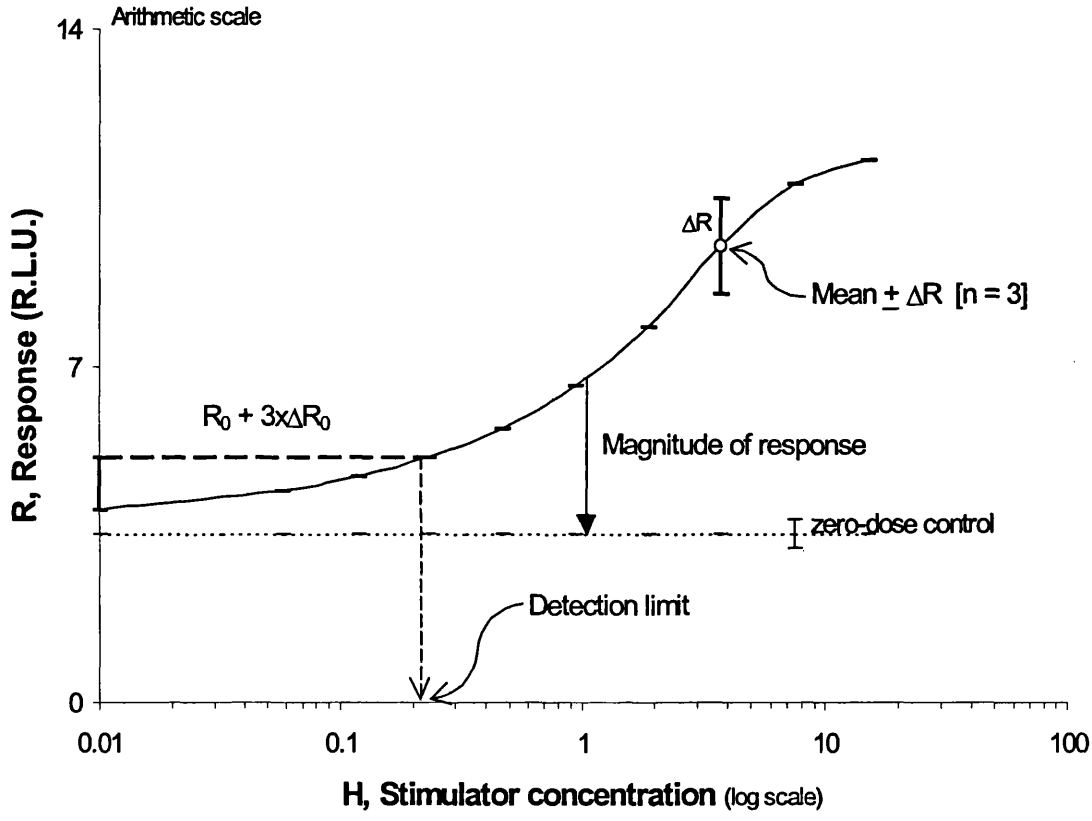

Arithmetic scale
14
7
0
R, Response (R.L.U.)
ΔR
Mean ± ΔR [n = 3]
R0 + 3xΔR0
Magnitude of response
zero-dose control
Detection limit
0.01
0.1
1
10
100
H, Stimulator concentration (log scale)

Figure 9.1b

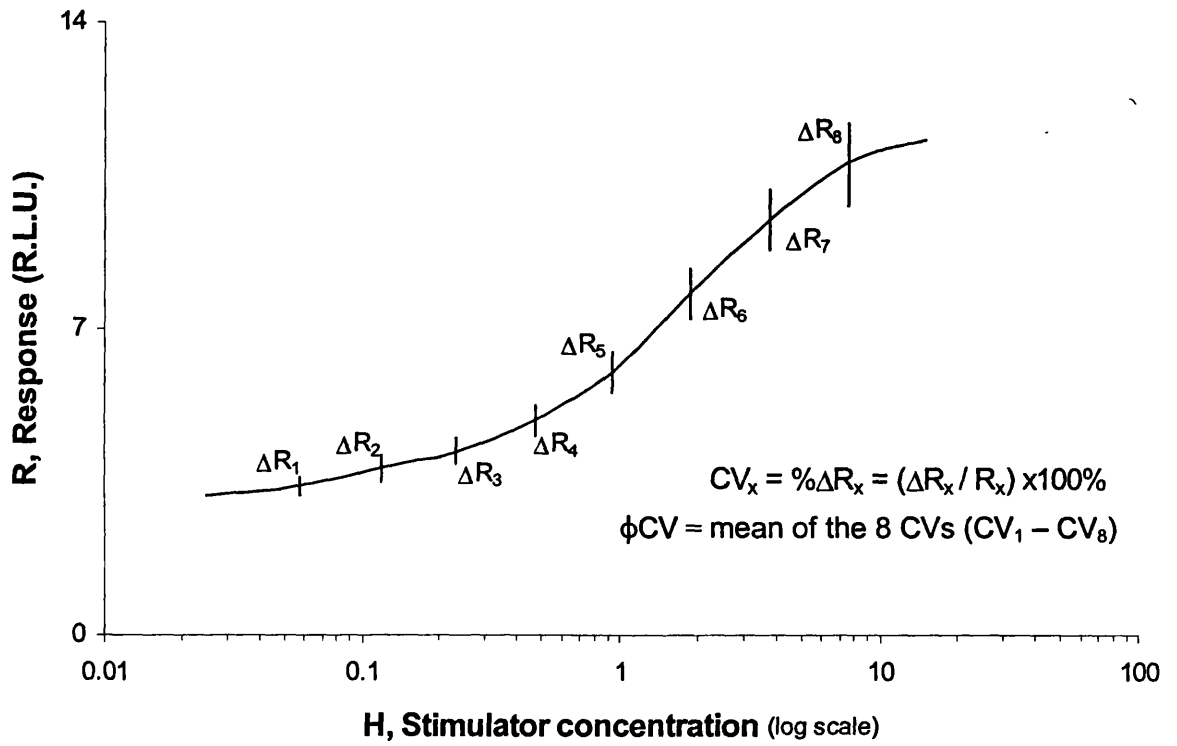

14
7
0
R, Response (R.L.U.)
0.01
0.1
1
10
100
H, Stimulator concentration (log scale)
ΔR1
ΔR2
ΔR3
ΔR4
ΔR5
ΔR6
ΔR7
ΔR8
CVx = %ΔRx = (ΔRx / Rx) x100%
ϕCV = mean of the 8 CVs (CV1 – CV8)

Figure 9.1c

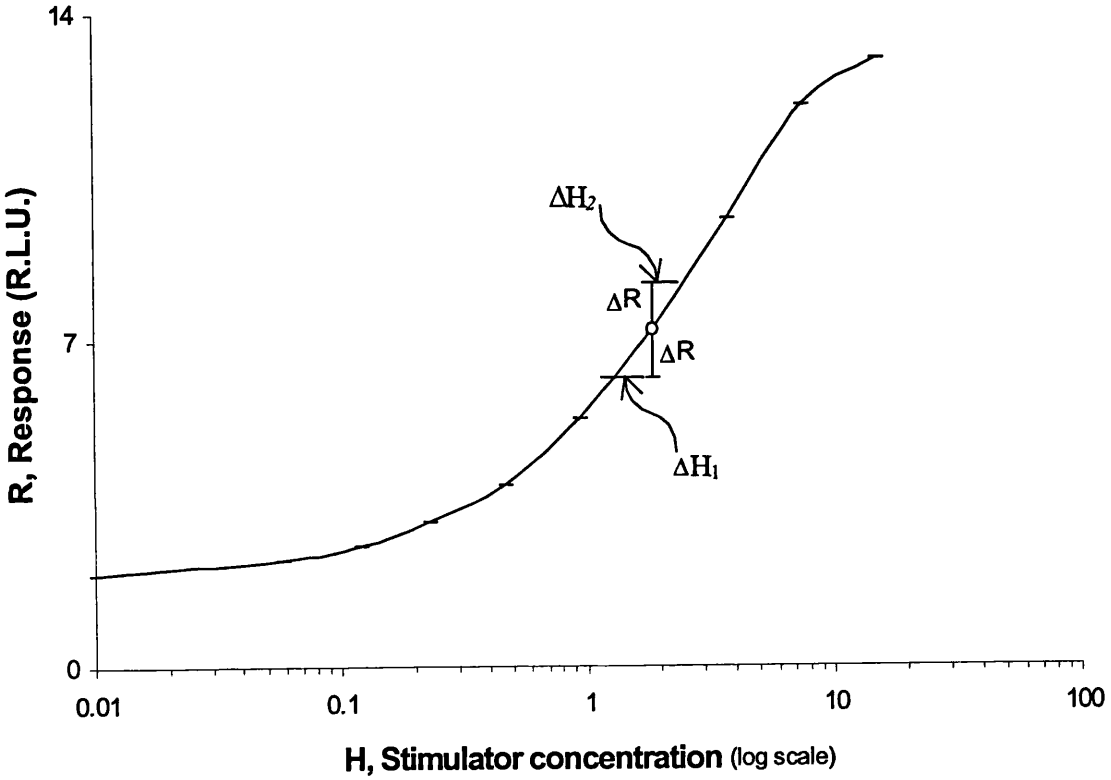

14
7
0
R, Response (R.L.U.)
0.01
0.1
1
10
100
H, Stimulator concentration (log scale)
ΔH2
ΔR
ΔR
ΔH1

Figure 9.1d

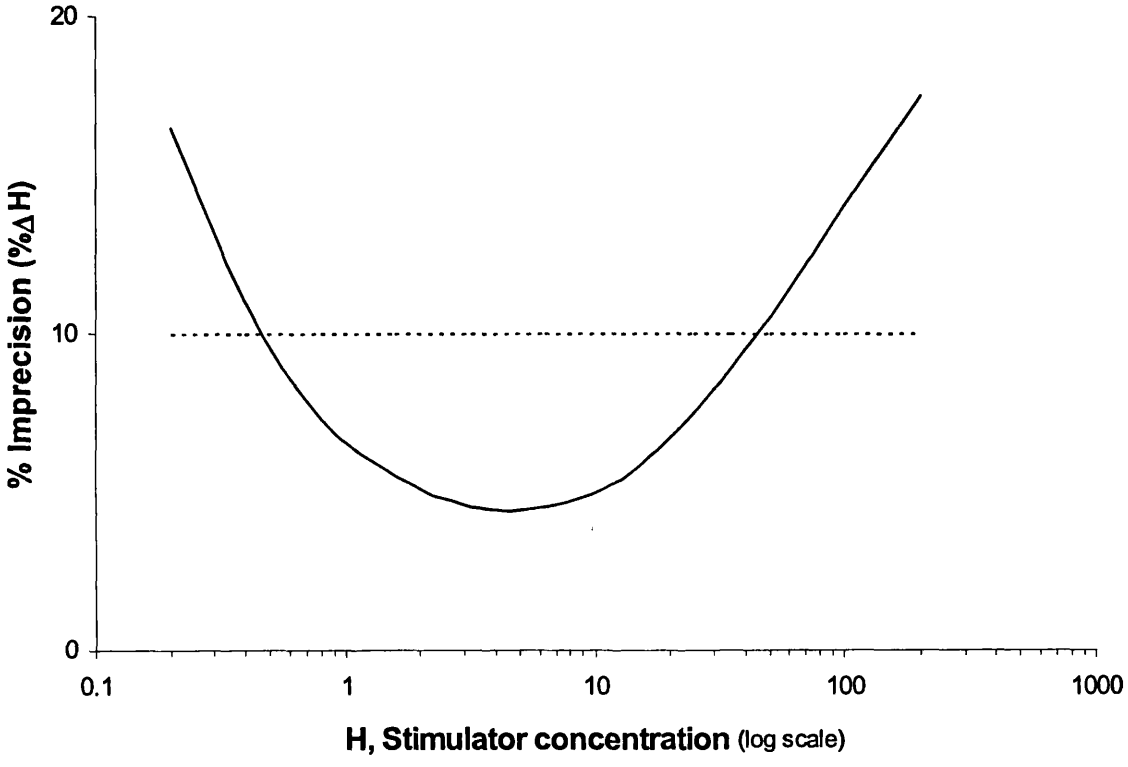
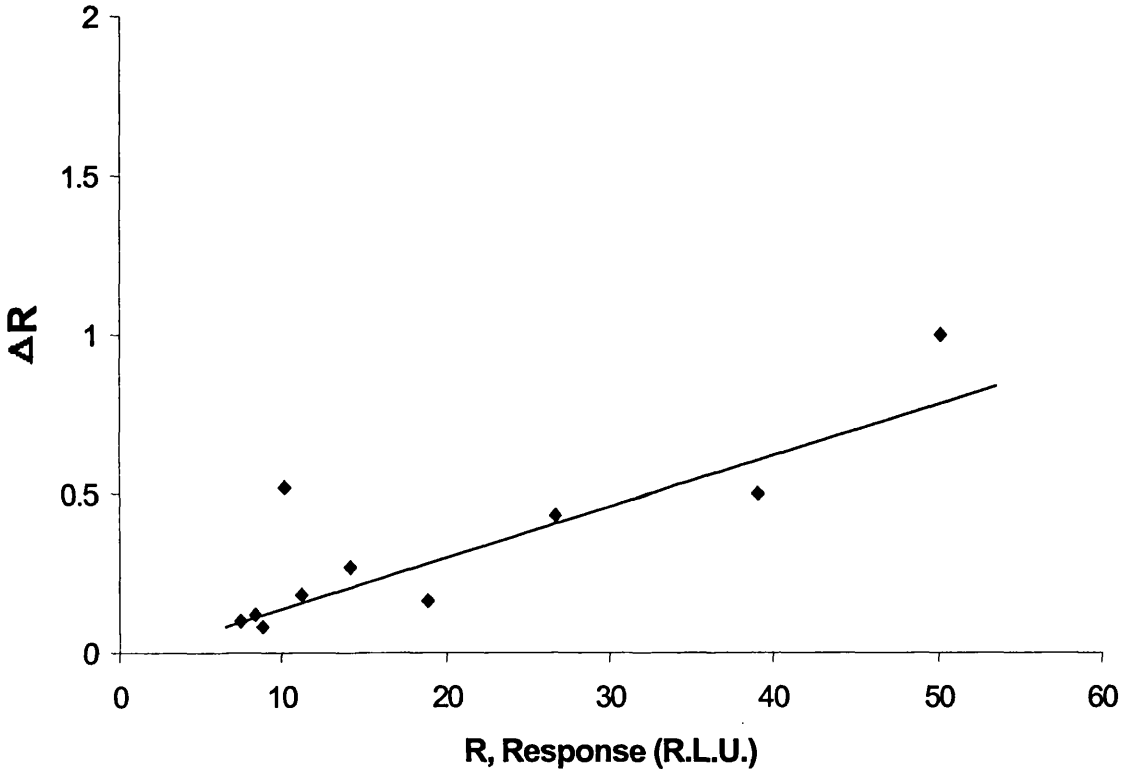

20
10
0
% Imprecision (%ΔH)
0.1
1
10
100
1000
H, Stimulator concentration (log scale)

Figure 9.1e



Statistical Analysis

As mentioned above, for most bioassays triplicate cultures were used and the light outputs could then be expressed as the ***Means ± S.D.*** of these triplicates. Where relevant, statistical analysis was performed to determine significance as detailed below.

The statistical significance of differences between two sets of results was tested by the ***Unpaired (two-sample) t-tests***. The pooled standard deviation, ***s***, was calculated as:

$$s = \sqrt{\frac{(n_1-1) \times s_1^2 + (n_2-1) \times s_2^2}{n_1 + n_2 - 1}}$$

where the first set of results gave a mean ± S.D. of $x_1 \pm s_1$ from n_1 values, and the second set of results gave a mean ± S.D. of $x_2 \pm s_2$ from n_2 values.

The test statistic ***t*** value was determined from:

$$t = \frac{x_1 - x_2}{s \times \sqrt{(1/n_1 + 1/n_2)}}$$

A ***p*** value was then obtained for a given ***t***, with $n_1 + n_2 - 2$ degrees of freedom (***df***) from a t-distribution table.

9.3 RESULTS

The protocols for the Bioassays A – D were described in the Section 9.2.2.1 and summarised in Table 9.1. This classification of our bioassay protocols emerged from numerous experiments aimed at securing an optimised bioassay. These experiments are detailed below. As mentioned in the Section 9.2.2.1, the bioassay consisted of three distinct phases. The strategy adapted in our investigation was to systematically vary conditions in each of the three phases. However, the preparation of the monolayer, i.e. Phase 1 of the bioassay, was generally the same for Bioassays A – D. The exception was in the experiments investigating the effects of pre-incubation "starvation" and cell plating density. One major change in the bioassay protocol, from that used for Bioassay A, was the use of the Promega System II for Bioassays B – D. This change became possible when the Promega System II became available. This only occurred during the course of our investigations.

Although the effects of numerous variables were investigated with each of the protocols, this cannot claim to be a fully comprehensive study. Some variables were investigated in only one of the bioassays. This was when we considered that a conclusive result in one bioassay system would be applicable to the other protocols. Thus, such a variable was not exhaustively investigated in the other bioassays. It should be noted that singlicate cultures were used in some of the preliminary experiments. This was due to the considerable expense of the Promega luciferase assay systems described in Section 9.2.2.3. Thus replicate cultures, ideally with n = 3, were only used in later studies when improvements in within assay errors were targeted. Finally, a summary of the variables to be investigated across Bioassays A – D is shown in Table 9.2.

9.3.1 Bioassay A

Selection of the best assay medium for Phase 2 i.e. the exposure of the cells to stimulators
Determining the optimal exposure time to stimulators
The effects of IBMX

9.3.2 Bioassay B

The most economic use of Promega System II during Phase 3
Preparation of the monolayer (Phase 1):
(i) The potential benefits of pre-assay "starvation"
(ii) The effect of varying the cell plating density
The influence of the serum composition of the assay medium:
(i) Determining the optimum % of horse serum incorporated in assay medium
(ii) The effect of including FCS in the assay medium
The effect of prolonged stimulation of cells
The effect of forskolin

9.3.3 Bioassay C

The effect of retaining culture medium during Phase 2
Preparation of the monolayer (Phase 1):
(i) The effect of preparing the monolayer in 50µl of culture medium
(ii) The effect of varying the cell plating density
Determining the optimal exposure time to stimulators
The effects of:
(i) Forskolin
(ii) IBMX
(iii) Combinations of IBMX and forskolin
(iv) Simultaneous stimulation by hTSH, TSAb and forskolin
Investigation for parallelism between hTSH and TSAb

9.3.4 Bioassay D

Reinvestigating the effects of the incorporation of a "starvation" step
Re-examination of the aspiration step at the end of Phase 1
Determining the optimal conditions for signal development using Promega System II:
(i) The emergence of systematic errors
(ii) Signal "drift" across the 96-well microtiter plate
(iii) The influence of the amount of luciferase reagent on signal stability
(iv) The influence of reducing the reading time/well on signal stability

Table 9.2 **A summary of the variables investigated in Bioassays A – D.** Listed above are the variables investigated in each of the bioassays to secure an optimized bioassay. Detailed descriptions of each experiment are described in Sections 9.3.1 – 9.3.4 for Bioassays A – D respectively.

9.3.1 Optimization of Bioassay A

Bioassay A was based upon the initial protocol received from N.I.H.. This included methods for the culture and preparation of the monolayers, i.e. Phase 1 (see Section 9.2.2.1) and the exposure of the microcultures to the stimulators, i.e. Phase 2. For Phase 3, a luciferase measurement system which required separate steps for cell lysis and signal development (Promega System I) was proposed. Our overall strategy was to use the N.I.H. protocol, which we refer to as N.I.H. Protocol 1, as a starting point and then to systematically investigate a succession of variables with a view to improving the system. The final outcome was Bioassay D (see Section 9.3.4).

Outline of N.I.H. Protocol 1.

- For Phase 1, CHO25LUC cells were harvested by trypsinization and monolayers were prepared by plating cells into the wells of 96-wells plates (clear or white) at $4x10^5$ cells/ml in culture medium (100µl/well).
- The monolayers were then allowed to grow till confluency. 24hr prior to Phase 2, the culture medium was aspirated and replaced with fresh culture medium (100µl/well). This completed Phase 1 and the monolayers were then committed to Phase 2.
- For Phase 2, the culture medium (100µl) was fully aspirated and the monolayers were washed twice with AM2 (200µl/well) prior to the addition of the stimulators which were also diluted in AM2.
- Exposure of the stimulators (TSH or Graves' patient sera) was for 2hr at 37°C in the standard gassed and humidified incubator. At the end of Phase 2, the wells

were fully aspirated and the monolayers were again washed twice with AM2 (200µl/well).

- For Phase 3, luciferase activities were determined using Promega System I. Cell lysis was achieved with the addition of the lysis buffer (50µl/well) followed by repetitive pipetting. Cell lysate (20µl) was added to a cuvette which was then placed in the luminometer. Luminescence was determined following the addition of the luminescent substrate (100µl) with the auto-injection function of the luminometer. The latter was programmed to read each cuvette for 10sec after a 2sec delay.

PRELIMINARY EXPERIMENT TO SELECT THE BEST ASSAY MEDIA

As described above the N.I.H. Protocol 1 used AM2 (see Section 9.2.2.2) as the medium for the exposure of the cells to stimulators i.e. Phase 2. This medium was also used to wash the cells before and after incubation with stimulators. Using Protocol 1, we initially confirmed that an increase in luminescence from the microcultures could be detected in response to hTSH (data not shown). However, close microscopic observation revealed that after washing with AM2, there was extensive monolayer detachment and loss of cells. This was particularly noticeable in the presence of IBMX ± 200mU/L hTSH (data not shown). Because uncontrollable cell detachment would clearly be a serious source of between-well variation, the use of AM2 was abandoned. Several different media (see Section 9.2.2.2) including culture medium, AM1 and Ham's F12 nutrient medium simply supplemented with penicillin and streptomycin i.e. with no serum supplements, were compared against AM2. The different media did not significantly alter the light outputs from the zero-

dose controls, and a 3.5- and 2.3-fold increase in R.L.U. in response to 200mU/L hTSH was observed when AM1 and Ham's F12 medium was used respectively (data not shown). This was larger than the fold-increase when AM2 had been used. Crucially, no detachment and hence the loss of cells was observed when media other than AM2 were used. Since the largest fold-response was obtained, with similar zero-dose control values, with AM1, this was selected as the assay medium for further studies with Bioassay A.

DETERMINATION OF THE OPTIMAL EXPOSURE TIME TO STIMULATORS

Using AM1 for Phase 2, a time course was performed to determine the optimal time for exposing the cells to a thyroid stimulator (Figure 9.2a). The cells were exposed to ± 200mU/L hTSH for 0 – 6hr. Light output increased from both the unstimulated cells and those activated with hTSH. For both, this plateaued effectively after 3 – 5hr with evidence of a decline in the signal at the longest exposure time (6hr). The magnitude of bioassay response to the TSH was judged to be maximal between 3 – 5hr (Figure 9.2b). As a consequence, an incubation time of 4hr was selected for future studies.

INVESTIGATION OF POSSIBLE POTENTIATION OF THE RESPONSE TO TSH BY THE USE OF IBMX

IBMX is a cyclic nucleotide inhibitor of the enzyme phosphodiesterase. It thus inhibits the degradation of cAMP by the enzyme and thereby sustains any increase in intracellular cAMP. Consequently, IBMX might be expected to enhance any response to hTSH stimulation. Possible synergy between IBMX and hTSH in the luciferase

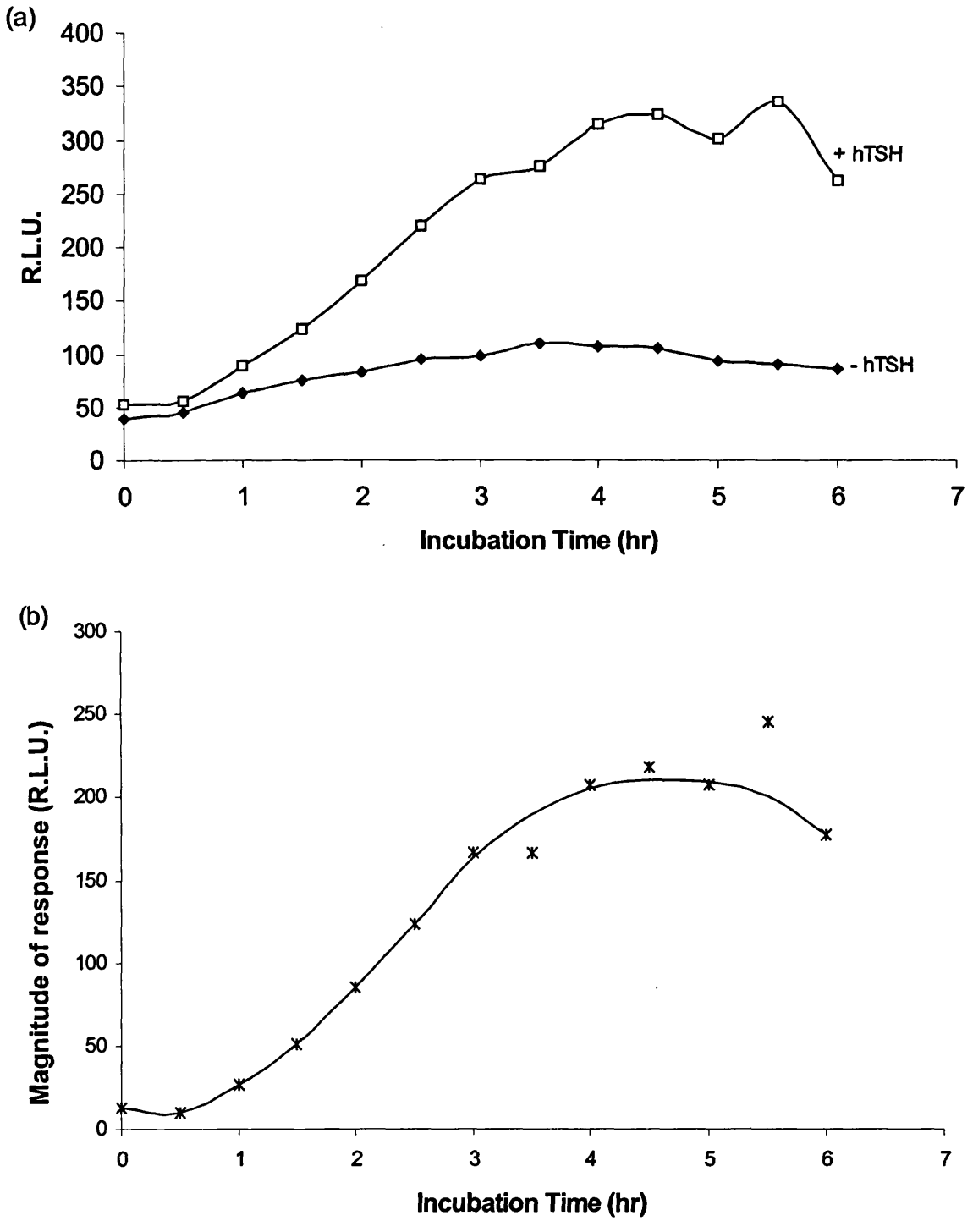


Figure 9.2 **Bioassay A: time course of luciferase stimulation by hTSH.** (a) Cells were exposed to 200mU/L hTSH (80/558; □) for 0 – 6hr; light outputs from unstimulated controls are also shown (◆). The R.L.U. of singlicate cultures have been plotted against time. (b) Transformation of (a) so that the magnitudes of response (see Section 9.2.3, Figure 9.1a) to TSH are plotted against time.

reporter gene bioassay was therefore investigated. Using AM1 as the assay medium, increasing light emission was observed with increasing concentrations of IBMX for both TSH-stimulated and unstimulated cells (Figure 9.3a). Maximal light output was reached at 0.25mM IBMX for both stimulated and unstimulated cells. Any potentiation of the magnitude of the response to hTSH (Figure 9.3b) appeared to be a complex function of IBMX concentration. Although numerically, the magnitude of response was maximum at 0.0625mM IBMX, it was not considered to be significantly larger than that observed in the absence of this phosphodiesterase inhibitor. We therefore concluded that no useful potentiation to TSH could be obtained by the addition of IBMX to Bioassay A.

DOSE-RESPONSE CURVE TO TSH

Response to hTSH 80/558

Using the conditions established above for Phase 2 (AM1, 4hr incubation, no IBMX), we examined the response to multiple incremental doses of hTSH (Figure 9.4). The dose of 400mU/L resulted in a well-defined maximal light output of 226 R.L.U., which was a 4.9-fold increase above the zero-dose control light output (45.7 R.L.U.). An unexpected and sharp decline in light output was observed at concentrations >400mU/L. We could not determine a theoretical detection limit for TSH determination from this study since only singlicate cultures had been used. However, it appeared that with Bioassay A, responses might be obtained with doses of hTSH of the order of 1mU TSH/L and above.

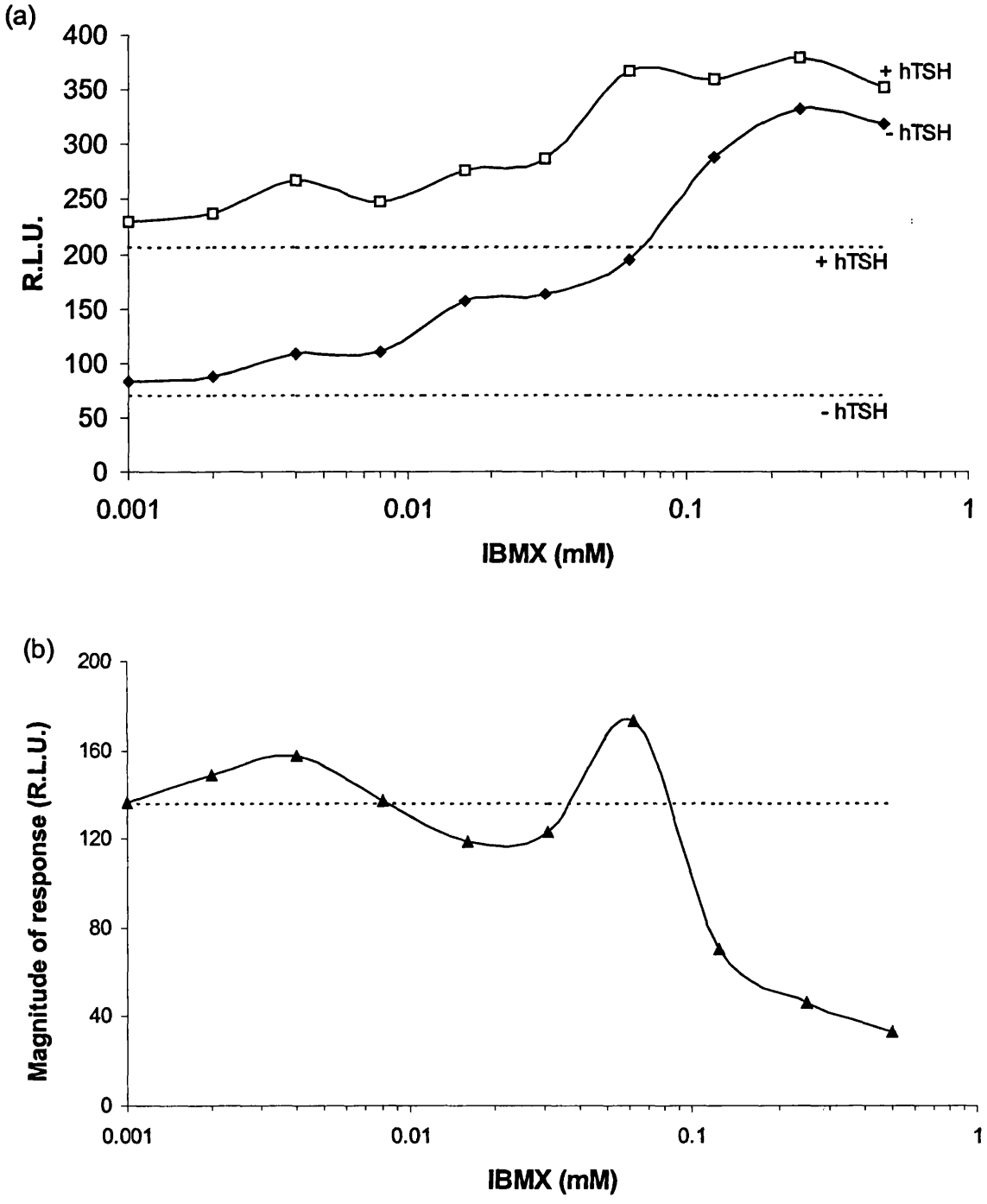


Figure 9.3 **Bioassay A: the investigation of possible potentiation of bioassay responses to hTSH by IBMX.** (a) The increase in luminescence in response to increasing concentrations of IBMX in the presence (□) and absence (◆) of 200mU/L hTSH 80/558. --- represents light outputs in the absence of IBMX. The R.L.U. of singlicate cultures have been plotted. (b) The magnitudes of response (see Section 9.2.3, Figure 9.1a) to TSH plotted against IBMX concentration. --- represents the magnitude of response (±TSH) in the absence of IBMX.

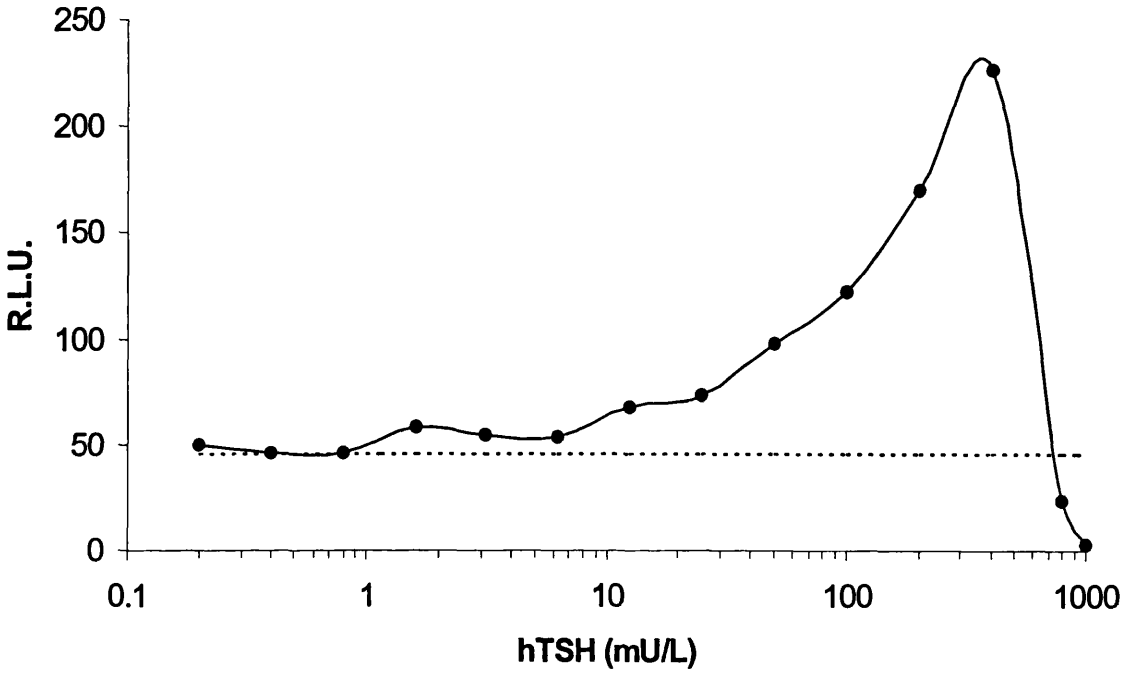


Figure 9.4 **Bioassay A: the increase in luminescence in response to increasing concentrations of hTSH (80/558).** --- represents the light output from the unstimulated control microcultures. The R.L.U. from singlicate microcultures have been plotted.

9.3.2 Optimization of Bioassay B

During the course of our work with Bioassay A, a second generation luciferase assay system (Promega System II), became available. This system offered several advantages. Firstly, a single reagent both lysed the cells and supplied the luciferase substrates in one step. Thus, it offered a major advantage over Promega System I, for which a separate and error-prone extraction of intracellular luciferase had been required. Secondly, because of the stability of the light output, the single reagent could be added manually and then the plate read in the luminometer, without the need for the auto-injection facility. We anticipated, therefore, that a protocol exploiting Promega System II would improve on Bioassay A. Indeed, since neither a tedious separate extraction was required nor the auto-injection step, sample throughput could be considerably enhanced. Consequently, numerous additional variables could be readily investigated to optimize conditions for Bioassay B as are described below.

PRELIMINARY INVESTIGATION INTO THE MOST ECONOMICAL USE OF PROMEGA SYSTEM II

We initially investigated the nature of a TSH dose-response curve using Promega System II (Figure 9.5). This was run according to the Promega protocol which recommended the addition of 100µl/well of the single step "Steady-Glo" luciferase reagent. The light output from the unstimulated cells was 17.7 ± 0.2 R.L.U. and this increased, in the presence of 500mU/L hTSH, to 96.3 ± 0.8 R.L.U.. Thus, a 5.4-fold increase in light production was induced by the highest dose of the stimulator tested (500mU/L). This fold-response was similar to that achieved with Bioassay A (Figure

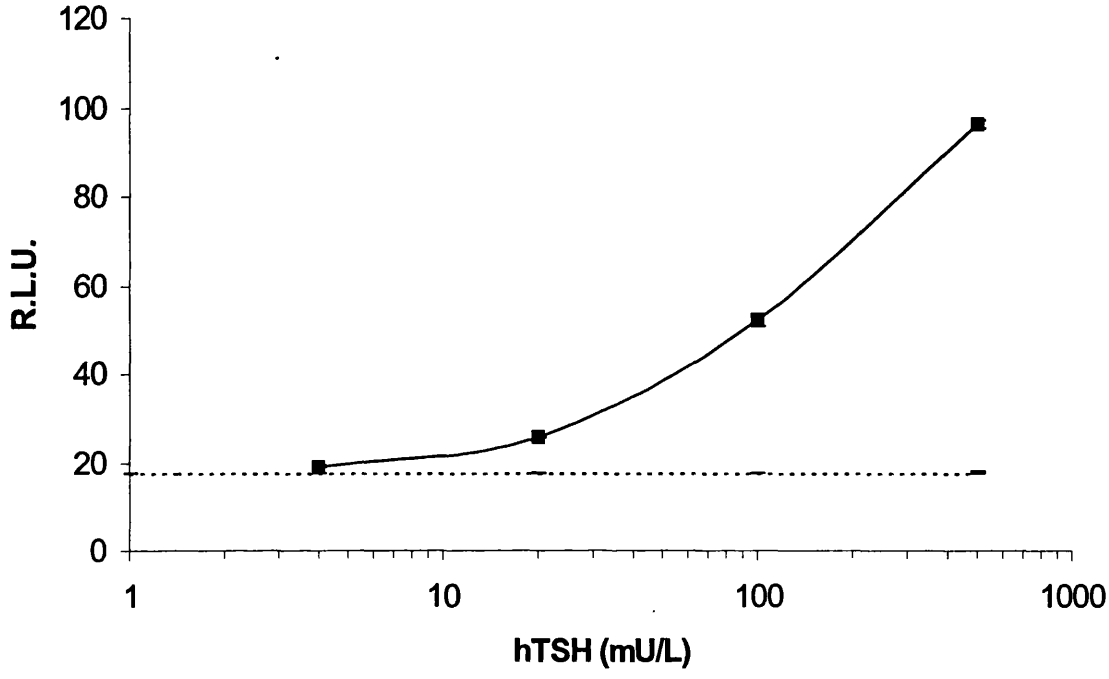


Figure 9.5 **Bioassay B: the increase in luminescence in response to increasing concentrations of hTSH (80/558) using Promega System II.** The assay was performed using the manufacture's recommended concentration of "Steady-Glo" luciferase reagent (100µl/well). The reagent (pre-equilibrated to room temperature) was added using a Repeater Eppendorf pipette in a consecutive sequence (wells B2 – C6). The reagent was then mixed for 10sec using a plate shaker and left for a further 10min at room temperature before placing in the Anthos Lucy 1 microplate luminometer. To quantify light emission, each well was read for 10sec following the same sequence that had been used for the addition for the "Steady-Glo" reagent. The mean ± S.D. of the light outputs from triplicate microcultures has been plotted. --- is the light output from the unstimulated control microcultures.

9.4). However, as we had anticipated, with this new system, the within replicate reproducibility was impressive. We attributed this to the use of the combined reagent, which, unlike with Bioassay A, avoided the separate extraction step, after Phase 2 of the bioassay. Another major advantage of the Promega System II was the stability of the light emission obtained with it. This made it possible to avoid using the auto-injection facility of the luminometer, and thus speeded up the response measurement.

Although the technical manual for this assay system recommended using 100µl of "Steady-Glo" luciferase reagent/well, the manufacturer also reported that there was a wide tolerance in the system, such that light outputs did not vary over a wide reagent concentration range (Promega technical manual #TM051). We tested this claim as shown in Figure 9.6. We confirmed that light output was strikingly constant despite a 5-fold variation in the volume of "Steady-Glo" reagent added to the wells. The fold-increase in the response to hTSH was 3.2 ± 0.2 (mean ± S.D.) as measured for the 5 reagent volumes tested. Thus there was only a small % variance in fold response (6.25%) and there was no evidence of a systematic change with reagent volume (Figure 9.6). We therefore confirmed the claim made by Promega for the unusual tolerance in this system. We further confirmed this by comparing two hTSH dose-response curves. These were set up identically except that in Phase 3, the "Steady-Glo" reagent was added at final concentrations that differed 3-fold (Figure 9.7). There was a slight reduction in light outputs at the lower reagent concentration (20µl/well). However, since this was consistent over the entire range of hTSH concentrations tested and was also seen with the unstimulated controls, the actual increment in bioassay signal to the increasing doses of hTSH, above that observed with the zero-dose unstimulated cells i.e. the magnitude of response (see Section 9.2.3, Figure

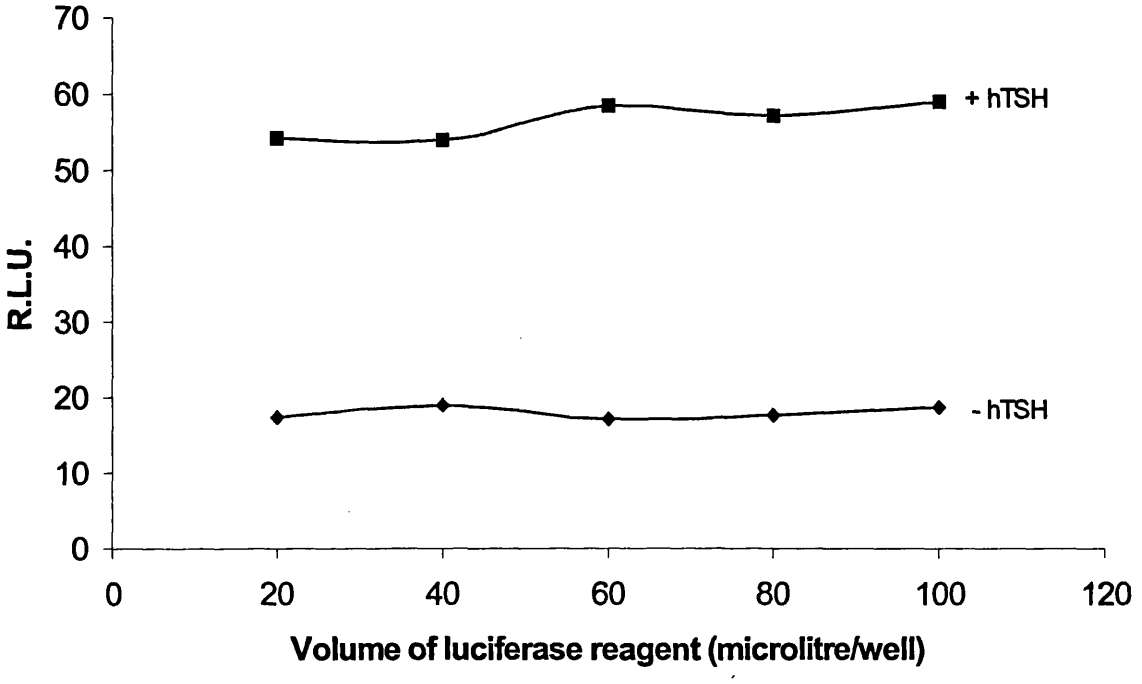


Figure 9.6 **Bioassay B: the stability of light output obtained despite a 5-fold variation in the volume of "Steady-Glo" luciferase reagent added** Microcultures were incubated in the presence (■) and absence (◆) of 100mU/L hTSH (80/558). For Phase 3, the volume of the "Steady-Glo" luciferase reagent added varied from 20 – 100µl/well. The light outputs from singlicate cultures have been plotted.

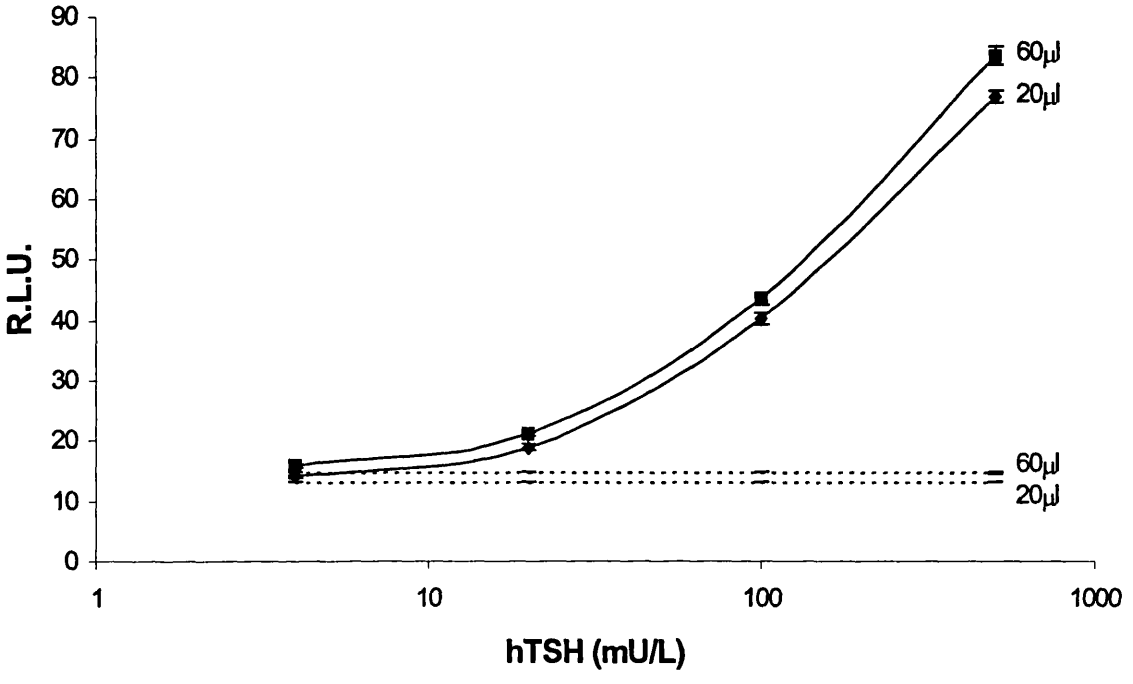


Figure 9.7 **Bioassay B: the effect of using different volumes of "Steady-Glo" luciferase reagent on dose-response curves to hTSH.** Microcultures were stimulated with increasing concentrations of hTSH (80/558). For Phase 3, either 20µl (◆) or 60µl/well (■) of luciferase reagent was added. --- represents the light outputs from unstimulated microcultures. The mean ± S.D. has been plotted for triplicate cultures.

9.1a) was virtually identical. Moreover, the favourable within-assay precision, first noted when 100µl had been used (Figure 9.5), had been retained (Figure 9.7). We therefore concluded that Bioassay B could be run using only 20µl of the "Steady-Glo" reagent/well. Since this was by far the most expensive reagent, this adaptation reduced the cost of the bioassay 5-fold.

In addition to reagent concentration tolerance, the "Steady-Glo" system was designed for high-throughput batch testing, since the glow luminescence signal produced was reported to have a half-life of >5hr. It was claimed that in CHO cells, a 25% decay in luminescence would be observed only 2.5hr after the addition of the "Steady-Glo" reagent to the wells (Promega technical manual #TM051). Clearly it was important to check that this stability was retained when using a lower concentration of reagent (20µl, Figure 9.8). The R.L.U. emitted from one triplicate set of wells were recorded over a period of 2hr. There was a gradual decrease in the signal over this period. A 3.5% decay was observed after 60min but after 120min, the signal had decreased by approximately 30%. This concurs with the signal kinetics reported by Promega, when 100µl of reagent had been added to each well, and we concluded that signal instability would not result from the use of only 20µl/well. However, as is reported much later (Bioassay D), this aspect required re-investigation, especially when patient sera were introduced into the system (see Section 10.3.5).

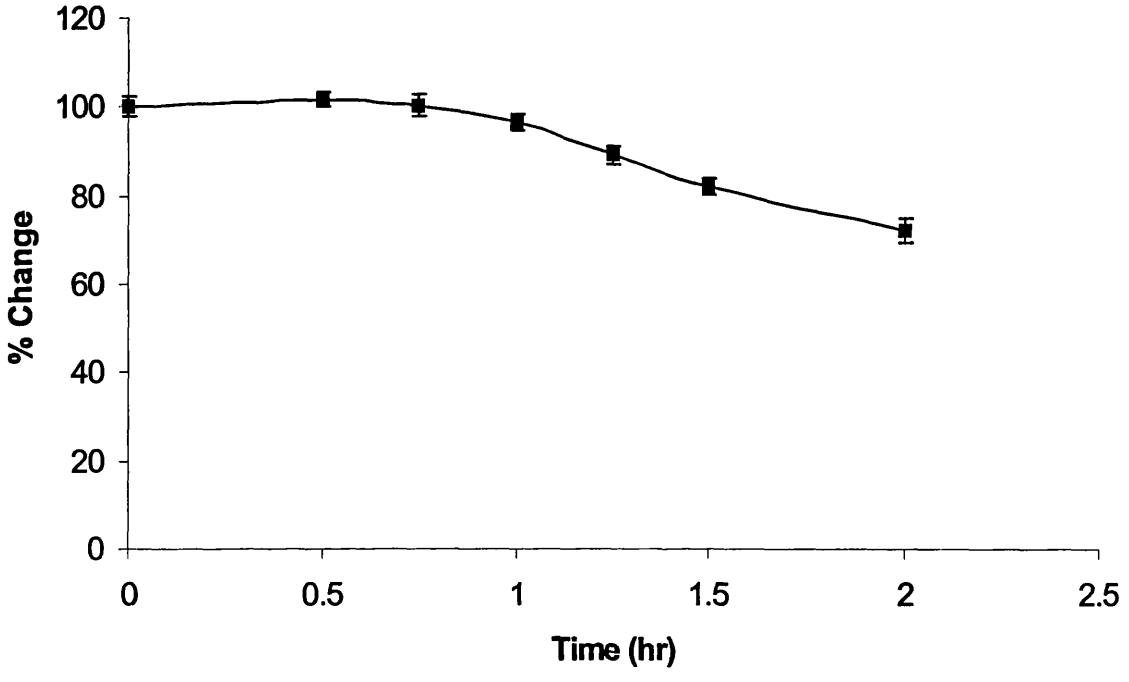


Figure 9.8 **Bioassay B: the stability of the glow luminescence signal produced using a lower concentration of luciferase reagent from Promega System II.** One set of triplicate microcultures in the presence of 250mU/L hTSH (80/558) was set up. After the addition of the "Steady-Glo" luciferase reagent (20µl/well as opposed to the recommended 100µl/well), light output was recorded over a period of 2hr at the selected times shown on the x-axis. The mean ± S.D. R.L.U. of the triplicate wells was obtained for each time point. The decline of the signal was then plotted as the % change ± S.D. in the magnitude of the initial reading at time 0, which was expressed as 100%.

AN INVESTIGATION OF POTENTIAL BENEFITS OF A PRE-ASSAY "STARVATION" STEP

Several attempts were made to improve the sensitivity of the bioassay i.e. the responses to low doses of TSH, via modifications in the protocol for preparing the monolayer i.e. Phase 1 (see Section 9.2.2) prior to the exposure of microcultures to the hormone (Phase 2). Much earlier work with TSH-responsive rat thyroid FRTL-5 cells had demonstrated that overnight maintenance in medium without any stimulators, increased the subsequent sensitivity of the cells to stimulation with TSH. Such cells were said to have become "quiescent" during the overnight pre-incubation (Vitti P *et. al.*, 1982). The resulting FRTL-5 monolayers had lower basal levels of cAMP and were also more responsive to low doses of TSH. We performed two investigations to try and similarly improve the sensitivity of bioassays based upon the CHO25LUC cells, using the Bioassay B protocol, as described below.

Firstly, cells were plated as described in Section 9.2.2.1.1. However, after 24hr, the culture medium, which contained 10% FCS, was removed and the cells were cultured overnight in AM1, in which 10% horse serum was substituted for the FCS. The next day, the cells were stimulated with increasing concentrations of hTSH prepared in AM1. The resulting dose-response curve (Figure 9.9a) was compared with that from cells which had not been "starved" i.e. had been cultured for the entire 48hr in 10% FCS, as was conventional. Increasing luminescence to increasing hTSH concentrations was observed for both sets of monolayers. Unexpectedly, greater light outputs were consistently observed in the monolayer which had been "starved". The greatest light outputs were observed after exposure to 500mU/L hTSH, in both sets. In

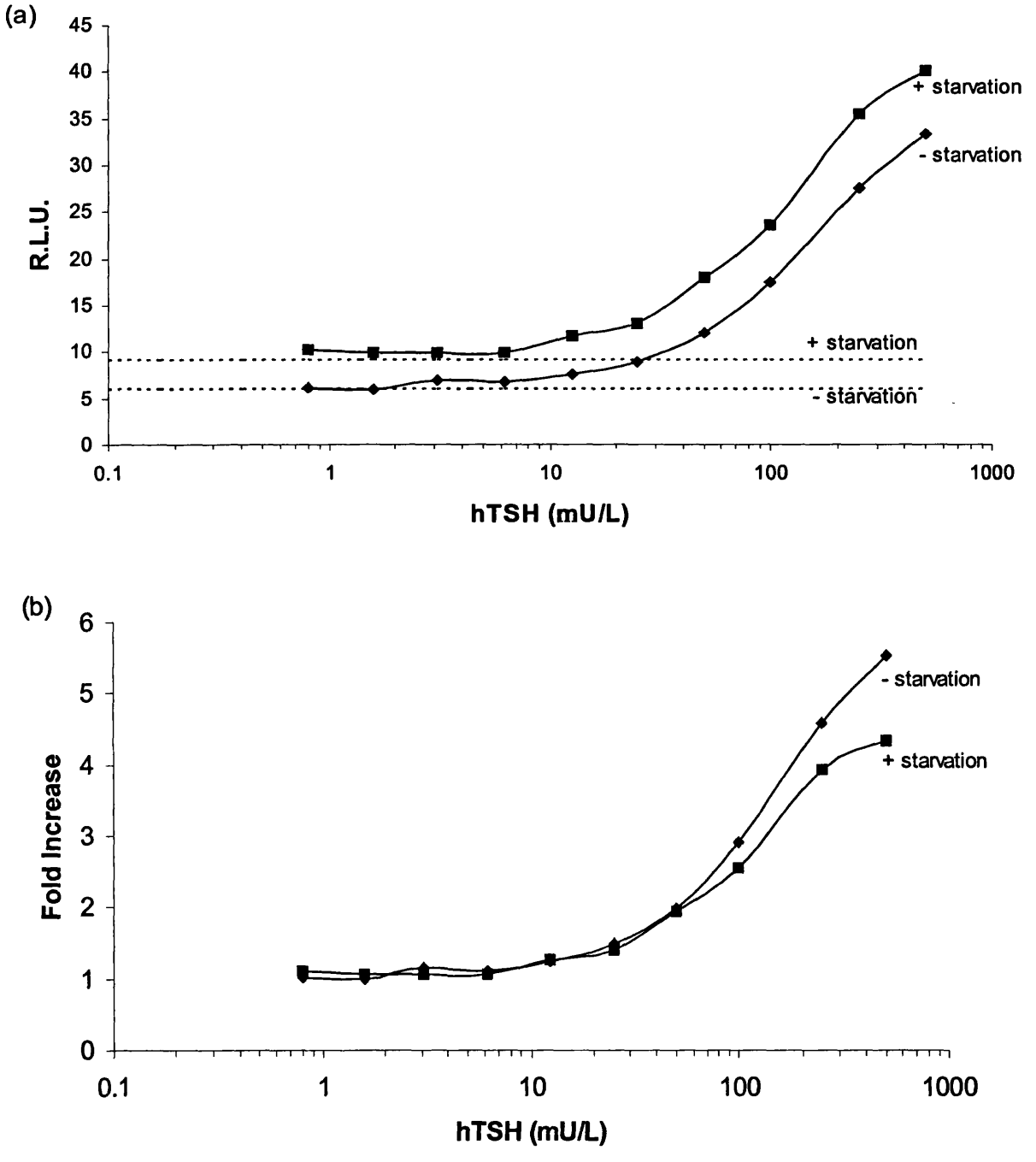


Figure 9.9 **Bioassay B: the effect of the incorporation of a pre-assay "starvation" step on the subsequent response to hTSH.** (a) Monolayers were prepared as described in Section 9.2.2.1.1. However 24hr after plating, a pre-assay "starvation" step was incorporated. For the next 24hr, the incubation was either continued in the usual cultured medium (◆), or AM1, in which the 10% FCS had been replaced with 10% horse serum, was substituted (■). The resulting monolayers were then exposed to hTSH (80/558), and luciferase assayed according to the standard protocol for Bioassay B (see Section 9.2.2.1.2). --- shows the unstimulated control light outputs as labelled. The means of duplicate cultures are plotted. (b) The results of (a) have been transformed to allow comparison of the fold increase in response to hTSH by cells with (■) and without (◆) "starvation".

response to this dose of TSH, a 5.5-fold increase above the control response by the "non-starved" cells was obtained compared to a 4.3-fold increase above the control responses by the "starved" cells (Figure 9.9b). However, the magnitudes of bioassay response were similar. Thus, withdrawal of the stimulators present in 10% FCS, neither lowered the unstimulated control values nor resulted in a greater increase in the fold-response to stimulation with a high dose of TSH (500mU/L). Most significantly, withdrawal of the FCS for 24hr failed to enhance the sensitivity of the bioassay i.e. the responses to low TSH doses (Figure 9.9).

Secondly, we tried a prolonged starvation period. Cells were plated and grown to confluency as described in Section 9.2.2.1.1. After the 48hr required to form the confluent monolayer, they were then maintained for a further 1 or 2 days in AM1 i.e. in the absence of FCS. The resulting monolayers were then stimulated with a relatively low dose of hTSH (10mU/L). No significant improvements in the magnitude of the responses to hTSH were observed following the prolonged starvation (data not shown).

INVESTIGATING THE EFFECT OF PREPARING THE MONOLAYER WITH LOWER CELL PLATING DENSITIES

In addition to investigating for possible beneficial effects of pre-assay starvation on bioassay sensitivity, we also investigated possible gains following the lowering of the cell plating density. It was considered possible that by reducing the number of cells in each well, the baseline control signals may be lowered without necessarily affecting the responses to a relatively low dose of hTSH (10mU/L). The number of cells/well,

in a constant plating volume (100µl), was varied with plating densities ranging from 0.25 – $4x10^5$ cells/ml. Although as expected, decreasing light outputs were observed with the unstimulated controls, with decreasing cell plating densities, the responses to 10mU/L hTSH were, unfortunately, reduced (Figure 9.10a). Overall, we found that the differences between stimulated and unstimulated light outputs i.e. the magnitudes of response (see Section 9.2.3, Figure 9.1a) were significantly improved at the higher cell plating densities (Figure 9.10b). As a consequence, we concluded that we were already working at the cell density ($4x10^5$ cells/ml) which gave the largest magnitude of bioassay response to hTSH, and that no gain in bioassay sensitivity could be achieved by lowering the cell plating density.

THE INFLUENCE OF THE SERUM COMPOSITION IN ASSAY MEDIUM

The optimum % of horse serum incorporated in assay medium

In Bioassay A, we established that AM1 was the most suitable assay medium for Phase 2, namely the exposure of the cells to the stimulators (see Section 9.3.1). AM1 arbitrarily contained 10% horse serum, but it had not been determined whether this concentration was optimal. We therefore investigated the effects of varying the % horse serum on the magnitudes of response to 50mU/L hTSH (Figure 9.11a). Increasing light output was obtained for both the non-stimulated cells and those activated with hTSH, with increasing horse serum in the medium. However, the largest magnitude of response was obtained with 10% horse serum (Figure 9.11b). Thus, it appeared that this was the best concentration of horse serum in AM1 for Bioassay B.

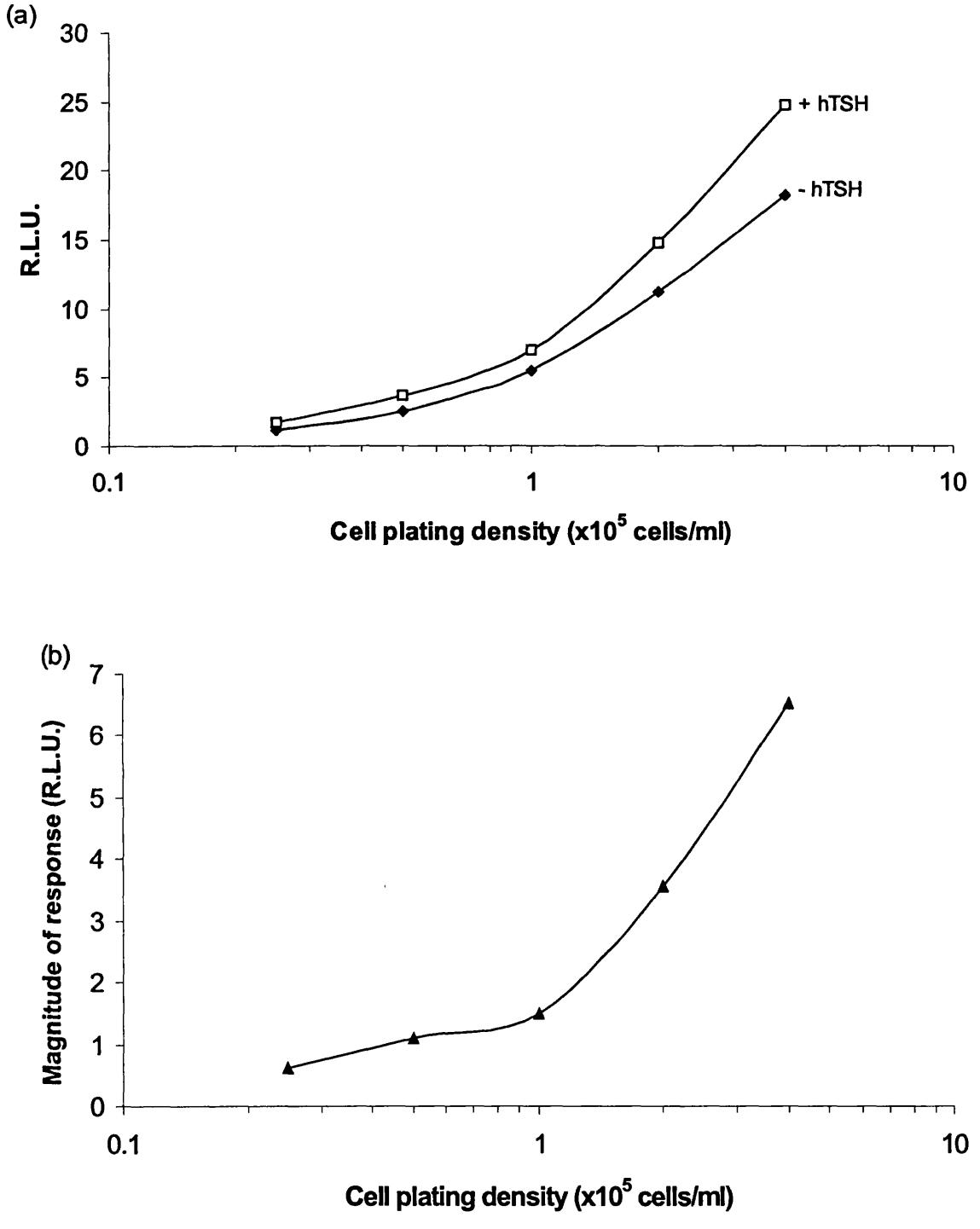


Figure 9.10 **Bioassay B: the effect of lower cell plating densities on light outputs from unstimulated and stimulated microcultures.** (a) Microcultures were prepared as described in Section 9.2.2.1.1 but with plating densities ranging from 0.25 – 4x10^5 cells/ml (100µl/well). Cells were then stimulated with (□) and without (◆) hTSH (80/558, 10mU/L). The means of duplicate cultures have been plotted. (b) The magnitudes of response (see Section 9.2.3, Figure 9.1a) plotted against plating density.

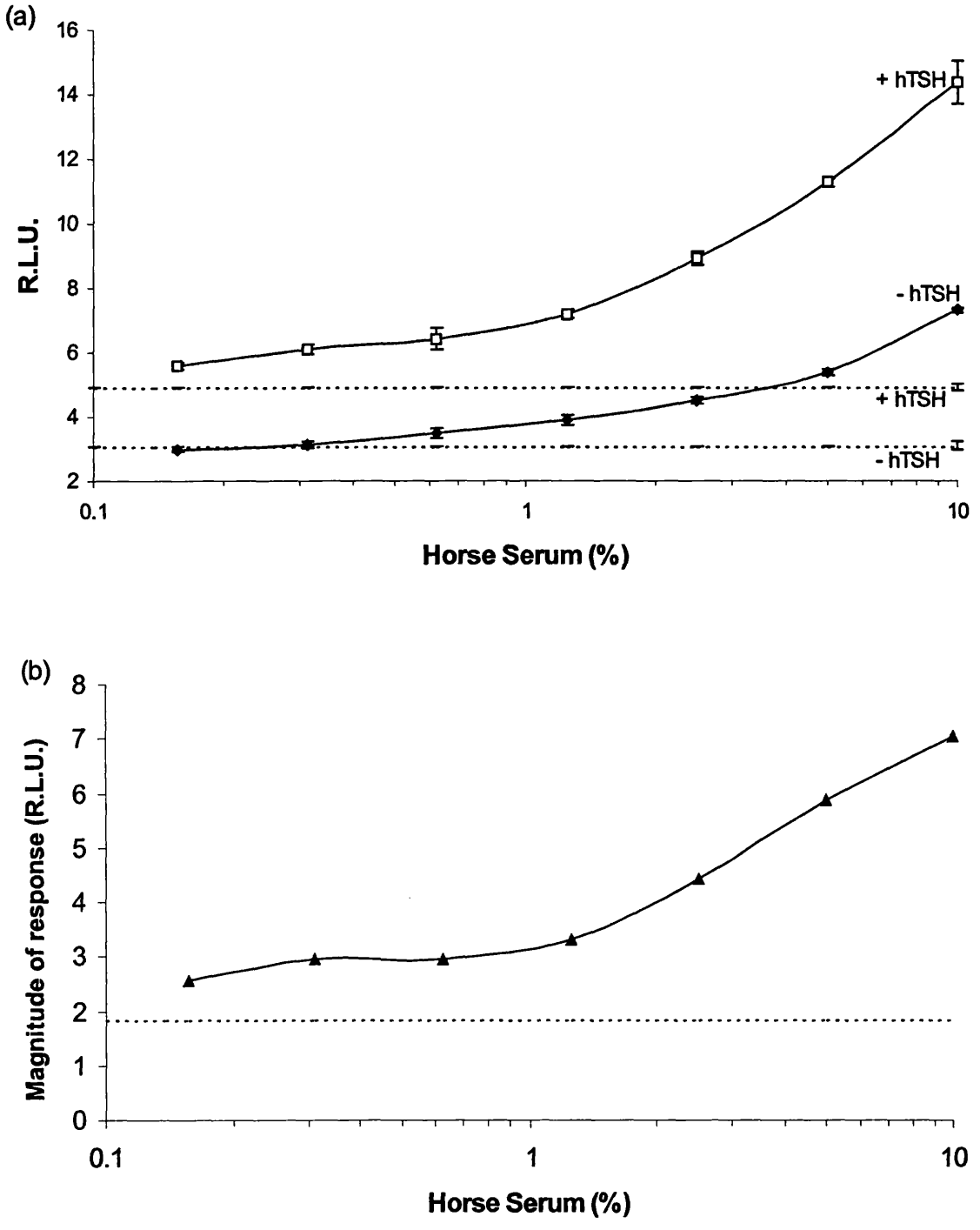


Figure 9.11 **Bioassay B: the effect of varying the concentration of the horse serum included in the assay medium on the response to hTSH.** (a) AM1 supplemented with varying concentrations of horse serum (0.15 – 10%) was used for Phase 2 i.e. the exposure of the cells to hTSH (80/558, 50mU/L; □); R.L.U. observed in the absence of hTSH (◆). --- represents the R.L.U. emitted in the total absence of horse serum (±TSH). Results are the mean ± S.D. for each set of triplicate microcultures. (b) The magnitudes of response (see Section 9.2.3, Figure 9.1a) to TSH plotted against the % of horse serum. --- represents the magnitude of response obtained when AM1 without horse serum had been used.

The effect on light emission of the inclusion of FCS in the medium used for Phase 2

We investigated the effect of the inclusion of FCS, during the 4hr exposure of the cells to TSH, on the responses to the hormone i.e. Phase 2. This was investigated by the generation of a series of assay media, based on AM1 containing 2.5% horse serum, which were further supplemented with a varying % of horse serum or FCS. The additional concentrations of FCS and horse serum used ranged from 0 – 10%. Thus the media finally contained a minimum and maximum of 2.5% and 12.5% of total serum respectively. These media were then used for Phase 2 i.e. the exposure of the cells to hTSH (100mU/L). Varying the serum composition in this way had little effect on light emission from the unstimulated and TSH stimulated monolayers, and the slight changes in R.L.U. emitted generally shifted in parallel (Figure 9.12a & b). Thus when the results were expressed as magnitudes of response to TSH (Figure 9.12c), no notable changes in this basic bioassay parameter were observed.

THE EFFECT OF PROLONGING THE EXPOSURE TIME OF THE MONOLAYERS TO LOW DOSES OF TSH

A period of 4hr had been established as the optimum exposure duration for TSH stimulation for Bioassay A (Figure 9.2a & b). However, it had been reported that, when working with monolayers of human thyroid cells, prolonging exposure times, e.g. to 16hr, can lead to enhanced bioassay sensitivity when responding to either hTSH or TSAb (Bidey SP *et. al.*, 1982). For the sake of completeness we tested the effect of prolonging the exposure time to relatively low doses of TSH using the protocol for Bioassay B (Figure 9.13). Cells were stimulated with relatively low doses

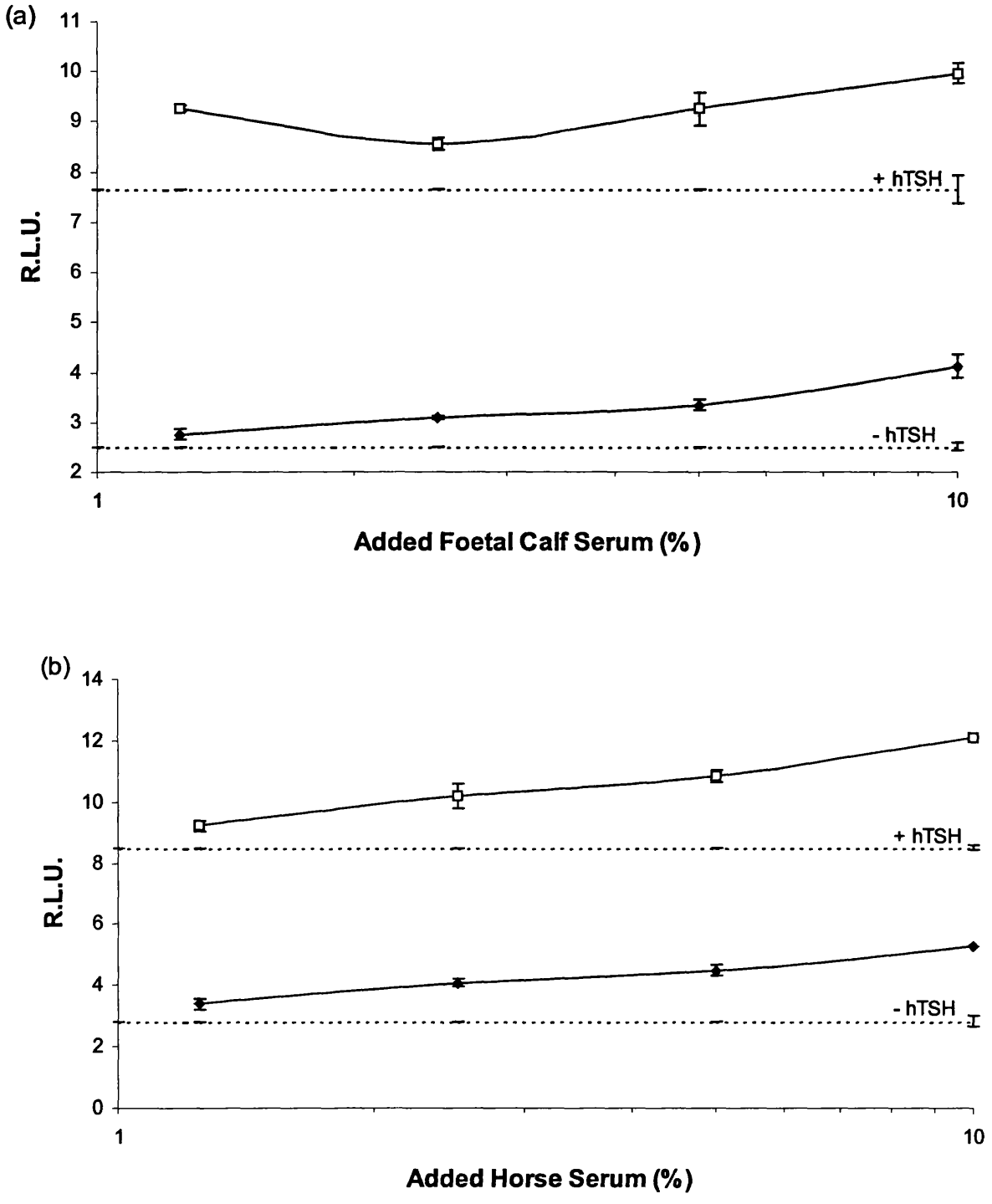


Figure 9.12 **Bioassay B: the effects of FCS together with horse serum on the response to hTSH.** During Phase 2, AM1 containing 2.5% horse serum was supplemented with varying concentrations of (a) FCS (1.25 – 10%) and (b) horse serum (1.25 – 10%). These media were used during the exposure of the cells to hTSH (80/558, 100mU/L, □); R.L.U. observed in the absence of hTSH (◆). --- represents the R.L.U. observed when 2.5% horse serum was the sole serum supplement. Results are the mean ± S.D. R.L.U. for each set of triplicate microcultures.

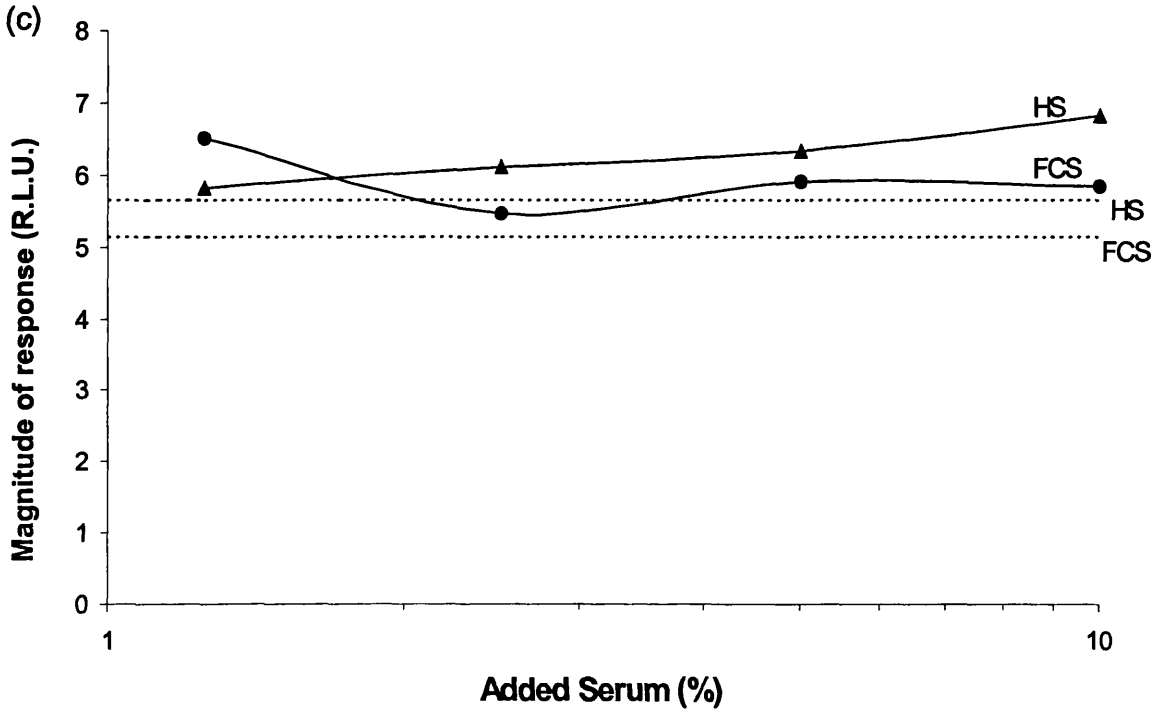


Figure 9.12 (c) The results shown in Figure 9.12(a) & (b) expressed as magnitudes of response (see Section 9.2.3, Figure 9.1a) to TSH. The magnitudes of response to TSH have been plotted against the % of added serum, either FCS (●) or horse serum (HS; ▲). --- represents the magnitudes of response obtained in assay media containing no additional serum, but solely the baseline 2.5% horse serum.

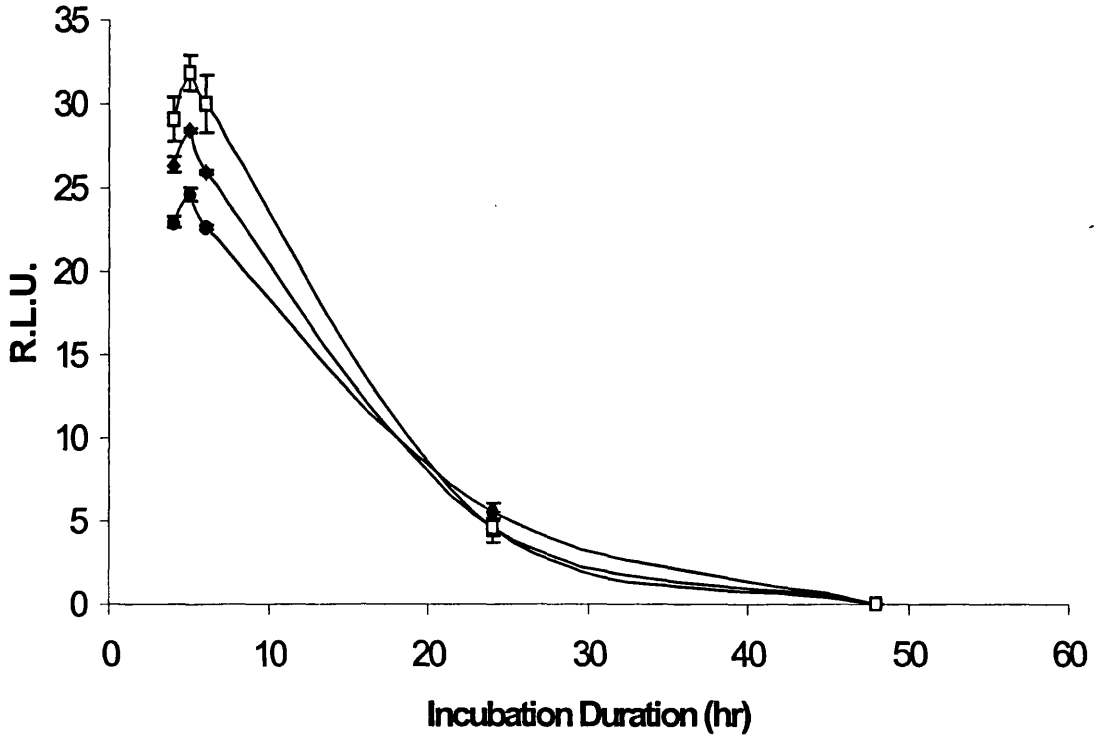


Figure 9.13 **Bioassay B: the effect of prolonging exposure time of the monolayers to low doses of hTSH on light output.** Cells were exposed to low doses of hTSH (80/558), at 5 (◆) and 10mU/L (□), for 4 – 48hr; light outputs from unstimulated controls are also shown (●). The mean R.L.U.± S.D. for each set of triplicate cultures exposed to each dose have been plotted against time.

of hTSH (5 and 10mU/L) for 4, 5, 6, 24 and 48hr. Slightly increased luminescence in response to both concentrations of hTSH, when compared to unstimulated controls, were observed after incubation times of 4, 5 and 6hr. Maximal light output was recorded after 5hr for both unstimulated cells and those exposed to hTSH. However, after prolonged stimulation of the cells (24 and 48hr), light production markedly decreased and was in fact negligible after 48hr. No response to the hormone was detected after these longer exposure times. We concluded that it was not beneficial for assay sensitivity to grossly prolong the exposure time and that the standard time of 4hr adopted previously remained optimal.

INVESTIGATION OF POSSIBLE POTENTIATION OF BIOASSAY RESPONSES USING FORSKOLIN

Forskolin, an AC activator, stimulates intracellular cAMP independently of cell surface receptors and their associated G-proteins. This diterpene activates the catalytic subunit of AC directly. Consequently, forskolin should be able to stimulate luminescence in the CHO25LUC cells in a dose dependent manner. A dose-response curve in response to forskolin is shown in Figure 9.14. The dose of 100μM gave the greatest light emission of 90.3 R.L.U., which was a 7-fold increase above the control response (12.9 R.L.U). The theoretical detection limit was not determined as singlicate cultures were used in this preliminary study. The curve appeared to plateau at 6.2μM forskolin.

Forskolin has been reported to potentiate responses when incubated simultaneously with thyroid stimulators such as TSH (Bidey SP *et. al.*, 1985). Possible synergistic

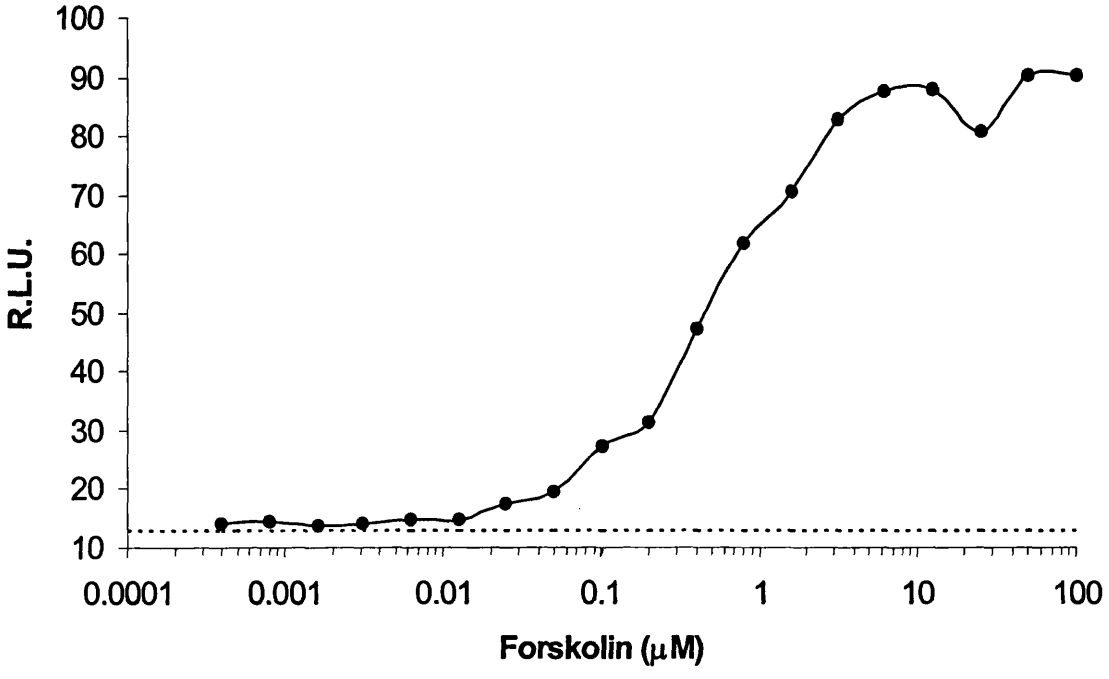


Figure 9.14 **Bioassay B: the increase in luminescence in response to increasing concentrations of forskolin.** --- is the light output observed with the unstimulated control. The R.L.U. of singlicate cultures have been plotted.

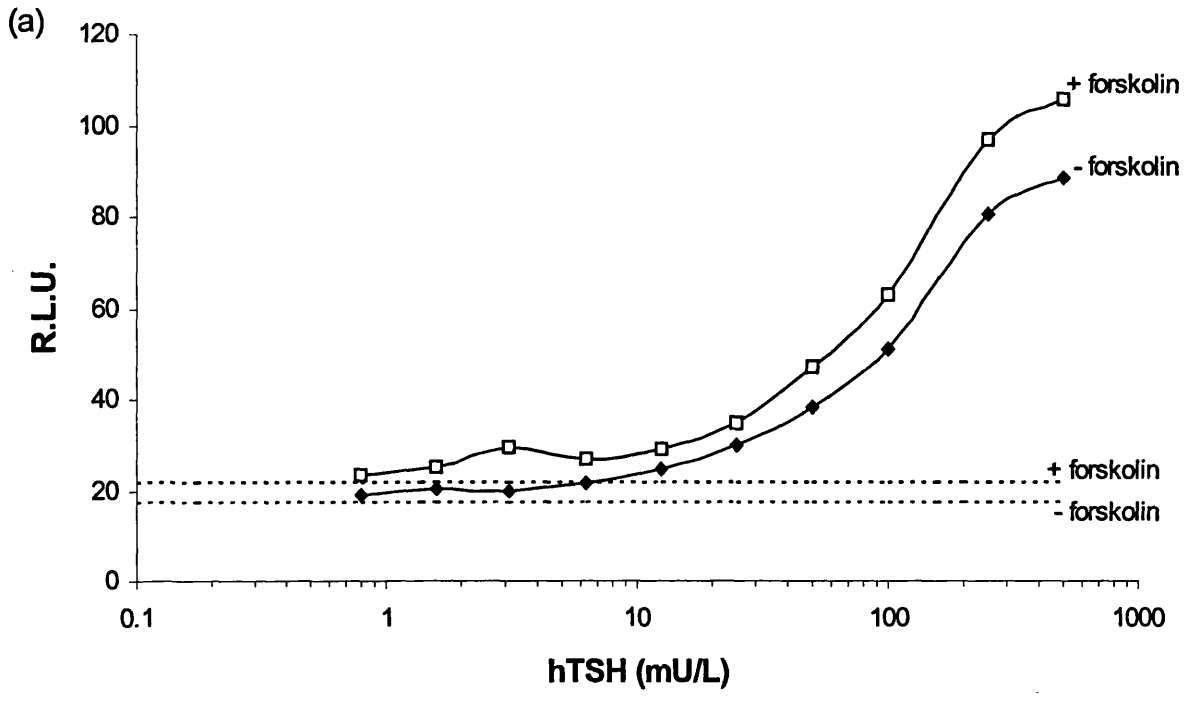
effects of forskolin on the response to hTSH stimulation were tested by the co-incubation of a low dose of forskolin (0.025μM), together with increasing doses of the hTSH. Light production was increased in the presence of forskolin throughout the hTSH dose-response curve (Figure 9.15a). Expression of the results as magnitudes of response (see Section 9.3.2, Figure 9.1a) showed no significant enhancement of the responses to the lower doses of hTSH (Figure 9.15b), apart from a slight, but transient, gain in response at 3mU TSH/L. We concluded that the inclusion of a low dose of forskolin in the bioassay would not improve bioassay sensitivity.

DOSE-RESPONSE CURVES TO THYROID STIMULATORS

Using the optimal conditions for Phase 2 obtained above (AM1, 4hr incubation, no forskolin), we investigated the responses to the following thyroid stimulators:

Response to hTSH (80/558)

Although the CHO25LUC cells had previously been shown to respond to hTSH stimulation (Figure 9.5 & 9.7), these preliminary dose-response curves were obtained with relatively few hTSH concentrations (n = 5). A more comprehensive dose-response curve was therefore established with 9 concentrations (Figure 9.16). The highest dose of 500mU/L gave the greatest light emission of 24.0 ± 0.9 R.L.U.. This was a 4.9-fold increase above the control response (4.9 ± 0.2 R.L.U). There was no evidence of a plateau being approached at this high dose suggesting that greater magnitudes of response could be obtained with yet higher concentrations. The detection limit, determined as the lowest TSH concentration which elicited light



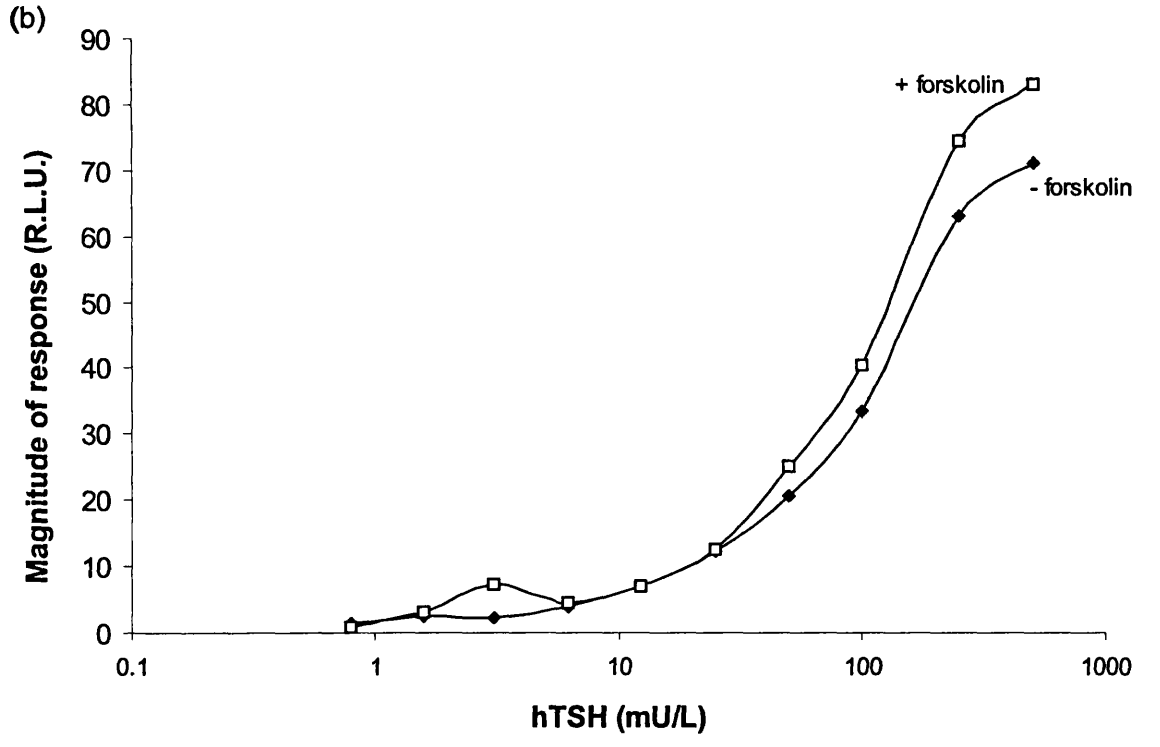


Figure 9.15 **Bioassay B: an investigation of possible potentiation of the bioassay response to TSH by forskolin.** (a) The increase in luminescence in response to increasing concentrations of hTSH (80/558) in the presence (□) and absence (◆) of a low dose of forskolin (0.025μM). --- shows the light outputs from the zero TSH controls. The R.L.U. of singlicate cultures have been plotted. (b) The magnitudes of response (see Section 9.2.3, Figure 9.1a) to TSH in the presence (□) and absence (◆) of forskolin plotted against hTSH concentration.

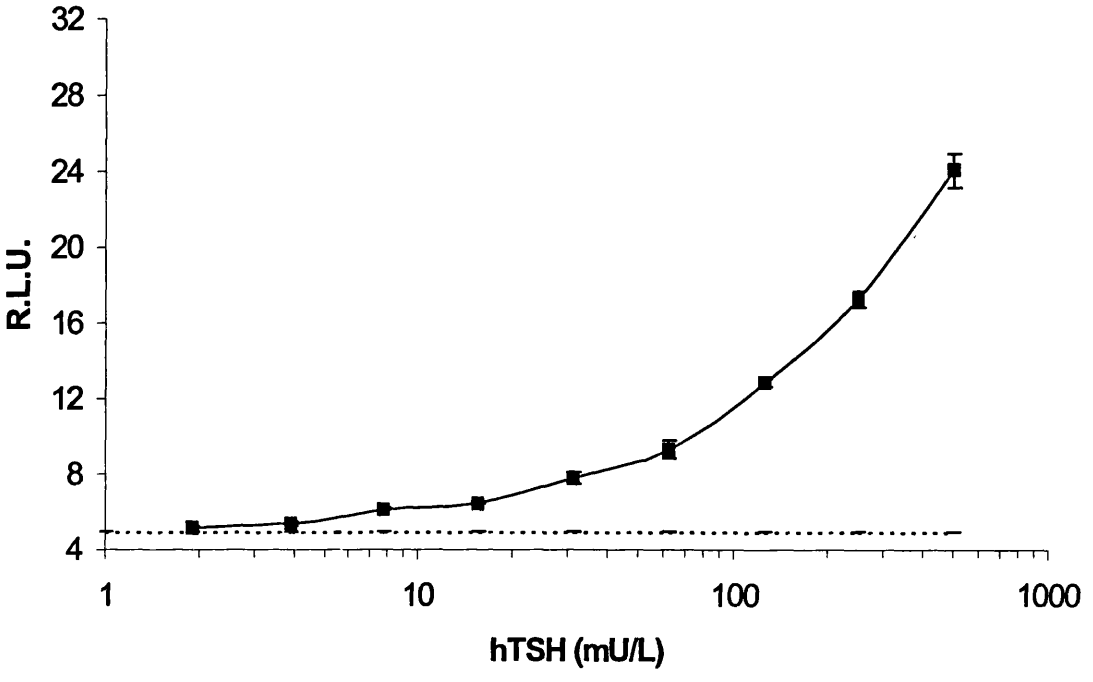


Figure 9.16 **Bioassay B: a comprehensive dose-response curve to hTSH (80/558).** --- is the light output from the unstimulated control microcultures. Results are the mean ± S.D. from triplicate microcultures.

emission 3 S.Ds. above the zero dose TSH control (see Section 9.2.3, Figure 9.1a), was established to be 4.6mU/L.

Response to thyroid stimulating antibodies

Two International Reference Preparations of TSAb were tested on CHO25LUC cells using Bioassay B. The dose-response curves of LATS-B (65/122) and TSAb (90/672) are shown (Figures 9.17 & 9.18 respectively). A maximum response of 11.1 ± 0.5 R.L.U was observed at 15.0mU/ml LATS-B. This represented a 2.3-fold increase above the unstimulated zero control (4.7 ± 0.1 R.L.U.). A theoretical detection limit of 0.16mU/ml was determined for LATS-B. With the second reference preparation for TSAb (90/672), an increase in luminescence was observed over the dose-range of 0.78mU to 25.0mU/ml of TSAb (Figure 9.18). The greatest light output of 17.0 ± 0.8 R.L.U. was recorded at 25.0mU/ml. This was a 2.8-fold increase above the control (6.0 ± 0.3 R.L.U.). An unexpected decline in response was observed at the highest concentrations (>25.0mU/ml) of this stimulator. A theoretical detection limit of 0.4mU/ml was determined for this First International Stimulator for TSAb, using Bioassay B.

9.3.3 Optimization of Bioassay C

The availability of the single step Promega System II resulted in Bioassay B, which was a significant improvement on the Protocol 1 devised by N.I.H.. However, despite our efforts to further optimize Bioassay B, a relatively high detection limit for TSH (~5mU/L) was only possible (Figure 9.16). Such sensitivity is poor compared with immunoassays, since with the second and third generation immunoassays for TSH,

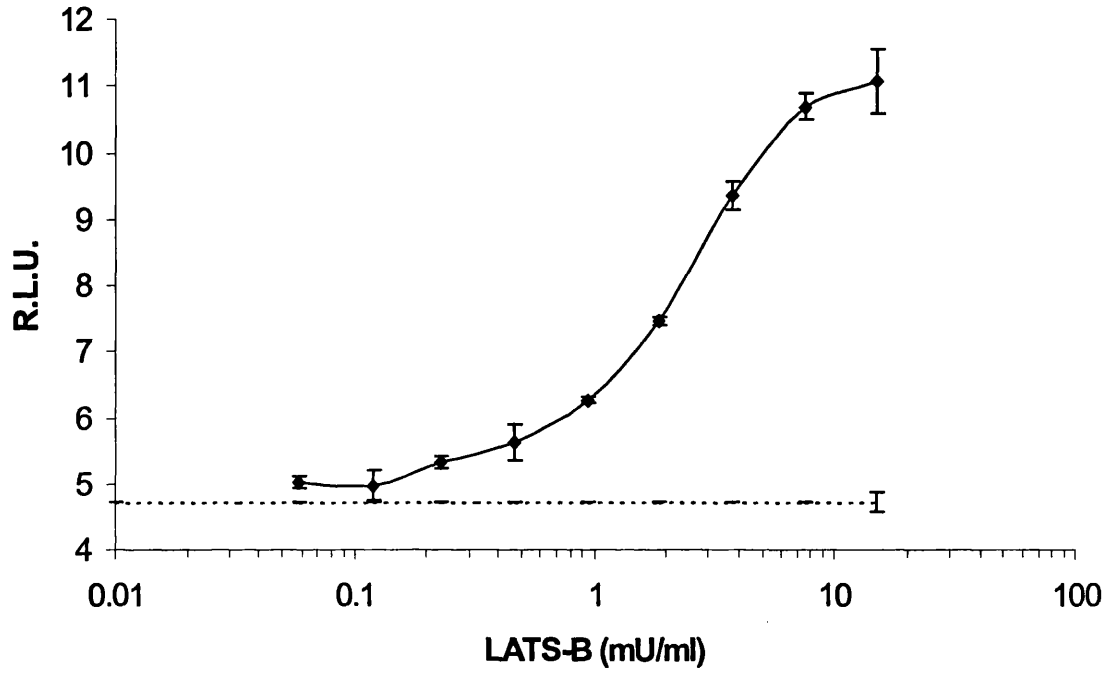


Figure 9.17 **Bioassay B: the increase in luminescence in response to increasing concentrations of MRC LATS-B Standard coded 65/122.** --- is the light output from the unstimulated control microcultures. Results are the mean ± S.D. for triplicate microcultures.

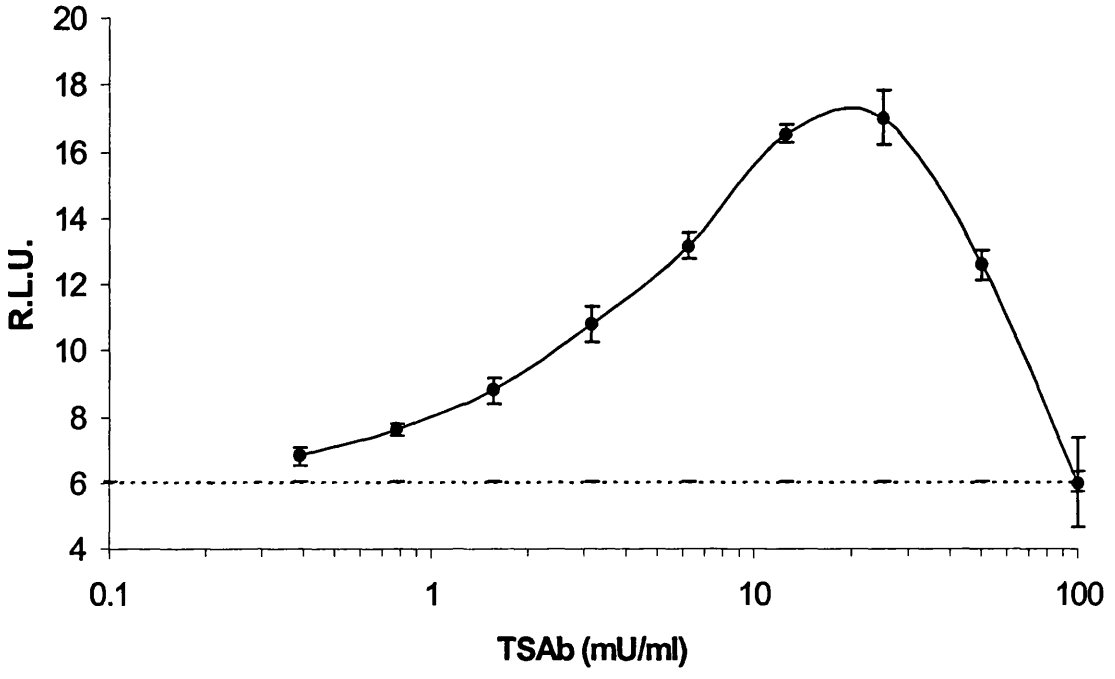


Figure 9.18 **Bioassay B: the increase in luminescence in response to increasing concentrations of the First International Standard for TSAb (90/672).** --- is the light output from the unstimulated control microcultures. Results are the mean ± S.D. for triplicate microcultures.

detection limits of <0.1mU/L TSH are attainable (Spencer CA *et. al.*, 1995). In Bioassay C, we adopted another strategy in our efforts to increase bioassay sensitivity. Emphasis was now placed on reducing the errors in the bioassay, since diminishing errors would themselves lead to an increased assay sensitivity. Lower detection limits would then be an inevitable consequence of the smaller response errors (S.D.s) at zero and low doses of TSH (Figure 9.1a). In addition, controlled reduction of response errors would not only enhance the bioassay sensitivity, but also improve the overall precision of the assay system (see Section 9.2.3, Figure 9.1c).

INVESTIGATING THE POSSIBILITY OF USING CULTURE MEDIUM AS THE ASSAY MEDIUM DURING PHASE 2

Manipulation of the medium above the monolayers, such as when the cell culture medium was aspirated at the end of Phase 1 in Bioassays A and B, inevitably disturbed the integrity of the cell monolayers. This clearly diminished monolayer reproducibility, increased between well variation and was a source of within-assay response errors. Theoretically, this could be avoided if it was possible to leave the culture medium used for Phase 1 during the exposure of the monolayers to the stimulators, i.e. Phase 2 of the bioassay (see Section 9.2.2). The essential question then became whether the presence of 10% FCS used to promote the growth of the monolayers during Phase 1, would adversely influence the magnitudes of the responses to the stimulators in Phase 2.

With Bioassay B, we had already made a preliminary investigation of the effects of using an assay medium supplemented with FCS during Phase 2 (Figure 9.12). This

had shown that there was no significant difference in the magnitude of response to a single dose of hTSH (100mU/L) in the presence of 10% FCS. Subsequently, a detailed comparison of TSH dose-response curves using either AM1 or culture medium, during Phase 2, was made (Figure 9.19a). (AM1 had been used for Bioassay B, and was identical to the culture medium used for Phase 1, except that for AM1, the 10% FCS had been replaced by 10% horse serum). Lower light emissions were consistently obtained when culture medium was substituted for AM1. A basal control response of 7.7 ± 0.1 and 11.5 ± 0.2 R.L.U. was observed for monolayers exposed to solely culture medium and AM1 respectively. No plateau was observed for either curve and the greatest light outputs of 30.1 ± 0.4 and 40.9 ± 0.9 R.L.U. were obtained with 500mU/L hTSH in the assays performed with culture medium and AM1 respectively. Furthermore, no significant differences in the % increase in the responses were observed over the concentration range of 0 – 31.25mU/L (Figure 9.19b). However at concentrations >31.25mU/L, slightly greater % increases were obtained when culture medium was used. At the maximum dose (500mU/L), an increase of 393.3% and 356.4% was attained in the assays performed with culture medium and AM1 respectively. Significantly, we noted that when cultured medium had been used during Phase 2, the within-assay response errors were reduced. The ϕCV (Figure 9.1b) for the dose-response curve with culture medium was, across the entire TSH dose range, only 1.9 ± 0.8%, whereas when AM1 had been used, this increased to 3.0 ± 1.9%. Although this improvement was statistically not very significant ($p<0.2$), we attributed it to the reduced disturbance of the monolayers when culture medium had been used compared to the obligatory complete aspiration required when AM1 had been used. Despite this overall increase in precision, with the zero and low doses of

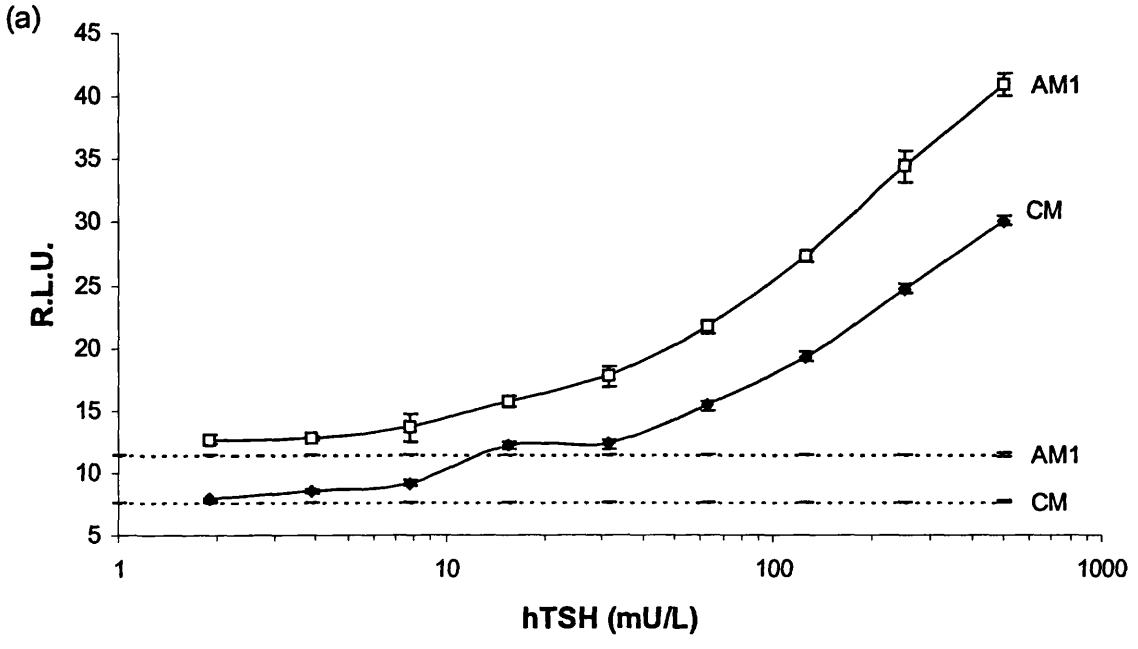


Figure 9.19 **Bioassay B & C: investigating the possibility of using culture medium as the assay medium for Phase 2.** (a). Comparing the effect of AM1 and culture medium (CM) as assay medium on the response to hTSH (80/558) stimulation. hTSH was serially diluted in CM (◆) or AM1 (□). For the monolayer used for the curve established in CM, at the end of Phase 1, only 50µl of the CM was removed. This was done so as to cause minimal disturbance to the underlying monolayers. The TSH dilutions, including zero dose control were then added as an additional 50µl, thus restoring the volume in the well to a total of 100µl. In contrast, the wells used for the curve established in AM1 followed the standard procedure for Bioassay B i.e. at the end of Phase 1, the CM was fully aspirated prior to the addition of the TSH serial dilutions (100µl). The TSH concentrations plotted are the final concentrations in the wells used for Phase 2. --- shows the light outputs from unstimulated controls. Results are the mean ± S.D. for triplicate microcultures.

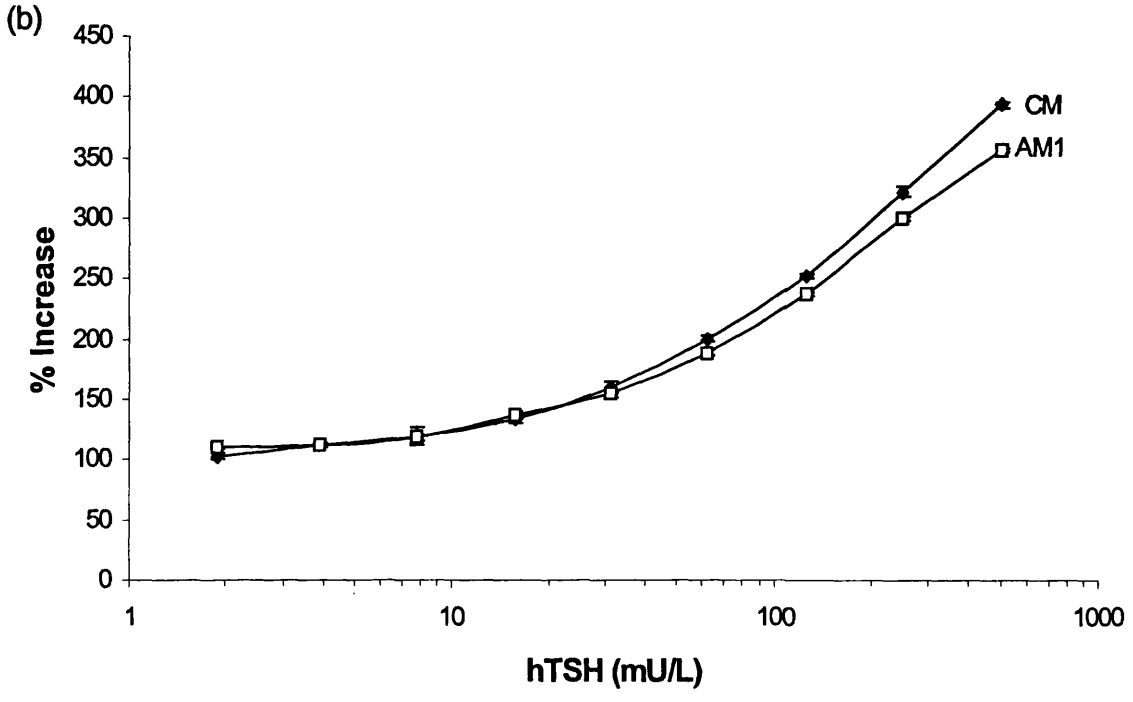


Figure 9.19 (b) The results shown in Figure 9.19(a) have been transformed to allow comparison of the % increase in response to hTSH above unstimulated controls, by cells in AM1 (□) and CM (◆). The unstimulated controls have both been normalized to 100%.

TSH, the errors incurred in these two particular assays were similar. This resulted in identical detection limits of the two curves of 1.9mU TSH/L.

In view of these encouraging results, we decided to adopt the protocol whereby the culture medium was used both for Phase 1 and 2 as described in the legend to Figure 9.19. We have designated this the standard procedure for Bioassay C (see Section 9.2.2.1.3).

AN INVESTIGATION IN THE EFFECT OF PREPARING THE MONOLAYER IN A REDUCED VOLUME (50µl) OF CULTURE MEDIUM

In an attempt to further eliminate the potential errors generated by the removal of the 50µl of culture medium from the wells (see above and Section 9.2.2.1.3), the monolayer was prepared in 50µl instead of 100µl/well of culture medium. As a result, hTSH in 50µl of fresh culture medium could be added directly to the wells. This was compared to a parallel study in which the protocol for Bioassay C described in Section 9.2.2.1.3 was followed (data not shown). The fold increases above the control responses were comparable between the two conditions in the concentration range of 0 – 250mU/L hTSH. At the highest dose, 500mU/L hTSH, a 7.6- and 6.9-fold increase above the respective control response was observed from cells cultured in 100µl and 50µl/well of culture medium respectively (data not shown). Furthermore, a ϕCV (see Section 9.2.3) of 4.2 ± 1.7% and 4.8 ± 2.1% was calculated when cells were cultured in 100µl and 50µl/well of culture medium respectively. We concluded that nothing significant was to be gained by adopting this minor modification to the protocol for Bioassay C.

INVESTIGATING THE EFFECT OF CELL PLATING DENSITY ON RESPONSES AND ERRORS OBTAINED WITH BIOASSAY C

Using Bioassay C, the effect of cell plating density on both the magnitude of the responses to hTSH and the response errors in the assay system were investigated. Since we had noticed a tendency for errors to increase with light output it was possible that smaller errors may be achieved if the magnitudes of the R.L.U. could be kept low. Cells were seeded at 2 and $4x10^5$ cells/ml in culture medium (100µl/well) and stimulated with hTSH after 48hr. The dose-response curves are shown in Figure 9.20a. Higher magnitudes of light output were consistently observed with the higher cell plating density ($4x10^5$ cells/ml). However, the fold increase with increasing concentrations of hTSH was similar except at the highest dose of TSH tested (250mU/L, Figure 9.20b). At this concentration, a 4.7- and 5.5-fold increase above the control response was observed with 2 and $4x10^5$ cells/ml respectively. When the ϕCV were calculated, we found that a lower % was observed with the plating density at $4x10^5$ cells/ml (1.8 ± 1.4%) compared to $2x10^5$ cells/ml (2.3 ± 1.4%). However, this was not a significant difference ($p>0.1$) and we therefore concluded that reduction of the cell density from the standard ($4x10^5$ cells/ml), did not result in an improved performance for Bioassay C.

DETERMINATION OF THE OPTIMAL EXPOSURE TIME TO THYROID STIMULATORS

Using the protocols for Bioassays A and B, an optimum incubation duration of 4hr has been established (e.g. Figure 9.2a & b). However, in these early studies, this had

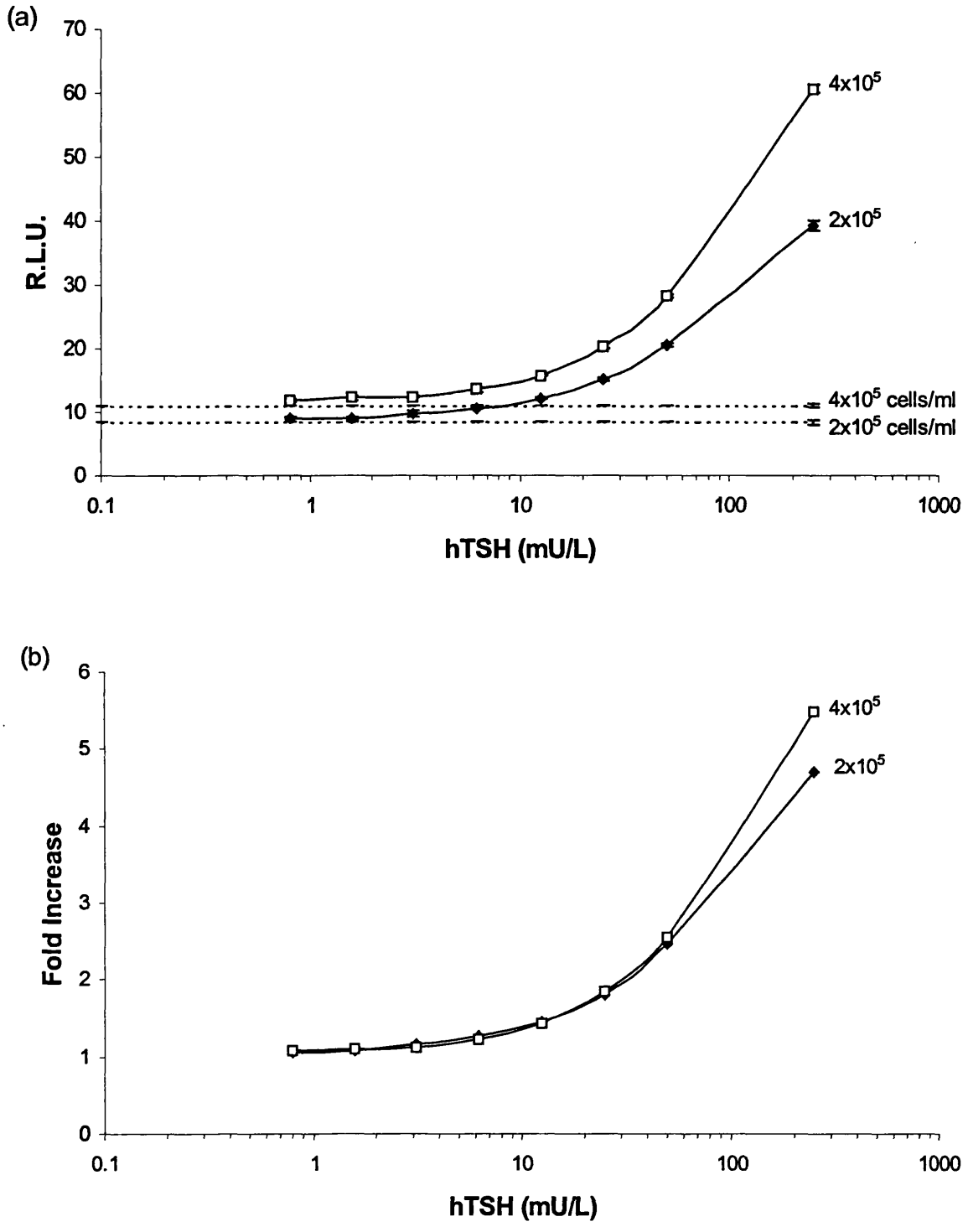


Figure 9.20 **Bioassay C: investigating the effect of a lower plating density on bioassay responses and errors.** (a) In Phase 1, monolayers were prepared at the densities of 2x (◆) and $4x10^5$ cells/ml (□). In Phase 2, they were stimulated with increasing concentrations of hTSH (80/558). --- represents the light outputs from unstimulated controls. Results are the mean ± S.D. for triplicate cultures. (b) The results of (a) have been transformed to compare the fold increase above the unstimulated controls in response to hTSH by cells seeded at 2x (◆) and $4x10^5$ cells/ml (□).

only been established in response to hTSH. Using Bioassay C, we investigated the optimal times for both TSH and TSAb. The cells were exposed to either hTSH (100mU/L) or TSAb 90/672 (12.5mU/ml) for 0 – 6hr (Figure 9.21a). For unstimulated cells, the highest R.L.U. was obtained after 3hr. However, maximal light output was observed after 4 and 5hr of incubation respectively when the cells were stimulated with TSAb and hTSH. Although the magnitude of bioassay response was maximal after 5hr for both stimulators (Figure 9.21b), there were only marginal differences between 4 and 5hr. As a consequence, the incubation time of 4hr was retained as the optimal exposure duration for future studies with Bioassay C. We note that the times courses for hTSH using either Bioassay A or Bioassay C are qualitatively similar (Figures 9.2 & 9.21). However the improvements in bioassay precision, gained by the successive modifications described to this point, are also apparent.

INVESTIGATION OF POSSIBLE POTENTIATION OF THE RESPONSE USING FORSKOLIN IN BIOASSAY C

In Bioassay B, the synergistic effect of a low dose of forskolin (0.025μM) with hTSH had been tested. No potentiation of responses had been observed at low concentrations of hTSH, but there had been slight enhancement of responses at concentrations >50mU/L (Figure 9.15a & b). Due to the significant adaptations in the modified protocol for Bioassay C, the possibility of forskolin enhancing the magnitudes of responses was reinvestigated.

Dose-response curves to forskolin were investigated in the presence and absence of a single, low dose of hTSH (1.6mU/L, Figure 9.22a). Maximum responses of 80.2 ± 0.7

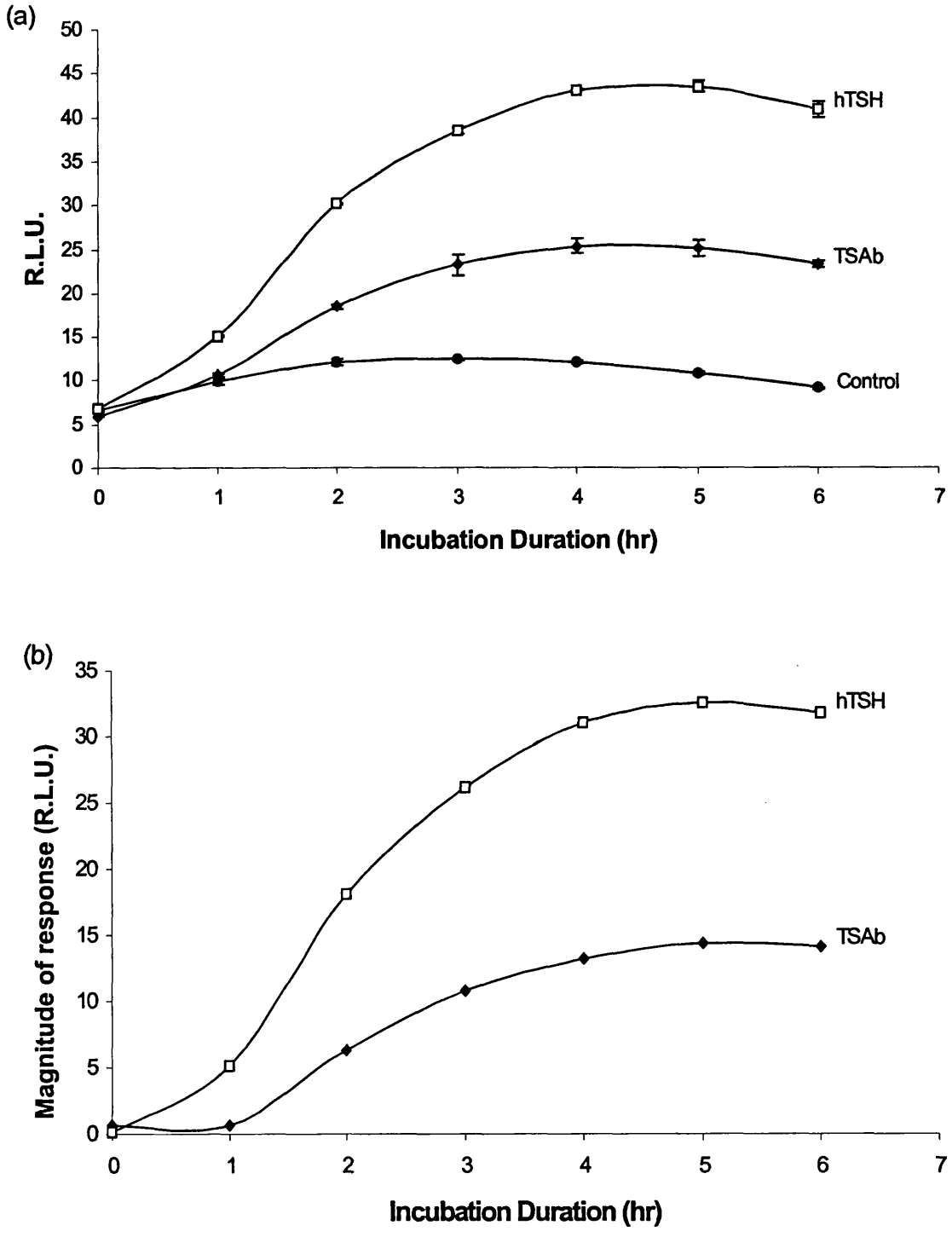


Figure 9.21 **Bioassay C: time courses of luciferase stimulation by hTSH (80/558) and TSAb (90/672).** (a) Cells were exposed to 100mU/L hTSH (□) and 12.5mU/ml TSAb (◆) for 0 – 6hr; light outputs from unstimulated controls have also been shown (●). Results are the mean ± S.D. for triplicate cultures. (b) The magnitudes of response (see Section 9.2.3, Figure 9.1a), for cells stimulated with hTSH (□) and TSAb (◆) have been plotted against time.

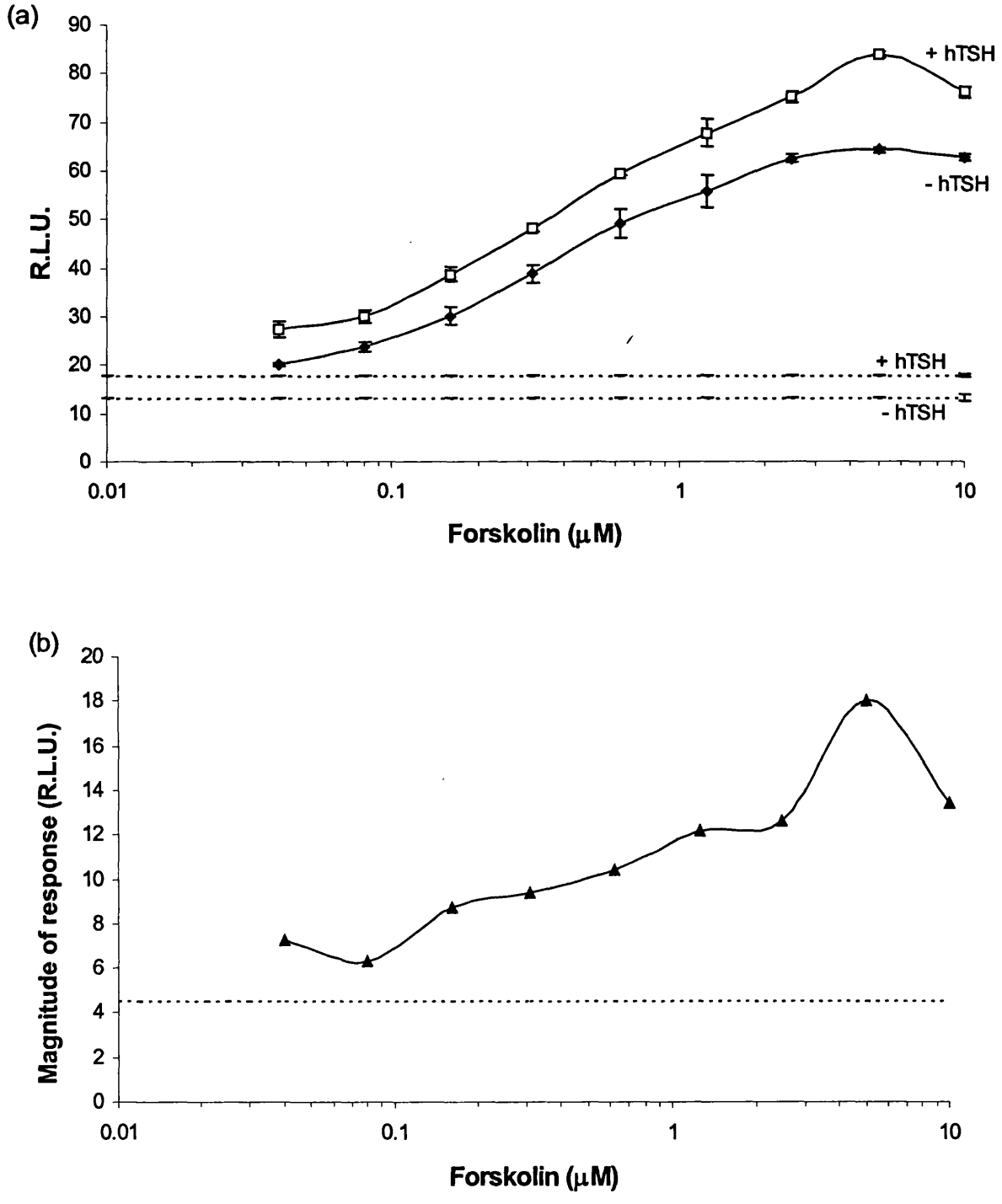


Figure 9.22 **Bioassay C: an investigation to determine an optimal forskolin dose for synergy with TSH.** (a) The increase in luminescence in response to increasing concentrations of forskolin in the presence (□) and absence (◆) of 1.6mU/L hTSH (80/558). --- shows the light outputs from controls with zero forskolin. Results are the mean ± S.D. for triplicate microcultures. (b) The magnitudes of response to TSH (see Section 9.2.3, Figure 9.1a) plotted against forskolin concentrations to determine the optimum dose of forskolin for synergy studies. --- represents the response to TSH in the absence of forskolin.

and 64.2 ± 0.5 R.L.U were observed after exposure to 5.0μM forskolin in the presence and absence of hTSH respectively. This was a 4.5- and 4.8-fold increase above the control responses (17.8 ± 0.2 and 13.3 ± 0.7 R.L.U.), with and without hTSH respectively. The greatest magnitude of response to TSH (see Section 9.2.3, Figure 9.1a), was also obtained at 5.0μM forskolin (Figure 9.22b). As a result, the relatively high dose of 5μM forskolin was determined to be optimal. Consequently this constant dose of forskolin (5μM) was co-incubated together with increasing doses of hTSH. Since the potentiation observed was subtle, we have included the results from two identical studies which are detailed below (Figures 9.23 & 9.24).

Study 1

In the presence of a relatively high constant dose of forskolin (5μM), the basal control was 74.5 ± 0.9 R.L.U. compared to 12.7 ± 0.4 R.L.U. in the absence of forskolin (Figure 9.23a). No plateau was approached in either TSH dose-response curve at the highest concentration of 200mU/L hTSH. In the absence of forskolin, a sharp increase in luminescence was observed at doses >10mU/L hTSH. In contrast, in the presence of forskolin, there was merely a gradual rise in light output over the entire hTSH concentration range (0.1 – 200mU/L). As a result, only a 1.4-fold increase above the control response was observed in the presence of forskolin compared to a 5.1-fold increase in the absence of forskolin. Nevertheless, a slight potentiation of the responses to lower doses of hTSH (≤10mU/L) was observed when the magnitudes of response (see Section 9.2.3, Figure 9.1a) were plotted against hTSH concentration (Figure 9.23b). However, there was no significant difference in the theoretical detection limits (see Section 9.2.3, Figure 9.1a) obtained. A detection limit of 0.8mU/L was obtained in the presence of forskolin compared to 1mU/L in the absence

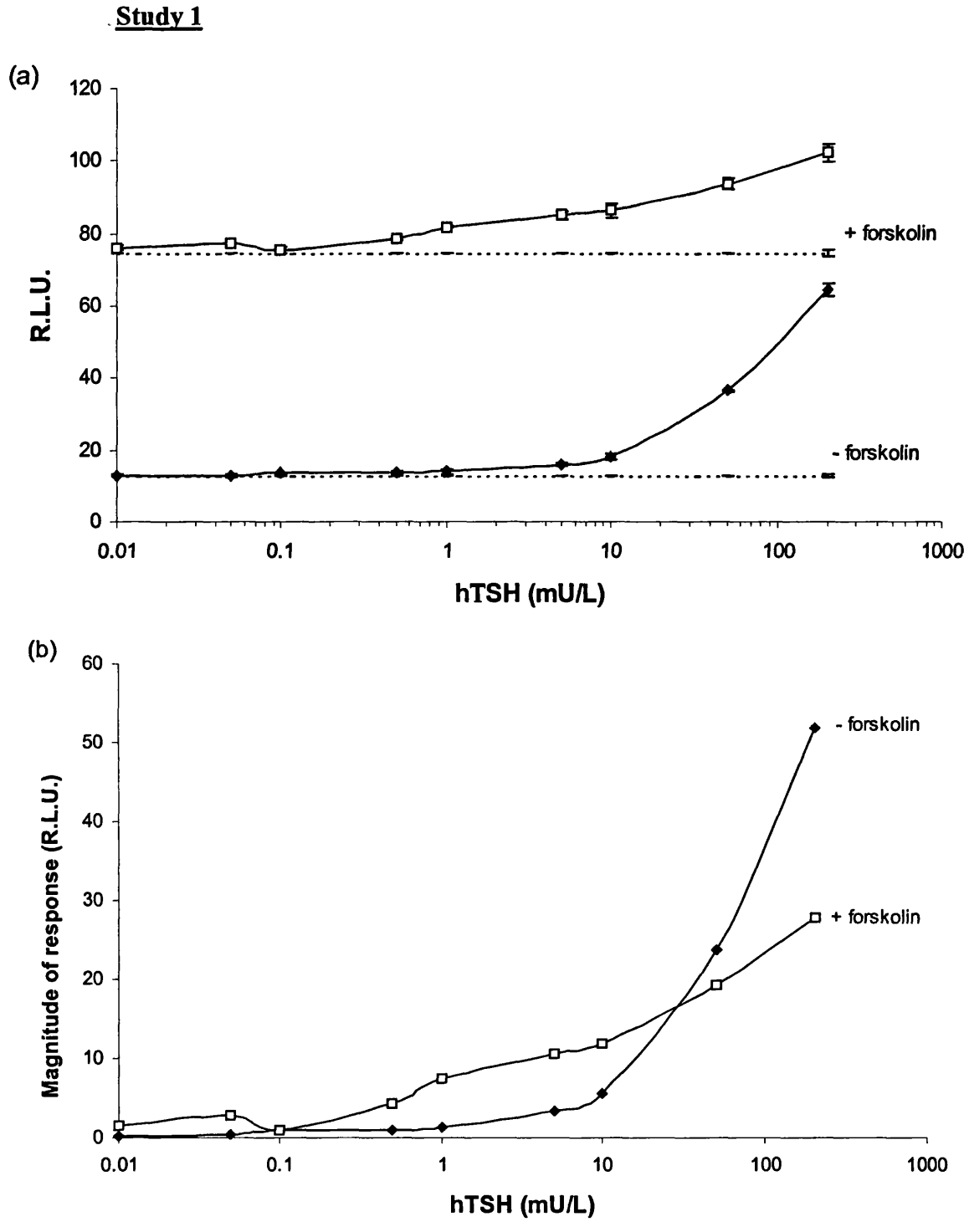


Figure 9.23 **Bioassay C: an investigation of the possible potentiation of bioassay responses to TSH using a predetermined optimal dose of forksolin.** (a) The increase in luminescence in response to increasing concentrations of hTSH (80/558) in the presence (□) and absence (◆) of 5μM forskolin. --- represents the light outputs from controls with zero TSH. Results are the mean ± S.D. for triplicate cultures. (b) The magnitudes of response (see section 9.2.3, Figure 9.1a) in the presence (□) and absence (◆) of forskolin plotted against hTSH concentration.

of forskolin. Thus, although it appeared that there was a slight synergistic effect when 5μM forskolin was added to lower doses of TSH, the diterpene did not increased the bioassay sensitivity.

Study 2

Strikingly similar results were observed for Study 2, which was a repeat of Study 1 (Figure 9.24).

Thus although in Bioassay C, forskolin did appear to potentiate responses to relatively low TSH doses, the effect was only slight and was only observed with a relatively high dose of forskolin (5μM). This inevitably resulted in a substantial increase (6-fold) in the bioassay zero-TSH control. In addition, the entire shape of the TSH dose-response curve was altered in the presence of forskolin and the loss of the magnitude of response would inevitably lead to a deterioration in precision of TSH measurements at doses >10mU/L. We therefore concluded that the addition of 5μM forskolin would not significantly benefit the overall performance characteristics of Bioassay C.

INVESTIGATION OF POSSIBLE POTENTIATION OF THE RESPONSES USING IBMX IN BIOASSAY C

In Bioassay A, we investigated for synergy between IBMX and TSH (Figure 9.3). Although we had concluded that there was no potentiation of the responses to TSH by IBMX, since such major modifications to our bioassay protocols had occurred since Bioassay A, we considered that a re-investigation with Bioassay C was advisable. As

Study 2

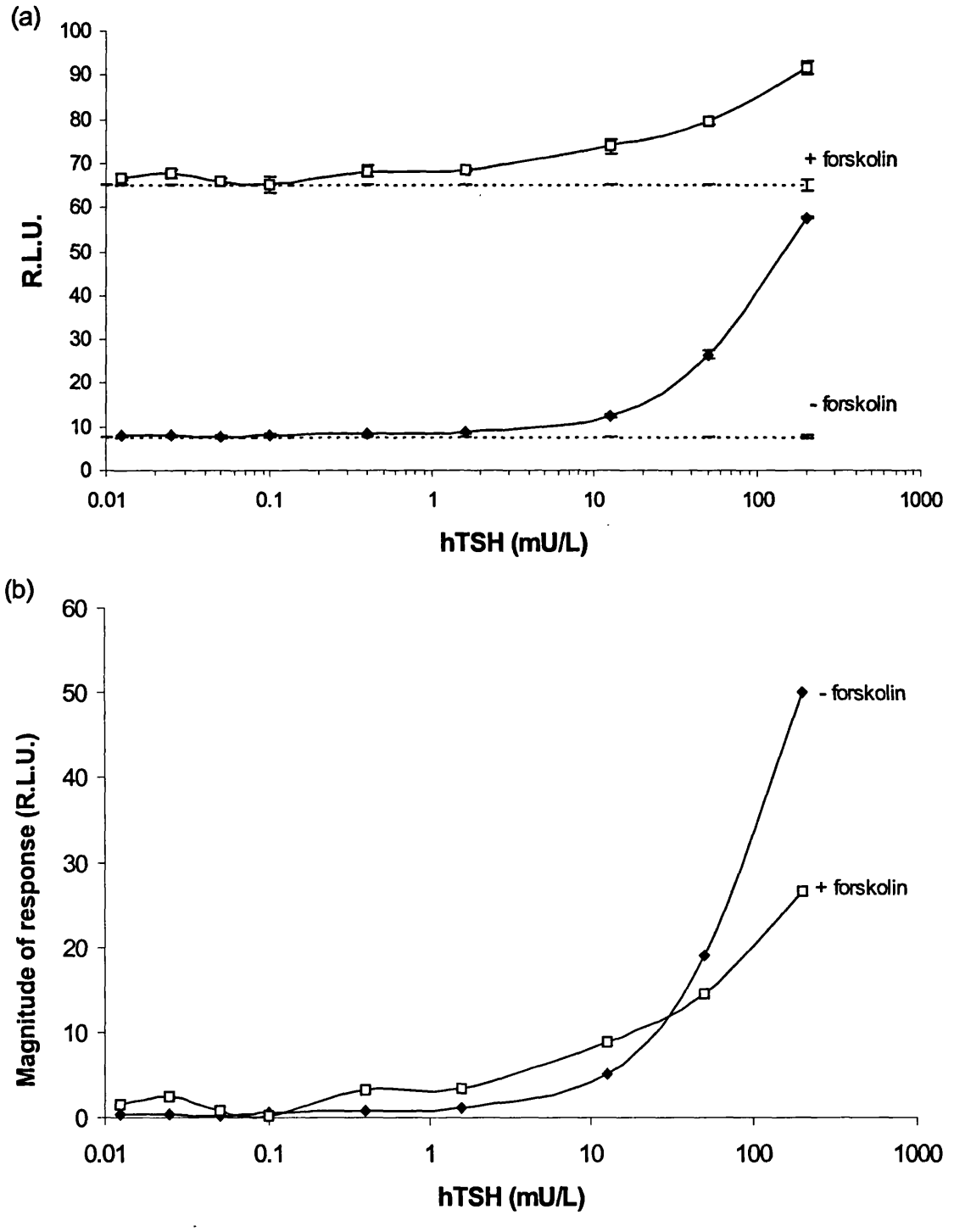


Figure 9.24 **Bioassay C: a reinvestigation of the possible potentiation of bioassay responses to TSH using a constant dose of forskolin.** For details see legend to Figure 9.23, since this is a replicate experiment.

mentioned previously, IBMX is a cyclic nucleotide phosphodiesterase inhibitor which inhibits the degradation of cAMP.

Dose-response curves to IBMX were investigated in the presence and absence of a single, low dose of hTSH (1.6mU/L, Figure 9.25a). The highest dose of 0.5mM gave maximal light emissions of 106.5 ± 0.4 and 91.2 ± 0.8 R.L.U. in the presence and absence of hTSH respectively. This represented a 6.1- and 6.6-fold increase above the control responses (17.5 ± 0.04 and 13.8 ± 0.4 R.L.U.), with and without hTSH respectively. The greatest magnitude of response to TSH (see Section 9.2.3, Figure 9.1a), was also observed with 0.5mM IBMX (Figure 9.25b). Thus, it appeared that IBMX has a similarly slight synergistic effect as had forskolin on the responses to low doses of TSH (see Figures 9.22 & 9.25). With both agents, relatively high concentrations were required to increase the responses to the low dose of TSH (1.6mU/L), and the increase gained was relatively small. Moreover, since high doses of IBMX and forskolin had proved optimal, their inclusion in the bioassay would have greatly increased the bioassay baseline.

INVESTIGATION OF POSSIBLE POTENTIATION OF RESPONSES TO TSH BY USING COMBINATIONS OF IBMX AND FORSKOLIN IN BIOASSAY C

As only slight potentiation of the responses to low doses of hTSH using either forskolin or IBMX had been observed (Figures 9.22 – 9.25), we investigated whether simultaneous addition of both of these reagents would lead to augmented potentiation. Monolayers of cells were prepared as described in Section 9.2.2.1.3. During Phase 2, they were exposed to mixtures of IBMX and forskolin in the

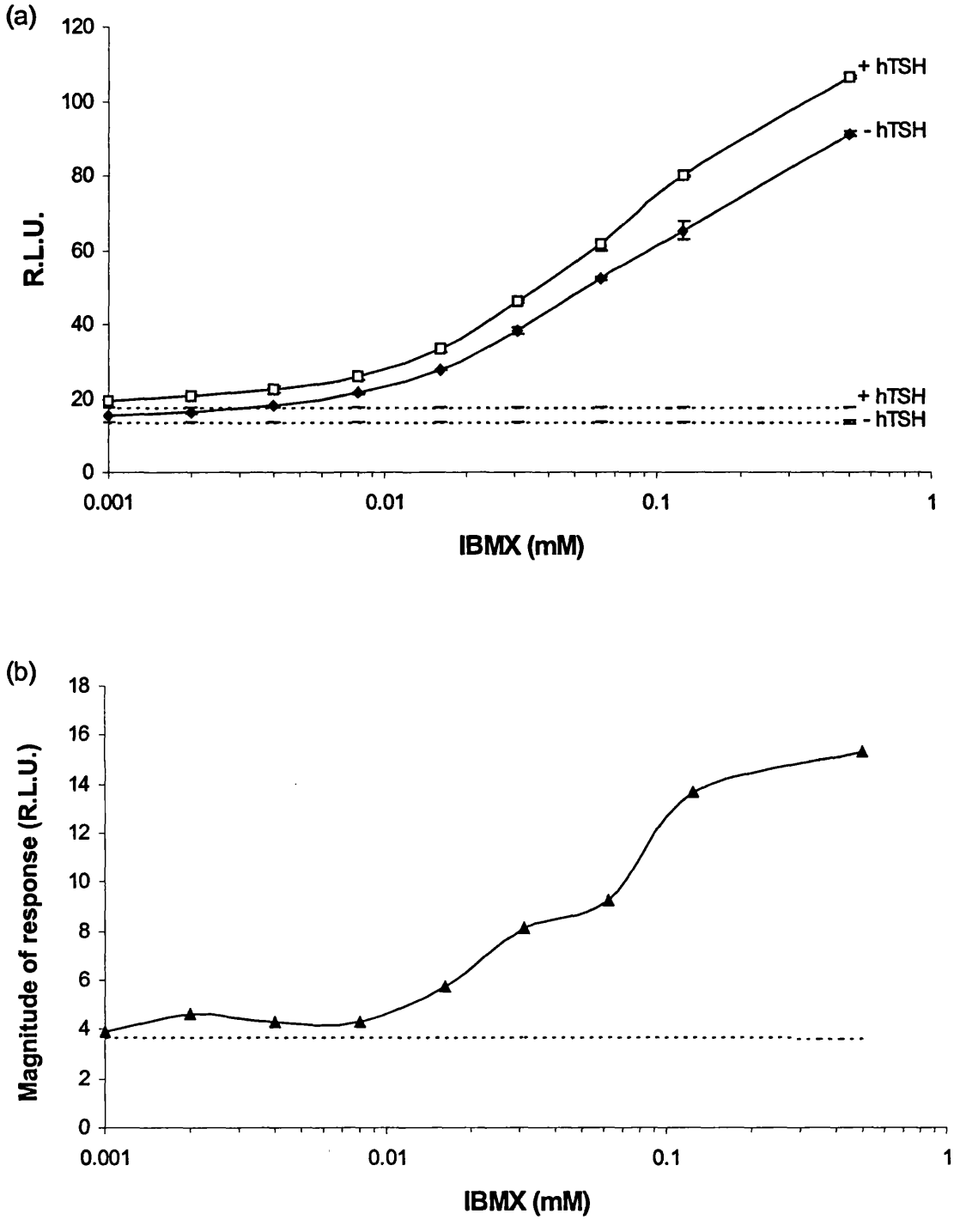


Figure 9.25 **Bioassay C: an investigation of possible potentiation of bioassay responses to TSH by IBMX.** (a) The increase in luminescence in response to increasing concentrations of IBMX in the presence (□) and absence (◆) of 1.6mU/L hTSH (80/558). --- shows the light outputs from controls with zero IBMX. Results are the mean ± S.D. for triplicate cultures. (b) The magnitudes of response to TSH (see Section 9.2.3, Figure 9.1a) plotted against IBMX concentrations. --- represents the response to TSH in the absence of IBMX.

presence and absence of a single, low dose of hTSH (1.0mU/L). IBMX concentrations ranged between 0 – 1.25mM (n = 10) and forskolin between 0 – 10μM (n = 6). For Phase 2, 50μl of culture medium was removed from the microcultures according to the standard protocol and 25μl of each IBMX and forskolin was added to give a final volume of 100μl/well. IBMX and forskolin incremental concentrations were added across (wells 2 – 11) and down (wells B – G) the microtiter plate respectively thus generating a matrix of singlicate concentration combinations. The constant dose of TSH (1mU/L) was added in the forskolin aliquots (25μl). The study was repeated three times and in each experiment, bioassay responses (± hTSH) increased in a dose dependent manner when IBMX or forskolin was added independently i.e. zero dose of the other stimulator (data not shown). This thus confirmed the results shown in Figures 9.22 and 9.25. A decline in light output was observed when the highest IBMX concentration (1.25mM), which had not been tested previously (Figure 9.25) was added. However, no notable augmentation in the potentiation of the response to TSH was gained by using any of the combined doses of IBMX and forskolin. Moreover a troublesome feature of the results was the finding that upon repeating the experiment with three separate subcultures of the cells, the concentration combinations yielding greatest potentiation changed with each experiment. Such instability had not been encountered before with this bioassay system, which in our experience is highly reproducible – see for example Figures 9.23 and 9.24. We therefore decided not to pursue this approach to enhancing the magnitudes of response of the bioassay to TSH.

INVESTIGATING THE RESPONSES OBTAINED WITH SIMULTANEOUS STIMULATION OF THE CELLS BY hTSH, TSAb AND FORSKOLIN

Using Bioassay C, the cells were stimulated with hTSH (100mU/L), TSAb (12.5mU/ml) and forskolin (1mM) either individually or in combinations of 2 or 3 of these agonists. These concentrations were selected as they gave approximately half maximal responses (see Figures 9.19, 9.22 & 9.29). The variables tested were as follows: (a) hTSH, (b) TSAb, (c) forskolin, (d) hTSH + TSAb, (e) forskolin + TSAb, (f) forskolin + hTSH and (g) all 3 stimulators together. The observed light outputs (R.L.U.), are shown in Figure 9.26. The additive values, calculated as the sums of the observed R.L.U. of the appropriate individual stimulators inclusive of the control component, are also shown. We found that the observed results corresponded closely to the calculated additive values. Thus no synergy was observed between these three contrasting agonists, and we concluded that their effects were simply additive.

INVESTIGATION FOR PARALLELISM BETWEEN TSH AND TSAb IN BIOASSAY C

With Bioassay C, we have developed an assay system which has good replicate reproducibility. Such favourable bioassay precision should allow us to investigate critically for parallelism between different thyroid stimulators and in particular between TSH and TSAb. It was hoped that parallelism would be established between TSH and TSAb, so that results from a clinical bioassay for TSAb based upon this system might eventually be expressed in TSH "equivalents". Because of the importance of this it was investigated on several separate occasions, first with

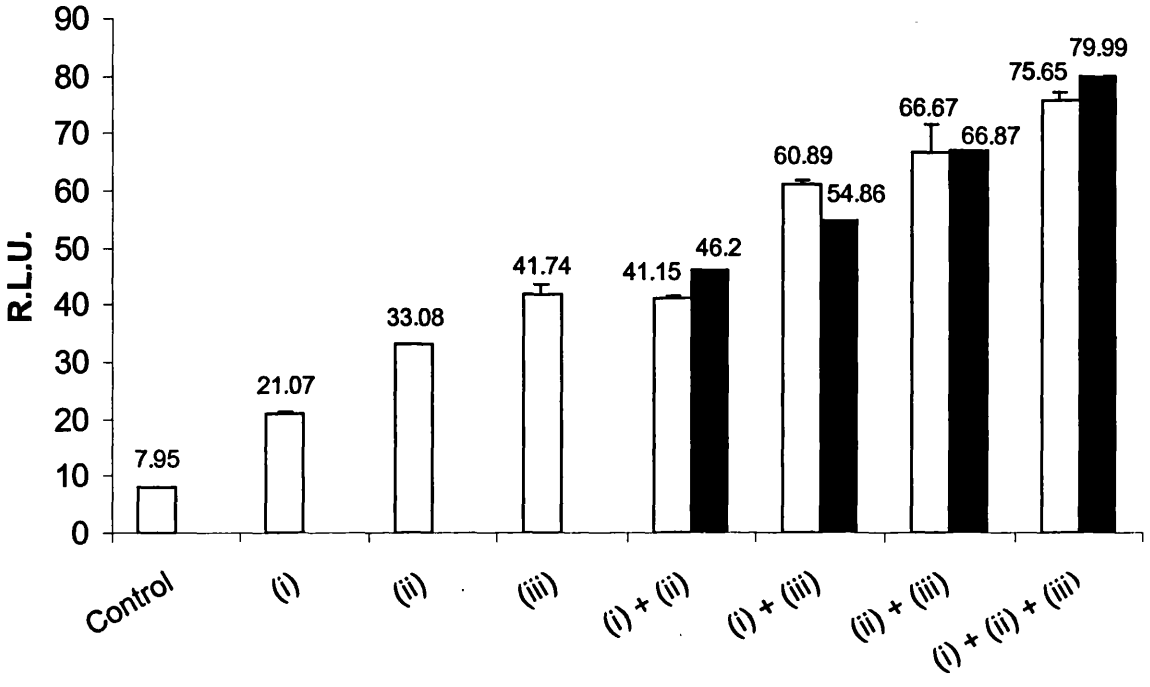


Figure 9.26 **Bioassay C: an investigation of possible synergistic effects of the simultaneous stimulation of the monolayers by different stimulators.** The 3 stimulators were: (i) 12.5mU/ml TSAb (90/672), (ii) 100mU/L hTSH (80/558) and (iii) 1μM forskolin. The monolayers were stimulated by the individual stimulators and in combinations as indicated above (also see text for details). The control shows the light outputs from unstimulated control microcultures. The observed R.L.U. values (□) have been plotted and compared to the calculated additive values (■). The actual numerical values for both sets of results are also denoted above. The observed R.L.U. values are the mean ± S.D. from triplicate microcultures.

Bioassay C, as described below, and later, in greater detail, with Bioassay D, and yet again in our detailed investigation of patient samples (see Section 10.3.6).

Investigation of parallelism between two contrasting vials of TSH preparation coded 80/558

Using Bioassay C, CHO25LUC cells had previously been shown to respond to stimulation with the hTSH preparation coded 80/558 in a dose-dependent manner, generating an ascending limb of a dose-response curve (Figure 9.19a, 9.20a, 9.23a & 9.24a). In this study, the dose-response curves of two contrasting vials of this TSH preparation were compared (Figure 9.27). These vials had been stored at different temperatures. The International Reference Preparation (80/558) had been consistently stored at -20°C for 20 years. However, the other vial, recoded 84/703, had been stored for 15 years at -80°C, after an initial storage period (4 years) at -20°C.

For parallelism studies, the freeze-dried preparations had been reconstituted in culture medium (see Section 9.2.2.2) and serially diluted in this medium. Maximal responses of 12.4 ± 0.3 and 12.5 ± 0.2 R.L.U. were observed at the highest concentration (1000mU/L) for 80/558 and 84/703 respectively. This represented a 3.6- and 3.4-fold increase above the light outputs from the control, unstimulated microcultures, these being 3.4 ± 0.1 and 3.7 ± 0.3 R.L.U. for 80/558 and 84/703 respectively. No evidence of approaching a plateau was observed at the highest concentration of either curve suggesting that greater magnitudes of response could have been obtained with concentrations >1000mU/L. Theoretical detection limits (see Section 9.2.3, Figure 9.1a) of <7.8 and 11mU/L were estimated for 80/558 and 84/703 respectively from these curves. As anticipated, the two dose-response relationships appeared to be

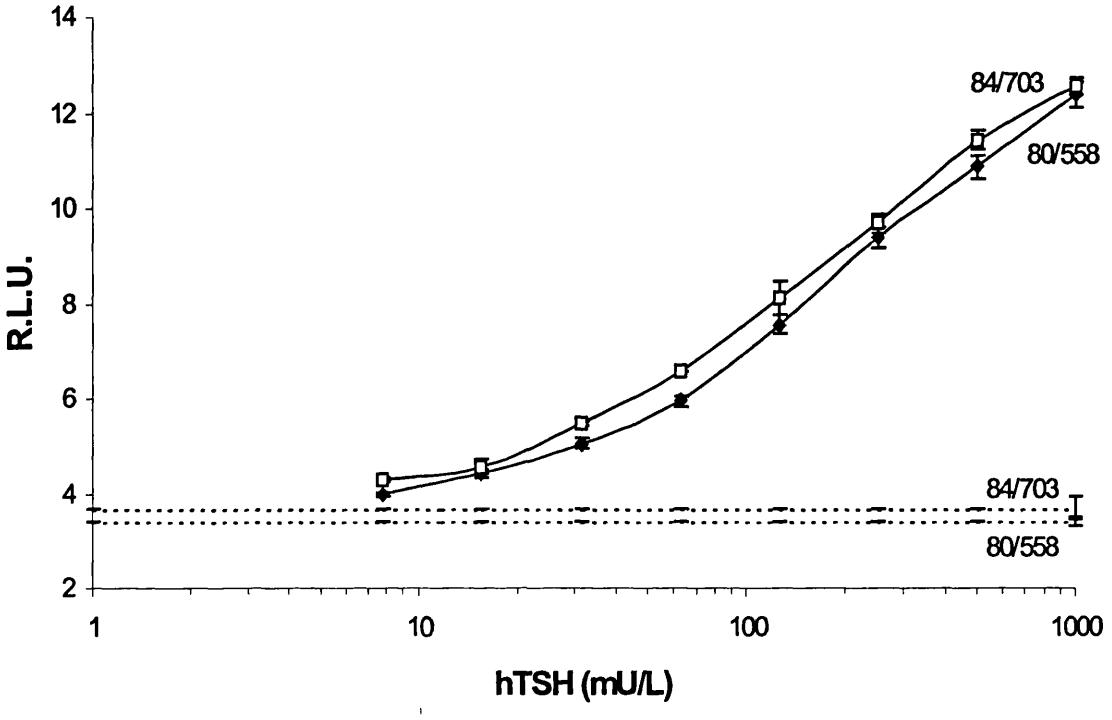


Figure 9.27 **Bioassay C: the increase in luminescence in response to increasing concentrations of two contrasting vials of hTSH.** The 2 hTSH vials were coded hTSH 84/703 (□), and 80/558 (◆) from NIBSC --- shows the light outputs from the unstimulated controls. Results are the mean ± S.D. for triplicate microcultures.

parallel. Thus this provided an example of Bioassay C being used to demonstrate parallelism, when the latter would be expected.

Dose-response relationship to TSAb preparation coded 90/672

This preparation of TSAb is the most potent international reference preparation available. However, we have already reported that there had been an unexpected and marked decline in the light output with concentrations >25mUml, when this preparation had been tested earlier in Bioassay B (Section 9.3.2; Figure 9.18). Since phosphate ions have been reported to inhibit luciferase (Promega technical manual #TM051, 1998), and we had noted that NIBSC had prepared 90/672 in a phosphate buffer, the influence of these ions was considered to be the most likely cause of the decline in response with high concentrations of TSAb. The freeze-dried preparation received from NIBSC had been reconstituted and serially diluted in non-phosphate containing culture medium (see Section 9.2.2.2). This explanation was confirmed by comparing dose-response curves to 90/672 before and after the preparation had been dialysed against non-phosphate containing Ham's F12 nutrient medium; the latter was the basic medium used for the preparation of culture medium. Before dialysis, as had been observed in Bioassay B, the dose-response curve to 90/672 in Bioassay C showed a maximum in the presence of 25mU/ml which then, with the highest dose (50mU/ml), declined (Figure 9.28). However, after dialysis no such decline was observed. This would be consistent with the removal of an inhibitor, which we assumed to be phosphate ions, from the preparation, by the dialysis.

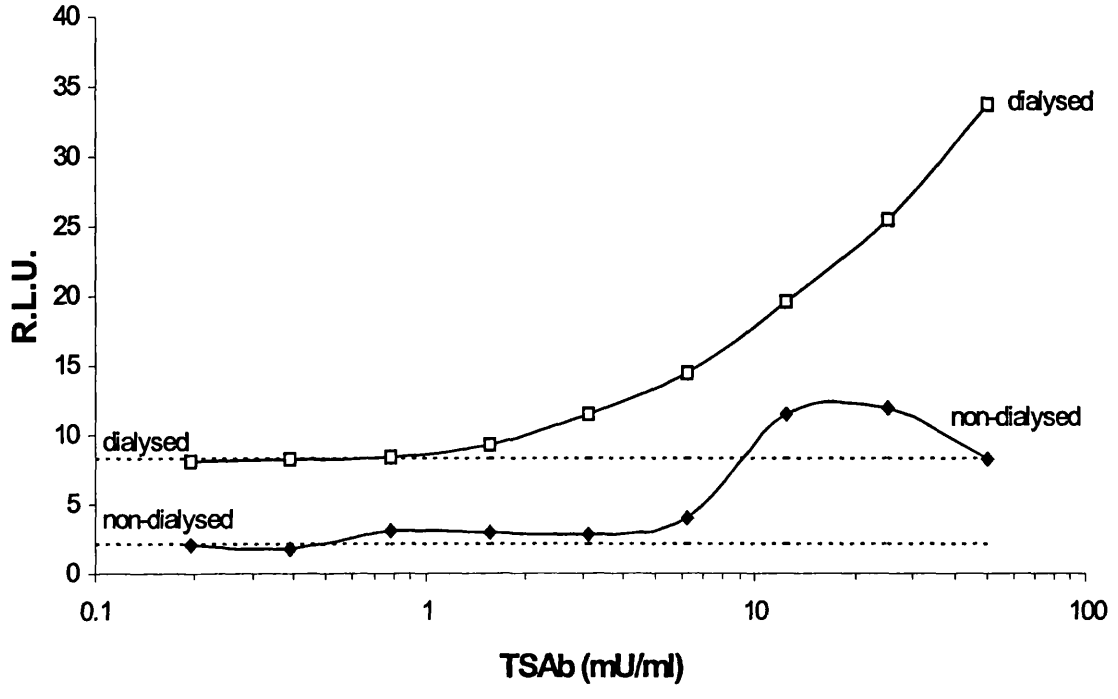


Figure 9.28 **Bioassay C: the effect of dialysis on the increase in luminescence in response to increasing concentrations of the First International Standard for TSAb (90/672).** Two vials of the freeze-dried preparation were reconstituted in culture medium (4ml). This was then subdivided, and part was used to prepare a serial dilution in culture medium. The remainder was dialysed against 1L of Ham's F12 nutrient medium. A low molecular weight cut-off dialysis membrane was used (6,000-8,000) and dialysis was carried out over a 24hr period at 4°C. This dialysed preparation was then used to prepare a second serial dilution in culture medium. The responses to the dialysed preparation (□) have been compared to the non-dialysed preparation (◆). These dose-response curves were obtained in two separate assays, which accounts for the difference in the unstimulated controls. --- shows the light outputs from the unstimulated controls. The means of duplicate microcultures have been plotted.

An investigation of parallelism between TSAb (90/672) and hTSH (80/558)

Having removed the inhibitory phosphate ions from the reconstituted 90/672 by dialysis, we then compared responses to hTSH (80/558) and dialysed TSAb (90/672) with both being serially diluted in culture medium (Figure 9.29). A response of 50.2 ± 1.0 R.L.U was obtained at the highest concentration of 500mU/L for hTSH. This represented a 6.7-fold increase above the unstimulated control (7.5 ± 0.1 R.L.U.). No evidence of an approaching plateau was observed at this high dose (500mU/L). For the dialysed TSAb (90/672), the greatest light output of 28.6 ± 0.9 R.L.U. was obtained after exposure to 50mU/ml. This was a 4.1-fold increase above the unstimulated control (7.0 ± 0.3 R.L.U.). No decline in response was observed at the highest concentration (50mU/ml). Theoretical detection limits of <1.9mU/L and 1.1mU/ml were estimated for hTSH and TSAb respectively from these dose-response curves. However, since the highest dose obtainable with the vial of TSAb (90/672) was only partially along the ascending limb of the dose-response curve to TSH obtained with Bioassay C, it was not possible, from this experiment, to conclude whether the two dose-response curves were or were not parallel.

9.3.4 Optimization of Bioassay D

We optimized Bioassay C using two strategies. Firstly we attempted to increase the magnitudes of response to stimulators, e.g. by testing for potentiators such as forskolin and IBMX. Secondly we had sought to reduce any response errors in the assay system, in particular by devising a protocol which caused minimal disturbance to the microcultures. The avoidance of complete aspiration of the culture medium at the end of Phase 1 was an example of this approach. This yielded a bioassay with

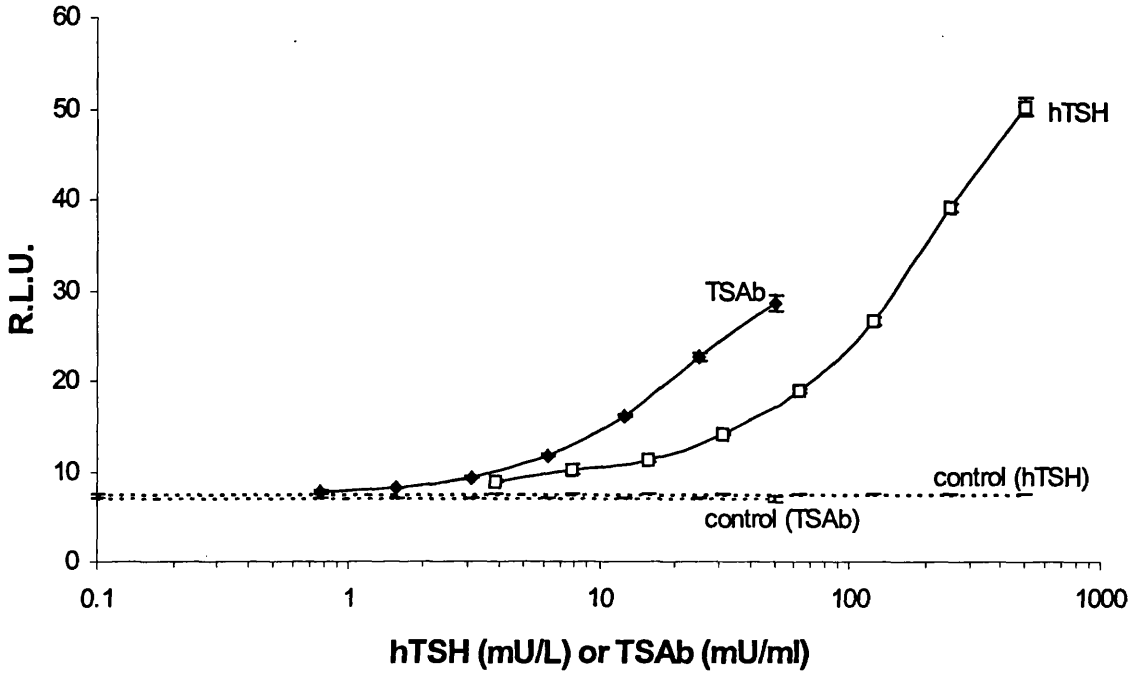


Figure 9.29 **Bioassay C: an investigation for parallelism between hTSH and TSAb.** Serial dilutions for the dialysed reconstituted stock of TSAb (90/672; ◆) used for Figure 9.28 and also reconstituted hTSH (80/558; □) were prepared in culture medium. Monolayers were then exposed to these stimulators and light outputs determined according to the protocol for Bioassay C (see Section 9.2.2.1.3). Results are the mean ± S.D. for triplicate microcultures. --- represents the light outputs from the unstimulated controls.

favourable between-well replication, as evidenced by the relatively small response errors in for example the two dose-response curves to hTSH shown in Figure 9.27. However, we remained concerned that only small magnitudes of response were being obtained with doses of hTSH which were at the upper end of the normal physiological range (5mU/L). This dose was, in fact, usually close to the detection limit of Bioassay C. Moreover, pilot studies with Bioassay C, investigating responses to sera from Graves' patients, which in much earlier work we had shown to be potent stimulators of adenylate cyclase in FRTL-5 cells, revealed that with Bioassay C only poor magnitudes of response could be obtained to these sera (see Section 10.3.3).

Following discussions with our collaborators at N.I.H. and a commercial laboratory with which they were also collaborating, namely DHI, we decided to test another protocol which they claimed was leading to good magnitudes of response with potent sera from Graves' patients. However, they complained that with this new protocol, the within-assay response errors which they obtained were frustratingly bad. Utilising the experience which we had gained from our work on refining the within-assay precision of Bioassays A – C, we investigated the new protocol which we will refer to as N.I.H. Protocol 2. Our critical investigations, which are detailed below, resulted in the development of Bioassay D, in which special attention has been paid to minimising within-assay errors.

Outline of N.I.H. Protocol 2

The most pertinent features were as follows:

- Monolayers were prepared by plating cells at a relatively high initial density (100µl at $7x10^5$ cells/ml in culture medium).

- After 24hr, the culture medium was aspirated and the monolayers were washed with a proprietary "starvation" medium obtained from DHI. The monolayers were then further incubated (24hr) with the "starvation" medium (100μl). This completed Phase 1 and the monolayers were then committed to Phase 2.
- The "starvation" medium (100μl) was fully aspirated and stimulators were added after dilution in a proprietary solution (AM3), which also had been obtained from DHI.
- Phase 2: exposure of the stimulators (TSH or Graves' patient sera) was for 4hr at 37°C in the standard gassed and humidified incubator. Graves' sera were added (110μl) as a 10% dilution in AM3 i.e. only 10μl sera was required per microculture.
- At the end of Phase 2, the wells were fully aspirated and Phase 3, namely cell lysis and luciferase activities were determined using Promega System I.

PRELIMINARY EXPERIMENTS WITH N.I.H. PROTOCOL 2

In view of our poor experience with Promega System I in Bioassay A, from the outset we substituted this with the one-step Promega System II. In a preliminary experiment described later in Section 10.3.3, we demonstrated that the use of AM3 as the assay medium for Phase 2 i.e. the exposure of the monolayers, resulted in much larger magnitudes of response to selected potent Graves' sera than had been observed with Bioassay C. The latter had used culture medium, as oppose to AM3, during Phase 2. Thus it appeared that AM3 was crucial for obtaining large magnitudes of response to the patient sera.

Following this initial observation, we embarked upon a series of investigations aimed at refining N.I.H. Protocol 2, to develop Bioassay D. We focussed, in particular, on minimising within-assay errors, as is described below.

Is the "starvation" step beneficial?

The 'starvation" step recommended in N.I.H. Protocol 2 necessitated complete aspiration of the medium above the monolayers on two separate occasions. In our view, this inevitably threatened the integrity of the monolayers and was a potential source of between-well variation, which could contribute substantially to the bioassay response errors. Evidence for this had previously been obtained with Bioassay C (Figure 9.19), when avoidance of complete aspiration resulted in the ϕCV (see Section 9.2.3, Figure 9.1b) for dose-response curves to TSH decreasing from 3.0 to 1.9%. We therefore compared the performance characteristics of dose-response curves to TSH and TSAb with and without the "starvation" step incorporated into N.I.H. Protocol 2.

Dose-response curves to TSH, with and without starvation (Figure 9.30) demonstrated that a significantly lower baseline light output ($p<0.001$) was obtained after starvation (2.4 ± 0.4 R.L.U.) than for the non-starved monolayers (5.5 ± 0.1 R.L.U.). With the highest dose of TSH tested (250mU/L), the light outputs were similar (56.3 ± 0.9 *v* 59.3 ± 1.1 R.L.U.). Similar relative findings were obtained for TSAb (Figure 9.31). The performance characteristics most relevant to deciding on any benefit to be gained by the "starvation" step have been summarised in Table 9.3. "Starvation" resulted in decreased light outputs from the zero-dose controls in both the TSH (2.4 ± 0.4 *v* 5.5 ± 0.1 R.L.U., $p<0.001$) and the TSAb (2.5 ± 0.07 *v* 6.0 ± 0.3 R.L.U., $p<0.001$) response

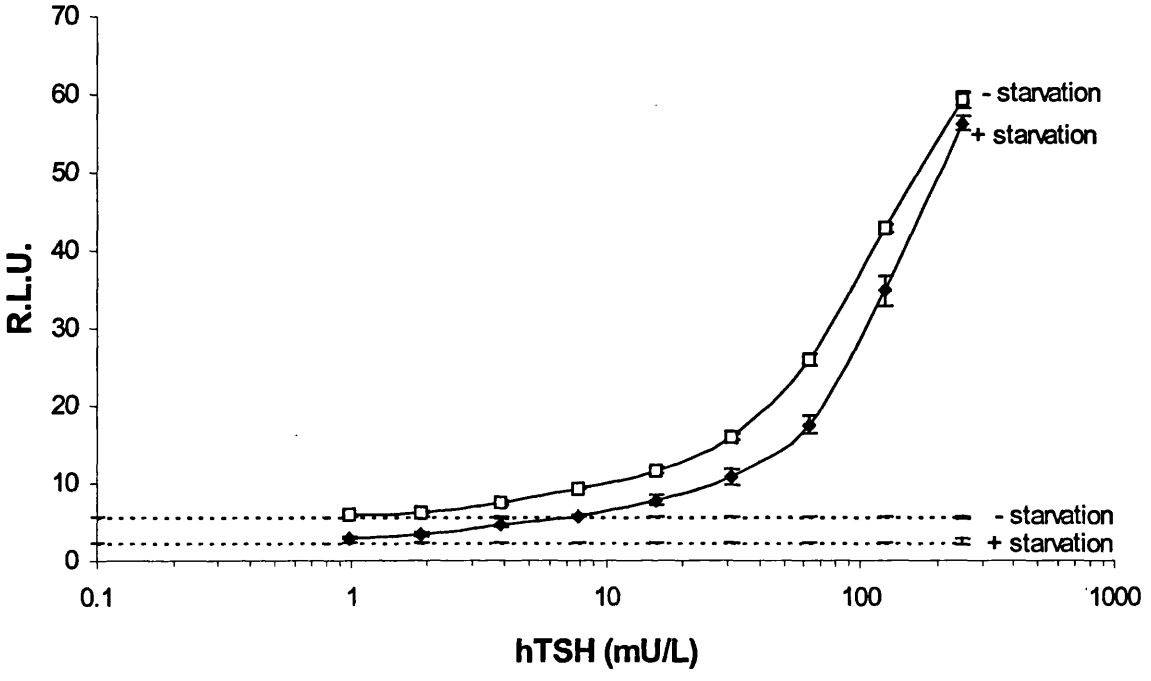


Figure 9.30 **Bioassay D: the effect of the incorporation of a "starvation" step on the subsequent response to hTSH (80/558).** Monolayers were prepared in Phase 1 either with or without a "starvation" step. Those subjected to a "starvation" step were initially plated in culture medium (100µl) at a relatively high density ($7x10^5$ cells/ml). After 24hr, the culture medium was completely aspirated and replaced with "starvation" medium and incubated for a further 24hr. In contrast, the cells which were not "starved" were plated as in Phase 1 for Bioassay C i.e. $4x10^5$ cells/ml (100µl/well) and they remained undisturbed for the full 48hr incubation period. The two sets of monolayers were then exposed to TSH, diluted in AM3, for 4hr, to complete Phase 2. For the "starved" and non-starved monolayers, the respective "starvation" and culture medium (100µl) was completely aspirated before the addition of TSH diluted in AM3. Luciferase activity was determined in Phase 3 using Promega System II, as for Bioassays B and C, with 20µl/well of the reagent being added. --- shows the light outputs from the unstimulated controls. Results are the mean ± S.D. for triplicate cultures.

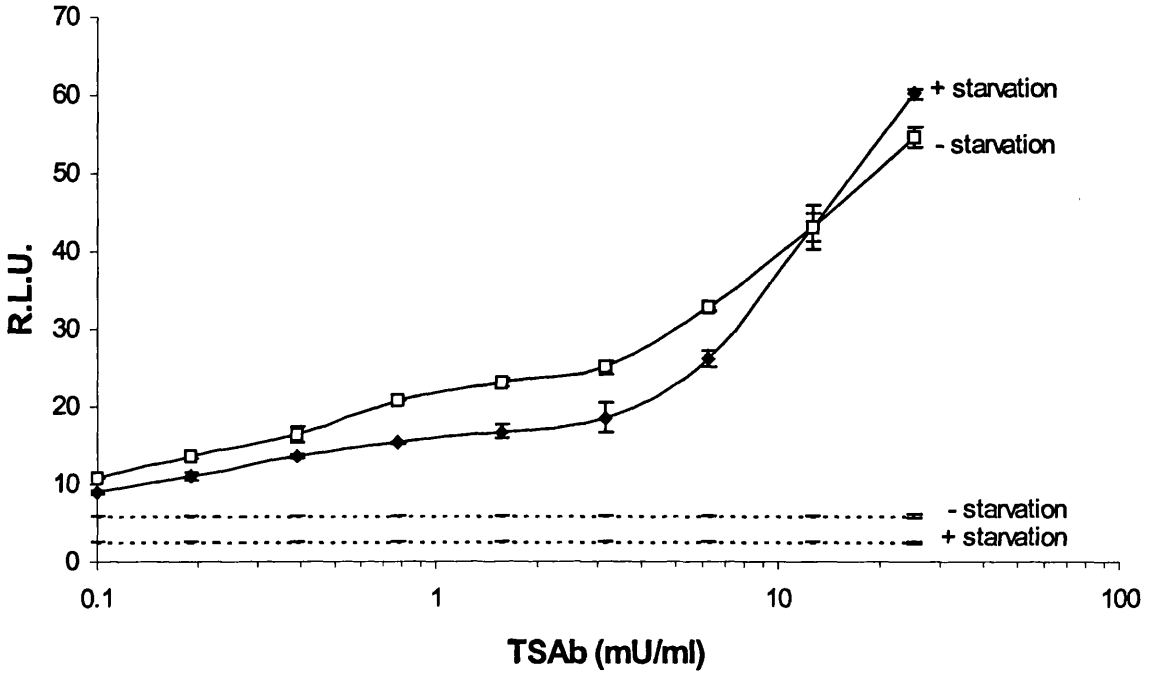


Figure 9.31 **Bioassay D: the effect of the incorporation of a "starvation" step on the response to TSAb (90/672).** The protocol was as described for Figure 9.30 except that the stimulator was TSAb (90/672) as oppose to hTSH (80/558). This had been previously reconstituted in culture medium and dialysed to remove phosphate ions.

	hTSH 80/558		TSAb 90/672	
	+ starvation	- starvation	+ starvation	- starvation
Zero dose control light output (R.L.U.)	2.4 ± 0.4	5.5 ± 0.1*	2.5 ± 0.07	6.0 ± 0.3*
The concentration which gave the greatest response	250mU/L	250mU/L	25mU/ml	25mU/ml
Greatest response (R.L.U.)	56.3 ± 0.9	59.3 ± 1.1***	66.2 ± 0.7	54.7 ± 1.4*
Magnitude of greatest response above control (R.L.U.)	53.9	53.8	63.7	48.7
Fold-increase above control	23.1	10.7	24.5	9.1
Detection limits	1.9mU/L	1.0mU/L	<0.098mU/ml	<0.098mU/ml
ϕCV (%)	6.8 ± 3.7	2.5 ± 1.0**	4.2 ± 2.9	2.7 ± 1.7

Table 9.3 **Bioassay D: the influence of the "starvation" step on selected performance characteristics of response curves to TSH and TSAb.** The full dose-response curves are shown in Figures 9.30 and 9.31. *, $p<0.001$; **, $p<0.01$; ***, $p<0.05$, - starvation v + starvation

curves. Although "starvation" increased the fold stimulation above these basal unstimulated light outputs (TSH, 23.1 *v* 10.7; TSAb, 24.5 *v* 9.1), this largely reflected the decrease in the baseline controls. It was notable that the magnitudes of bioassay response (see Section 9.2.3, Figure 9.1a) above the zero-dose controls were less influenced by starvation. Moreover, as anticipated, avoidance of the "starvation" step resulted in a consistent decrease in the ϕCV (see Section 9.2.3, Figure 9.1b) of each dose-response curve (TSH, 6.8 ± 3.7 *v* 2.5 ± 1.0%, $p<0.01$: TSAb, 4.2 ± 2.9 *v* 2.7 ± 1.7%, $p>0.1$). This will have contributed to the lowering of the detection limit for hTSH, although we demonstrated no corresponding improvement in the sensitivity to TSAb.

Since avoidance of the starvation step improved the ϕCV and had relatively small effects on the magnitudes of response, we considered that there was no substantial gain by "starvation". In addition, the one advantage to "starvation", namely the lowering of the zero dose basal control, was later found to be obliterated when 10% patient serum was added (see Section 10.3.4). In the presence of 10% human serum, the zero dose controls increased approximately 3.5-fold and their lowering by the "starvation" step was no longer observed. Thus we no longer "starved" the microcultures during Phase 1 for any of our future studies with Bioassay D.

The removal of the "starvation" step from the protocol of Bioassay D, as it did not offer any additional advantages, did not affect the superior bioassay performance characteristics of Bioassay D. A comparison of selected performance characteristics between Bioassays C and D is shown in Table 9.4. Although higher light outputs were observed from the unstimulated controls with Bioassay D, the table highlighted the

	hTSH 80/558		TSAb 90/672	
	Bioassay C	**Bioassay D**	**Bioassay C**	**Bioassay D**
Zero dose control light output (R.L.U.)	3.4 ± 0.1	5.5 ± 0.1**	2.8 ± 0.1	6.0 ± 0.3**
Response to a reference dose of TSH (250mU/L) or TSAb (25mU/ml) (R.L.U.)	9.4 ± 0.2	59.3 ± 1.1**	7.7 ± 0.5	54.6 ± 1.4**
Magnitude of response (R.L.U.)	6.0	53.8	4.9	48.6
Fold-increase above zero dose control	2.8	10.7	2.8	9.1
Detection limit	<7.8mU/L	1.0mU/L	1.5mU/ml	<0.098mU/ml
ϕCV (%)	2.2 ± 0.4	2.5 ± 1.0*	4.2 ± 2.1	2.7 ± 1.7*

Table 9.4 **Bioassay D: a comparison between the performance characteristics of Bioassay C versus Bioassay D.** The table highlights the improvements in the magnitudes of response to both TSH and TSAb, when AM3 is used as the dilutent for Phase 2 (Bioassay D) as opposed to culture medium (Bioassay C). In particular, the use of AM3 resulted in much greater magnitudes of response to low doses of TSAb e.g. 1mU/ml, but little effect on those to low doses of TSH. *, $p>0.1$; **, $p<0.001$; Bioassay C *v* Bioassay D

improvements in the magnitudes of response to both TSH and TSAb. Furthermore, much lower detection limits (see Section 9.2.3, Figure 9.1a) were obtained using Bioassay D, thus, demonstrating the increased sensitivity of the bioassay. The low ϕCV (see Section 9.2.3, Figure 9.1a) attainable with Bioassay C, remained achievable with Bioassay D, despite the obligatory disturbance of the monolayers with this protocol.

A re-examination of the aspiration step at the end of Phase 1

The use of AM3 in Bioassay D, as opposed to culture medium in Bioassay C, had proved clearly beneficial to the magnitude of response for Bioassay D (Table 9.4). However, this necessitated a change of medium in the wells. We therefore reinvestigated the problem of the inevitable disturbance to the monolayers caused by the standard aspiration method used to remove the culture medium and its replacement with AM3. We compared the standard method, accomplished with an Eppendorf pipette, against a method successfully employed previously for precise ESTA bioassays, based upon monolayers of FRTL-5 cells (Ealey PA *et. al.*, 1988), namely plate inversion, tipping and blotting. A direct comparison between the two methods revealed that using the tipping method, an acceptable ϕCV (see Section 9.2.3, Figure 9.1b) across the 96-well microtiter plate of only 2.7 ± 1.5% was obtained. In this particular experiment, a comparable variance was achieved with the aspiration method (2.6 ± 1.1%). However since tipping was faster and more suitable for multiplate assays, it was adapted for all future studies. Consequently, for all future studies, at the end of Phase 1, culture medium was removed by plate tipping.

DETERMINATION OF THE OPTIMAL CONDITIONS FOR SIGNAL DEVELOPMENT USING PROMEGA SYSTEM II

The emergence of systematic errors

One, almost inevitable, consequence of the progressive reduction of random errors by developing Bioassays A – D, was that when the results from the most refined of these systems, namely Bioassay D, were scrutinised, systematic errors were revealed. Thus, in the results from the microtiter plate shown in Figure 9.32, set up to examine for evidence of positional variation, two systematic errors were apparent. One was a tendency for, in any given triplicate, the light output from the first well to be the highest. For example, this occurred in 12 out of the 19 triplicate sets shown in Figure 9.32 and was a persistent feature in numerous other assays. The second was for mean light outputs from replicated triplicates to vary according to their position on the plate. For example, light outputs of 9.2 ± 0.6 and 14.0 ± 0.2 R.L.U. ($p<0.001$) were obtained for normal sera when tested twice in different positions on the plate (wells B6-8 *v* G10-12). There was, however, no evidence for the "edge-effects" sometimes encountered in microtiter plate assays of this nature, when for example the results from column 12, on the edge of the plate were compared with those from column 10 and 11.

Such systematic errors could have been due to several sources. Firstly, we considered that they could have been a product of our method of plating the cells at the start of Phase 1. This was accomplished by dispensing the cell suspension (100µl/well) from a Repeater Eppendorf pipette. To test this possibility, we varied the dispensing pattern with regard to the sequence of wells filled with the pipette. Since this had no effect on

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Zero Control				Normal sera				hTSH (20mU/L)		
		4.76	4.88	4.86		9.54	9.55	8.58		15.96	15.53	15.47
C		PS Sample 7				PS Sample 1				PS Sample 27		
		23.89	21.66	21.38		14.02	11.86	12.39		8.60	7.08	8.31
D		AH serum				PS Sample 2				PS Sample 20		
		39.52	33.10	39.19		17.01	15.68	15.68		74.74	71.71	72.64
E		PS Sample 22				PS Sample 9				PS Sample 1		
		11.48	10.25	10.77		37.12	35.47	33.60		13.81	15.81	17.24
F		PS Sample 23				PS Sample 27				AH serum		
		57.83	53.06	54.09		9.88	9.99	9.22		46.18	44.14	43.33
G		PS Sample 4				PS Sample 20				Normal sera		
		41.43	38.49	36.82		79.0	80.19	81.86		14.03	13.78	14.20
H		Zero Control										
		5.56	5.42	5.63								

Figure 9.32 **Bioassay D: the results from a representative microtiter plate set up to examine for evidence of positional variation.** Cells were seeded and cultured in the microtiter plate according to the standard protocol (see Section 9.2.2.1.4). Sets of triplicate microcultures were exposed to selected controls and stimulators. Several of these were repeated at contrasting positions on the plate as indicated e.g. the zero-dose control was tested in wells B2-4 and again in wells H2-4. All sera was tested at a final concentration of 10%. Exposure was for 4hr (110µl/well). Luminescence was detected 10min after the addition of 20µl of luciferase reagent. The light output, in the form of R.L.U., has been recorded for each microculture as indicated above. PS = Patient Serum

the positional errors (data not shown), it was eliminated as their cause. Secondly, we investigated whether the systematic error in the triplicate, whereby the first of each set tended to be higher than the remaining two readings, could have been due to carry-over of signal from a well of the triplicate immediately preceding the first well. Although there was little evidence that high light outputs from one set of triplicates were associated with this systematic error, we further investigated for carry-over by leaving "spacer" wells between each set of triplicates (Figure 9.32). However, the systematic error persisted and signal carry-over was therefore eliminated as the cause. Finally, we investigated whether kinetic instability in the signal development during Phase 3 could underlie these systematic errors.

Signal "drift" across a 96-well microtiter plate

We set up a "same-treatment" plate, in which replicate microcultures in each of the 96 wells were merely exposed to AM3 during Phase 2 i.e. they were all unstimulated controls. Luciferase reagent (20μl) was then added to each well and light outputs were measured after 10min. Since each well was read for 10sec, it took ~16min to read the entire plate. The light outputs were observed to increase progressively across the plate as shown in Figure 9.33.

One explanation for this could have been that since AM3, used for Phase 2, had been added in sequence from wells A1 to H12, the earlier wells had been exposed to AM3 for longer than the others. Longer exposure to AM3 could have resulted in a lower baseline signal. However, since the time of addition of AM3 to the entire plate was only about 2min, and the incubation time for Phase 2 was 4hr, this time difference was considered negligible.

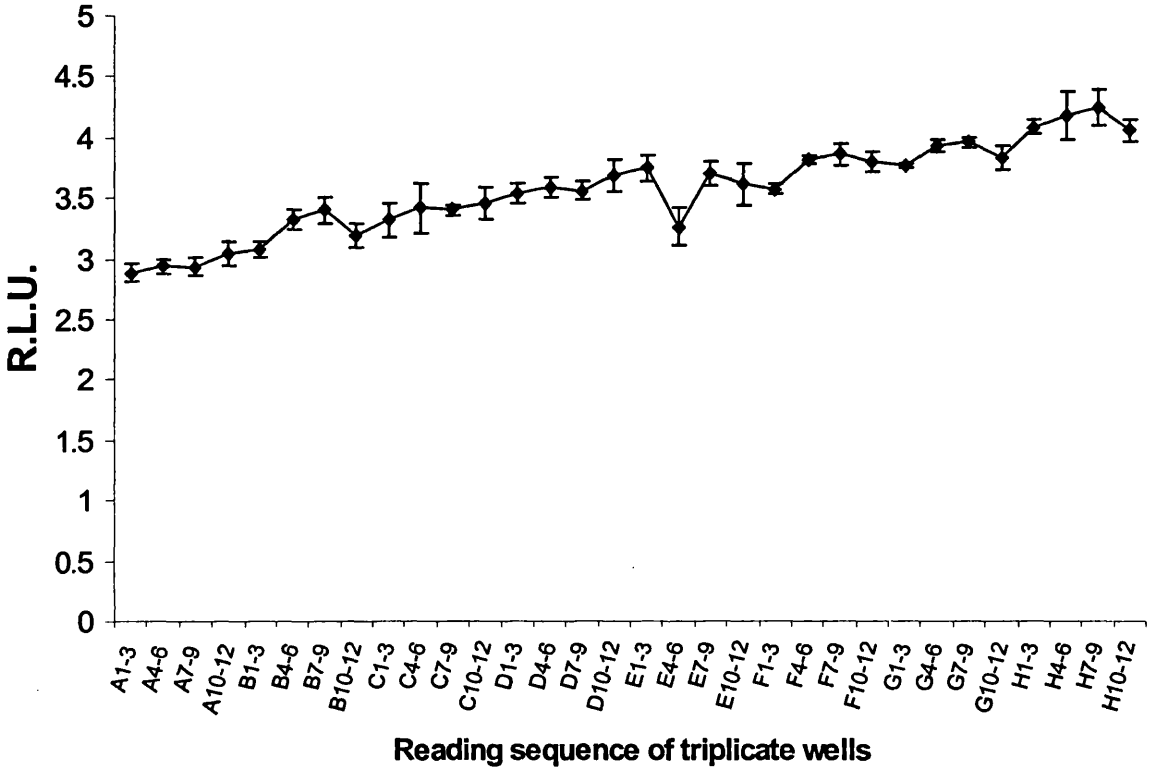


Figure 9.33 **Bioassay D: signal "drift" across a 96-well microtiter plate.** Cells were seeded and cultured in the plate according to the standard protocol (see Section 9.2.2.1.4). All 96 wells were treated identically with AM3 only, being added to each well for 4hr during Phase 2 i.e. they were all unstimulated controls. Luminescence was detected 10min after the addition of 20µl of luciferase reagent. Each well was read for 10sec with the reading sequence being as shown on the x-axis i.e. starting with the well A1 and finishing with well H12. The total time required to read the plate was ~16min. Light outputs have been plotted as mean ± S.D. for sets of triplicate microcultures as indicated e.g. A1-3. This mimics the plate design used for all our studies e.g. dose-response curves, time courses etc.

Another possible explanation was that light output changed with time after addition of the luciferase reagent to the microtiter plate. The first triplicate (wells A1-3) was read 10min after the addition of the luciferase reagent to the final well (H12), whereas, since it took ~16min to read the entire plate in the luminometer, a much longer period had elapsed before light output was measured from the final triplicate (wells H10-12). If this explanation was correct, a progressive change in light output, would be expected, as had been observed (Figure 9.33).

To test this possibility further, another "same-treatment" plate was set up. In this experiment all the microcultures were exposed to hTSH (100mU/L) during Phase 2. Light outputs from the plate was read on 3 successive occasions, namely 0, 0.5 and 1hr after the addition of the luciferase reagent to the final well (H12, Figure 9.34a). A progressive increase in light output across the plate was again observed for the first reading, confirming the observation made with the unstimulated controls (Figure 9.33). However, signal "drift" was much less noticeable for the second and third readings (Figure 9.34a). This suggested that the origin of the problem was an early rise in light output, following the addition of the luciferase reagent, which then stabilized later. This was confirmed by plotting light outputs from three selected sets of triplicates against time (Figure 9.34b). A marked increase in light output was seen for wells A1-3, over the first hour but after this the signal stabilized (between 1 and 1.5hr). By 2.75hr, it had however declined. The latter was presumably due to the signal fading expected with Promega System II investigated previously (Figure 9.8). In contrast, only a minimal early increase in light output was observed for triplicates F4-6 and H10-12, prior to a parallel signal decline at the later readings (Figure 9.34b). These results thus confirmed that there was signal instability immediately after the

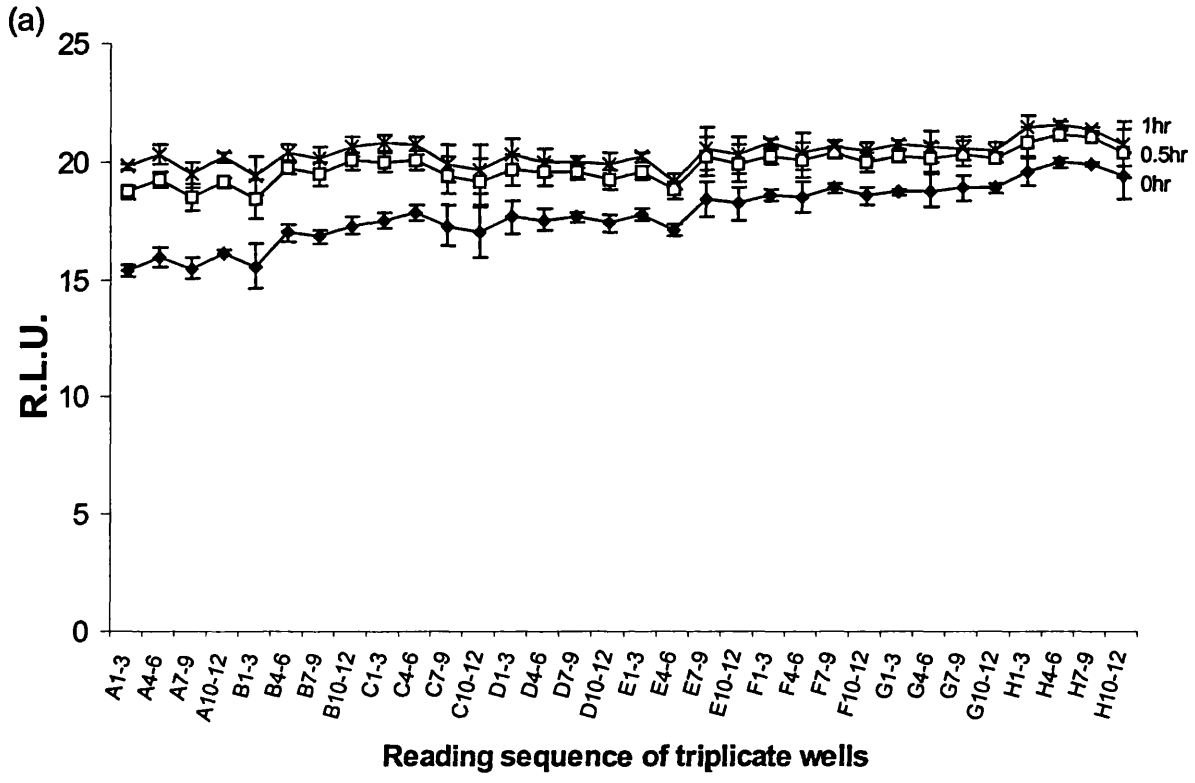


Figure 9.34a **Bioassay D: signal "drift" across a 96-well plate.** The effect of signal "drift" across the 96-well plate in which the monolayers were prepared according to the standard protocol for Phase 1 and then stimulated with hTSH 80/558 (100mU/L) for 4hr during Phase 2. Luminescence was measured at 0 (◆), 0.5 (□) and 1.0hr (×) after the addition of 20µl of luciferase reagent to the final well (H12). Each well was read for 10sec with the reading sequence being as shown on the x-axis. The total time required to read the whole plate was ~16min. Results are the mean ± S.D. for light outputs from each set of triplicate microcultures as indicated e.g. A1-3, A4-6 etc.

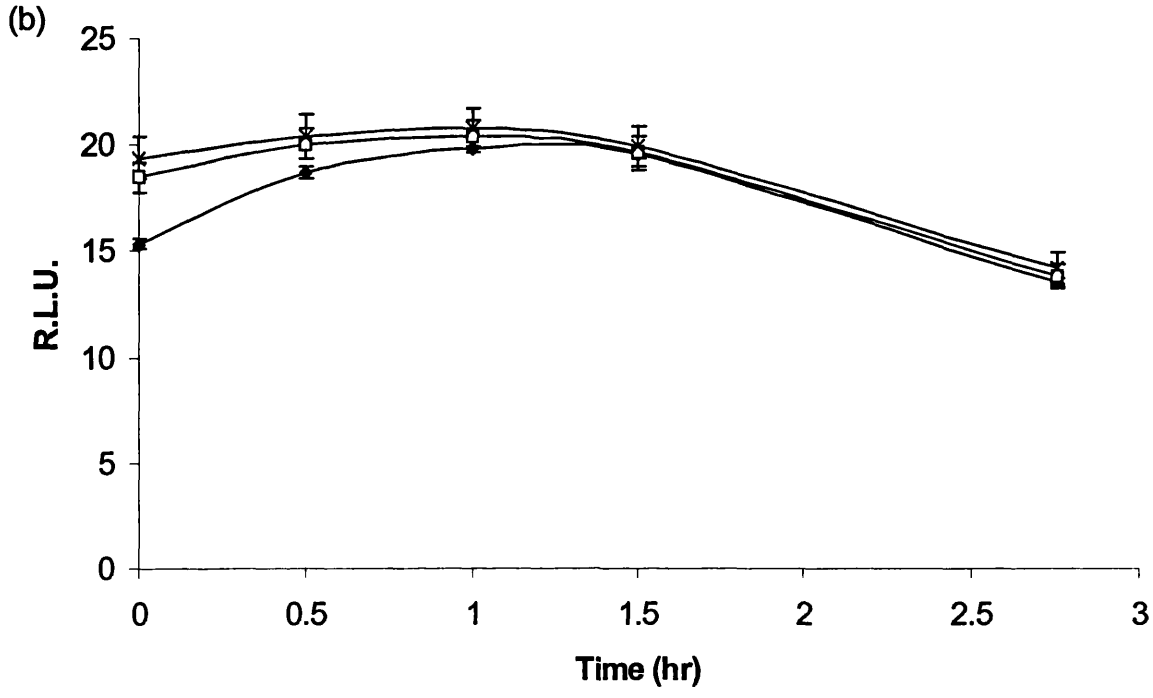


Figure 9.34b **Bioassay D: selected underlying time courses taken from Figure 9.34a.** The changes in the signals from three sets of selected triplicate microcultures shown in (a) have been plotted against time since the addition of luciferase reagent to the last well (H12). In addition, results from further readings obtained after 1.5 and 2.75hr are shown. The selected triplicates were A1-3 (◆), F4-6 (□) and H10-12 (×). Results are the mean ± S.D. for each set triplicate microcultures.

addition of the luciferase reagent. However, relative stability was attained if, as in wells F4-6, about 10min or for wells H10-12, about 16mins, had been allowed to elapse before reading light outputs. From Figure 9.34b, it can be seen that greatest signal stability was obtained after about 1hr.

An investigation for any influence of the amount of luciferase reagent on signal stability

Multiple microcultures were set up which were either (a) unstimulated or (b) stimulated with hTSH (20mU/L) during Phase 2. For Phase 3, one of four selected volumes of luciferase reagent was added to the microcultures, and multiple successive timed readings were then made (Figures 9.35a & b). Again an early rise in signal was obtained, followed by a plateau, prior to the anticipated decline in light output. However it was evident that the greatest signal instability at the early readings, was observed when only 20μl of reagent had been added. Interestingly, the highest amount of luciferase reagent (100μl) did not give rise to the highest light output. This was achieved when 40 or 50μl was added. However, signal instability was consistently observed with each of the volumes of luciferase reagent added.

The influence of reducing the reading time/well on signal stability

In the preceding section, we demonstrated that the kinetics of signal development was complex. An early rise in light output was followed, after about 1hr, by a "window' of stability which lasted about 1hr, prior to a decline in signal. To minimise the effects of this signal instability on "drift", it seemed advisable, after the addition of the luciferase reagent, to allow time to elapse prior to reading light outputs. Additional studies (not shown) identified 40min as being a sufficient time. In addition, it would

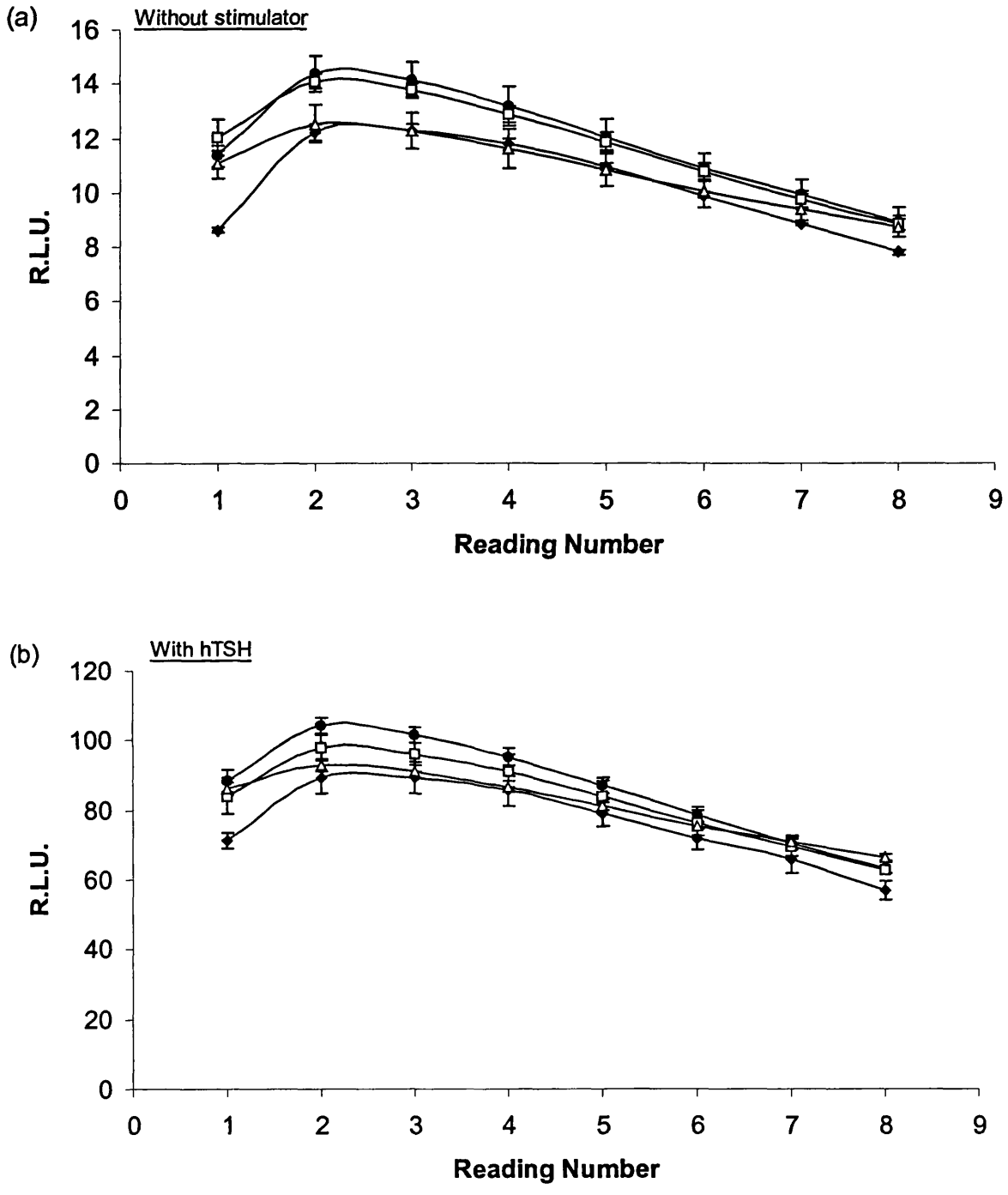


Figure 9.35 **Bioassay D: the effect of varying the amount of luciferase reagent on the time course of the development of the luminescent signal.** The time course of signal development was determined for sets of triplicate microcultures (a) without and (b) with hTSH (20mU/L, 80/558) stimulation during Phase 2. Luminescence was determined 10min after the addition of 20 (◆), 40 (●), 50 (□) and 100µl (△) of luciferase reagent/well in a sequence of timed readings as indicated on the x-axis. Each well was read for 10sec and the total reading time required for the whole plate was ~16min. Thus, Reading 1 was 10min after the addition of the reagent and Reading 2, 26min etc. Results are the mean ± S.D. of the light outputs for each set of triplicate microcultures.

clearly be advantageous if the reading of an entire microtiter plate could be accomplished in as short a time as possible. Consequently, we investigated the effect on signal "drift' of shortening the reading to 1, 3 and 5sec/well (Figures 9.36a & b). Shortening the reading time would not be expected to influence absolute R.L.U. values since these are in fact always expressed as "per second", throughout our studies (see Section 9.2.3). This was confirmed and it appeared that 1sec was an adequate time to accumulate the photons from each well. However, it was immediately apparent that rapid plate reading resulted in virtual elimination of assay drift. The % variation in triplicates for the unstimulated cells, expressed as ϕCV (see Section 9.2.3, Figure 9.1b) across the microtiter plate after a reading time of only 1sec, was 2.3%, whereas after reading times of 3 and 5sec, it was 3.8 and 3.7% respectively. Similarly, when stimulated with hTSH, the ϕCV for 1, 3 and 5sec reading times were 1.5, 3.1 and 3.3%. Moreover, scrutiny of the results with the shorter reading times revealed that the distribution of the 3 individual values in each triplicate were no longer subject to systematic errors whereby that first reading tended to be the highest. However, the reason for this improvement was not apparent.

We therefore concluded that assay "drift" could be eliminated if the protocol was changed so that (a) 40min was allowed to elapse, after the addition of the luciferase reagent, prior to reading any plate in the luminometer and (b) reading times were shortened from 10 to 1sec/well. In addition, due to results from parallel studies obtained with human sera (see Section 10.3.6), it was decided to increase the amount of luciferase reagent added/well from 20 to 50μl.

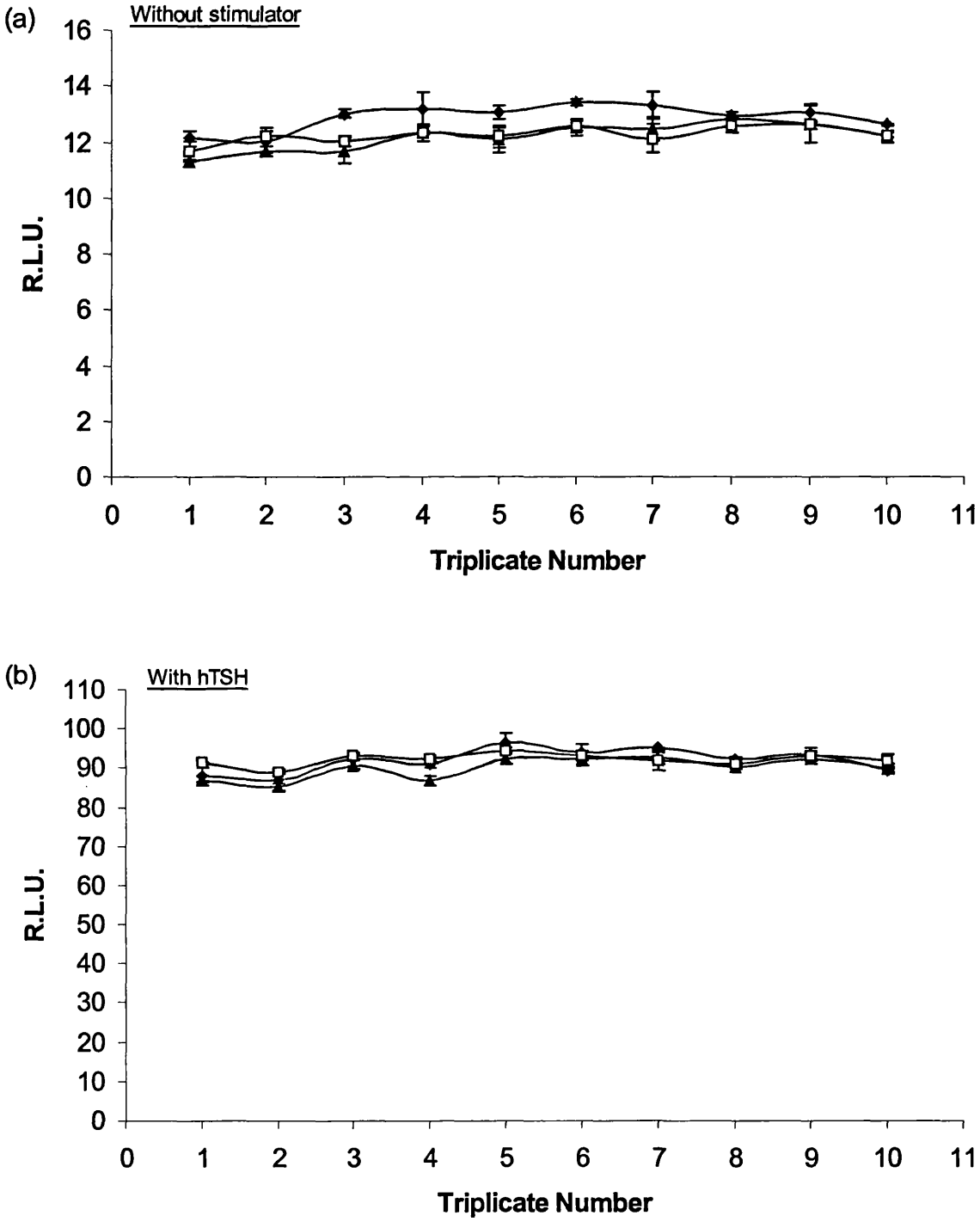


Figure 9.36 **Bioassay D: the effect of decreasing the signal reading time/well on assay "drift".** The effect of signal "drift" across three 96-well microtiter plates in which cells were (a) unstimulated and (b) stimulated with hTSH (80/558, 20mU/L). Each identical 96-well plate contained alternate triplicate cultures of unstimulated and stimulated wells (10 triplicates of each). Luminescence was determined 40min after the addition of 50µl/well of luciferase reagent. The plates were read once at a preset reading time of 1 (□), 3 (▲) or 5sec/well (◆) i.e. one plate has a reading rate of 1sec/well, the second, 3sec/well etc. Results are the mean ± S.D. R.L.U. for each set of triplicate microcultures.

BIOASSAY D: RESPONSE TO hTSH 80/558 USING THE REVISED PROTOCOL

To conclude, we show a representative dose-response curve to TSH (Figure 9.37a) using the finalized protocol for Bioassay D which has been systematically established as described above. Greatest light emission (338.7 ± 1.0 R.L.U.) was observed at the highest TSH dose tested (500mU/L), this being a 9.3-fold increase above the control response of 36.4 ± 0.8 R.L.U.. The raised baseline in this experiment, was expected; it is because the TSH had been diluted in AM3 supplemented with 10% normal human serum. As will be shown in Results Section 10, the inclusion of human serum consistently leads to an increase in light outputs from the unstimulated controls. The magnitude of response to this dose of TSH (~300 R.L.U.) was large and approaching a plateau. Of particular note is the observation that the ϕCV of the response error (see Section 9.2.3, Figure 9.1b), calculated across the entire dose-response curve, was only 1.6 ± 1.1%.

An Imprecision Profile (see Section 9.2.3, Figure 9.1d) was generated from this dose response curve (Figure 9.37b). This demonstrated that using the revised protocol developed as Bioassay D, impressive precision could be obtained. The % imprecision of TSH measurement was estimated to be as low as 2.5% for TSH concentrations of 20 – 30mU/L and remained below 10% for doses as high as 320mU/L. This precision is comparable to that achievable with immunoassays, and is the consequence of a protocol which yields minimal response errors combined with substantial magnitudes of response to TSH. However, despite these favourable performance characteristics, the detection limit (see Section 9.2.3, Figure 9.1a) was estimated to be relatively high (<5mU/L) and within the normal physiological range.

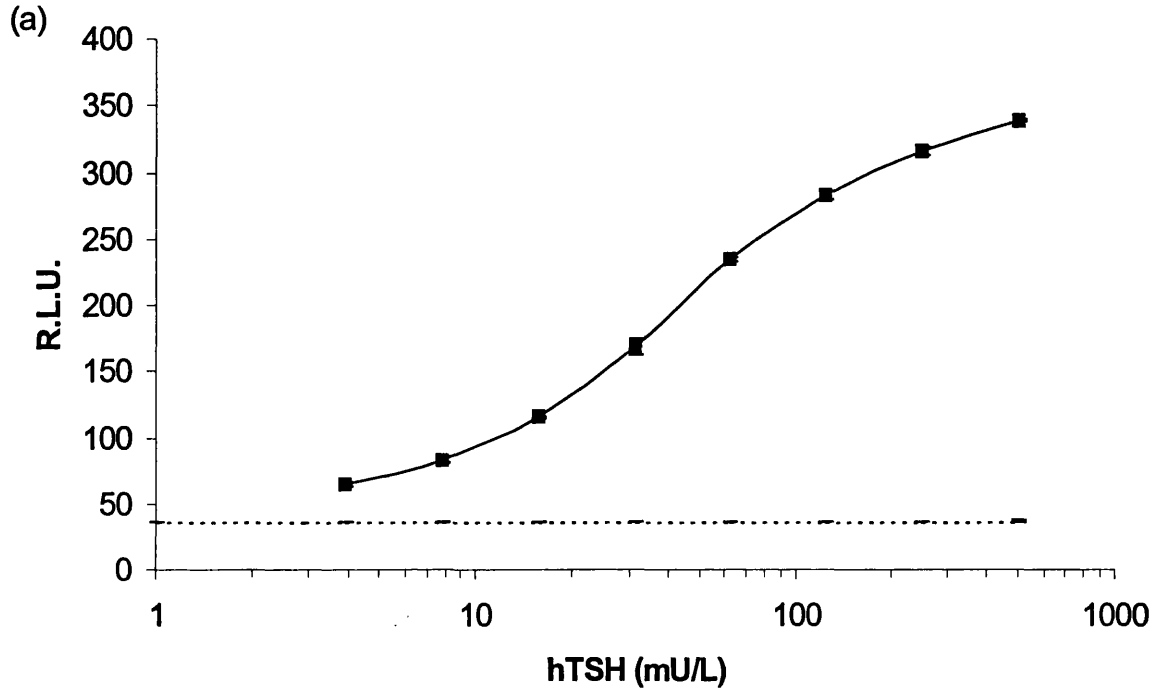


Figure 9.37a **Bioassay D: the increase in luminescence in response to increasing concentrations of hTSH (80/558).** The assay was performed using the finalized bioassay protocol described in Section 9.2.2.1.4 i.e. no "starvation" step was used in Phase 1, AM3 was the dilutent for Phase 2 and for Phase 3, light outputs were determined 40min after the addition of 40µl/well of Promega System II luciferase reagent, with each well being read for only 1sec. --- is the light output from unstimulated control microcultures. Results are the mean ± S.D for triplicate microcultures.

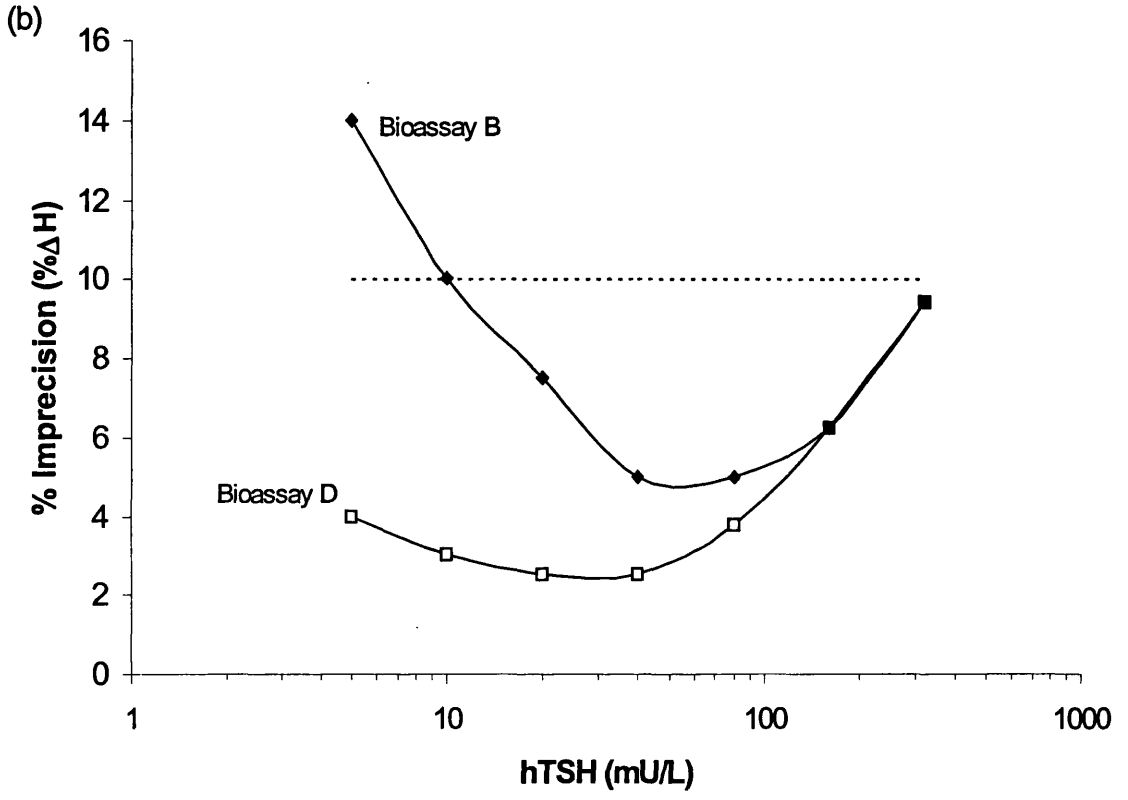


Figure 9.37b **Bioassay D: an imprecision profile constructed from the dose-response curve shown in Figure 9.37a (□).** The % imprecision was estimated as described in Section 9.2.3 and Figures 9.1c-e. For comparison we also show the imprecision profile constructed from a representative dose-response curve for Bioassay B (◆; Figure 9.16).

On Figure 9.37b, we also showed an Imprecision Profile generated from a representative dose-response curve (Figure 9.16) using Bioassay B. With the latter, the imprecision was less favourable for TSH doses ranging from 5 – 80mU/L. This was a consequence of a higher ϕCV of the response errors (2.6 ± 1.5%) and a smaller magnitude of response (e.g. for 500mU TSH/L, this was only ~20 R.L.U. for Bioassay B compared with ~ 300 R.L.U. for Bioassay D). These characteristics summerize the improved bioassay performance which has resulted from the development of Bioassay D. Demonstration of the **consistently** favourable performance of Bioassay D is shown in Figure 9.38. This is a cumulative imprecision profile generated from 4 representative dose-response curves to TSH using Bioassay D. The average % imprecision of TSH measurement was estimated to be as low as 3.1% for TSH concentrations of 40 – 80mU/L and only exceeded 10% for doses as high as 320mU/L (10.9%). This showed that the precision of Bioassay D observed in Figure 9.37b is not the result of the selection of the "best" dose-response curve. Figure 9.38 confirmed that precision similar to that obtained in Figure 9.37b could be reproduced with any given Bioassay D provided that the assay conditions remained constant. This consistent performance of Bioassay D offers confidence in our decision to use it in the screening of sera from GD patients for TSAb (see Section 10.3.8).

9.4 CONCLUSIONS

We have summarized the development of a reporter gene luminescence bioassay for thyroid stimulators, TSH and TSAb, using the CHO cell line CHO25LUC. Our successive systematic investigations, based upon a progression of protocols (Bioassays A – D), have attempted to optimize the bioassay performance

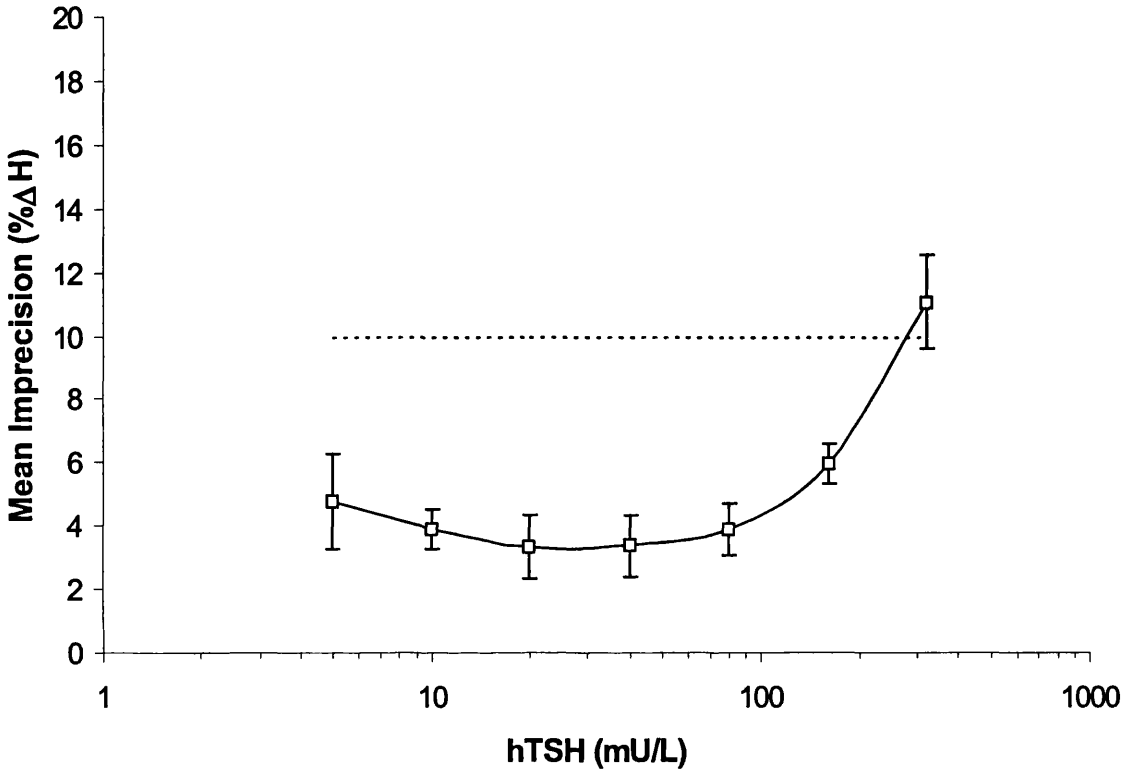


Figure 9.38 **Bioassay D: a cumulative imprecision profile constructed from hTSH dose-response curves**. The % imprecision for 4 dose-response curves to hTSH (80/558) obtained using Bioassay D was estimated as described in Section 9.2.3 and Figures 9.1c-e. The results shown are the mean % imprecision (ΔH) ± S.D. (n = 4).

characteristics by ensuring conditions for the largest magnitude of response combined with a protocol which minimized response errors. In the next Section, we finally describe the adaptation of the optimized bioassay for the measurement of thyroid stimulators in the presence of human serum.

Section 10

The detection of Thyroid Stimulating Antibodies by the CHO25LUC Luminescence Bioassay

10.1 INTRODUCTION

The numerous different approaches to the development of a reliable TSAb bioassay have been outlined in Section 3.5 of the Introduction. Despite the technical advances made over the past few decades, no *in vitro* bioassay suitable for large-scale clinical use has yet been developed. The ideal bioassay should be precise, sensitive to low physiologically relevant doses of thyroid stimulators, technically undemanding and suitable for mass testing of patient sera.

As reported in Section 9, we have progressively developed and optimized four luminescence bioassays for TSH (Bioassays A – D), based upon the CHO cell line CHO25LUC. In Bioassay D, we concluded that we had succeeded in developing a bioassay optimized to suit our current purpose. In this Section, we summarise our attempts to adapt this system for the detection of TSAb in serum samples from GD patients.

10.2 MATERIALS AND METHOD

10.2.1 Materials

The materials used here, in this section, were the same as those used in Section 9. The details had been previously described in full in Section 9.2.1. The TBI Binding assay was obtained from RSR Limited, Cardiff, UK.

10.2.2 Methods

10.2.2.1 Protocols for Bioassays C & D

10.2.2.1.1 Bioassay C

This bioassay involved cells exposed to stimulations in culture medium (see Section 10.2.2.2) and has been described in detail in Section 9.2.2.1.3.

Briefly:

- Phase 1: CHO25LUC cells were seeded into the inner wells of the white 96-well microtiter plates at $4x10^5$ cells/ml (100µl culture medium/well). The plates were then incubated for 48hr prior to Phase 2.
- Phase 2: After 48hr, 50µl of culture medium was removed from each well and thyroid stimulators prepared in culture medium were added (50µl/well). Serum samples were added (10µl) together with 40µl fresh culture medium to give a 10% final in well concentration. Exposure was for 4hr.
- Phase 3: Luminescence was determined using Promega System II i.e. the single step Promega Steady-Glo luciferase assay system (Promega Code E2510, also see Section 9.2.2.3). Light output was measured with the Anthos Lucy 1 microplate

luminometer (10sec/well) 10min after the addition of the luciferase reagent (20µl/well).

10.2.2.1.2 Bioassay D

This bioassay involved cells exposed to stimulators in AM3 (see Section 10.2.2.2) and had been fully detailed in Section 9.2.2.1.4.

Briefly:

- Phase 1: The cell monolayers were prepared as described in Bioassay C above with the exception that all 96 wells were seeded.
- Phase 2: After the 48hr incubation, the culture medium (100µl) was removed and replaced with thyroid stimulators in AM3 (110µl), which was a proprietary solution provided by DHI. Graves' sera were added (110µl) as a 10% dilution in AM3 i.e. only 10µl sera was required per microculture. Incubation was for 4hr.
- Phase 3: Luminescence was determined using Promega System II (50µl/well). Unless stated otherwise, light output was measured 40min after the addition of the luciferase reagent using the luminometer. Each well was read for only 1sec.

10.2.2.1.3 N.I.H. Protocol 2

This protocol had been provided by our collaborators at N.I.H. and DHI. The protocol was detailed in Section 9.3.4 in which cells were exposed to stimulators in AM3 (see Section 10.2.2.2).

Briefly:

- Phase 1: Monolayers were prepared by plating cells at a relatively high initial density (100µl at $7x10^5$ cells/ml in culture medium). After 24hr, the monolayers

were washed with a proprietary "starvation" medium obtained from DHI. The monolayers were then further incubated (24hr) with the "starvation" medium (100µl).

- Phase 2: The "starvation" medium (100µl) was fully aspirated and stimulators (TSH or Graves' patient sera) were exposed for 4hr after dilution in the proprietary solution, AM3. Graves' sera were added (110µl) as a 10% dilution in AM3 i.e. only 10µl sera was required per microculture.
- Phase 3: Luminescence was determined using Promega System I.

10.2.2.2 Media used for Phase 2

The different media used in Phase 2 of the bioassay i.e. the exposure for the cells to the stimulators in this section are summarised below.

Culture Medium	This medium is the same as that used in the routine culture of CHO25LUC cells. It comprises Ham's F12 nutrient medium supplemented with 10% FCS and antibiotics penicillin and streptomycin (see Section 5.1.3).
Assay Medium 3 (AM3)	This is a proprietary solution obtained from DHI.
Assay Medium 4 (AM4)	This medium is AM3 (as above) supplemented with 10% human pooled euthyroid sera. This medium was used in the investigation for parallelism between thyroid stimulators with Bioassay D.

10.2.2.3 Potency Index Study

We investigated the possibility of expressing TSAb in patient serum as TSH "equivalents", i.e. as a Potency Index (P.I.) in mU/L. This required that in the bioassay, each microtiter plate included a TSH dose-response curve, as a standard curve, in conjunction with test patient sera. The mean R.L.U. observed for each test patient serum would then be interpolated from the TSH standard curve and expressed as a P.I.. In our study, 4 sequential bioassays (i – iv) were performed to investigate the reproducibility of the results. In each bioassay, using Bioassay D with the protocol detailed above (see Section 10.2.2.1.2), 9 selected patient sera were tested in each microtiter plate, together with TSH standards.

TSH Standard Curve

As explained above, each microtiter plated included a TSH dose-response curve. Care was taken to minimize between-assay variations by the bulk preparation of a master series of TSH standards. Bulk aliquots of selected concentrations of the International Reference Preparation hTSH (80/558), ranging from 0 – 500mU/L, were pre-prepared by doubling dilution in AM4 (see Section 10.2.2.2), gravimetrically. Sub-aliquots (400µl) were then snapped frozen in liquid nitrogen and stored at -20°C. On the day of the bioassay, the aliquots were thawed at room temperature and thoroughly mixed before addition to appropriated microcultures. The standard curves were set up using triplicate cultures for each TSH concentration, thus, the mean R.L.U. ± S.D. for each triplicate was used to plot the standard curves.

Patient sera

Initially a group of 9 patient sera were selected for the developmental work with the bioassay. As with TSH, to minimize between-assay variation, pre-prepared aliquots of each sample, appropriately diluted, were prepared and frozen. The 9 sera were PS1, 9, 17, 20, 23, 25, 68, 80 and AH. These represented the full range of stimulation potency with TSI values, determined in a preliminary study, ranging from 1.4 to 5.5 (see Section 10.2.3). Each serum was initially diluted to 10% in bulk-preparations, in AM4, and then sub-aliquoted (400µl). The aliquots were then snap frozen in liquid nitrogen and stored at -20°C. On the day of the bioassay, the aliquots were thawed at room temperature and thoroughly mixed before adding to the wells. The mean R.L.U. ± S.D. for triplicate cultures for each sample was used to interpolate a TSH "equivalent" from the TSH standard curve, this value being termed a Potency Index (P.I.). In addition, the observed mean R.L.U. for each sample were also expressed as a TSI (see Section 10.2.3).

10.2.2.4 Detection of TSAb activity in patient sera

The following categories of patient sera were included in our study. Informed consent had been obtained from all patients.

A. Negative controls i.e. euthyroid or non-thyroid disease controls, together with sera from hypothyroid patients and those suffering from either an isolated hot nodule or multinodular goitre. Three pools of euthyroid sera, designated UKN, USN and MMI, were also used. UKN was a pool of 12 euthyroid volunteers, USN was obtained from our collaborators (DHI) and MMI was a pool of sera from 12 euthyroid multiple myeloma patients.

B. Potential Stimulators

(i) Sera from hyperthyroid patients attending the Thyroid Clinic at UCH. They were collected over a period of 12 months.

(ii) Sera from a National Survey of Graves' patients and their relatives (n = 287). These were kindly supplied by the Freeman Hospital in Newcastle.

GD was diagnosed by the finding of biochemical hyperthyroidism, together with evidence of one of the following: (a) significant thyroid-associated ophthalmopathy (American Thyroid Association Class 3 or worse), (b) diffuse increase in thyroid uptake on a radionuclide scan, and/or, in the case of the National Survey, (c) positive serum TSH binding inhibitory antibodies.

10.2.2.5 Protocol for Binding Assay

42 patient sera obtained from the Thyroid Clinic at UCH were tested in a TBI Binding Assay according to manufacturer's instruction. The assay, a TRAb ELISA assay, was based upon the competition between TRAbs in patient serum samples and TSH, in the form of TSH-biotin, for TSHRs coated on the ELISA plate wells. TRAbs were detected by their ability to inhibit the binding of TSH-biotin to the same receptors. The amount of bound TSH-biotin was then measured by a colorimetric reaction as a result of the addition of streptavidin peroxidase (SA-POD) and the peroxidase substrate tetramethyl benzidine (TMB). Briefly, to start, the strips of ELISA plate wells were firmly fitted into the frame provided. Then 20μl of start buffer was pipetted into each well, followed by 100μl/well of the negative and positive controls provided, 4 calibrators and test sera, each being tested in duplicate. The plate was then covered

and incubated for 2hr at 20-25°C on a plate shaker. After the 2hr, the samples were discarded by inverting the plate and the wells were washed once with wash buffer which was also discarded by inversion and tapping of the plate on an absorbent surface to remove any excess buffer. This was then followed by the addition of 100µl of reconstituted TSH-biotin into each well and a further incubation of 20min at room temperature without shaking. Once again, after the 20min, the unbound TSH-biotin was discarded and the wells were washed once with wash buffer. Then 100µl of SA-POD was pipetted into each well and incubated for another 20mins on the bench without shaking. Again, the excess SA-POD was discarded and the wells washed twice with wash buffer followed by once with distilled water. For the colorimetric reaction, 100µl of TMB was pipetted into each well followed by incubation for 20mins in the dark to allow the colour to develop. Finally to stop the reaction, 50µl of stop solution was added to each well. Optical densities (O.Ds.) were then read at 450nm, with a reference wavelength of 655nm, using a microtiter plate reader (Bio-Rad, Model 3550, Richmond, CA).

The results, referred to as TBII values, were then calculated and expressed as a % of TSH inhibition using the following formula:

$$100 \times 1 - \frac{\text{test sample absorbance at 450nm}}{\text{kit negative control absorbance at 450nm}}$$

10.2.3 Quantitative assessment of bioassay performance characteristics

As discussed in Section 9, for the quantitative assessment of the performance of the bioassays, several criteria have been used. These were as follows:

- Light outputs were expressed as ***Relative Light Units*** (R.L.U.). They are shown either as the mean R.L.U. for duplicate microcultures or, when appropriate, expressed as mean ± standard deviation (S.D.) of triplicate microcultures.
- The theoretical ***Detection Limits*** of stimulators
- ***Magnitudes of response***
- ***Fold-responses***
- Estimates of within assay errors as ***ϕCV***

All the above criteria had been defined in Section 9.2.3 (Figures 9.1a-e). In addition to the above, results from patient sera were also expressed as a ***Thyroid Stimulation Index (TSI)***. This was calculated as: Mean R.L.U. for test serum / Mean R.L.U. for the euthyroid control serum. Furthermore, statistical analysis to determine significance was also performed as detailed in Section 9.2.3.

10.3 RESULTS

We have described in Section 9 the progressive development of four Bioassays for TSH (A – D). We demonstrated a gradual improvement in performance with each successive bioassay, in particular leading to increased bioassay precision. Bioassay D appeared to be best suited for adaptation for the measurement of thyroid stimulators in human sera. However, we initially began our investigations into the effects of human sera using Bioassay C. Unfortunately, with this protocol, we failed to detect significant stimulation with sera from several Graves' patients, apart from one extremely potent patient (LM). N.I.H. Procotol 2 (see Section 10.2.2.1.3) subsequently became available and was developed into Bioassay D which we then adapted for the large scale testing of TSAb in patient sera.

10.3.1 Preliminary investigation: the effect of human serum using Bioassay C

Since we had no prior knowledge of the effect of human sera on the CHO25LUC cells, it was necessary to establish not only the tolerance of these cells to human serum but also the optimum % of serum to use in a CHO25LUC luminescence bioassay. We first tested the effects of a range of serum concentrations on responses obtained with Bioassay C (Figure 10.1). We compared the responses obtained with a serum from a patient with florid GD (LM), against our in-house negative control (UKN), this being a serum pool prepared from 12 euthyroid individuals. With both sera, the luminescence increased as the serum concentrations increased from 0.625 – 10%, after which a plateau was approached. However, much larger increases in light output were observed with serum LM with a plateau in light output 3.1-fold above that observed with the UKN. This was consistent with our previous finding that this serum was a very potent stimulator of cAMP production in FRTL-5 cells (Yateman ME, unpublished data). We also show the results from another serum (PS1), taken from a patient with mild GD, which only induced light outputs slightly above those obtained with UKN. However, again a plateau was approached at 10% serum. We therefore concluded from this preliminary experiment, that the bioassay system was capable of responding to TSAb in patient serum, and that the optimal serum concentration for further testing was 10%. However, it was evident that with this concentration of serum, the bioassay baseline i.e. light output with the euthyroid serum, would rise compared with light outputs from microcultures which had not been exposed to human serum.

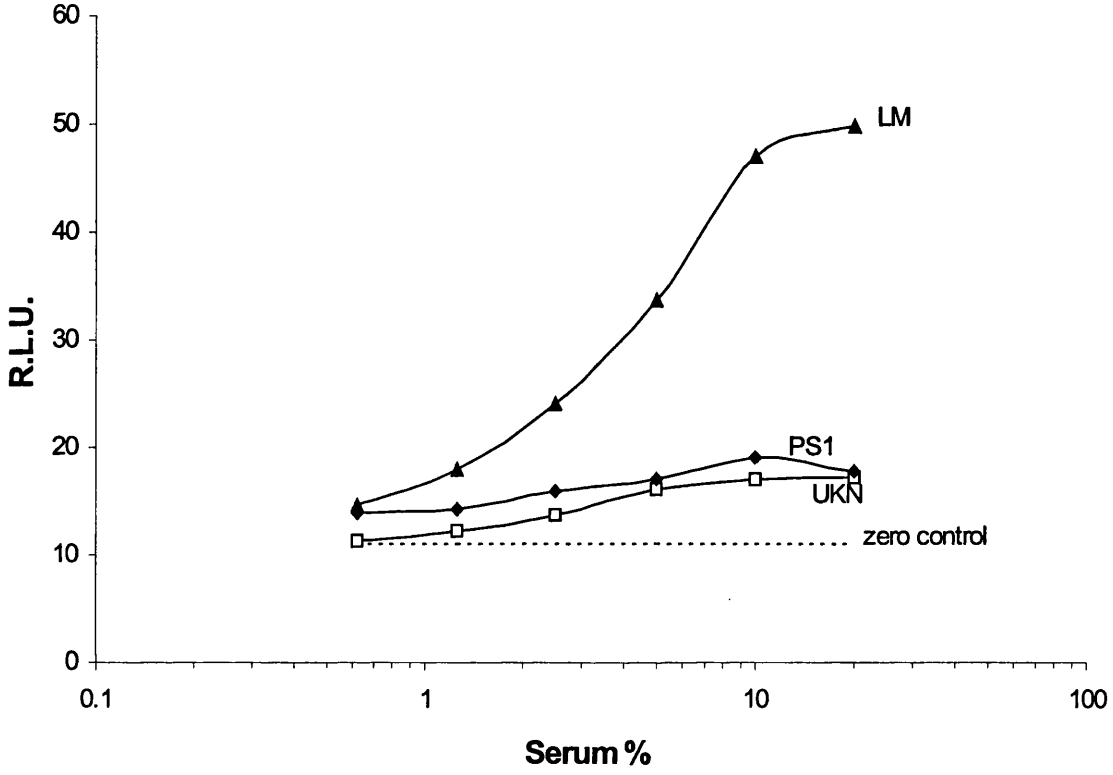


Figure 10.1 **Bioassay C: investigating the effect of varying concentrations of 3 contrasting human sera.** Two patient sera, PS1 (◆) and LM (▲), were compared to the euthyroid negative control pool UKN (□). Each serum was diluted in culture medium to generate the range of serum concentration (0 – 20%). The bioassay was performed according to the standard protocol described in Section 10.2.2.1.1 --- shows the light outputs from the control microcultures to which no human serum had been added. The mean R.L.U. of duplicate microcultures have been plotted.

10.3.2 Investigation of possible potentiation of patient sera responses using IBMX in Bioassay C

In Section 9, using Bioassay C, attempts had previously been made to investigate possible IBMX potentiation of responses to TSH (Figure 9.25). Although slight synergistic effects were observed with relatively high concentrations of IBMX, we concluded that synergy did not benefit the performance of the bioassays. However, since we had not been able to detect any stimulation in response to several Graves' patient sera (PS1-5) using Bioassay C (data not shown), we investigated the possible enhancement of their responses with IBMX.

8 patient sera (PS1-8) were tested and compared to our in-house euthyroid negative control sample (UKN) in the presence and absence of IBMX (Figure 10.2a & b). In the presence of a relatively high dose of IBMX (0.31mM), the UKN sample yielded a high light output of 88.6 ± 1.1 R.L.U. compared to 13.2 ± 0.1 R.L.U. in the absence of IBMX (Figure 10.2a). This striking rise in light output due to IBMX was also observed in the responses to all the patient sera tested. The addition of 0.31mM IBMX caused an average 6.5-fold increase in the observed light emissions. Despite the increase in light outputs in the presence of IBMX, this did not enhance the responses to the patient sera above the negative control. No marked differences were observed between the TSI (see Section 10.2.3) obtained for each serum in the presence and absence of IBMX (Figure 10.2b). We concluded that the addition of IBMX did not potentiate the responses of the test sera and as a result did not contribute to the detection of TSAb in these patient sera. Thus, there was no benefit in pursuing possible potentiation of responses in the CHO25LUC luminescence bioassays using IBMX.

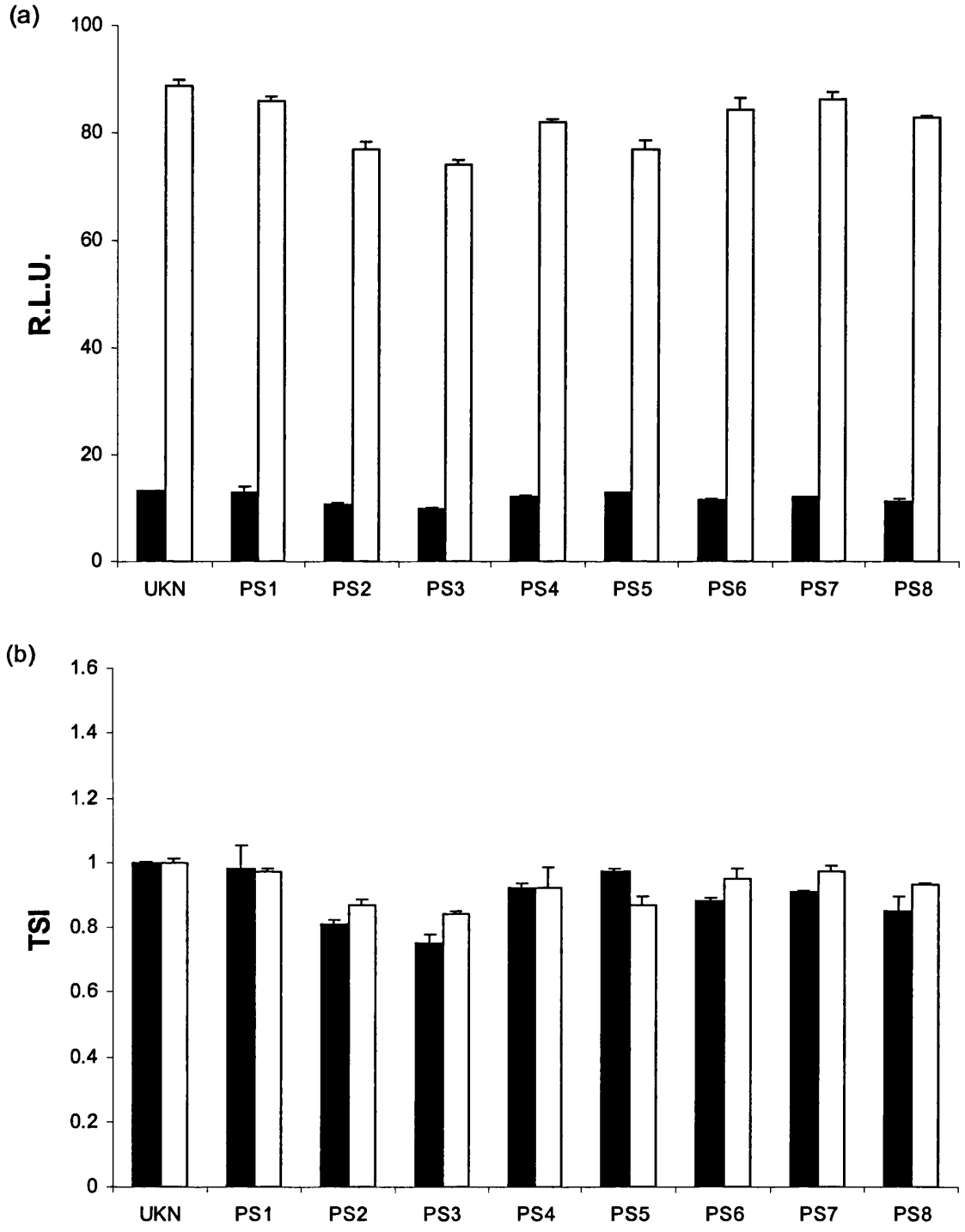


Figure 10.2 **Bioassay C: an investigation of possible potentiation of bioassay responses to GD patient sera by IBMX.** (a) The increase in luminescence in response to the euthyroid control sera (UKN) and 8 patient sera in the presence (□) and absence (■) of 0.31mM IBMX. The bioassay was performed according to the standard protocol described in Section 10.2.2.1.1. Results are the mean ± S.D. R.L.U. for triplicate cultures. (b) The results in (a) have been transformed to compare TSI values (see Section 10.2.3) in the presence (□) and absence (■) of 0.31mM IBMX.

10.3.3 Preliminary comparison between the N.I.H. Protocol 2 and Bioassay C

We reported in Section 9 that our collaborators at N.I.H. and DHI claimed that N.I.H. Protocol 2 gave good magnitudes of response with potent sera from Graves' patients. In Section 9.3.4, we improved this protocol and developed Bioassay D. We now describe how we adapted this system to detect TSAb in sera from Graves' patients. However initially, prior to the optimization of N.I.H. Protocol 2, we compared its performance characteristics to those of Bioassay C in a preliminary study described here.

Firstly, the differences in performance characteristics of N.I.H Protocol 2 and Bioassay C in response to hTSH (80/558) and TSAb (90/672) were compared (Figure 10.3 & Table 10.1). In this study, the monolayers were exposed to 3 doses each of TSH (10, 50 & 250mU/L) and TSAb (1, 10 & 25mU/ml). A lower light output ($p<0.001$) was observed for the unstimulated control microcultures with N.I.H. Protocol 2 (2.1 ± 0.1 R.L.U.) than with Bioassay C (4.5 ± 0.1 R.L.U.). Although, there was a dose-dependent increase in response to TSH and TSAb in both assays, considerably higher light emissions were obtained using N.I.H. Protocol 2. With the lowest dose of TSH tested (10mU/L), the light outputs were similar (8.8 ± 0.7 *v* 7.0 ± 0.2 R.L.U., $p<0.05$). However, in contrast, at the highest dose of TSH tested (250mU/L), a considerably higher light output was obtained with N.I.H Protocol 2 compared to Bioassay C (52.8 ± 1.1 *v* 17.8 ± 0.8 R.L.U.). Similarly, in response to the highest dose of TSAb tested (25mU/ml), a greater light output was observed with N.I.H. Protocol 2 (48.3 ± 2.9 *v* 12.4 ± 0.3 R.L.U.). Moreover, and importantly, in

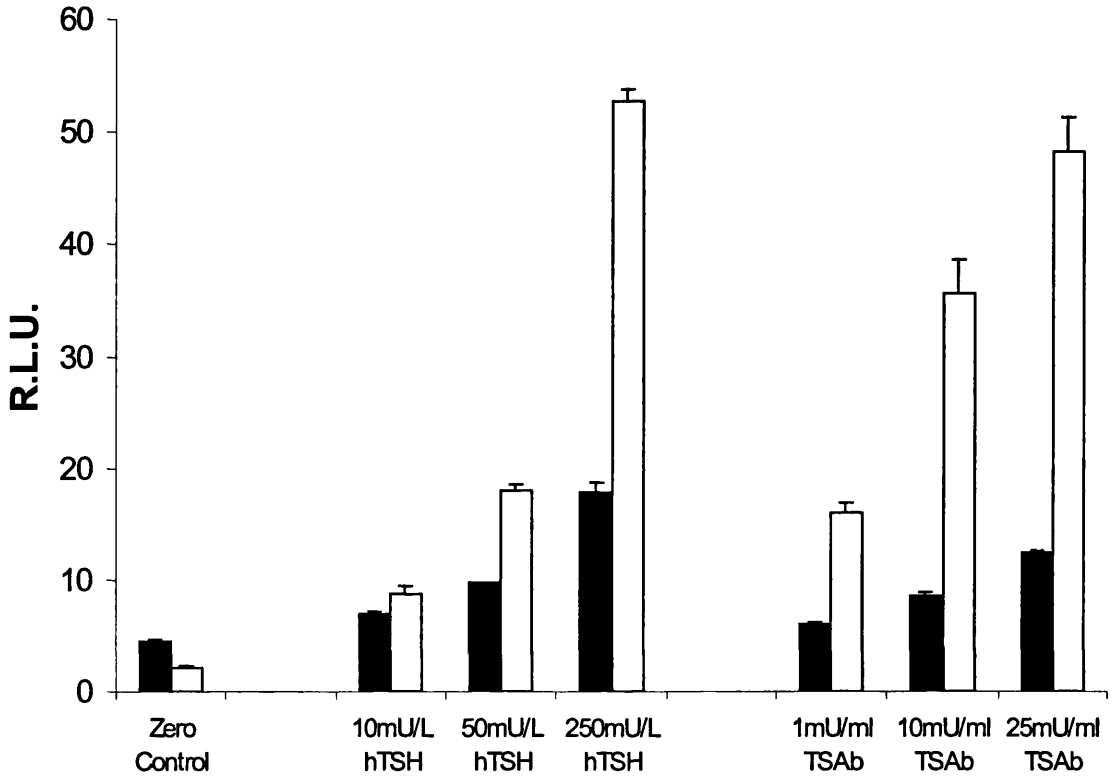


Figure 10.3 **Bioassay C & N.I.H Protocol 2: a preliminary study comparing bioassay responses to TSH and TSAb using Bioassay C or N.I.H Protocol 2.** A bar chart showing the responses, detailed in Table 10.1, to hTSH (80/558) and TSAb (90/672), and the unstimulated controls, obtained using Bioassay C (■) and the N.I.H Protocol 2 (□). Results are the mean ± S.D. R.L.U. for triplicate microcultures.

	Bioassay C			N.I.H. Protocol 2		
Test sample	**R.L.U. ± S.D.**	**Magnitude of response (R.L.U.)**	**Fold increase**	**R.L.U. ± S.D.**	**Magnitude of response (R.L.U.)**	**Fold increase**
Basal Control	4.5 ± 0.1	—	—	2.1 ± 0.1**	—	—
TSH						
10mU/L	7.0 ± 0.2	2.5	1.6	8.8 ± 0.7*	6.7	4.2
50mU/L	9.8 ± 0.01	5.3	2.2	18.0 ± 0.5	15.9	8.6
250mU/L	17.8 ± 0.8	13.3	4.0	52.8 ± 1.1	50.7	25.1
TSAb						
1mU/ml	6.1 ± 0.2	1.6	1.4	15.9 ± 1.0	13.8	7.6
10mU/ml	8.6 ± 0.3	4.1	1.9	35.5 ± 3.1	33.4	16.9
25mU/ml	12.4 ± 0.3	7.9	2.8	48.3 ± 2.9	46.2	23.0

Table 10.1 **Bioassay C & N.I.H Protocol 2: a preliminary study comparing bioassay responses to TSH and TSAb using Bioassay C or the new N.I.H Protocol 2.** This table summarised the results obtained in response to hTSH (80/558) and TSAb (90/672) using Bioassay C and the bioassay performed using the N.I.H. Protocol 2. The bioassays were performed according to the standard protocols described in Section 10.2.2.1. The stimulated responses are compared to the basal unstimulated controls responses observed in both assays. Results are the mean ± S.D. R.L.U. for triplicate microcultures. These R.L.U. are shown graphically in Figure 10.3. *, $p<0.05$ and **, $p<0.001$ for N.I.H. Protocol 2 *v* Bioassay C

response to the lowest dose of TSAb tested (1mU/ml), a ~2.5-fold increase in light output was observed with N.I.H. Protocol 2 (15.9 ± 1.0 *v* 6.1 ± 0.2 R.L.U.). As a result of a lower unstimulated basal light emission accompanied by greater light outputs with N.I.H. Protocol 2, larger fold-responses were inevitably obtained compared to Bioassay C (Table 10.1). Notably, the increase in fold-response was much larger at higher doses of the thyroid stimulators (TSH 250mU/L, 25.1- *v* 4.0-fold; TSAb 25mU/ml, 23.0- *v* 2.8-fold). These results suggested that N.I.H. Protocol 2 did lead to greatly improved magnitudes of response in response to thyroid stimulators and, more significantly, that the assay appeared to have an increased sensitivity to low doses of the stimulators, and in particular to TSAb.

These results heightened our confidence with regard to the detection of TSAb in patient sera. In the same study, 10 sera from hyperthyroid patients and 3 euthyroid control samples (UKN, USN & MM1) were also tested in the two bioassays and their responses compared (Figure 10.4a & Table 10.2). The euthyroid samples yielded similar light outputs with each bioassay. We had anticipated the lower zero control responses, when using the N.I.H. Protocol 2, due to its "starvation" step, as was observed. In contrast, slightly higher light outputs were observed after exposure to the 3 euthyroid samples (UKN, USN and MM1) with the N.I.H. Protocol 2 (8.0 ± 1.0, 9.3 ± 1.5 & 8.4 ± 1.0 R.L.U.) compared to Bioassay C (7.1 ± 0.2, 5.4 ± 0.1 & 6.0 ± 0.2 R.L.U.). When tested in Bioassay C, no significant increase was observed in the light outputs, compared to UKN, in response to patient sera with the exception of the exceptionally potent serum LM. This gave an increase of 6.1 R.L.U. above the euthyroid control (UKN). In contrast, using N.I.H. Protocol 2, there were significant increases in light outputs to all the patient sera, when comparing responses to UKN

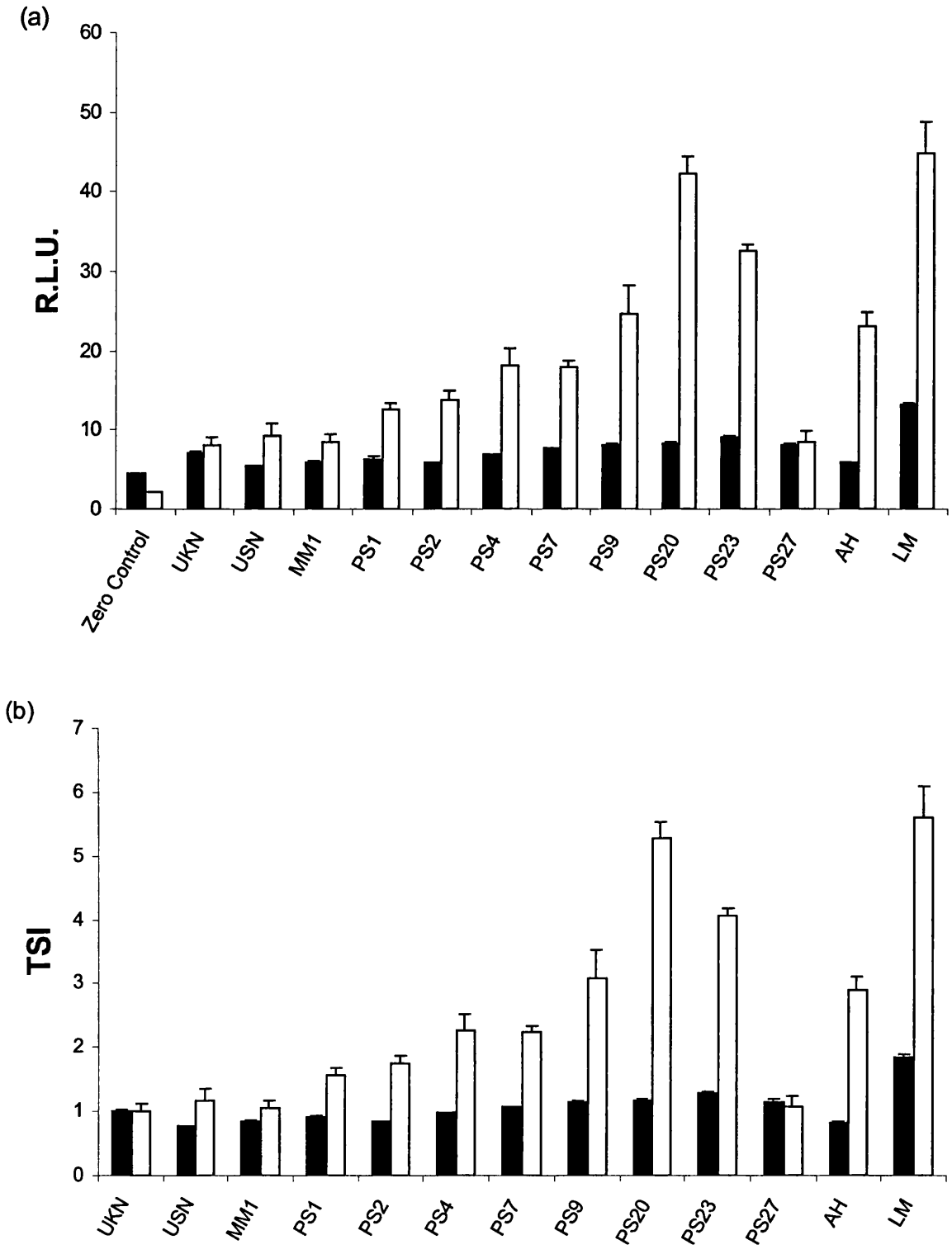


Figure 10.4 **Bioassay C & N.I.H Protocol 2: a preliminary study comparing bioassay responses to 10 selected sera from hyperthyroid patients.** (a) shows the light outputs, detailed in Table 10.2, following exposure to 3 euthyroid controls (UKN, USN & MM1) and 10 patient sera, using Bioassay C (■) and N.I.H Protocol 2 (□). Results are the mean ± S.D. R.L.U. for triplicate cultures. The zero controls have no added human serum. (b) The results in (a) have been transformed to compare TSI values (see Section 10.2.3) using either Bioassay C (■) or N.I.H. Protocol 2 (□).

Test sample	Bioassay C R.L.U. ± S.D.	Bioassay C Magnitude of response (R.L.U.)	Bioassay C TSI	N.I.H. Protocol 2 R.L.U. ± S.D.	N.I.H. Protocol 2 Magnitude of response (R.L.U.)	N.I.H. Protocol 2 TSI
Basal Control (no serum)	4.5 ± 0.1	—	—	2.1 ± 0.1**	—	—
Euthyroid controls						
UKN	7.1 ± 0.2	—	1.0	8.0 ± 1.0 ****	—	1.0
USN	5.4 ± 0.1	-1.7	0.76	9.3 ± 1.5 ***	1.3	1.2
MM1	6.0 ± 0.2	-1.1	0.84	8.4 ± 1.0 ***	0.4	1.1
Patient serum						
PS1	6.4 ± 0.2	-0.7	0.9	12.6 ± 0.8*	4.6	1.6
PS2	6.0 ± 0.03	-0.7	0.84	13.9 ± 1.0*	5.91	1.7
PS4	6.9 ± 0.08	-0.2	0.97	18.1 ± 2.2*	10.1	2.3
PS7	7.7 ± 0.1	0.6	1.1	17.9 ± 0.8*	9.9	2.2
PS9	8.1 ± 0.1	1.0	1.1	24.8 ± 3.5*	16.8	3.1
PS20	8.3 ± 0.3	1.2	1.2	42.1 ± 2.2*	34.1	5.3
PS23	9.1 ± 0.2	2.0	1.3	32.5 ± 1.0*	24.5	4.1
PS27	8.2 ± 0.1	1.1	1.1	8.6 ± 1.3 ****	0.6	1.1
AH	5.9 ± 0.1	-1.2	0.82	23.1 ± 1.7*	15.1	2.9
LM	13.2 ± 0.2	6.1	1.9	44.7 ± 4.0*	36.7	5.6

Table 10.2 **Bioassay C & N.I.H Protocol 2: a preliminary study comparing bioassay responses to euthyroid sera and sera from 10 hyperthyroid patients, when tested with either Bioassay C or N.I.H Protocol 2.** The table summarises the results obtained in response to euthyroid controls (UKN, USN & MM1) and 10 patient sera. The bioassays were performed according to the standard protocols described in Section 10.2.2.1. The light output from each patient sera were compared to those from the UKN control observed in each respective assay. Results are expressed as the mean ± S.D. R.L.U. for triplicate microcultures, magnitude of response above the UKN and also TSI. These are shown graphically in Figure 10.4. *, $p<0.001$; **, $p<0.01$; ***, $p<0.05$; ****, $p>0.10$ for N.I.H. Protocol 2 *v* Bioassay C

except for PS27. It is significant that all the sera were from GD patients, except for PS27, which was from a patient suffering from transient thyrotoxicosis due to hyperemesis gravidarum. This serum would not therefore have been expected to have circulating TSAb. With the sera from patients with GD, impressive magnitudes of responses, ranging from 4.6 – 36.7 were obtained.

TSI values (see Section 10.2.3) were calculated for the patient samples using the euthyroid control sample UKN as the reference. Except for PS27, all the sera from GD patients had a TSI >1.5 using N.I.H Protocol 2 (Table 10.2). For LM, which previous studies had shown to be exceptionally potent, a TSI of 5.6 was obtained with N.I.H. Protocol 2 whilst with Bioassay C, it was only 1.9. These results clearly demonstrated that N.I.H Procotol 2 was superior to the optimized Bioassay C, for the detection of TSAb in human sera.

10.3.4 Is the pre-assay "starvation" step beneficial when human serum is introduced?

The original N.I.H. Protocol 2, described in Section 10.2.2.1.3, was significantly different from the finalised protocol for Bioassay D. One major difference was the incorporation of a "starvation" step, prior to Phase 2 (see Section 9.2.2), for N.I.H. Protocol 2. However, as discussed in Section 9.3.4, our aim was to minimize potential errors within the assay system and it seemed obvious that the additional manipulation of the microcultures required for the "starvation" step could be a major source of error. The lack of benefit of the "starvation" step had been demonstrated in Section 9 (see Section 9.3.4; Figures 9.30, 9.31 & Table 9.3) when we concluded that it was

advantageous to eliminate the "starvation" step from the protocol for the TSH bioassay. Although, "starvation" did result in lower unstimulated baseline light outputs, thus leading to a slight increase in sensitivity, it also contributed to the errors observed in the assays (Table 9.3). Without the "starvation" step, significantly improved ϕCVs were observed (2.5 ± 1.0% *v* 6.8 ± 3.7%, $p<0.001$ from Table 9.3) and there were only relatively minor effects on other performance criteria of the bioassay in the absence of "starvation". We concluded that "starvation" did not benefit the performance of Bioassay D and thus it was removed from our final protocol. However, we needed to check the effects of "starvation" after human serum had been introduced into the system.

We investigated the performance characteristics of N.I.H. Protocol 2 in the presence of human sera with and without the "starvation" step. Light outputs were compared for microcultures exposed to sera from 12 thyrotoxic patients, 3 euthyroid controls (Section 10.3.3), a single dose of hTSH (20mU/L) and control medium (AM3) with no added human serum (Table 10.3). A lower unstimulated baseline (4.6 ± 0.1 R.L.U.) was observed with "starvation" compared to the microcultures which had not been starved (6.2 ± 0.1 R.L.U.). Similar to the results observed previously (Table 9.3), in response to TSH (20mU/L), a 5.4 and 3.4-fold increase above the control responses were obtained with and without "starvation" respectively. This confirmed that N.I.H Protocol 2 has increased responses to TSH, since with Bioassay C an average 1.5-fold increase in response to 20mU/L TSH had been observed.

It was notable that in the presence of the euthyroid control sera, light outputs were consistently greater after "starvation" (Table 10.3). Light emissions of 17.4 ± 2.0, 14.4

N.I.H. Protocol 2

	+ Starvation			- Starvation		
Test Sample	**R.L.U. ± S.D.**	**Magnitude of response (R.L.U.)**	**Fold increase**	**R.L.U. ± S.D.**	**Magnitude of response (R.L.U.)**	**Fold increase**
Basal control†	4.6 ± 0.1	—	—	6.2 ± 0.1*	—	—
hTSH (20mU/L)	25.0 ± 0.9	20.4	5.4	21.2 ± 0.8 **	15.0	3.4

Test serum	**R.L.U. ± S.D.**	**Magnitude of response (R.L.U.)**	**TSI**	**R.L.U. ± S.D.**	**Magnitude of response (R.L.U.)**	**TSI**
UKN	17.5 ± 2.0	—	1.0	11.3 ± 0.7 **	—	1.0
USN	14.4 ± 2.3	-3.1	0.83	9.1 ± 0.4 ***	-2.2	0.81
MM1	10.9 ± 1.5	-6.6	0.63	8.1 ± 1.1 *****	-9.4	0.72
PS22	11.8 ± 1.2	-5.7	0.68	7.4 ± 0.9 **	-3.9	0.66
PS27	14.2 ± 0.9	-3.3	0.81	8.4 ± 0.4 *	-2.9	0.74
PS1	22.7 ± 3.3	5.2	1.3	17.0 ± 2.7 ****	5.7	1.5
PS2	19.5 ± 3.0	2.0	1.1	15.8 ± 2.4 *****	4.5	1.4
PS4	31.0 ± 0.7	13.5	1.8	23.6 ± 2.0 **	12.3	2.1
PS7	32.9 ± 7.9	15.4	1.9	24.6 ± 1.6 *****	13.3	2.2
PS9	38.4 ± 3.1	20.9	2.2	28.7 ± 0.7 **	17.4	2.5
AH	50.1 ± 0.8	32.6	2.9	45.1 ± 0.8 **	33.8	4.0
PS20	77.2 ± 1.5	59.7	4.4	71.2 ± 1.6 **	59.9	6.3
PS23	58.5 ± 7.9	41.0	3.4	54.5 ± 5.2 *****	43.2	4.8
PS33	16.4 ± 1.1	-1.1	0.9	11.5 ± 1.4 **	0.2	1.0
PS34	17.7 ± 0.2	0.2	1.0	11.2 ± 1.2 *	-0.1	0.98

Table 10.3 **N.I.H. Protocol 2: the influence of the "starvation" step on selected performance characteristics of responses to TSH and human sera.** The table summarises the results obtained in response to hTSH 80/558 (20mU/L), euthyroid controls (UKN, USN & MM1) and 12 patient sera using N.I.H. Protocol 2 i.e. with "starvation" step, and the bioassay performed without this step. Results are the mean ± S.D. R.L.U. for triplicate microcultures. † No added human serum; *, $p<0.001$; **, $p<0.01$; ***, $p<0.05$; ****, $p<0.1$; *****, $p>0.1$, for – starvation *v* + starvation

± 2.3 and 10.9 ± 1.5 R.L.U. compared to 11.3 ± 0.7, 9.1 ± 0.4 and 8.2 ± 1.1 R.L.U. were observed in response to the euthyroid samples UKN, USN and MM1 with and without "starvation" respectively. Thus, in contrast to "starvation" lowering control light outputs in the absence of human serum, the addition of human serum (10%) reversed the situation. Light outputs were also consistently higher in response to the 12 patient sera after "starvation". This suggested that "starvation" enhanced the sensitivity of the monolayers to non-specific serum effects. However, although lower light outputs were observed in the absence of "starvation", the magnitudes of response, above the relatively low euthyroid controls, were generally retained (Table 10.3).

Using the sample UKN as the reference, the TSI of each patient sample with and without "starvation" were calculated (Table 10.3). This showed that, with sera selected to provide a relevant range of TSI, similar values were observed in both assays. Potent sera resulted in greater TSI when "starvation" was not used (e.g. PS20 TSI of 6.3 *v* 4.4). Furthermore, a slightly improved ϕCV was observed when a "starvation" step was not included (7.1 ± 5.1% *v* 8.8 ± 6.4%, $p>0.10$). Although this was not a significant difference in this particular experiment, we considered that removal of the error-prone "starvation" step from the protocol would generally improve bioassay ϕCVs and therefore overall precision. Thus, it appeared that in the presence of human serum the "starvation" step did not benefit the assay performance. Most importantly, this experiment clearly demonstrated that the presence of human serum (10%) reversed the one potentially important positive influence of "starvation". Although, "starvation" resulted in a lowering of the basal controls ran without serum, it also appeared to cause an increased sensitivity to non-specific serum effects in the

presence of human sera. As a consequence, the one beneficial effect of the "starvation", namely a reduction in the light outputs of the negative control, was not retained in the presence of human serum. Consequently, we again concluded that the removal of the "starvation" step was beneficial. We renamed the bioassay protocol, which eliminated the "starvation" step, Bioassay D.

10.3.5 Determination of the optimal conditions for signal development using Promega System II in the presence of human serum

During the development of Bioassay D, we had worked to reduce the within-assay response errors (Section 9.3.4). Scrutiny of the results revealed that when using Promega System II, systematic errors were associated with the development of the luminescence signal. We had observed positional errors (Figure 9.32) and signal drift across the 96-well microtiter plate (Figures 9.33 & 9.34) when luminescence was determined only 10min after the addition of luciferase reagent (20µl/well). As a result, we then investigated several variables in our attempts to eliminate these problems with Bioassay D. Detailed investigation of the time courses of the developing light signals revealed dynamic changes in the signal with time (Figure 9.34b). The results showed signal instability immediately after the addition of the luciferase reagent, so that light output increased with time, plateauing after ~1hr and subsequently declining. Furthermore, we also examined the influence of the amount of added luciferase reagent on signal stability (Figure 9.35a & b); although there were dose-related modifications, the underlying signal instability persisted at all concentrations tested.

It was important to reinvestigate signal instability in the presence of human sera (10%). Multiple microcultures were set up and exposed to sera from 6 hyperthyroid patient (PS 38, 48, 58, 59, 63 & 81) during Phase 2 of Bioassay D. The TSAb potencies of these sera had previously been determined to range from mild to highly positive. For Phase 3, one of four selected volumes of luciferase reagent was added to the microcultures (20, 40, 50 & 100µl/well), and multiple successive timed readings were then made (Figures 10.5a-f). A similar pattern in the changes in the signals was observed for all of the sera. An early rise in the light output was followed by a plateau and finally a gradual decline in the signal. However, the degree of instability in the signals varied between individual sera. The early rise in signal was particularly prominent when only 20 or 40µl of the luciferase reagent had been added. Furthermore, light outputs attained with 20µl of reagent were the lowest whereas the highest light outputs were obtained when 50 or 100µl of reagent was used, although 100µl did not always yield the highest output. With the larger amounts of reagent (50 or 100µl), the plateau was repeatedly reached between Readings 2 and 3 i.e. 26 - 42min after the addition of the reagent indicating that this time-window would yield the most stable readings.

Comparison between Figure 9.35a/b and Figures 10.5a-f, revealed that the instability in signal development was much greater when 10% human serum has been added to Bioassay D. Moreover the magnitudes of the changes in light output against time, were larger for some sera than others. This serum-specific effect was in agreement with some previous observations in preliminary studies with Bioassay D. From these we had found that (a) positional variations were observed with a given serum, when it

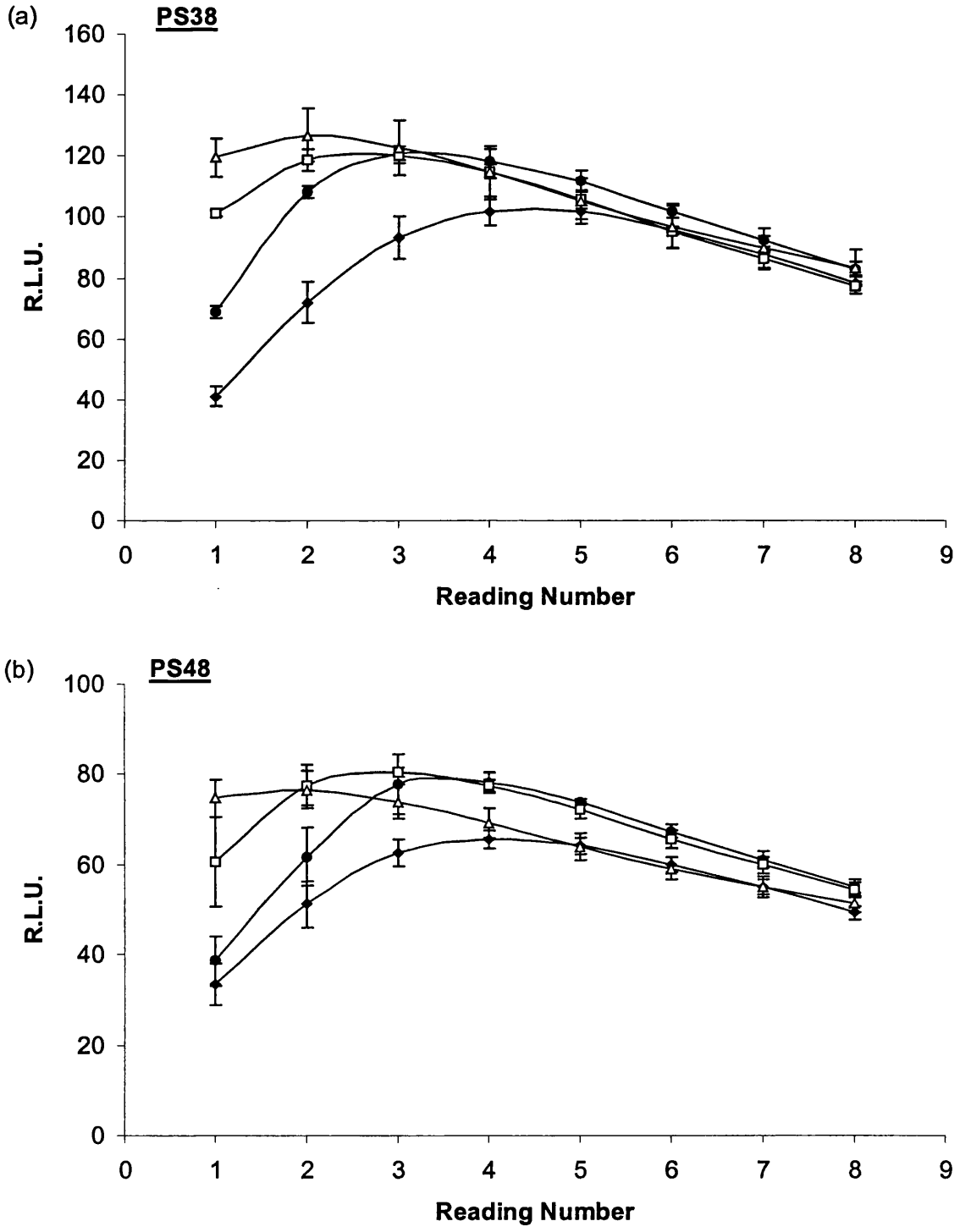


Figure 10.5a & b **Bioassay D: the effect of varying the amount of luciferase reagent on the time course of the development of the luminescence signal.** The time course of signal development was determined for sets of triplicate microcultures in the presence of either serum sample PS38 (a) or PS48 (b). Luminescence was determined 10min after the addition of 20 (◆), 40 (●), 50 (□) and 100µl (△) of luciferase reagent/well in a sequence of timed readings as indicated on the x-axis. Each well was read for 10sec and the total reading time required for the whole plate was therefore ~16min. Thus, Reading 1 was 10min after the addition of the reagent and Reading 2, 26min etc. Results are the mean ± S.D. of the light outputs for each set of triplicate microcultures.

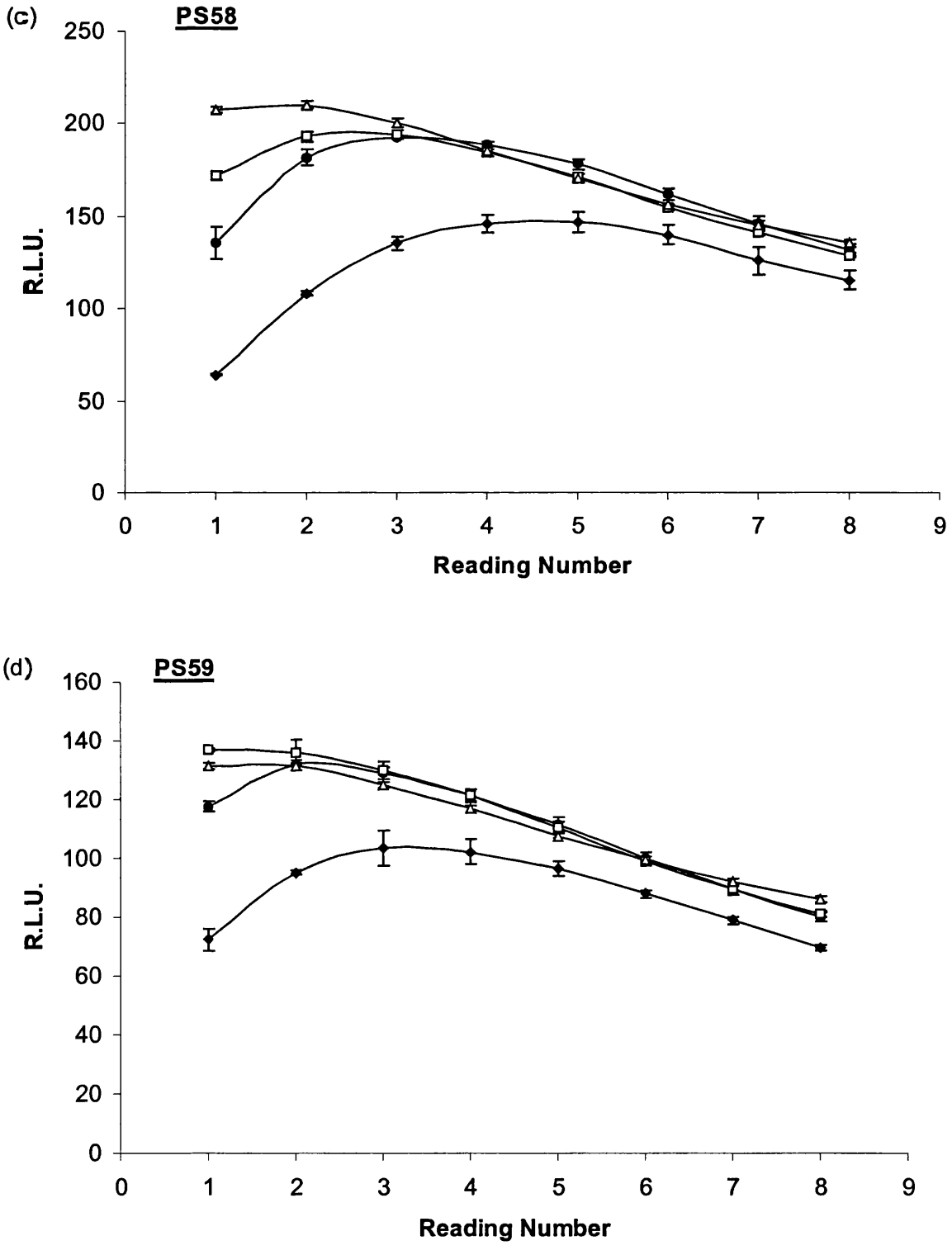
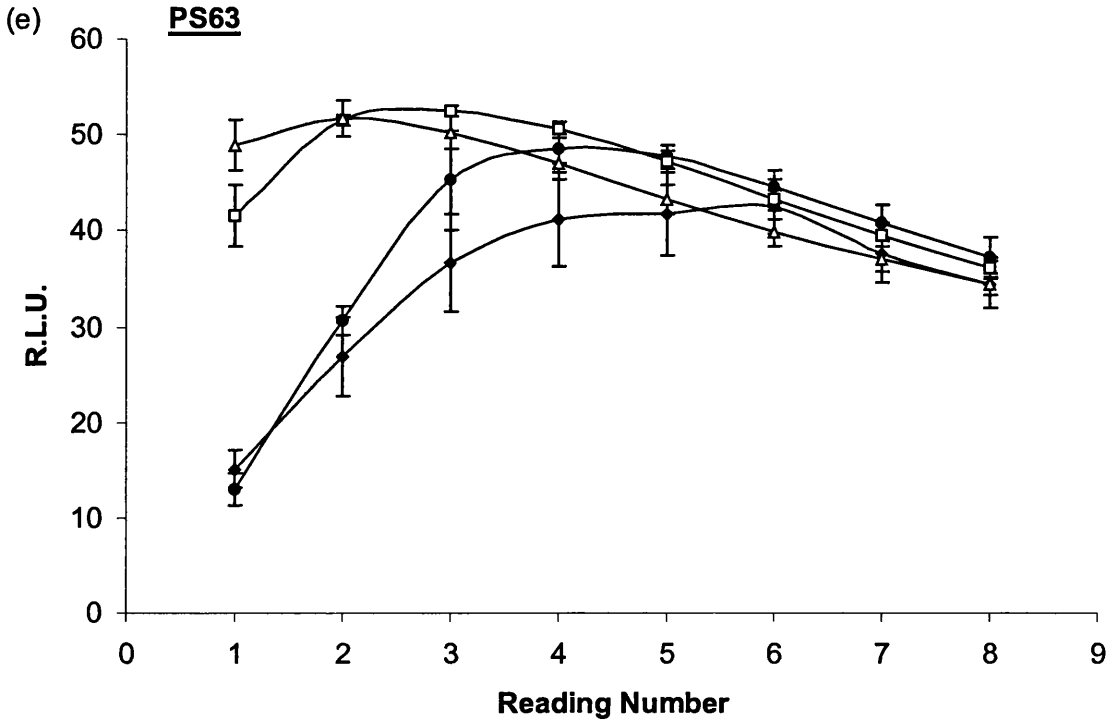


Figure 10.5c & d **Bioassay D: the effect of varying the amount of luciferase reagent on the time course of the development of the luminescent signal.** The time course of signal development was determined for sets of triplicate microcultures in the presence of either serum sample PS58 (c) or PS59 (d). For details see legend to Figure 10.6a & b, since this is part of the same experiment.



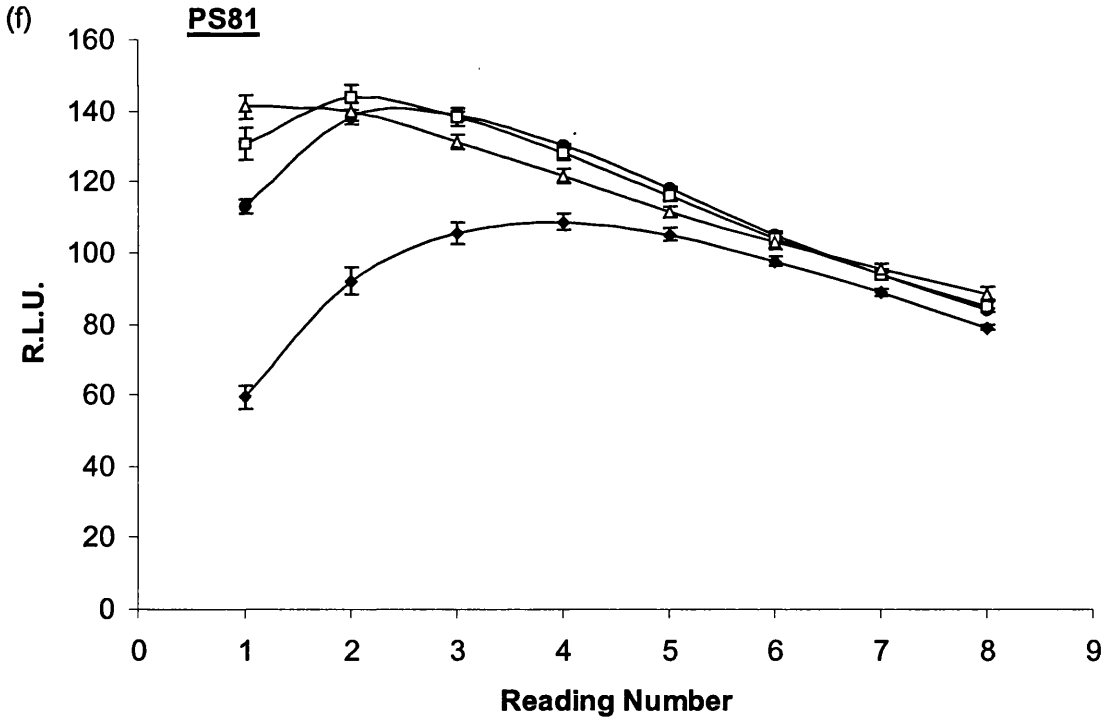


Figure 10.5e & f **Bioassay D: the effect of varying the amount of luciferase reagent on the time course of the development of the luminescent signal.** The time course of signal development was determined for sets of triplicate microcultures in the presence of either serum sample PS63 (e) or PS81 (f). For details see legend to Figure 10.6a & b, since this is part of the same experiment.

was tested at several positions on the plate and (b) this problem was more serious for some sera than others.

In addition to the influence of the volume of reagent on signal stability, we had also examined the influence of reducing the reading time/well (Figure 9.36), with regard to stability of Bioassay D. We concluded that it was advantageous to reduce the reading time to 1sec/well. The final optimized protocol (Table 9.1), with a delay after adding the reagent and reading for only 1sec/well, thus best utilized the short period of a stable signal. This eliminated both positional errors and signal "drift" (see Section 9.3.4).

10.3.6 Investigation for parallelism between thyroid stimulators

We had previously attempted to investigate for parallelism between TSH and TSAb using Bioassay C, but with inconclusive results (see Section 9.3.3). As anticipated, dose-response curves for two contrasting vials of hTSH (coded 80/558 and recoded 84/703) were parallel (Figure 9.27). However, the attempt to establish parallelism between TSH and the dialysed TSAb preparation (coded 90/672) proved inconclusive (Figure 9.29). In Bioassay D, we have established a superior bioassay compared to Bioassay C (see Section 9.3.4, Table 9.4). Bioassay D has enhanced responsiveness to thyroid stimulators, particularly TSAb. Thus, we decided to use this improved assay system, with the protocol described in Section 10.2.2.1, to re-investigate for parallelism between, not only reference preparations of TSH and TSAb, but also between sera from different patients with GD.

An investigation of parallelism between TSAb (90/672) and hTSH (80558)

For this parallelism study, the freeze-dried preparations of TSAb (90/672) and hTSH (80/558) had been reconstituted in culture medium. The reconstituted TSAb preparation was then dialysed against non-phosphate containing Ham's F12 nutrient medium to remove the phosphate ions within the preparation, since phosphate ions had been reported to inhibit luciferase activity (Promega technical manual #TM051, 1998). We had demonstrated with Bioassay C that the removal of the phosphate ions by dialysis had eliminated this inhibition (Figure 9.28). Following dialysis, the dialysed TSAb preparation as well as the reconstituted TSH preparation were serially diluted in AM3 which was the assay medium used in Phase 2 (see Section 9.2.2) of Bioassay D.

The dose-response curves to hTSH and dialysed TSAb were compared (Figure 10.6). The greatest light output of 59.3 ± 1.1 R.L.U. was obtained at the highest concentration of 500mU/L for hTSH. This represented a 10.7-fold increase above the unstimulated control (5.5 ± 0.1 R.L.U.). There was no evidence of approaching a plateau at this dose. For the dialysed TSAb (90/672), a highest response of 54.6 ± 1.4 R.L.U. was obtained in response to the maximal dose of 25mU/ml. At this high concentration (25mU/ml), there was a 9.1-fold increase above the basal control (6.0 ± 0.3 R.L.U.). Theoretical detection limits (see Section 9.2.3) of 1.0mU/L and <0.1mU/ml were estimated for hTSH and TSAb respectively from these curves. When these results are compared against those for two vials of TSH, where parallelism would be expected (Figure 9.27), it was clear that parallelism was not observed between TSAb and TSH (Figure 10.6). However, we acknowledge that with the conditions for Phase 3 used for this particular experiment, the signal readings were

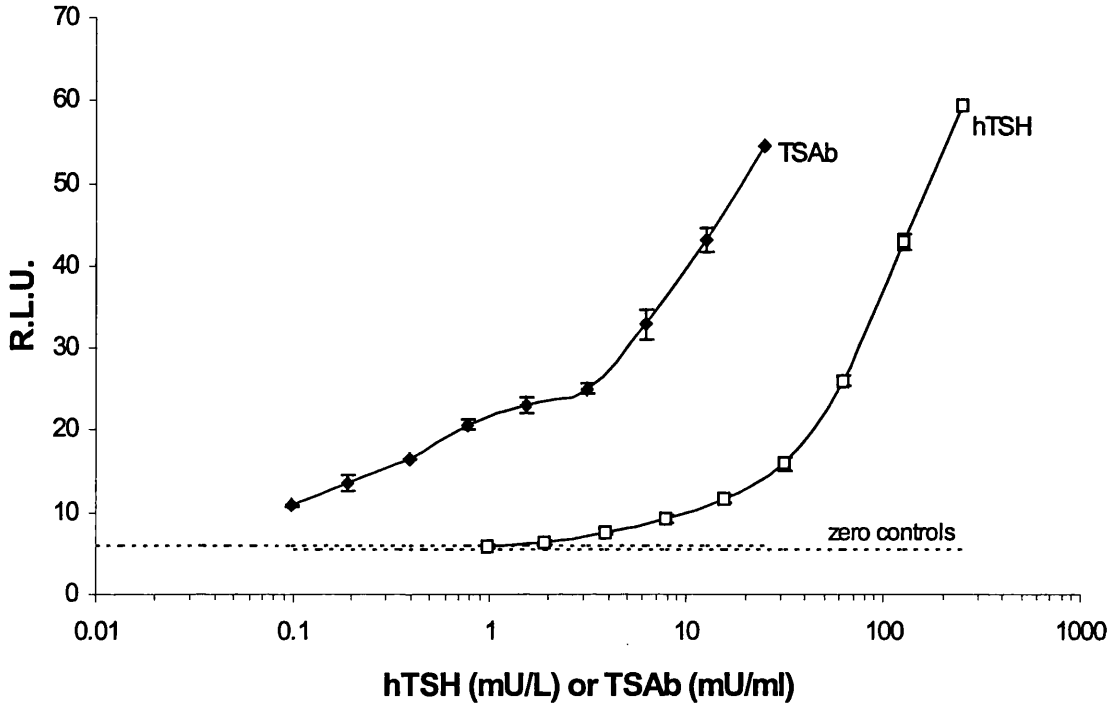


Figure 10.6 **Bioassay D: an investigation for parallelism between hTSH and TSAb.** Serial dilutions for the dialysed reconstituted stock of TSAb (90/672; ◆) and the reconstituted hTSH (80/558; □) were prepared in AM3. Briefly, dialysis of TSAb was achieved as follows: four vials of the freeze-dried preparation were reconstituted in culture medium (4ml). This was then dialysed against 1L of Ham's F12 nutrient medium. A low molecular weight cut-off dialysis membrane was used (6-8,000MWt). Dialysis was carried out over a 48hr period at 4°C. Monolayers were then exposed to these appropriate dilutions of TSH or TSAb for 4hr and light outputs were determined 10min after the addition of luciferase reagent from Promega System II (20µl/well). Reading times were 10sec/well. Results are the mean ± S.D. R.L.U. for triplicate cultures. --- represents the light output from the basal unstimulated control.

vulnerable to slight distortion due to instability in the signal development, as discussed in Section 10.3.5.

An investigation for parallelism between hTSH (80/558) and a selected patient serum

Since the patient serum introduced 10% human serum into the system, to establish matrix equivalence between TSH and the patient serum, when added to Bioassay D, the TSH was diluted in an assay medium containing 10% human serum. This diluent was termed AM4. It was AM3 supplemented with 10% human pooled euthyroid sera (see Section 10.2.2.2) and was used as the assay medium for Phase 2 i.e. the exposure of monolayers to stimulators.

A vial of the International Reference Preparation hTSH (80/558) was reconstituted in AM4 and serially diluted in the same medium gravimetrically. The human serum sample selected for this study, PS20, was from a patient (TL) who was suffering from GD with severe ophthalmopathy. The serum was also serially diluted in AM4 gravimetrically to generate a series of test samples with a range of patient serum concentrations (0 – 10%). Since AM4 was the diluent, the concentration of human serum in all wells was consistently maintained at 10%.

The dose-response curves in response to TSH and the human serum (PS20) are shown in Figure 10.7. A greatest light output of 56.7 ± 1.7 R.L.U. was observed in response to the highest concentration of TSH (500mU/L). This represented a 4.9-fold increase above the basal control (11.5 ± 0.2 R.L.U.). There was evidence suggesting the beginning of a plateau. A theoretical detection limit of <2mU/L was estimated from

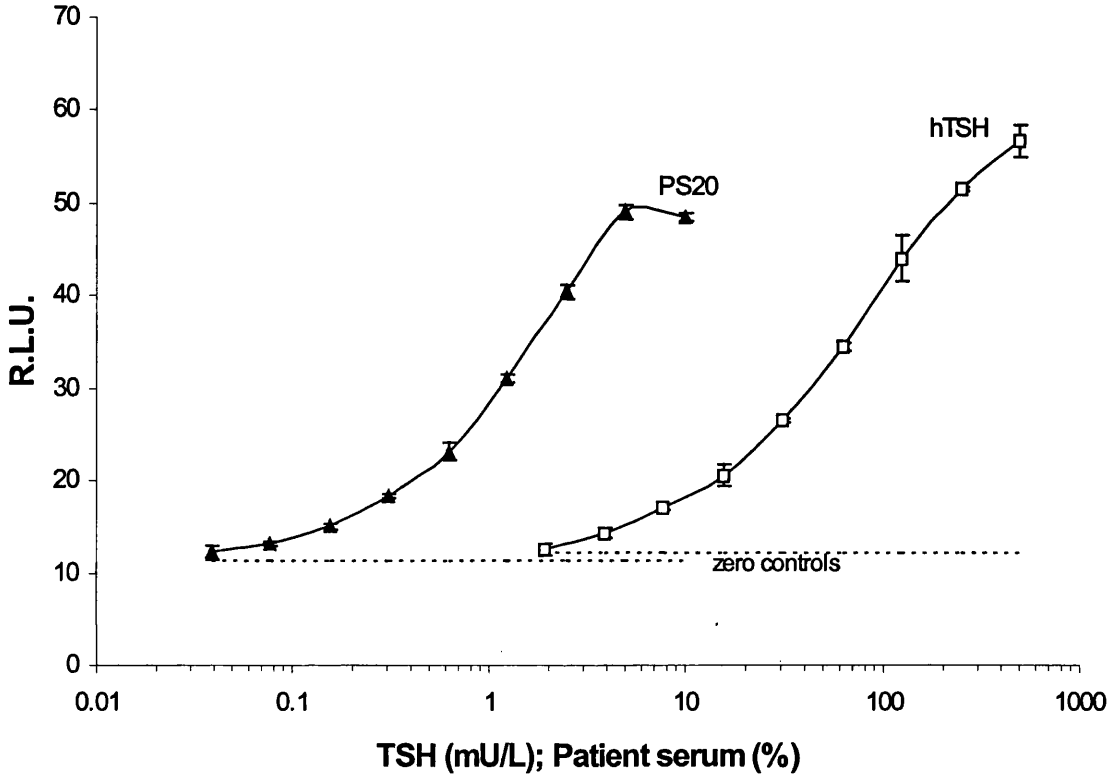


Figure 10.7 **Bioassay D: an investigation for parallelism between hTSH and a selected patient serum.** Serial dilutions of hTSH (80/558; □) and patient serum PS20 (▲) were prepared gravimetrically in AM4 (see Section 10.2.2.2). Monolayers were then exposed to these stimulators for 4hr and light outputs determined 1hr after the addition of luciferase reagent from Promega System II (40µl/well). Reading times were 1sec/well. Results are the mean ± S.D. R.L.U. for triplicate cultures. --- represents the light outputs from the unstimulated control.

the dose-response curve. With the patient serum (PS20), increasing light emissions were also observed with increasing serum concentrations but a plateau was reached at 5% patient serum when light output was only 49.1 ± 0.8 R.L.U.. This was well below the response for the highest dose of TSH (56.7 ± 1.7 R.L.U.). Since throughout the entire experiment, the human serum concentration was maintained at 10%, the presence of human serum in the patient sample could not account for the clear lack of parallelism observed between TSH and the TSAb in the patient serum. Moreover, in this experiment the conditions used for signal development during Phase 3 were not liable to "drift" or positional variation. We therefore concluded that the TSH and patient TSAb had not yielded parallel dose-response curves.

Extended investigations for parallelism between further selected patient sera using Bioassay D

In Figures 10.8 and 10.9, we show additional studies investigating for parallelism between different selected Graves' patient sera. All samples had been gravimetrically diluted in AM4 and the bioassay conditions were identical to those used for Figure 10.8. Parallelism was not observed between any of the sera.

We note that PS20 and PS81 were from the same patient. The patient was initially suffering from severe thyroid associated ophthalmopathy. After sample PS20 had been taken, the patient was successfully treated with immunosuppressants, and PS81 was taken 6 months later, when the eyes had greatly improved. Clearly, there had been a decrease in the potency of PS81, compared to PS20. Presumably this was a consequence of a reduction in TSAb due to the immunosuppression. However, it is of

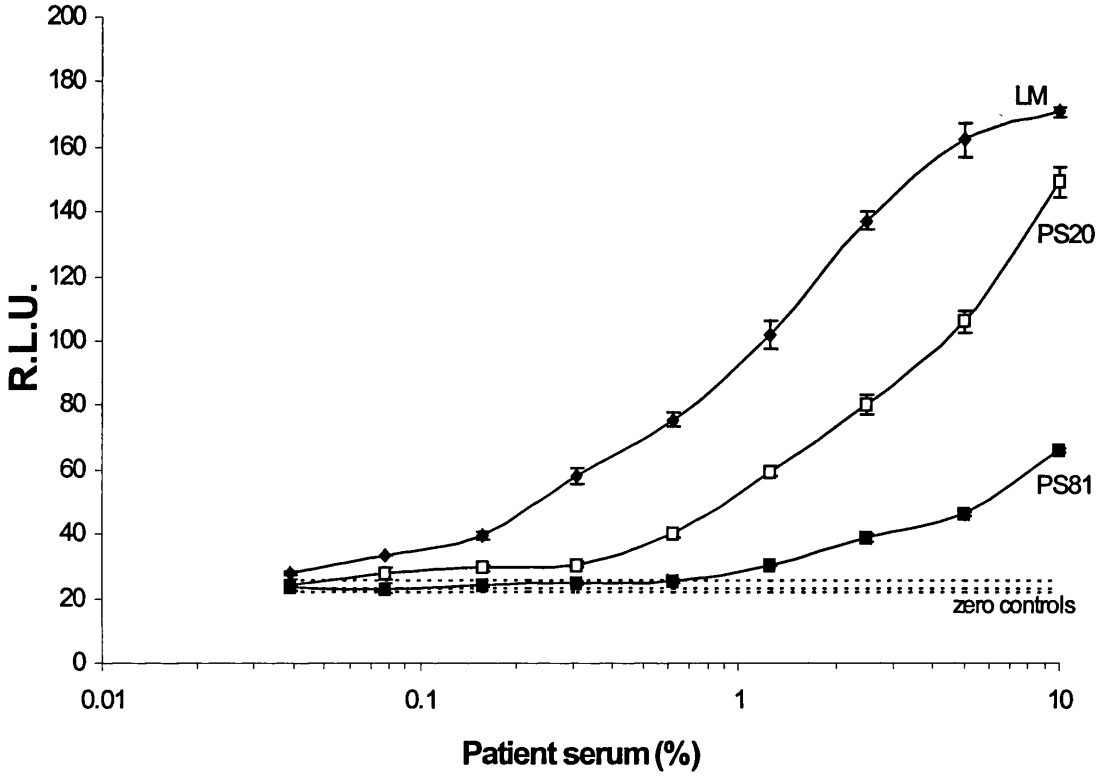


Figure 10.8 **Bioassay D: an investigation of parallelism between 3 selected patient sera.** In this study, the three Graves' patient sera used were samples PS20 (□) & 81 (■) which were from a single patient (TL) and the other from LM (◆). PS20 had been collected 6 months before PS81. In the intervening time, the patient had been successfully treated for Graves' ophthalmopathy by immunosuppression. The sera were serially diluted in AM4 (see Section 10.2.2.2) gravimetrically. Monolayers were then exposed to these stimulators for 4hr and light outputs were determined 1hr after the addition of luciferase reagent from Promega System II (40µl/well). Reading times were 1sec/well. Results are the mean ± S.D. R.L.U. for triplicate cultures. --- represents the light outputs from the control microcultures with only 10% euthyroid sera i.e. AM4.

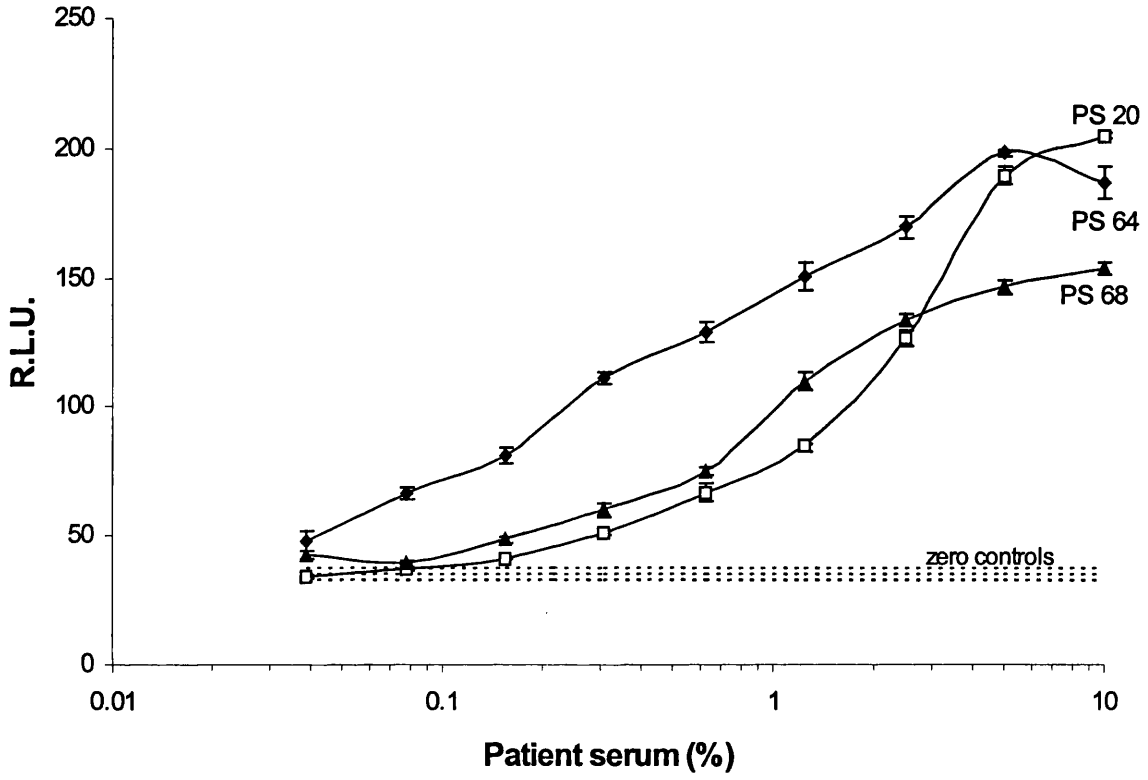


Figure 10.9 **Bioassay D: a further investigation of parallelism between 3 selected patient sera.** In this study, the three Graves' patient sera used were samples PS20 (□), 64 (◆) and 68 (▲). The sera were serially diluted in AM4 (see Section 10.2.2.2) gravimetrically. Monolayers were then exposed to these stimulators for 4hr and light outputs were determined 1hr after the addition of luciferase reagent from Promega System II (40µl/well). Reading times were 1sec/well. Results are the mean ± S.D. R.L.U. for triplicate cultures. --- represents the light outputs from the control microcultures with only 10% euthyroid serum i.e. AM4.

interest that even these two samples, taken from the same patient, did not appear to yield parallel dose-response curves.

Thus using both Bioassay C (see Section 9.3.3) and D, which were precise and highly reproducible assay systems, we concluded that parallel dose-response relationships could not be demonstrated between (i) TSH and the International Reference Standard for TSAb (coded 90/672), (ii) TSH and a Graves' patient sera or (iii) different patient sera.

10.3.7 Potency Index Study

Once Bioassay D, with its impressive within-assay reproducibility, had been established, we were able to critically investigate for parallelism between different thyroid stimulators and in particular between TSH and different TSAb. As demonstrated in the preceding Section (10.3.6) no such parallelism was observed. This precluded our expressing the potencies of different TSAb, by interpolation from TSH dose-response curves, as "TSH equivalents" i.e. their potencies being given as equivalent doses of TSH which provoked equal magnitude of responses. However, we did not abandon this approach entirely without further investigations. Acknowledging that non-parallelism between the stimulators existed, nevertheless we tested whether it would be useful to express relative potencies of different TSAbs as a "potency index" (P.I.). For this, one would keep the concentrations of TSAb to be tested constant e.g. 10% human serum in the bioassay well. One then interpolates an equivalent TSH dose which had provoked an equal light output, from the TSH dose-response curve run on the same microtiter plate as the test sera. Although the P.I. would not be an "absolute"

measurement, since due to the non-parallelism it would change if a different concentrations of a given TSAb were to be tested, it was hoped that, if tested in successive bioassays, it would provide a more stable measurement than the index conventionally used, namely TSI. This was because it would correct for variations in the responsiveness of individual cultures to thyroid stimulators. We tested this hypothesis as is described below.

Four independent bioassays were ran in succession using Bioassay D. Two were set up on Day 1, and two on the immediately following day (Day 2). Thus, the only difference between Day 1 and Day 2 assays was that the latter used cells maintained in batch culture for 24hr longer. For Phase 1, the plating densities ($4x10^5$ cells/ml) were the same for all 4 bioassays. A TSH dose-response curve was set up on each microtiter plate, and 9 selected patient sera, together with a euthyroid pool, were also tested on each plate.

The results (Table 10.4) revealed that when the relative potencies of the different patient sera were expressed as P.I.s, there was an exceptionally large between-assay variation, with a mean CV of 27.5 ± 14%. In contrast, when the results were expressed as TSI values, this declined to 16.3 ± 5.8%. Thus it appeared that when expressing the results as P.I.s, variations in responsiveness of the 4 different cultures to TSH, did not correct for variations in responsiveness to TSAbs. In other words, the responsiveness of a given culture to TSH was governed by different factors from TSAb. We concluded that expressing the result as P.I.s was not a useful strategy.

	TSI					P.I. (mU equivalent TSH/L)				
Test Sera	Assay: i	ii	iii	iv	Between assay CV (%)	Assay: i	ii	iii	iv	Between assay CV (%)
PS1^{+}	1.8	1.8	1.4	1.4	14.5	5.6	3.3	2.1	2.5	47
PS25^{+}	2.1	2.3	1.8	1.9	13.7	8.3	7.3	6.4	7.6	11.5
PS9^{++}	4.6	5.1	3.5	3.4	14.8	46	40	29	26	44
PS17^{++}	3.8	4.3	3.3	3.2	11	31	28	26	23	10.7
PS23^{++}	6.8	7.7	4.9	5.8	16.2	142	119	59	87	22.4
PS80^{++}	3.8	4.0	3.2	3.4	9.4	31	24	26	26	11
AH^{++}	3.9	4.7	2.4	2.9	29	32	33	14	18	40
PS20^{+++}	8.0	9.0	6.4	7.1	20	326	351	130	181	26
PS68^{+++}	5.9	6.7	4.7	4.9	18.9	90	75	58	57	35
	Mean Between Assay CV = 16.3 ± 5.8%					Mean Between Assay CV = 27.5 ± 14.0%				

Table 10.4 **Bioassay D: investigation of between-assay variation with 4 successive independent bioassays.** This table summarises the results, in terms of both TSI values and potency indices (P.I.), in response to 9 patient sera tested in four separate bioassays (assays i – iv). Patient sera of varied potency (mild, moderate and highly positive were indicated by $^{+}$, $^{++}$ and $^{+++}$ respectively) were tested. Details of the method used for the study had been described in Section 10.2.2.3 and the standard protocol for Bioassay D was used (see Section 10.2.2.1.2). The results were expressed as both TSI and P.I. values.

The mean **within**-assay variations for the 4 different bioassays were estimated to be 2.1 ± 1.5, 1.4 ± 0.9, 1.3 ± 1.1 and 1.5 ± 1.0% respectively. This impressively small within-assay precision is born-out in the Imprecision Profiles for these bioassays shown in Figure 9.38. Thus we have a situation of poor between-assay variation e.g. 16.3 ± 5.8% when the results are expressed as TSIs, from assays which have impressive within-assay reproducibility. This indicated that changes in the individual cultures used for the 4 assays were introducing instability into the system. In support of this we noted that the results shown in Table 10.4, from the 4 different bioassays could be grouped. Careful inspection of TSI values, shows that there was close agreement between bioassays (i) and (ii) and again between bioassays (iii) and (iv) i.e. the one extra day in bulk culture, had resulted in a shift in TSI values, which then contributed to the overall larger between-assay variations (16.3 ± 5.8%). It appeared therefore that although Bioassay D was precise, it was not "rugged", since a deliberate minor change in culture conditions had resulted in a relatively large shift in TSI values.

This was tested by setting up 4 sequential bioassays and taking care that the culture conditions were kept as constant as was possible. Each was set up on a separate week i.e. the overall time span of the investigation was 4 weeks. We therefore investigated the stability of Bioassay D when ran over a period of one month. It was immediately apparent (Table 10.5) that the between-assay variation improved, having a mean value of only 9.5 ± 3.7%, when the results were expressed as TSI values compared with the previous value of 16.3 ± 5.8%; the significance of the difference was $p<0.05$. This therefore confirmed our impression that Bioassay D was not "rugged", and to obtain

TSI

Test Sample	Assay: 1	2	3	4	Mean ± S.D.	Between assay CV (%)
QC1	1.0	1.3	1.1	1.1	1.13 ± 0.13	11.5
QC2	2.2	1.9	2.0	2.0	2.03 ± 0.13	6.4
QC3	4.0	3.7	4.1	3.6	3.85 ± 0.24	6.2
QC4	2.9	2.8	3.2	3.2	3.03 ± 0.21	6.9
QC5	0.8	0.9	0.7	1.0	0.85 ± 0.13	15.3
QC6	2.9	2.4	2.7	3.1	2.78 ± 0.30	10.8

Mean between assay CV = 9.5 ± 3.7%

Table 10.5 **Bioassay D: reinvestigation of between-assay variation with 4 successive independent bioassays.** This table summarises the results, in terms of TSI values in response to 6 quality control (QC) samples tested in four separate bioassays (assays 1 – 4). QC samples were selected patient sera of varied potency. Details of the method used for the study had been described in Section 10.2.2.3 and the standard protocol for Bioassay D was used (see Section 10.2.2.1.2). The results were expressed as TSI.

between-assay variation which reflected the good within-assay precision, one needed to keep all bioassays condition as constant as possible.

10.3.8 Detection of TSAb in human serum samples

To conclude our work, using Bioassay D adapted for use with 10% human serum, we screened two groups of Graves' patients for the frequency of TSAb stimulation. This represented a preliminary clinical application of the reporter gene bioassay, exploiting its high capacity for sample throughput.

Euthyroid control samples

Sera from 20 euthyroid individuals with no history of thyroid disease were bioassayed to determine a reference range which would hopefully allow us to differentiate between euthyroid subjects and patients with thyroid disease. The TSI values of the 20 sera ranged from 0.6 to 1.8. The mean ± S.D. obtained from these TSI values was 1.0 ± 0.24. Thus the euthyroid reference range was determined to be 0.5 – 1.5, this being 2 S.Ds below and above the mean value. It was noted that a TSI value of 1.8 was obtained with just one of these sera. Statistically this might be expected, since 5% of TSI values would be expected to lie outside this reference range. As an additional negative control group, we also investigated the TSI values of 14 sera from patients with Type 2 Diabetes. The mean ± S.D. of these was 0.9 ± 0.1 and all were <1.5.

We noted that when tested with the two prototypes of this reporter gene bioassay (Watson PF *et. al.*, 1998; Evans C *et. al.*, 1999), the upper limit of the euthyroid reference range was also reported to be 1.5.

Samples from a UK survey of Graves' patients and their relatives

We were fortunate to obtain samples collected as part of a survey of Graves' patients and their relatives, conducted throughout the UK (Imrie H *et. al.*, 2001). A total of 106 euthyroid relatives gave TSI values of 0.91 ± 0.22 (Figure 10.10). 24.5% of the GD patients (n = 155) were positive for TSAb (i.e. had TSI values >1.5) with TSI values ranging from 0.5 to 5.2. In contrast, only 1 out of 26 hypothyroid relatives investigated was positive. The others had a mean TSI of 1.02 ± 0.27, which was close to that of the euthyroid controls.

Samples from patients attending the Thyroid Clinic at UCH

A total of 100 patients were collected over a period of 18 months (Figure 10.11). All 9 patients classified as suffering from autoimmune hypothyroidism were negative (TSI <1.5) in the bioassay. In contrast, 32 of the 76 (42.1%) Graves' patients tested were positive (TSI >1.5). The TSI values of the Graves' patients ranged from 0.7 – 5.6. Only 2 of the 13 patients classified as suffering from multinodular goitre were marginally positive with TSI values of 1.6 and 1.7 respectively. Both patients with solitary toxic nodules were negative.

It was notable that for one patient (TL), who presented with severe eye signs which improved after immunosuppressive treatment, the TSI values declined over a period of 9 months in accordance with the improved clinical condition (Figure 10.12). We have already compared detailed dose-response curves to two of these samples PS20 and PS81 (Figure 10.8).

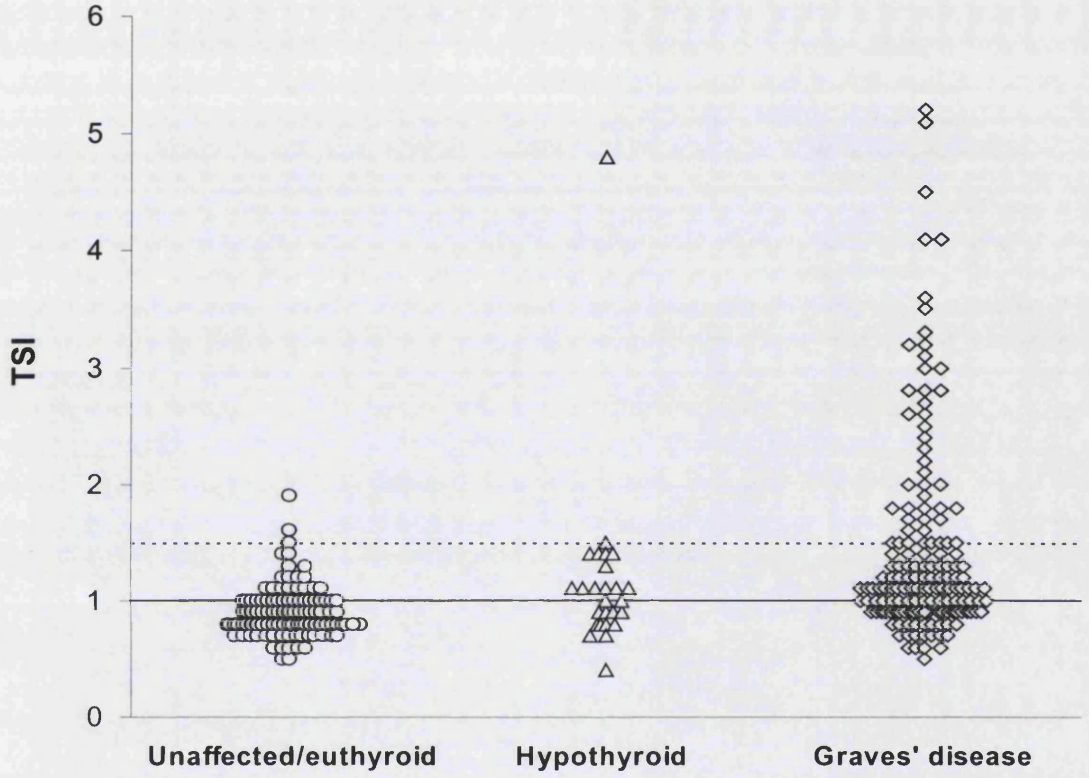


Figure 10.10 **Bioassay D: the thyroid stimulation index (TSI) values of the serum samples obtained from the UK National Survey.** A total of 287 samples were screened using Bioassay D in a double-blind study. The samples had been classified as described in Section 10.2.2.4 and were as follows: (a) 106 unaffected / euthyroid controls (○), (b) 26 hypothyroid patients (△) and (c) 155 patients with Graves' disease (◇). The results are the mean R.L.U. for duplicate cultures, expressed as TSIs (see Section 10.2.3). A cut-off TSI value of 1.5, indicated with ----, was considered to be the upper limit of the euthyroid reference range as determined from our in house euthyroid controls.

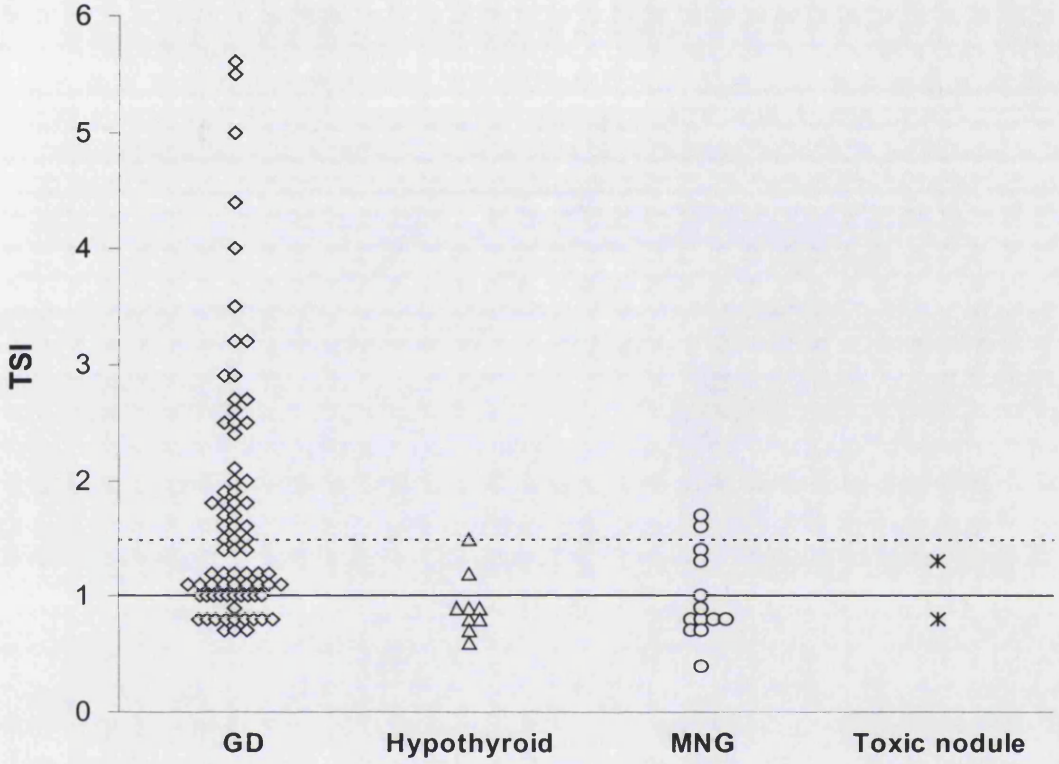


Figure 10.11 **Bioassay D: the thyroid stimulation index (TSI) values of the serum samples collected from patients attending the Thyroid Clinic at UCH.** A total of 100 samples were obtained and screened using Bioassay D. The samples had been classified as described in Section 10.2.2.4 and were as follows: (a) 76 Graves' disease (GD) patients (◇), (b) 9 hypothyroid patients (△), (c) 13 multinodular goitre (MNG) patients (○), and (d) 2 patients with solitary toxic nodule (∗). The results are the mean R.L.U. for duplicate cultures, expressed as TSIs (see Section 10.2.3). A cut-off TSI value of 1.5, indicated with ----, was considered to be the upper limit of the euthyroid reference range as determined from our in house euthyroid controls.

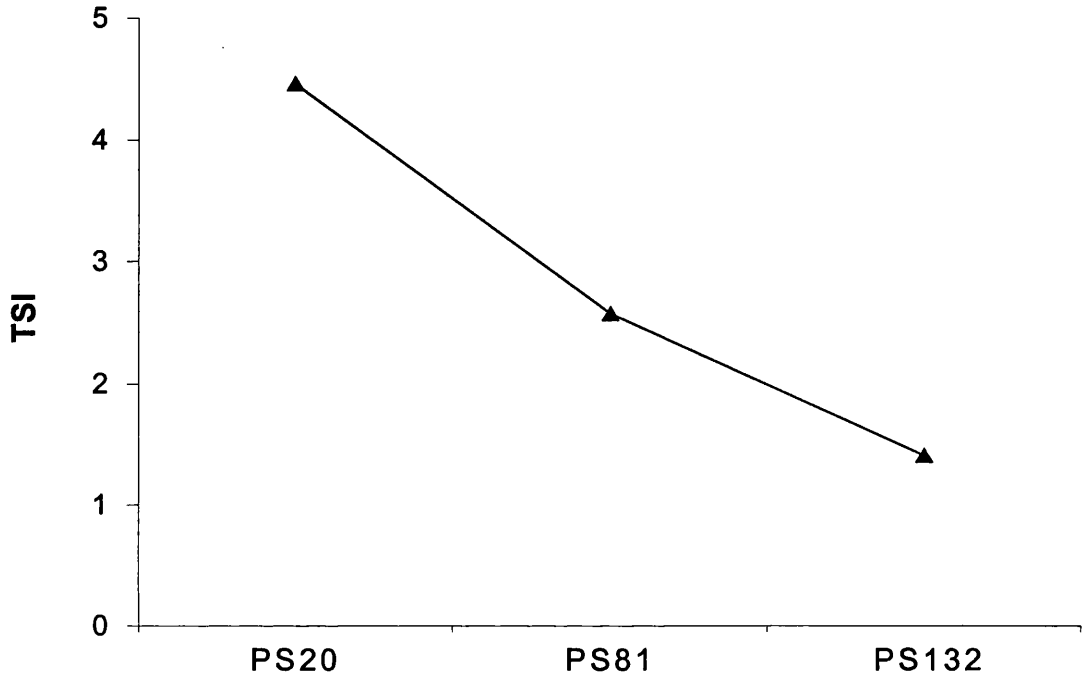


Figure 10.12 **Bioassay D: decrease of TSI values for a hyperthyroid patient (TL) with severe Graves' ophthalmopathy, after immunosuppressive treatment.** 3 successive serum samples (PS20, 81 & 132) were taken from this GD patient (TL) who was initially suffering from severe thyroid associated ophthalmopathy. After sample PS20 had been taken, the patient was successfully treated with immunosuppressants, and PS81 was taken 6 months later, when the eyes had greatly improved. The final sample PS132 was then taken another 3 months after PS81. Results are the mean R.L.U. for duplicate cultures expressed as TSI values (see Section 10.2.3). They were assayed in one bioassay.

We also tested selected patients (n = 42), who had a range of TSI values, with the assay for TBII (Figure 10.13; Section 10.2.2.5). No correlation was found between the TSI and the TBII values ($r = 0.19$, $p>0.05$).

10.4 CONCLUSIONS

We have demonstrated that Bioassay D may be successfully adapted for use with human sera. The favourable within-assay precision of this bioassay allowed us to critically test for parallelism between different patient TSAbs and TSH. None was found. We were therefore unable to express stimulation with patient sera in "TSH equivalents" and resorted to the conventional use of TSI values.

Despite the exceptionally precise nature of the bioassay, with within-assay ϕCV of ~2%, we found that between-assay variation was much larger (16%) and that TSI values were vulnerable to "shifts" with what appeared to be relatively innocuous changes in the bioassay protocol. We therefore concluded that the bioassay was not "rugged".

In a preliminary study, we screened different patient groups for the presence of TSAb. Apart from one hypothyroid patient, the only patients positive in the bioassay were those with GD, a minority of which had TSI values >5. We therefore concluded that this high throughput bioassay has a good potential for being suitable for clinical exploitation where it could be used to detect TSAb in patient sera and sharpen a diagnosis for autoimmune hyperthyroidism i.e. GD.

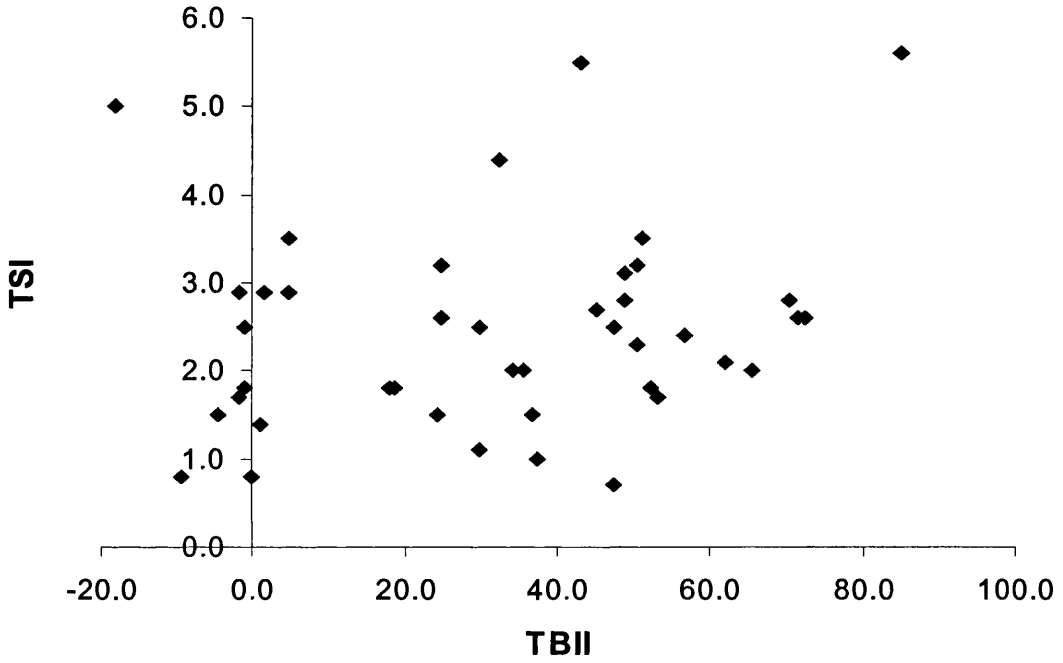


Figure 10.13 **Bioassay D: a comparison of the TSI and TBII values measured in both Bioassay D and a TBI assay.** 42 sera obtained from the Thyroid Clinic at UCH, tested previously using Bioassay D (see Section 10.2.2.4) were tested in the TBI binding assay (see Section 10.2.2.5). Samples were tested in duplicate in each of the assays and the results are expressed as the means. A negative TBII value indicated that TSH binding was slightly increased, rather than decreased, by a test serum. The correlation coefficient between these results was calculated to be $r = 0.19$, $p>0.05$.

CHAPTER 4

CONCLUDING DISCUSSION

Ever since the first discovery of TSAbs, which was about 5 decades ago, repeated attempts have been made to assess the usefulness of their measurement for the management of a patient with GD. There are two demanding prerequisites for this. Firstly, and most obviously, the TSAb measurement technique, which will underpin any such clinical investigation, must be adequately sensitive, specific and precise. Secondly, the patients themselves must be well characterized and the course of their disease followed for a long period e.g. for 5 years. To date, no study has entirely satisfied these demands, although progressive refinements in assay techniques and clinical protocols have been made over the years, due to the effort of numerous research laboratories and clinical groups. This is demonstrated by a very recent and ambitious study (Maugendre D & Massart C, 2001). This involved sample collection over an 8 year period, following a total of 140 patients for up to 4½ years each. It used both a binding assay and a TSAb bioassay based upon the measurement of cAMP in cultures of human thyrocytes. Ultimately, few results from individual patients were actually shown and the authors, as so many have done previously, finally concluded that although neither assay can be used with confidence to predict the course of the disease in an **individual** patient, anticipated trends could be found for different patient groups. For example, the group which relapsed after 18 months of treatment with antithyroid drugs was more positive in both assays, according to statistical criteria, than those who entered remission. Such an outcome is consistent with the view that persistent inadequacies with existing assay techniques are the root cause of this frustration and lead to the not infrequent patient "misclassification" reported by these authors. It is this fundamental problem which we have attempted to address in the current work.

Over the last decade, reporter gene technology based upon luciferase signalling has advanced considerably. It is these advances, particularly in signal stability, which we have exploited for the development of our luminescence bioassay for thyroid stimulators. Both the pioneering work with the FSH bioassay (Albanese C *et. al.* 1994), and the two early attempts at luminescence bioassay for thyroid stimulators (Watson PF *et. al.*, 1998; Evans C *et. al.*, 1999) used flash technology. The necessity for a separate luciferase extraction step together with the instability of the signal, resulted in systems which were far from precise.

Our work has been based upon the recently developed Steady-Glo (Promega) technology, which does not require an extraction step. Through Bioassays A – D we have progressively refined the protocol, aiming at maximising precision and as a consequence, sensitivity. Our achievement is best summarised in the cumulative precision profile (Figure 9.38), which shows that not only have we been able to minimise bioassay imprecision to as little as 3.1%, but that this is reproducible when tested in a sequence of bioassays. In comparison, using transfected CHO cells and the cumbersome, error prone cAMP measurement system, Massart C *et. al.* (2000) recently reported within-assay imprecision of 10 – 15%. Moreover, Evans C *et. al.* (1999), who used a prototype luminescence bioassay based upon flash technology, reported within-assay imprecision of 9.3 – 11.5%. Using the latter system, Watson PF *et. al.* (1998) claimed a within-assay precision of 5%, but we would comment that (a) only this single value was reported, whereas, inevitably, there must be a range of values over different doses, as best exemplified by our imprecision profile (Figure 9.38) and (b) no results were given to support their claim.

We consider that the major features of the protocol responsible for our attaining improved precision were as follows:

(i) **minimal disturbance to the target cell monolayers**. In some protocols e.g. Bioassay C, we avoided supernatant changes completely, this maximising the integrity and reproducibility of the monolayers in the wells. However, for Bioassay D, the final protocol adopted, because of the necessity to change to AM3 or AM4 for Phase 2, i.e. the exposure of the monolayers to the stimulators, we had to accommodate one medium change. This was accomplished by tipping and blotting, since we considered aspiration too disruptive and likely to damage the monolayers, thereby increasing between-well variation.

(ii) **Use of the Steady-Glo system, referred to as Promega System II, for Phase 3 i.e. signal development**. We discovered that even with this system, the advantages of which have already been discussed above, we experienced positional variation when samples were repeatedly tested within one microtiter plate. We critically assessed the signal stability of this system and found that changes of signal with time, after the addition of the Promega reagent, were responsible for the positional variation and assay drift. We minimised this by increasing the volume of reagent added, from 20µl to 50µl, by delaying signal measurement for 40 minutes after the addition of the reagent and thirdly, by reducing the reading time from 10 to 1 second/well. The latter resulted in any given plate being read within 2 minutes, from start to finish, and thus eliminated drift due to changes of signal with time during plate reading itself.

Once we had achieved such good within-assay precision, we were able to carefully investigate for parallelism between the dose-response curves to different thyroid stimulators. We demonstrated that, although, as might be expected, parallelism was observed between different vials of the International Standard for TSH (Figure 9.27), no such parallelism could be demonstrated between TSH and different TSAbs. This was unfortunate since it precluded the possibility of expressing the potency of a given patient's TSAb in terms of a "TSH equivalent". The latter would obviously have been immediately meaningful to our clinical colleagues who are used to assessing patient thyroid status in terms of circulating TSH concentrations. Parallelism between different TSAbs has been claimed by Massart C *et. al.* (2000). Relying upon cAMP measurement in transfected CHO cells, which is error prone, they state that "dilution curves for TSAb assay performed on CHO transfected cells were parallel straight lines as shown for sera of two patients". However, these results were not in practice "shown" and thus it is not possible to assess the certainty of their claim.

Although, with our refined protocol, we were able to obtain within-assay precision comparable to that obtainable with immunoassays, we were frustrated to find that between-assay precision was relatively poor. We found that between-assay variation was vulnerable to what might appear to be innocuous changes in bioassay conditions. For example, merely preparing the monolayers from cells which had been kept in growth medium for 4 rather than 3 days, resulted in a considerable shift in potencies, as determined by TSI values (Table 10.4). Interestingly we were not able to adjust for the alteration in absolute responses, by interpolating the results from TSH dose-response curves; this actually further increased between-assay variation. This indicates that changes in the physical conditions of different batches of monolayers

lead to changes in absolute responses to a stimulator such as TSH, as may well be expected, but that the alterations in responsiveness to TSH are different from those to TSAb. Thus, we concluded that although we had achieved a bioassay for thyroid stimulators which was capable of impressive within-assay precision, acceptable between-bioassay precision was only attained if we were very careful to adhere to a closely defined bioassay protocol. We concluded that the bioassay could not therefore be described as "rugged".

Other investigators, using the more error-prone system based upon cAMP extraction and separate measurement, have reported a need to tightly control culture conditions (Massart C *et. al.*, 2000). Since they were also working with CHO cells transfected to express the TSH receptor, it is possible, as suggested by (Massart C *et. al.*, 2000) that the problem originates with the CHO cells themselves. It is difficult to envisage what might cause such inherent instability. Future development of the bioassay would require investigation of this fundamental problem and, if a resolution cannot be found, changing from CHO cells as the host. Obviously, good between-bioassay variation is required if a patient is to be followed over 9 – 12 months, whilst on an initial course of antithyroid drugs, in an attempt to use the bioassay as a prognostic indicator for remission after withdrawal of the therapy.

At the outset of this work, it had been hoped that luminescence technology, which nowadays uses ultrasensitive detection systems and has inherent signal amplification, would lead to an increase in sensitivity of the bioassay for TSH. Immunoassays for this hormone have been transformed over the last 20 years, in terms of their sensitivity, with third generation immunoassays able to detect as little as 0.01mU

TSH/L (Spencer CA *et. al.*, 1995) i.e. well below the normal circulating concentrations. Unfortunately, this expectation for the bioassay has not been realised. We tried several means of increasing the magnitudes of response to lower doses of TSH (e.g. 1mU/L), by the inclusion of IBMX and/or forskolin, monolayer "starvation" regimes, optimisation of cell plating densities and time of incubation with the stimulator, but none were successful. As an additional strategy, we refined the protocol to minimise between-well variation so that response errors were as small as possible. This increase in precision improved the detection limit, lowering it from ~5mU/L (Figure 9.16) to ~1mU/L (Figure 9.30). However, despite this, the detection limit remained within the normal physiological range for TSH, which was already attainable with previous bioassays relying upon cumbersome and error-prone cAMP extractions and separate measurements (Ealey PA, Yateman ME & Marshall NJ, unpublished results). In the two previous descriptions of luciferase reporter gene bioassays for thyroid stimulators, little attention was paid to optimising sensitivity. Watson PF *et. al.*, (1998) used exceptionally high TSH doses e.g. 5000mU/L as did Evans C *et. al.* (1999) e.g. 1000mU/L.

Using Bioassay C, despite reasonably adequate magnitudes of response to TSH and also to a very potent TSAb, such as the International Standards and an in-house positive Quality Control (LM), virtually nil responses were observed with samples from other patients with GD. This situation was transformed with Bioassay D, when AM3 was substituted as the stimulator dilution medium i.e. for Phase 2 of the bioassay. Although AM3 increased the magnitudes of response to TSH, it had an even greater influence on responses to TSAbs. This medium has been formulated by our collaborators at DHI and for commercial proprietorial reasons they have not been able

to divulge its composition to us. However, it is likely that it is a hypotonic medium, analogous to that used by Kasagi (Kasagi K *et. al.*, 1982), because this has been shown to increase responses to TSAb more than TSH. Thus here we have an example of a shift in physical condition for the bioassay which influences TSAb differently from TSH. As we suggested previously, such a phenomenon might underpin our inability to reduce between-assay variation by expressing the relative potencies of TSAbs as "TSH equivalents" or potency indices.

Once we had obtained a means of increasing the magnitude of bioassay response to TSAbs, we were able to exploit the high throughput of Bioassay D by screening 421 patients and controls for the presence of detectable TSAb. We first had to adapt the bioassay for use with human sera. We showed that 10% serum was optimal. This had the benefit that only 10μl of unfractionated patient serum was required per determination. Using this, TSI values for 20 euthyroid sera were 1.0 ± 0.24. Thus the upper limit of a euthyroid TSI was determined to be 1.5. This was in exact agreement with the limits determined by both prototype reporter gene bioassays (Watson PF *et. al.*, 1998; Evans C *et. al.*, 1999). Of the 35 patients with autoimmune hypothyroidism investigated, all except one fell within this range. The one exception gave a high TSI of 4.8. Since this patient was obtained via the UK Survey of GD patients and their relatives and not the UCH Thyroid Clinic, we were unable to investigate them further. However, when we asked for the records to be doubled-checked, no evidence could be found for a misclassification of thyroid status. Moreover, a repeated aliquot from the master stock held at the Freeman Hospital yielded a similar result in a subsequent bioassay. We are consequently left to speculate that this patient may contain both TSAb and TSH-blocking antibodies. The latter might prevail *in vivo* but, using 10%

serum under *in vitro* conditions, TSAb may then stimulate in our bioassay, despite the presence of blocking antibodies. Evans and co-workers recently adapted their reporter gene bioassay to detect TSH-blocking antibodies in human serum (Jordon NJ *et. al.*, 2001). During this work, using deliberate mixtures of blocking and stimulating antibodies, they demonstrated that they could obtain either a stimulatory or an inhibitory response, or even a nil response, by varying the proportions of the two types of antibodies. Moreover, in previous unpublished studies, our own laboratory recorded a repeatedly potent stimulatory response in a bioassay based upon the direct measurement of cAMP, for sera from a patient classified as hypothyroid (unpublished results). The patient was additionally notable since she had twice given birth to thyrotoxic neonates. It is also possible that such patients, with hypothyroidism despite the presence of TSAb, could result from coincident deterioration of thyroid gland function itself. In this way, the TSAb would be without a target tissue.

A substantial proportion of the Graves' patients from the UK Survey (24.5%, n = 155) had TSI values >1.5 and were therefore positive for TSAb. Numerous studies have reported that ~95% of untreated Graves' patients are positive for TSAb (e.g. Costagliola S *et. al.*, 1999; Maugendre D & Massart C, 2001). However, since we were unable to distinguish treated from untreated patients from the information available to us, we could not make a comparable assessment. Likewise, a reasonable proportion of the Graves' patients from the UCH Thyroid Clinic (42.1%, n = 76) were positive for TSAb, and thereby contrasted with those with multinodular goitres, solitary toxic nodules, and autoimmune hypothyroidism.

Clearly, these clinical results must be regarded as a preliminary investigation with the newly developed Bioassay D. However, they look promising and merit a future detailed clinical study analogous to that recently reported by Maugendre and Massart (2001), who used the less precise earlier bioassay system based upon the direct measurement of cAMP. Ideally, patients samples need to be collected from well defined Graves' patients, both before treatment and also whilst undergoing the preliminary therapy with antithyroid drugs, in particular to ascertain whether the bioassay can be used (a) to definitively diagnose GD and (b) as a prognostic indicator of remission of this autoimmune condition. As demonstrated by Maugendre and Massart, such dedicated longitudinal studies require patient samples to be collected over a number of years. We would suggest that whilst this was taking place, a future study should be instigated to try to uncover the fundamental problem which resulted in the poor between-assay variation when working with the transfected CHO cells. As already discussed, to fully exploit the impressive within-assay precision achieved with our system, it may prove necessary to change the host cells used for this reporter gene luciferase bioassay.

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