ISOLATION AND CHARACTERISATION OF GENES CODING FOR CANDIDATE SPERM ANTIGENS FOR CONTRACEPTIVE VACCINES

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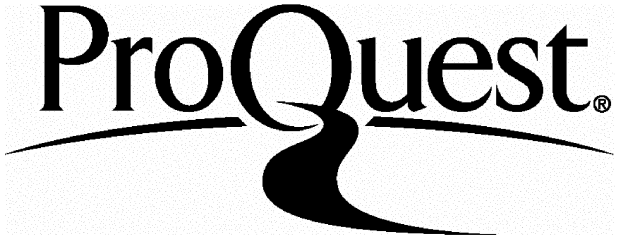

ABSTRACT

Specific protein components of the mammalian sperm acrosome are potential antigens for the development of an immunocontraceptive vaccine. In order to identify such acrosomal components, a series of specific antisera has been generated by immunising rabbits with purified acrosomal membrane fraction prepared from hamster epididymal spermatozoa.

Initial characterisation of the antisera by immunocytochemistry was performed on formaldehyde fixed, frozen sections of hamster and baboon testis and epididymis. Only post-meiotic germ cells in the testis were recognised by immune sera and staining was limited to the acrosomal region of spermatozoa. To analyze proteins recognised by these sera, baboon testis extract was resolved on 10% SDS-PAGE. Western-blot analysis indicated that rabbit antisera reacted to particular protein bands in the extracts even after pre-absorption of anti-coliform antibodies from each polyclonal serum.

Diluted rabbit polyclonal antisera designated R7 and R10, inhibited significantly sperm-zona binding and fertilisation in a hamster *in vitro* fertilisation (IVF) assay.

A combination of R7 and R10 antiserum was used as a probe for screening a human testis λgt11 expression cDNA library. This screen resulted in the selection of over 70 clones. Cloned cDNA inserts were isolated by amplification through the use of the polymerase chain reaction. Cloned inserts were characterised by restriction enzyme digestion and oligonucleotide probing techniques. β-galactosidase fusion proteins expressed by these clones were tested for their effect on fertilisation in a hamster IVF. One clone (HA5-2), showed significant blocking of fertilisation and marked

reduction of sperm-zona binding. Two other clones (HA6-2 and HB4-1), showed marked reduction of fertilisation and considerably lower levels of sperm binding. Sequence data from the 1.75 kb cloned HB4-1 insert revealed it to consist of two domains. The first 1132 nucleotides (nt) with >96% homology to human testis specific lactate dehydrogenase (LDH-C4) gene followed by the second downstream sequence 34 nt away from the end of LDH-C4 gene with >71% homology to *Chlamydomonas* caltractin gene.

The findings suggest that these clones encode functional molecules involved in sperm-zona binding, and hence the latter are possible candidates for contraceptive vaccine development.

DEDICATION

TO MY PARENTS

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ABBREVIATIONS

APS	Ammonium persulphate
AR	Acrosome reaction
bp(s)	Base pair(s)
BPB	Bromophenol blue
BSA	Bovine serum albumin
BWW	Biggers, Whitten and Whittingham buffer
cDNA	Complementary DNA
Ci	Curie
cpm	Counts per minute
CTAB	Cetyl trimethyl ammonium bromide
da	Dalton
DGZP-32	32 kilodalton deglycosylated zona pellucida protein
DMSO	Dimethyl sulphonic acid
dNTPs	de-oxyribonucleoside triphosphates
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetate
GTE	Glucose tris EDTA buffer
h	Hour
hCG	Human chorionic gonadotropin
IPTG	Isopropyl-β-D-thiogalactopyranoside
IVF	*In vitro* fertilisation
kb(s)	Kilobase (s)
kbp	Kilobase pairs
kDa	Kilodalton
LMP	Low melting point
M	Molar
M_r	Molecular weight
mA	Milliampere
mAb	Monoclonal antibody
MCS	Multiple cloning site
mg	Milligram
ml	Millilitre
mM	Millimolar
mm	Millimetre
mmol	Millimole
mU	Milliunit
NP-40	Nonidet-P40
ng	Nanogram
nm	Nanometre
nt	Nucleotide
OD	Optical density
OLB	Oligo labelling buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming unit
PID	Pelvic inflammatory disease
PMSG	Pregnant mare's serum gonadotrophin
RT	Room temperature
SDS	Sodium dodecyl sulphate
^{32}PαdCTP	^{32}P-labelled α-deoxycytidyl triphosphate
PEG	Polyethylene glycol

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	Saturated sodium chloride sodium citrate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBS/T	TBS containing Tween-20®
TE	Tris EDTA
TEMED	N,N,N',N'-tetramethyl-ethylene diamine
TFMS	Trifluoromethyl-sulphonic acid
Tween-20®	Polyoxyethylene sorbitan monolaurate
μl	Microlitre
μg	Microgram
μmol	Micromole
X-GAL	5-bromo-4-chloro-3-indoly-β-D-galactopyranoside
ZBP	Zona binding protein
ZP (ZP1, ZP2, ZP3)	Zona pellucida proteins (1, 2, 3)

CHAPTER 1

GENERAL INTRODUCTION

1.1 TRENDS OF GLOBAL POPULATION

In 1850, the world population was one billion, in 1930 two billion, 1961 three billion and in 1976 four billion. The estimated world population for April 1992 was 5.48 billion. If the present demographic statistics persist, the world population will double in 47 years and the eventual population will be 24 billion by the year 2300 **(Tuckwell and Koziol, 1992)**. Ninety-five percent of this global population growth is concentrated in the developing countries **(United Nations, 1984)**. This means further pressure on already scarce resources with a subsequent lowering of the standard and quality of living in these countries. In the light of this population explosion, a continued search for a safe, acceptable and reliable means of fertility regulation is an absolute necessity.

1.2 CONTRACEPTIVE METHODS IN CURRENT USE

The main methods of contraception in developing and developed regions of the world are shown in Figure 1.1. Female contraception accounts for 73% of global contraceptive methods.

1.2.1 Voluntary sterilisation

Voluntary surgical sterilisation is the most widely used contraceptive method in the world, with about 80 million couples currently using it to control their fertility (Table 1.1). It is permanent and this is regarded by many couples who have completed their desired family as more of an asset than a liability. Even in many developing countries, the chances of having one's offspring live to adulthood are considerably better today than previously, due to improved medical care, expanding health services, and better

nutrition.

1.2.1.1 Voluntary male sterilisation (Vasectomy)

Vasectomy involves blocking the passage of sperm through the vas deferens usually by ligating and cutting both ducts. It was first performed in the 19th century (see **Wolfers and Wolfers, 1974**), but was not used as a contraceptive method until this century and only recently became widely available. In the United States and Europe, vasectomy first became more widely used in the late 1960s when adverse publicity about the safety of oral contraceptives coincided with a feminist campaign to encourage greater male responsibility on fertility control (**Wortman and Piotrow, 1973**). While vasectomy continues to be popular in most countries, the proportion of total sterilisations that are vasectomies is dropping as new, simplified, female sterilisation procedures become available (**Green, 1978**). In developing countries vasectomy is often among the least used family planning methods due to the neglect by many planning programmes. World-wide, an estimated 42 million couples rely on vasectomy (Table 1.2). By comparison, nearly 140 million rely on female sterilisation (Table 1.3). Vasectomy is a major family planning method only in six developed countries (US, New Zealand, Australia, Great Britain, Canada, and the Netherlands), and in three developing countries (China, India, and South Korea). In many countries, vasectomy is hardly used, and few people have heard of the procedure compared with other methods (**Liskin *et al.*, 1992**).

1.2.1.2 Voluntary female sterilisation

Female sterilisation involves the blocking of both Fallopian tubes so that the egg released by the ovary each month cannot be fertilised by the spermatozoon. Although it still remains a more complicated procedure (and hence carries higher operative risks for the patient than, for example, vasectomy) new techniques and equipment

have greatly increased the availability of tubal ligation in rural areas. Currently, there are two ways to reach the Fallopian tubes. Mini-laparotomy consists of making a small incision in the abdomen, moving each tube to the incision, before ligation and the removal of a segment of each tube. Laparoscopy consists of inserting a laparoscope (operating telescope), into the abdomen through a small incision. Through the laparoscope, the surgeon observes the oviducts and usually applies clips, rings, or heat to block them. Both these methods are effective and relatively safe **(Church and Geller, 1990)**. Voluntary female sterilisation is now the world's most widely used family planning method and one of the fastest growing. An estimated 138 million women of reproductive age used this method in 1990 (43 million more than in 1984). Millions more are expected to ask for the method in the next decade **(Church and Geller, 1990)**.

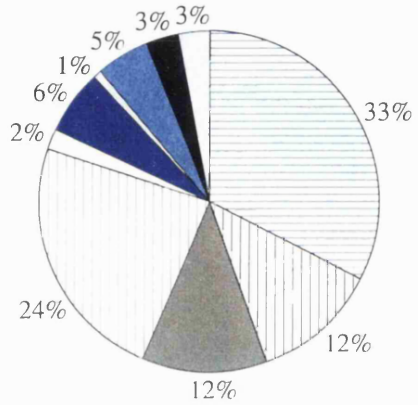
1.2.2 Female contraception

1.2.2.1 Steroidal contraceptives

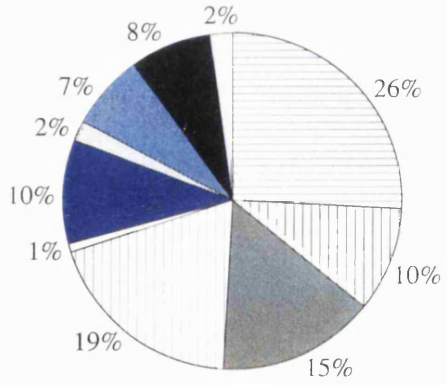
Steroidal contraceptives, are long-acting methods of contraception that have been developed and refined over the past 10-20 years (see **Newton, 1993**). From the classical long acting progestogen-only depot preparations there is now a range of delivery systems. These include combined and progestogen only injection systems and vaginal rings which may also contain either progesterone only or oestrogen and progesterone in combination. Subcutaneous implants at present containing only a progestogen, offer a range of duration of contraception from 2-5 years **(Croxatto *et al.*, 1988, Kurumaki *et al.*, 1985)**.

FIGURE 1.1

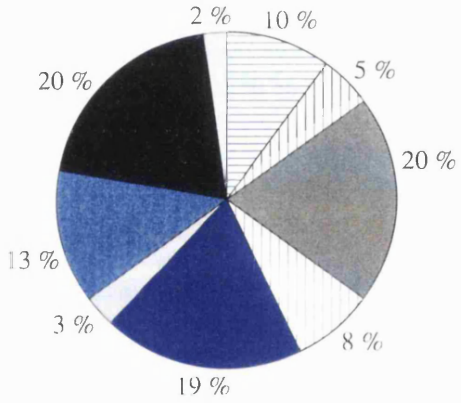
FIGURE 1.1 PREVALENCE OF DIFFERENT CONTRACEPTIVE METHODS IN DEVELOPING AND DEVELOPED REGIONS OF THE WORLD



Less developed regions



World



More developed regions

Female sterilization
Male sterilization
Pill
Intrauterine device
Injectable
Condom
Vaginal barrier methods
Rhythm
Withdrawal
Other methods

TABLE 1.1 ESTIMATED NUMBER OF COUPLES USING BIRTH CONTROL, WORLDWIDE BY METHOD, 1970 AND 1977 (after **Green, 1978**)

	1970 (millions)	1977 (millions)
Voluntary sterilisation	20	80
Oral contraception	30	55
Condom	25	35
IUD	12	15
Other methods*	60	65
Total	147	250
Abortion (annual incidence)	40	40

* Diaphragms, spermicides, rhythm, withdrawal, etc.

TABLE 1.2 WORLDWIDE USE OF VASECTOMY 1991. ESTIMATED USE AMONG MARRIED COUPLES OF REPRODUCTIVE AGE (after **Liskin *et al.*, 1992**)

Regions	% of couples relying on vasectomy	Number of couples relying on vasectomy (in thousands)
DEVELOPING AREAS		
Asia		
China	8	18,100
India	7	13,000
Other Asia and Pacific	1	800
Latin America	0.7	400
Near East and North Africa	*	**
Sub-Saharan Africa	*	**
All developing Areas	5	32,300
DEVELOPED AREAS		
Australia and New Zealand	13	400
Europe (includes Eastern Europe and former USSR)	3	3,000
North America	13	5,200
Other developed	2	600
All developed areas	5	9,200
WORLD	5	41,500

* Less than 0.5% of couples relying on vasectomy

** Fewer than 1,000 couples relying on vasectomy

TABLE 1.3 ESTIMATED NUMBER OF MARRIED WOMEN OF REPRODUCTIVE AGE VOLUNTARILY STERILISED FOR CONTRACEPTIVE PURPOSES, 1990
(after **Church and Geller, 1990**)

Regions	% of MWRA	Number of MWRA (in thousands)
DEVELOPING REGIONS		
Africa	1	800
Asia and Pacific		
China	30	63,200
Indian subcontinent	18	41,800
Other Asia	10	7,200
Latin America and Caribbean	17	9,400
Near East and North Africa	2	700
All developing Areas	18	123,000
DEVELOPED AREAS		
Europe		
East (includes USSR)	1	600
North (Scandinavia)	6	200
South	2	300
West	7	2,100
United States	23	7,500
All developed Areas	8	14,600
WORLD	16	137,700

MWRA= married women of reproductive age.

1.2.2.1.1 The pill

The idea of oral contraception with hormones dates back to 1920s (**Greep, 1984**). Not until the 1940s and 1950s, however, did inexpensive, orally effective hormones became available (**Djerassi, 1981**). Oral contraceptives (OCs) began to appear in developing countries in the mid 1960s, but high prices put them beyond the reach of all but just a few women (**Ravenholt and Piotrow, 1969**). OC use has continued to grow in the 1970s and 1980s. In developed countries, estimated 24 million married women used OCs in 1988 (Table 1.4). This represents almost 14 percent of married women of reproductive age. In developing countries, there were an estimated 39 million married women using OCs, or 6 percent of married women of reproductive age, in 1988 compared with 25 million in 1981. With more than 60 million users by 1988, 38 million of them in developing countries (Table 1.2), OCs trail only voluntary sterilisation and IUDs in worldwide use. The most popular pill today is the low-dose oestrogen-progestin pill. Low-dose pills contain only one quarter or less oestrogen and substantially less progestin than the first pills.

Widely used types of pills include: combined oestrogen-progestin, progestin only, and multiphasics. Progestin-only and multiphasics are special types of low-dose OCs. The progestin-only "mini pill" was developed in the early 1970s as a substitute to oestrogen pills since the latter had thromboembolic side effects. Each mini pill tablet contains 0.3-0.6 mg of norethindrone progestins or 0.03-0.0375 mg laevonorgestrel. Multiphasics are the newest OCs developed, and which became widely used in the 1980s. Their doses change during the pill cycle in order to provide menstrual cycle control, keep hormone doses low, and still prevent ovulation (**Apelo and Suplido, 1985; Ellsworth, 1986**).

The biggest benefit of the pill is its effectiveness. The pill may also reduce endometrial and ovarian cancer. It may further help in preventing anaemia, ectopic

TABLE 1.4 ORAL CONTRACEPTIVE USE WORLD WIDE 1988, ESTIMATED PRIMARILY FROM SURVEY AND SALES DATA (after Wharton and Blackburn, 1988).

Regions	Estimated percentage of MWRA using OCs	Estimated number of Married OC Users (in millions)
DEVELOPED AREAS		
Australia and New Zealand	30	0.9
Europe		
East (includes USSR)	6	3.5
North (Scandinavia)	21	0.6
South	14	2.8
West	29	8.8
Japan	1	0.2
North America	18	7.0
South Africa	15	0.6
Subtotal	14	24.4
DEVELOPING AREAS		
Asia		
China	5	10.1
Indian sub continent	2	32.0
Other Asian	13	9.2
Latin America and Caribbean	16	8.3
Near East and North Africa	12	4.4
Tropical Africa	5	3.3
Subtotal	6	38.5
TOTAL	8	62.9

MWRA = Married Women of Reproductive Age.

pregnancy, painful menstruation, benign breast tumours and ovarian cysts. Although the pill increases the risk of thrombo-embolism, stroke and heart attack, these conditions are mainly seen in older women who smoke cigarettes and use higher dose pills (**World Health Organisation, 1990**).

When used properly, OCs rank with injectables, the more recently developed hormonal implants, and the latest generation of IUDs as almost perfectly effective, reversible contraception.

1.2.2.1.2 Injectable preparations

These include progestogen-only preparations such as medroxyprogesterone acetate, 150 mg every 12 weeks, norethisterone enanthate 200 mg every 8-10 weeks, and combined injectables such as oestradiol valerate 5 mg plus medroxyprogesterone acetate 25 mg (Cyclofem®) and Oestradiol cypionate 5 mg plus Norethisterone enanthate 25 mg (Mesigyna®).

1.2.2.1.3 Vaginal rings

Vaginal rings also fall in two categories: (i) Progestin-only systems which include laevonogestrel 20 μg/day (Femring®), 3-ketodesogestrel 25 μg/day and progesterone 15 mg/day. (ii) Combined ring systems examples of these include ethyl oestradiol 12 μg/day plus 3-ketodesogestrel 120 mg/day and norethisterone acetate plus ethyloestradiol.

1.2.2.1.4 Implants

Implants includes, Norplant®-6 (6 capsules of laevonorgestrel), Norplant®-2 (2 covered rods of laevonorgestrel) and Implanon® (1 rod of 3-ketodesogestrel).

1.2.2.1.5 Hormone releasing IUDs

An example of this is levonova (20 μg/day laevonorgestrel). The efficacy of these methods is high, with failure rates of as low as 0.1 per 100 woman years. However, with progestogen-only systems, a significant proportion of women develop unpredictable menstrual bleeding and this is the commonest reason for discontinuation **(Newton and Hall, 1989; World Health Organisation, 1990)**.

The method of action of progestogen-only systems is primarily cervical mucus blockade and prevention of sperm transport across the cervix. Ovarian effects ranging from complete inovulation to disordered luteal phase, persistent follicles and disorganised hormone production add to the contraceptive effect in more than half of the treatment cycles, but cause some of the menstrual disturbance **(Landgren and Diczfalusy, 1982; Eissa *et al.*, 1987)**.

All these long acting methods are essential to family planning programmes, offering highly acceptable, and in some cases novel methods with high efficacy **(Jackson and Newton, 1989)**.

1.2.2.2 Barrier methods

1.2.2.2.1 Intrauterine devices (IUDs)

The intrauterine devices are substances that are inserted inside the uterus for contraceptive purposes. The contraceptive efficacy of IUDs is at least as good as and possibly better than that of the pill **(Edelman and van Os, 1990; van Look and von Hertzen, 1993)**. Since the introduction of copper-T **(Lippes *et al.*, 1976)**, when used as a regular method of contraception, they act primarily by preventing fertilisation and implantation **(World Health Organisation, 1987)**. A wide range of IUDs is available world-wide with frequent new developments aimed at improving efficacy or

overcoming specific problems (**Population information programme, 1988**). Although inert devices are widely used in some countries (notably China where stainless steel IUDs predominate), in developed countries, copper-containing medicated devices predominate. These IUDs consist of inert plastic holders impregnated with copper or pharmacological compounds. Copper, together with its stabilization over a silver core, have been the most notable changes of the last few years. The resulting range of Nova T®, Novagard®, Multiload 375® and TCu380A, provide contraception as effective as that offered by the combined pill but also permit a longer intrauterine lifespan of these devices before removal and refitting are required.

Some side effects and complications associated with IUD include a degree of pain and bleeding in addition to a risk of pelvic inflammatory disease (PID) (**Morris and van Wagenen, 1966**).

The inert plastic devices exert their contraceptive effect by a local mechanical irritation of the endometrium, causing changes of the mucosa and preventing the implantation of the fertilised zygote (**Shaw *et al.*, 1981**). The medicated IUD's, on the other hand, release chemical substances such as progesterone and laevonorgestrel, which inhibit fertilisation (**El-Maghoub, 1983**). The major drawbacks that IUDs have suffered are high expulsion rate though the recently developed IUD's have threads fixed at the arms of the pessary to avoid this problem (**Thiery *et al.*, 1983**). The other problem has been the disturbance it causes on the menstrual cycle leading to pelvic inflammation, haemorrhage, spotting and anaemia, a problem mainly encountered by the medicated copper IUD users (**Koch, 1983**).

1.2.2.2.2 Diaphragm

The diaphragm, is a dome-shaped rubber cap with a flexible metal ring which is placed in the vagina and covers the cervix. The dome provides a mechanical barrier on the cervix thereby preventing sperm from entering the uterus. The diaphragm use generally includes the simultaneous use of spermicidal jelly (creams) and therefore incorporates both physical and chemical barriers. It confers protection against cervical gonorrhoea and hospitalisation for pelvic inflammatory disease (PID) **(Austin *et al.*, 1984; Kelaghan *et al.*, 1982)**. However, diaphragm use is associated with increased rate of urinary infection and also bacterial vaginosis, possibly due to the detergent effect of the associated spermicide **(Hooton *et al.*, 1989; Vessey *et al.*, 1987)**.

1.2.2.3 Vaginal spermicides

Vaginal spermicides consist of two basic components: the carrier and the spermicide itself. The carrier an inert base, usually a foam, or a jelly and transports the spermicidal agent into the vagina against the cervix. The spermicide is a chemical agent that immobilises and kills spermatozoa. Vaginal spermicides rely on surfactants as the active agent. The most commonly used compound in the U.K. is the neutral surfactant, nonoxynol-9 **(Chantler, 1992)**. Other spermicidal products use either the structurally related compound octoxynol, or an alternative type of non-ionic surfactant, *p*-di-isobutyl phenoxypropyl ethoxyethanol. World-wide, the cationic surfactant benzalkonium chloride, the anionic detergent sodium docusate (dioctyl sodium sulphosuccinate), and the neutral surfactant Mengfegol™ (*p*-methanylphenyl polyoxyethylene) are also used. Surfactants have a common mode of spermicidal action. These detergents act on the lipid components of the membrane of the middle piece and the tail regions of the spermatozoa. This results in a rapid disruption of the membranes and loss of motility **(Wilborn *et al.*, 1983)**. Being a non-selective

mechanism, any unprotected lipoprotein membrane will be susceptible to disruption, including those of vaginal epithelium and micro-organisms.

Contraceptive sponges have no mechanism for maintaining a barrier over the cervix and are therefore assumed to mainly act as a reservoir for the release of spermicide, hence they are considered as inert vehicles for the spermicide.

1.2.3 Male contraception

1.2.3.1 Condoms

The condom is a rubber cap or collagenous tissue sheath which is made to fit over an erect penis. In 1990 an estimated 45 million married couples were using condoms presumably for family planning (**Liskin *et al*, 1992**). These 45 million amount to about 5% of all couples with the wife of reproductive age, and about 9% of all married couples using some form of family planning. Although condoms are a major method in some places such as Japan and the Scandinavian countries, they rank near the bottom in terms of world-wide contraceptive use. Roughly an equal percentage of couples rely on voluntary male sterilisation. A greater percentage of couples rely on voluntary female sterilisation (13%), IUDs (10%), and oral contraceptives (8%). Only female barrier methods, such as spermicides and diaphragms, rank lower, at (1%) of married women of reproductive age (**United Nations 1989**). However,condoms will provide two-way protection preventing contact with semen, infectious secretions and some genital ulcers (**Minuk *et al.*, 1986**). Several reviews (**Grimes and Cates, 1990**), have shown protection of both male and female against gonorrhoea, urethritis, PID and human immunodeficiency virus (HIV).

1.2.3.2 Coitus interruptus

Coitus interruptus or withdrawal, is a method of birth control in which the penis is withdrawn from the vagina before ejaculation. Obviously, this procedure demand a great deal of self control, considering the desire to achieve deeper penetration at the time of impending orgasm (**Hatcher *et al.*, 1974**). Many men are physiologically or psychologicallyunable to practice coitus interruptus either because they do not perceive the imminence of ejaculation or because the can not or choose not to withdraw in time (**Masters and Johnson, 1966**). Because of this coitus interruptus is unreliable as a method of contraception but is often used due to religious beliefs.

1.2.3.3 Gossypol and other polyphenolic compounds

Gossypol (GSP), is a phenolic bisequiterpene aldehyde extracted from the seed of a cotton plant and has been used as a male antifertility agent due to its ability to suppress spermatogenesis (**Poso *et al.*, 1980**). Polyphenols are compounds with two or more phenol functional groups linked together by covalent bond(s). Many of these compounds occur naturally in plant extracts and have been shown to exercise inhibitory effects on mammalian sperm functions (**Kumar *et al.*, 1989**). The physiological role of these compounds in plant system is well known but their fate in animals remains unclear (**Cadman, 1960; Crickshank and Perrin, 1964**). There is evidence that they act as uncouplers of mitochondrial phosphorylation (**Abou-Donia, 1976; Poso *et al.* 1980**); are inhibitors of glycolytic and tricarboxylic acid cycle enzyme activities (**Tso and Lee, 1982**), and that they are inhibitors of sperm specific enzymes including lactate dehydrogenase-C4 (**Tso and Lee, 1981**). Polyphenols are oxidised by enzymes like phenol oxidase and peroxidases which generate superoxide radicals (**Elstner, 1984**). Thus, they may interfere with sperm function by enhancing this superoxide generation, which results in eventual loss of sperm motility (**Kumar *et al.*, 1989**).

Gossypol has a high incidence of irreversibility (**Liu *et al.*, 1987; Liu and Lyle, 1987**), and potentially serious side effects such as hypokalaemia, and because of this, its use for contraception has been discontinued. It is hoped that a synthetic analogue of gossypol will eventually be found that retains its antifertility activity while eliminating its pharmacologically undesired properties.

1.3 CONTRACEPTIVE VACCINE DEVELOPMENT

Immunisation for contraceptive purposes (immunocontraception) is possible by raising antibodies that interfere with the processes of reproduction. For example, immunisation against antigens of gametes (oocytes or spermatozoa), may prevent fertilisation, while immunisation against trophoblast antigens may prevent implantation of the embryo. Three major approaches to contraceptive vaccine development are being pursued at the present time. The most advanced approach, which has already reached the stage of phase 2 clinical trials, involves the induction of immunity against human chorionic gonadotrophin (hCG - for review see **Aitken *et al.* 1993**). Vaccines are being engineered by linking tetanus or diptheria toxoids to a variety of hCG-based peptides centred on the β-subunit. Clinical trials have revealed that such preparations are capable of stimulating the production of anti-hCG antibodies. However, the long term consequences of such immunity in terms of efficacy or safety are unknown.

The alternative approaches to contraceptive vaccine development involve the induction of immunity against gamete-specific antigens found on the surface of the human spermatozoon and the zona pellucida (ZP), or antigens found on the embryo or extra-embryonic membranes. There is an abundance of experimental and clinical

data to suggest that this approach is feasible although biochemical characterisation and synthesis of candidate antigens is still incomplete. This review will be limited to discussion of gamete-specific antigens.

1.3.1 Sperm antigens

Immunisation of male and female animals with extracts of whole spermatozoa is known to cause infertility (**Menge *et al.*, 1979**). Men and women who spontaneously produce anti-sperm antibodies may also be infertile but otherwise healthy (**Boettcher, 1979**). Although the precise identity of the sperm antigens involved in auto-immunity are not yet known, such observations have led to the proposal that sperm proteins might be useful antigens in the development of a contraceptive vaccine (**Anderson and Alexander, 1983**).

Desirable sperm antigens for contraceptive vaccine development can be identified and isolated in several ways. These include: 1) sperm antigens which can be identified by naturally occurring sperm antibodies in the sera of infertile patients (**Jackson *et al.*, 1975; Teuscher *et al.*, 1982**); 2) sperm proteins of known sperm specific enzymes such as lactate dehydrogenase C4, acrosin, hyaluronidase (**Goldberg, 1973a; Topfer-Petersen *et al.*,1990**); and 3) sperm surface antigens reactive to polyclonal or monoclonal antibodies that affect normal sperm functions (**Naz *et al.*, 1984; O'Rand *et al.*, 1984; Moore and Hartman, 1984; Ellis *et al.*, 1985; Primakoff *et al.*, 1988a**).

Some of the major sperm antigens that have been investigated as candidates for contraceptive vaccine development are detailed below.

1.3.1.1 Lactate dehydrogenase C4

Lactate dehydrogenase C4 (LDH-C4 or LDH-X), is composed of a unique lactate dehydrogenase subunit (C). It is only synthesised in the testis during active spermatogenesis and is the major LDH isozyme of spermatozoa (**Zinkham *et al.*, 1964**). The sperm specific isozyme antigen has been isolated and purified in crystalline form from several animals. It is immunologically distinct and differs greatly both in its biochemical and enzymatic properties from somatic LDH isozymes composed of A and B subunits of somatic LDH (**Millan *et al.*, 1987**).

Antibodies to LDH-C4 do not cross-react to other LDH isozymes but do cross-react to LDH-C4 from other mammalian species (**Goldberg, 1973b**). Active immunisation of male and female rabbits against purified LDH-C4 antigen results in an immune response (**Goldberg, 1973a**). Anti-LDH-C4 antibodies partially suppress fertility in experimental animals including female mice and baboons (**Goldberg *et al.*, 1981a; Isahakia and Bambra, 1992**).

Human testis cDNA to LDH-C4 has been cloned and sequenced (**Millan *et al.*, 1987**). The deduced amino acid sequence to human LDH-C4 is as different from rodent LDH-C4 (73% homology), as it is from human LDH-A4 (76% homology) and porcine LDH-B4 (68% homology).

The mechanism whereby LDH-C4 inhibits pregnancy in female animals immunised with this isozyme is unclear. One idea is that suppression of fertility is achieved by cellular-lytic activity of the antibodies on spermatozoa (**Goldberg, 1973b**). This argument has been supported by the studies of **Shelton and Goldberg (1986)** who correlated the cell mediated cytotoxic response to LDH-C4 immunisation with a reduction in fertility. Recently, antibodies developed against synthetic peptide

antigens (designated MC_{5-15} and $MC_{211-220}$) derived from mouse LDH-C4, have been shown to bind both to purified enzyme and enzyme on the surface of sperm itself (**Beyler *et al.*, 1985**). The binding patterns from solid-phase radioimmunoassay has revealed similarity in binding of LDH-C4 to mouse and human spermatozoa. These results indicate that the amino acid sequences MC_{5-15} and $MC_{211-220}$ are the antigenic sequences on the LDH-C4 molecule that are exposed for immunoglobulin recognition against spermatozoa. LDH-C4 satisfies an essential requirement of a sperm antigen in that it provokes antibodies that are absolutely germ cell specific and do not cross-react with somatic isozymes (**Goldberg, 1975**). Furthermore, LDH-C4 is never synthesised by females and is hidden effectively from the immune system of the male by the blood-testis barrier.

1.3.1.2 Rabbit sperm antigen (RSA-1)

The rabbit sperm membrane auto-antigen-1, was initially documented by **Bigelow (1967)** and later by **O'Rand and Romrell (1977)**. This protein, now known as RSA-1, has been isolated and characterised as a fibrous tropomyosin-like sialoglycoprotein of molecular mass 13 kDa (**O'Rand and Porter, 1979**). Similar antigens have been reported on boar and guinea pig spermatozoa (**Topfer-Peterson *et al.*, 1985; Huang and Yanagimachi, 1984**). Furthermore, RSA-like molecules have been identified on human and mouse spermatozoa (**O'Rand and Irons, 1984**). RSA-1 has been identified as a surface antigen by immunofluorescence and immunoperoxidase localisation using monospecific antiserum (**O'Rand and Romrell, 1981**). These studies demonstrated that RSA first appeared on the surface of pachytene spermatocytes, and increased in amount as spermatozoa developed. The sialoglycoprotein moiety contains galactose and is a receptor for lectin *Ricinus communis* (**O'Rand and Porter, 1979**). The carbohydrate moiety of RSA-1 is also unique in containing xylose, a pentose sugar (**O'Rand and Porter, 1982**). Moreover,

on ejaculated spermatozoa RSA-1 has been found to occur as a complex of proteins, one of which, at least, originates from the seminal fluid (**O'Rand and Romrell, 1981.; O'Rand *et al.*, 1988**), Additional characteristics of RSA-1 are as follows: 1) RSA consists of a family of membrane glycoproteins, four of which have low molecular weight of 14, 16, 17, and 18 kDa respectively; and an aggregate at 68-70 kDa; 2) RSA family members are functionally similar, they block the cytotoxic activities of the autoimmune sera; 3) RSA-1 or its complex plays an important role during penetration of spermatozoa through the ZP, and is hence named also the zona binding protein (ZBP), and 4) family members of RSA bind solubilised ZP on nitrocellulose and on to the intact zona under *in vitro* fertilisation condition so as to prevent spermatozoa from binding. Immunisation of female rabbits with RSA-1 leads to reduction in fertility (**O'Rand *et al.*, 1984**).

1.3.1.3 SP-10 Antigen

SP-10 is one of three acrosomal proteins classified as "primary vaccine candidates" by a World Health Organization Task Force on Contraceptive Vaccines (**Anderson *et al.*, 1987**). It was first detected within the developing acrosome of round spermatids in the human testis (**Herr *et al.*, 1990a**). In mature, ejaculated spermatozoa SP-10 is specifically localised to the intra-acrosomal compartment but appears to be associated with the inner surface of plasma membrane (**Herr *et al.*, 1990b**). The SP-10 extracted from ejaculated human spermatozoa presents as a series of polymorphic immunogenic polypeptides with molecular mass ranging from 18-34 kDa (**Herr *et al.*, 1990b**), with isoelectric points of about pH 4.9. Western blot analysis of sperm extracts indicate that baboon and macaque SP-10 display multiple immuno-reactive forms similar to human SP-10 (**Herr *et al.*, 1990a**). A human cDNA for this intra-acrosomal protein has been cloned and sequenced (**Wright *et al.*, 1990**). Baboon and macaque SP-10 cDNAs also have been cloned and sequenced

(**Freemerman *et al.*, 1993**). A comparison of the deduced SP-10 amino acid sequences of human, baboon and macaque revealed a high degree of homology in these primate species (**Freemerman *et al.*, 1993**).

1.3.1.4 Mouse sperm antigen (MSA-63)

MSA-63 is a highly conserved acrosomal antigen reactive to a monoclonal antibody HS-63 which was generated against intact human spermatozoa. The purified antigen consists of a group of three proteins with molecular weights ranging from 42-50 kDa (**Liu *et al.*, 1989**). The purified antigen was used for immunisation of mice and rabbits and the antisera recognised the sperm acrosome on mature and testicular germ cells, but not somatic tissues. The iso-immune sera exhibited strong inhibition of mouse *in vitro* fertilisation and human sperm penetration of zona-free hamster eggs. A cDNA corresponding to a part of the MSA-63 mRNA has now been cloned from a mouse testis cDNA library using specific rabbit polyclonal antisera as probes (**Liu *et al.*, 1990**). A high degree of homology was observed between MSA-63 and the known human sperm antigen, SP-10, both at nucleotide and amino acid levels.

1.3.1.5 Fertilisation antigen-1 (FA-1)

The fertilisation antigen-1 (FA-1), is a sperm specific glycoprotein that has been purified from human and murine sperm membranes using mono- and polyclonal antibodies (MA-24; **Naz *et al.*, 1984**). Active immunisation of female rabbits with FA-1 causes marked reduction of fertilisation (**Naz, 1987**). Sera from immuno-infertile patients, but not from fertile individuals contain antibodies reactive to FA-1 (**Naz, 1990**). Human sperm FA-1 has been shown to bind to porcine ZP3 thereby neutralising its sperm-ligand activity in sperm-zona binding bioassay (**Naz *et al.*, 1991**).

Recent evidence revealed FA-1 to be devoid of proteolytic or acrosin activity, and even its immuno-affinity purified monoclonal antibody (mAb) lacks the inhibition to acrosin activity (**Kaplan and Naz, 1992**). The mAb, however, completely blocked human sperm penetration of zona-free hamster ova. Although the mechanism of inhibition of fertilisation by mAb still remained unclear, it probably involves the inhibition of sperm capacitation (**Kaplan and Naz, 1992**) or even the sperm acrosome reaction (AR). Currently, cDNA encoding human FA-1 is being cloned and sequenced to investigate whether a recombinant FA-1 protein might be a candidate molecule for immuno-regulation of human fertility and infertility.

1.3.1.6 PH-20

PH-20 is an integral membrane protein with a relative molecular mass of 64 kDa and was identified also using a monoclonal antibody. It is present on both the plasma membrane, and following the AR, on the inner acrosomal membrane of guinea pig sperm (**Primakoff *et al.*, 1985; Cowan *et al.*, 1986**). It has been purified from an octyl-glucoside extract of guinea pig sperm (**Primakoff *et al.*, 1988a**). Active immunisation with this sperm antigen resulted in complete and reversible suppression of fertility in guinea pigs (**Primakoff *et al.*, 1988b**).

1.3.1.7 The 95 kDa sperm protein (p95)

The 95 kDa sperm protein was identified in the mouse sperm using monoclonal antibody generated against hamster sperm heads (**Moore *et al.*, 1987**). The target antigen is involved in the prevention of binding of human spermatozoa to the ZP. It has been demonstrated to be selective in its binding to the ZP (sperm) receptor, ZP3 (**Leyton and Saling, 1989a**) and is therefore the candidate sperm receptor protein involved in primary binding to the ZP (**Burks and Saling, 1992**). The 95 kDa protein has characteristics of a protein tyrosine kinase (PTK). Tyrosine phophorylation of

p95 occurs during capacitation of mouse sperm and is enhanced by ZP binding **(Leyton and Saling, 1989a)**. Consistent with the hypothesis that p95 is a receptor for ZP3, this protein has been localised to the plasma membrane in fractionated preparations of spermatozoa.

1.3.1.8 The 56 kDa Sperm protein

The 56 kDa protein has been identified in the mouse and reported to mediate in primary sperm-ZP binding **(Bleil and Wassarman, 1990)**. ZP3 radio-labelled with ^{125}I-Denny-Jaffe cross-linking reagent reacted with a 56 kDa component on acrosome-intact mouse spermatozoa. Furthermore, this 56 kDa sperm protein was among those isolated using ZP3-affinity chromatography columns.

3.11.9 GA-1 antigen

This is a membrane antigen from rabbit testicular germ cells and spermatozoa purified using an anti-sperm monoclonal antibody **(Menge *et al.*, 1987)**. The antisera raised against this antigen imposed partial inhibition of post-fertilisation events but not the initial fertilisation steps.

1.3.1.10 Sperm acrosomal enzymes

A number of sperm acrosomal enzymes may also be antigen candidates for vaccine development. The first contact of spermatozoa with the egg occurs at the surface of the ZP. Before penetration, spermatozoa must bind to the ZP and complete the AR **(Topfer-Petersen *et al.*, 1990)**. The binding event and AR are complex processes that enable the spermatozoon to reach the plasma membrane of the oocyte. The molecules involved have not been elucidated fully. Evidence suggest the existence of complementary receptor molecules on both gametes to be of critical importance for these interactions **(O'Rand, 1988)**. Two enzymes, hyaluronidase and acrosin, have

been shown to be associated with the acrosomal matrix (**de Vries *et al.*, 1985**), and are thought to mediate in processes leading to fertilisation but their exact function remain obscure.

1.3.1.10.1 Hyaluronidase

Hyaluronate 3-glycanohydrolase, commonly known as hyaluronidase, refers to an enzyme that causes hydrolysis and depolymerization of hyaluronic acid. Its predominant substrate is hyaluronic acid and, to a lesser extent, chondroitin sulphates. Hyaluronic acid, a natural substrate of hyaluronidase, is present in the extracellular spaces of cumulus mass and corona radiata. Hyaluronidase can disperse the follicle cell layer from oocytes of the mouse, the rat, the hamster and the cumulus mass of the rabbit (**Joyce *et al.*, 1986**). This may enable the motile spermatozoon to pass through the cumulus mass. Women with unexplained infertility and infertile rabbits immunised with spermatozoa were reported to possess serum antibodies against hyaluronidase (**Metz *et al.*, 1972**). Non-human hyaluronidase has been purified by a number of investigators from male genital tract sources, including testis, spermatozoa and seminal plasma (**Joyce *et al.*, 1985**).

All hyaluronidase found in seminal plasma is sperm-derived, and none is secreted by the accessory sex glands (**Chang, 1947; Masaki and Hartee, 1962**). The spermatozoon hyaluronidase is identical to that found in the testis, but appears to differ from the lysosomal hyaluronidase of non-genital tract tissues and cells.

The purified enzyme from bull spermatozoa has a molecular weight of 60-65 kDa, is optically active at acidic pH, and requires certain cations for activity (**Zaneveld *et al.*, 1973**). There is only a brief communication regarding the purification of hyaluronidase from human testicles (**Erickson and Martin, 1978**). This is because human testicular hyaluronidase has been difficult to extract from frozen semen samples as it

is very unstable under the usual semen preservation conditions (-196^{o}C). The spermatozoon hyaluronidase is rapidly inactivated upon release either spontaneously or on extraction, and a large reduction in hyaluronidase activity occurs when human spermatozoa are stored under various conditions (**Joyce *et al.*, 1985**).

Spermatozoa of most mammalian species have been shown to traverse the cumulus matrix and corona radiata without undergoing the AR (**Talbot, 1985**). Furthermore, capacitated spermatozoa of other species which are known to be lacking in hyaluronidase such as those from sea urchin, frog (**Talbot, 1985**) and rooster (**Yanagimachi, 1988**) penetrate the mammalian cumulus complex without difficulty. This data indicate that the acrosomal hyaluronidase might not be essential for cumulus penetration, but could facilitate this process (**Talbot, 1985**). In keeping with this premise, the immunisation with hyaluronidase does not result in a significant reduction of fertility (**Morton and McAnulty, 1979**).

1.3.1.10.2 Acrosin

The best studied proteolytic enzyme from male reproductive tract fluids is the acrosomal trypsin-like protease, acrosin (**Polakoski and Zaneveld, 1976**). Acrosin is isolated in the sperm acrosome as an inactive precursor, pro-acrosin with a molecular mass of 53-55 kDa which appears first in early spermatids and increases in concentration during further spermatid differentiation (**Mansouri *et al.*, 1983**). In freshly ejaculated intact spermatozoa, pro-acrosin is highly stable. Under conditions where pro-acrosin auto-activation is expected to be negligible, such as acidic pH and in the presence of proteinase inhibitors, no preformed acrosin activity could be detected (**Cechova *et al.*, 1988**). The purified zymogen auto-activates at pH 8.0 and this activation is accelerated by either trypsin or acrosin at an enzyme:zymogen ratio of 1:20 (**Garner and Cullison, 1974**).

The biological function of acrosin as a zona penetrating enzyme has been controversial in the past. However, in the last few years new information concerning structure and properties has led to a better understanding of its special role in fertilisation (**Topfer-Petersen *et al.*, 1990**). In rabbits, antibodies formed in response to systematic immunisation with acrosin were found only in low amounts in oviductal fluids (**Syner *et al.*, 1979; De Ioannes *et al.*, 1990**).

The 53-55 kDa pro-acrosin appears to undergo a proteolytic clip in the N-terminal region leading to the active α-acrosin (**Cechova *et al.*, 1988**) and additionally in the C-terminal region (**Fock-Nuzel *et al.*, 1984**) leading to a 38 kDa active β-acrosin, which seems to be the most stable form of the enzyme. The processing of 53-55 kDa α-acrosin to the mature 38 kDa β-acrosin results in the liberation of an extremely hydrophobic polypeptide of approximately 85 amino acids from the C-terminal region.

Acrosin is a multifunctional enzyme combining several functional properties within a single molecule: 1) the catalytic triad of the proteinase; 2) hydrophobic domains responsible for the special acrosome membrane-associating character of the enzyme and 3) the carbohydrate binding sites by which the molecule can bind to the ZP. Acrosin with its unique structural and functional properties, may be instrumental in all three stages of ZP penetration i.e. binding (via its hydrophobic domains), digestion (pro-acrosin /acrosin activation) and sperm release (**Topfer-Petersen *et al.*, 1990**).

1.3.1.10.3 Other acrosomal enzymes and components

A number of investigators have studied the biochemical requirements and events involved in the AR (reviews **Meizel 1985, Yanagimachi 1981**). It is generally accepted that calcium ion influx is important for the induction of the AR, and that calcium ionophores such as A23187 enhance the reaction. Phospholipase A, an

acrosomal membrane enzyme together with the products of its hydrolysis of membrane phospholipids (lysophospholipids and arachidonic acid) are suggested to be involved in the AR in the hamster and the guinea pig (**Ohzu and Yanagimachi 1982**). Arachidonic acid is an unsaturated fatty acid that can be metabolised by the enzyme cyclooxygenase (prostaglandin synthetase), ultimately forming prostaglandins and thromboxanes. Evidence indicate that the fertilizing capacity of spermatozoa *in vivo* can be hindered by administration of cyclooxygenase inhibitors to female mice and rabbits (**Lau *et al.*, 1973; Lau and Saxena 1979**). These compounds (prostaglandins), can alter the biochemical activity of somatic cell membranes by acting as calcium ionophores or binding site regulators and have a role in exocytotic events (**Hall and Behrman 1982**). For these reasons, it has been hypothesised (**Joyce *et al.*, 1987**) that the metabolism of arachidonic acid by spermatozoa is important for fertilisation and, more specifically, for the AR.

1.3.1.11 β-1,4-Galactosyl-transferase

The enzyme β-1,4-Galactosyltransferase (GalTase) has been viewed traditionally as a biosynthetic component of the Golgi complex. However, it is found also on the surface of many cells where it can bind its specific glycoside substrate on adjacent surfaces or the extracellular matrix (**Miller *et al.*, 1992**). In the mouse spermatozoon, GalTase has been reported to initiate fertilisation by binding to terminal N-acetylglucosamine residues on the ZP glycoprotein ZP3 one of the three glycoproteins found in the egg zona pellucida (see below and **Miller *et al.*, 1992; Bleil and Wassarman, 1980b**). It is suggested that, after initial binding, ZP3 aggregates a receptor (probably GalTase) which activates G-protein-dependent exocytosis of the sperm AR (**Florman *et al.*, 1984; Macek *et al.*, 1991**). The acrosome contents consequently digest a penetration slit on the zona matrix through which sperm reach the egg. Following the AR, GalTase is retained on spermatozoa by redistribution to the lateral

surface of sperm head (**Lopez and Shur, 1987**). The function of GalTase in this new position remains unclear (**Miller *et al.*, 1993**). Since spermatozoa retain GalTase during the AR, rather than release it with its associated plasma membrane, it is probable that GalTase serves some function during or after the AR (**Lopez and Shur, 1987**). The scenario presented currently by Shur's group is that GalTase binds ZP3 initiating primary binding; this leads to aggregation of GalTase and induction of the AR (**Macek *et al.*, 1991**). During the AR, the sperm remains associated with the zona matrix by redistributing GalTase to the lateral sperm surface, still complexed with ZP3 oligosaccharides. This occurs so rapidly that the acrosomal contents are exposed without releasing the spermatozoon from the zona surface. Hence, the fully acrosome-reacted spermatozoon adheres to the ZP by its lateral surface, where it engages in secondary binding to other zona glycoproteins (ZP2) using previously identified sperm components (**Jones *et al.*, 1988; Lanthrop *et al.*, 1990**). At this point, zona penetration begins. Acrosomal β-N-acetylglucosaminidase may be necessary to prevent GalTase from re-associating with exposed N-acetylglucosamine residues, which would impede either the initiation or progression of zona penetration.

1.3.2 Oocyte antigens

1.3.2.1 Zona pellucida antigens

Mammalian oocytes are surrounded by a unique extracellular matrix, the zona pellucida. ZP is synthesised by the oocytes during the early stages of ovarian follicular development and is secreted to form an extracellular matrix that surrounds the oocytes, the ovulated egg and the pre-implantation embryo. The ZP mediates species specific events at fertilisation, provides a major block to polyspermy, and protects the growing embryo as it passes down the oviduct prior to implantation (**Yanagimachi 1988; Wassarman, 1988**). The mouse ZP has been well

characterised and is known to consist of three sulphated glycoproteins ZP1, ZP2 and ZP3 with molecular mass of 200, 120 and 83 kDa respectively (**Bleil and Wassarman, 1980a,b; Shimizu *et al.*, 1993**). ZP proteins are synthesised coordinately in the growing oocyte and secreted to form a filamentous matrix in which dimers of ZP2 and ZP3 are cross-linked by ZP1, giving the structural appearance of "beads on a string" (**Greve *et al.*, 1982, Shimizu *et al.*, 1993**). Unlike the testis, the ovary is not an immunologically privileged organ, and all cell types are exposed to a full range of serum components, including antibodies. Consequently, antibodies against ZP glycoproteins may play a role in infertility. Clinical data indicate that antibodies to ovarian antigens, including those of the ZP, may be responsible for a significant proportion of these cases and for associated infertility problems (**Shivers 1975; Damewood *et al.*, 1986; Mignot *et al.*, 1989**). Of the three zona glycoproteins, ZP3 is the best characterised. It is synthesised as a 44 kDa polypeptide chain, to which several asparagine linked (N-linked) and serine-threonine saccharides (O-linked) are attached covalently. A cDNA encoding mouse (**Ringuette *et al.*, 1988**), and human (**Chamberlin and Dean, 1990**) ZP3 has been cloned.

1.3.2.2 Immunisation with zona pellucida proteins

Antibodies raised against cumulus free mouse ova (**Tsunoda, 1977**), and isolated mouse zona pellucida (**Tsunoda and Chang, 1976; Henderson *et al.*, 1988**), were found to block fertilisation. The presence of unique antigenic determinants on the outermost surface of the ZP appears to be associated with the presence of a high concentration of carbohydrate residues (**Oikawa *et al.*, 1975**). Chemical removal of these oligosaccharides completely destroys the ability of mouse zona glycoprotein ZP3 to serve as a sperm receptor, while the free carbohydrate side chains exhibit sperm receptor activity (**Wassarman, 1988**).

The contraceptive potential of vaccines based on cross-reactive porcine zona antigens has been supported by active immunisation experiments involving a number of different primate species (**Sacco *et al.*, 1983; Gulyas *et al.*, 1983; Henderson *et al.*, 1988**). The porcine ZP glycoprotein families ZP1, ZP2 and ZP3, has been shown to exhibit marked microheterogeneity associated with the deferential glycosylation of the peptide cores (**Hedrick and Wardrip, 1981**). Removal of these carbohydrate side chains with trifluoromethyl sulphonic acid (TFMS), generates five backbone polypeptides with molecular masses of approximately 66, 52, 36, 32, and 16 kDa. The molecules at 32 and 36 kDa represent the predominant core polypeptides of ZP3 (**Henderson *et al.*, 1987**). These purified deglycosylated zona polypeptides have been used to raise polyclonal antibodies which have been assessed subsequently for their ability to interfere with sperm-zona interaction *in vitro* and *in vivo*. Polyclonal antisera against all core peptides disrupt sperm-zona interactions although the most profound inhibition occur with the anti-32 kDa preparations. Results from active immunisation study with the deglycosylated 32 kDa ZP protein (DGZP-32) using a primate model, the common marmoset, were poor (**Aitken *et al.*, 1990**). Three out of four experimental animals developed low antibody titres, and in the three animals, induction of immunity was associated with a reversible disruption of ovarian cylicity. Such sporadic disruption of ovarian function has now been observed in a wide variety of primates species including the baboons (**Dunbar *et al.*, 1989**).

Although initial results of immunisation with zona pellucida appeared attractive in terms of contraception, recent studies have indicated that ovarian dysfunction occurs due to oophoritis (inflammation of the ovary). Unless zona antigens can be modified to avoid this complication, it is unlikely that they can be used for human contraception.

1.4 VASECTOMY AND SPERM ANTIBODIES

At puberty, new sperm antigens make their appearance on the sperm surface during spermatogenesis. At this time, the spermatozoon is isolated within the epithelium and lumen of the seminiferous tubule by the blood testis barrier. The latter is formed by the tight junctions between adjacent Sertoli cells and isolates the developing germ cells from the immune system. The adult is not tolerant therefore to sperm antigens **(Dym and Caviacchia, 1977)**.

Humoral sperm-reactive antibodies have been detected in about 50% of men who have undergone vasectomy (**Shulman *et al.*, 1972**). The presence of these antibodies within reproductive tract secretions, noted at the time of vasovasostomy, has been correlated with an impaired chance of subsequent infertility (**Linnet and Hjort, 1981**). There is evidence also that at least some of the antibodies present in sera of auto-immune men are directed against antigens intrinsic to sperm rather than those derived at ejaculation (**Bronson *et al.*, 1984**).

Anti-sperm antibodies are related to, rather than being an absolute cause, of infertility and hence they reduce but not always totally prevent the likely occurrence of pregnancy. Individuals sensitised to spermatozoa show wide variations in reproductive failure. First, serum concentrations of these antibodies vary between men and women. Second, these antibodies may be both transudates from blood or secretions of sub-mucosal plasma cells within the reproductive tract (oviductal, uterine, or cervical-vaginal: **Uehling, 1971**; **Murdoch *et al.*, 1982**).

The effect of anti-sperm antibodies on fertility after vasectomy reversal is still controversial. Recent evidence suggests that the presence of anti-sperm antibody in

serum (IgG) or seminal plasma (IgA) of vasovasostomised men does not rule out the possibility of inducing pregnancy; on the other hand , if pregnancy is not induced, the explanation may be the presence of such antibodies (**Meinertz *et al.*, 1990**). The isotype of these antibodies, as well as their specificity to the sperm surface and their presence within the reproductive tract, must be determined for each individual. In the near future, it should be possible to identify the antigens reactive to these antibodies.

1.5 TOWARDS THE DEVELOPMENT OF A MALE CONTRACEPTIVE VACCINE

1.5.1 General Considerations

The development of vaccines based on sperm antigens represent a novel approach to contraception. The feasibility of this approach is based on two lines of evidence. The first type of evidence come from numerous studies of involuntary infertility in humans due to the presence of anti-sperm antibodies present in men and women (**Menge, 1980**). This involvement of circulating antibodies in infertility has been known for over 30 years (**Isojima, 1969**). The second line of evidence comes from immunisation studies of male and female animals (from various species), using extracts of spermatozoa or testis. Immunisation has been shown to result in significant reduction of fertility, by affecting sperm transport and function, fertilization, and even embryo failure and/or pre-implantation embryo mortality (**Menge, 1970; Alexander *et al.*, 1990**). As previously discussed, a number of immunogenic sperm-specific antigens have been identified and extensively characterised. It may be possible now to mass produce these antigens by recombinant genetic technology for use in contraceptive trials. But knowledge of the complexities

of the immune system and the manipulation of the immune response especially in the reproductive tissues, lags behind considerably (**Anderson, 1986**).

Two considerations must be made in using sperm-specific antigens for the development of immunocontraceptive vaccines. First is the efficacy and second is its reversibility. The general consensus is that a vaccine which is less than 90% effective would not be acceptable (**Anderson *et al.*, 1987**). For sperm antigens, [other than the study of **Primakoff *et al* (1988b)** which has not been confimed with further trials], it can be argued that purified or semi-purified preparations are not wholly effective in inhibiting fertility. Whole spermatozoa as immunogens are usually more effective but cannot be used for the development of an anti-sperm vaccine (in humans at least) due to two main factors: (i) A number of antigens of germ cells are also present on somatic cells. Antibodies against such components might produce auto-immune conditions detrimental to health; (ii) Intact spermatozoa may not result in antibodies against surface proteins that are weakly immunogenic, but are involved specifically in fertilization. For instance, immunisation of mice with epididymal spermatozoa leads to the formation of anti-sperm antibodies against various antigens, but not against the weak immunogens like LDH-C4 (**Tung *et al.*, 1979**).

At the biochemical level, it has been very difficult to isolate, characterise and produce in quantities individual sperm surface antigens for use in a monospecific vaccine (**Goldberg *et al.*, 1981b**). Promising, but often very confusing, results have been obtained primarily because of the ill-defined nature of antigen preparations. Therefore, there is an acute need for readily available, well characterised sperm molecules that would overcome these problems and could be used for an extensive programme of research and development of immunocontraceptive vaccines.

1.5.2 Recombinant DNA approach

The introduction of recombinant DNA technology over the last decade has allowed rapid progress in the analysis of gene structure and function, and in the production of potentially useful polypeptides in bacteria *Escherichia coli* and other vectors. In many cases, the successful strategy toward isolating a particular eukaryotic gene has been first to isolate a DNA copy of the messenger RNA encoded by that gene (a cDNA clone). The recombinant DNA is generated by a series of steps, starting with the synthesis of cDNA, ligation into a vector, and transformation of bacterial cells. A cDNA library representing the mRNA population is constructed using polyadenylated RNA extract from the appropriate tissue or cell type. Double-stranded cDNAs prepared from a complex poly $(A)^+$ RNA populations are ligated into λ-derived vectors (λ-gt10, λ-gt11, λ-ZAP), depending on which one is appropriate for subsequent experimental procedure to be followed. Only when the library DNA is in bacterial cells as a set of independent and replicon-competent transformants can effective size of the library be estimated. The cDNA clone of interest is then identified within the population of cDNA clones by screening the library with synthetic oligonucleotide probes, cDNA probes representing differentially expressed mRNAs, or an antibody probe (**Huynh *et al.*, 1985**). Often the experimentally limiting step had been lack of a suitable probes (nucleic acid or antibody), with which to screen for those clones containing relevant genes. Various methods have been developed to detect antigens produced by recombinant phage (**Hanahan and Meselson, 1980**). Several immunological approaches to screening for cDNA clones are now possible and isolation of protein-encoding genes from large recombinant DNA libraries can be achieved routinely, using antibodies to detect antigens produced by specific recombinants (**Young and Davis, 1983a**)

Antibodies directed against specific antigens either polyclonal or monoclonal have been used in the past to screen particular expression cDNA library, in bacterial or mammalian cell hosts. The gene encoding antigens such as SP-10, LDH-C4 and MSA-63, have all been cloned and their sequence determined in this way (**Wright *et al.*, 1990; Millan *et al.*, 1987; Liu *et al.*, 1990**).

The production of recombinant antigens representing spermatozoa proteins would be advantageous for two main reasons. From cellular stand-point, expression of these proteins, usually membrane antigens would allow further definition of their roles in physiology. At biochemical level, molecular biology would allow analysis of structure-function relationship of specific antigens (**Sutcliffe *et al.*, 1990; Chin, 1990**). The effects of recombinant antigens either as extracelluar proteins or as surface antigens on sperm-egg interaction may also be evaluated (**Moore *at al.*, 1993**). On the other hand, antibodies could be produced against synthetic oligopeptides corresponding to the deduced amino acid sequences of a given antigen. Thus, the application of molecular biology requires a full understanding of the biochemical, biological, and immunological characteristics of the specific antigens.

1.6 AIMS OF THE STUDY

The primary concern of this project is to develop a strategy that utilises a combination of immunological, biochemical, physiological and the recombinant DNA techniques that would identify and characterise sperm-specific vaccine candidate(s). In addition, since there is an urgent need for non-human primate model for contraceptive vaccine development, a broader concern of the work was to use the baboon tissue to ensure that sperm-specific molecules would be isolated and that would

be candidate antigens for both species.

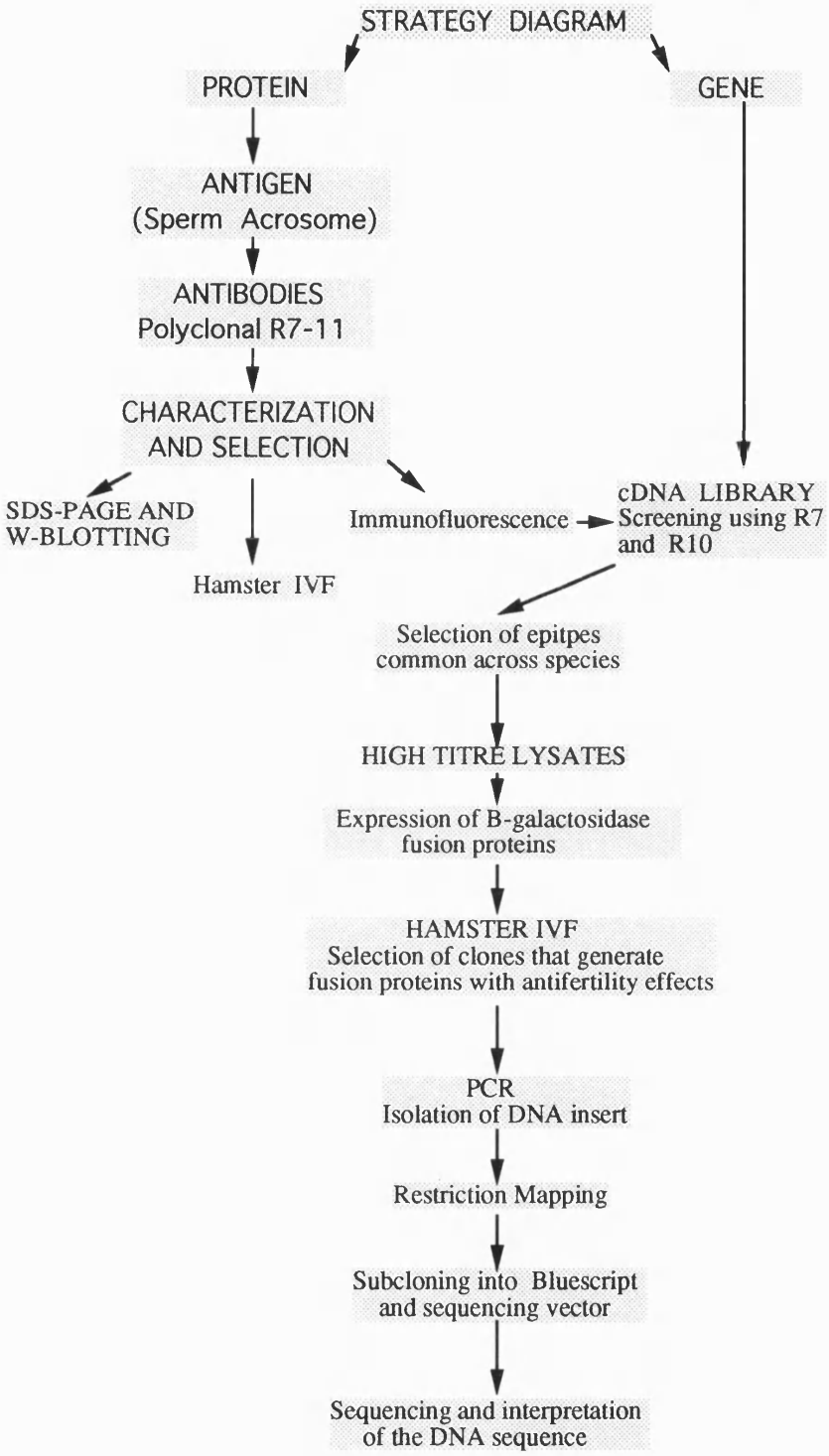
The baboon is an appropriate non-human primate model for contraceptive vaccine development and testing because:- (1) It is an old world primate and therefore more closely related to man than some other monkey (e.g South American marmoset). (2) A breeding colony exists in the home laboratory of the investigator (Institute of Primate Research, Nairobi, Kenya). (3) It is a large monkey and therefore surgical manipulations of the reproductive tracts can be easily performed.

The specific aims of the study are given below and the work strategy presented in Fig. 1.1

1. To use various immunological, biochemical and *in vitro* fertilization assays to characterise a battery of rabbit polyclonal antisera raised against purified hamster acrosomes.
2. To select appropriate polyclonal sera and use them as immunological probes to screen a human testis cDNA library.
3. To characterise the selected clones using molecular biological methods and undertake additional *in vitro* fertilization assays to confirm that the selected clones were relevant to contraceptive vaccine development.
4. To determine the nucleotide sequence of selected clone(s), analyze the information and identify the gene(s) involved.
5. To express fusion proteins of the chosen clones and to test in an *in vitro* fertilization assay.

FIGURE 1.2

FIGURE 1.2 STRATEGY OF RESEARCH


STRATEGY DIAGRAM
PROTEIN
GENE
ANTIGEN
(Sperm Acrosome)
ANTIBODIES
Polyclonal R7-11
CHARACTERIZATION
AND SELECTION
SDS-PAGE AND
W-BLOTTING
Hamster IVF
Immunofluorescence
cDNA LIBRARY
Screening using R7
and R10
Selection of epitpes
common across species
HIGH TITRE LYSATES
Expression of B-galactosidase
fusion proteins
HAMSTER IVF
Selection of clones that generate
fusion proteins with antifertility effects
PCR
Isolation of DNA insert
Restriction Mapping
Subcloning into Bluescript
and sequencing vector
Sequencing and interpretation
of the DNA sequence

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Baboons were kept at the Institute of Primate Research, Nairobi, Kenya. Testes and excurrent ducts (epididymides and vas deferens) were obtained by hemi-castration and stored in liquid nitrogen for transport to U.K. Syrian hamsters were obtained from Bantim and Kingman Ltd. (England) and were maintained at the Institute of Zoology on a 12:12 hours, light:dark cycle. Tissue samples were obtained after the animals were killed by overdose of sodium pentobarbital (Euthatal), May and Baker, Dagenham, U.K.

2.1.2 Bacteria

E. coli strain Y1090, lacU169, proA+, lon, araD139, strA, supF (trpC22::Tn10) (pMC9). This strain contains high levels of *lac* repressor, is deficient in *lon* protease and will modify but not restrict DNA. The cells were maintained on LB agar plates containing ampicillin (50 μg/ml) at 4°C and then used for plating and replication of λ gt11 phage genomes. *E. coli* strain JM101, lacpro, thi, supE, F'traD36, proAB, $lacI^{q}z$. The stock bacteria were grown at 37°C and maintained on minimal media plates at 4 °C and then used for the growth and replication of plasmids (bluescript SK and M13 mp 18 sequencing vector).

2.1.3 Vectors

Adult human testis λgt11 (*lac5 nin5 cI857 S100)* cDNA library was obtained from Cambridge Bioscience, Cambridge U.K. Bluescript® SK(+) was a gift from Dr Alison

Moore, Institute of Zoology. Ribonuclease A, M13 mp18 and M13 mp19 replicative form were obtained from Boehringer Mannheim Corporation Ltd., U.K.

2.1.4 Enzymes

All restriction endonucleases, T4 DNA ligase, Calf intestinal alkaline phosphatase (CIAP), Klenow fragment DNA polymerase, Proteinase K, Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), buffers were obtained from Northumbria Biochemicals Ltd (NBL) U.K.

2.1.5 Radiochemicals

Radiochemicals were purchased from New England Nuclear Inc., Boston, U.S.A. These were [α-^{32}P] dCTP (3000 Ci/mmol), [α-^{35}S] dATP (800-1500 Ci/mmol).

2.1.6 Buffers, solutions and growth media

The following section gives details of general solutions used. Solutions specific to a particular method are described in the appropriate section.

- 100x Denhardt's solution consisted of 2% (w/v) polyvinyl pyrolidine 360,000, 2% (w/v) BSA and 2% (w/v) Ficoll 400,000.
- H-broth consisted of 1% (w/v) bactotryptone, 0.8% (w/v) NaCl.
- H-Agar and H-top agar consisted of H-broth containing 1.2% and 0.8% (w/v) bacto-agar respectively.
- L-broth (LB) consisted of 1% (w/v) bactotryptone, 0.5% (w/v) bacto yeast extract, 1% NaCl in H_2O. The pH was adjusted to pH 7.2 with NaOH.
- L-broth containing ampicillin (50 μg/ml in water, sterilised by passage through a

0.22 μm Nalgene filter)

- LB-agar and LB-agarose consisted of LB containing 1.5% (w/v) bacto-agar and 0.7% (w/v) Ultrapure™ agarose respectively.

- M9 salts consisted of 0.6% (w/v) $NaHPO_4$, 0.3% (w/v) KH_2PO_4, 0.1% (w/v) NH_4Cl and 0.05% (w/v) NaCl.

- Minimal media plates were prepared by dissolving 1.5% (w/v) bacto-agar in M9 salts.

- Oligolabelling buffer (OLB) was made up from solutions **O, A, B,** and **C** as follows. Solution **O**: (1.25 M Tris-HCl pH 8.0, 0.125 M $MgCl_2$), **A**: (solution **O** [1 ml], β-mercaptoethanol [18 μl] dNTPs A, T, G [5 μl of each at 0.1 M]), **B**: 2 M Hepes made to pH 6.6 with sodium hydroxide and kept at 4°C. **C**: Hexanucleotides (Pharmacia Cat. No. 27-2166-01) 50 O.D> units in 550 μl of TE. Working OLB solution was made by mixing solutions **A, B,** and **C** in the ratio 10:25:15 (enough for 15 reactions) and was stored at -20°C in 50 μl aliquots.

- Phosphate buffered saline (PBS) pH 7.2 contained 135 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 15 mM KH_2PO_4 in H_2O.

- Pre-hybridisation buffer [0.01 M EDTA, 6x SSC, 5x Denhardt's, 0.1% SDS, 10% (w/v) dextran sulphate and 100 μg/ml salmon sperm DNA (denatured by heating to 100°C for 5 minutes)].

- 20x SSC consisted of 3 M NaCl and 300 mM trisodium citrate.

- TE (pH 7.5) consisted of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0).

- Tris-borate electrophoresis buffer (TBE) consisted of 100 mM Tris-HCl pH 8.3, containing 100 mM boric acid, and 2 mM EDTA.

- Tris-acetate electrophoresis buffer (TAE) consisted of 40 mM Tris-HCl, containing 1 mM EDTA (adjusted to pH 8.0 with glacial acetic acid).

- 2TY consisted of 1.6% w/v Tryptone, 1.0% w/v yeast and 5% w/v sodium chloride.

- All solutions were sterilised by autoclaving at 15 lb/sq.in. on a liquid cycle unless otherwise stated.

2.1.7 Oligonucleotides

Complementary pairs of oligonucleotides consisted of λgt11 sequences were synthesised on an Applied Biosystems model 381A oligonucleotide synthesiser. λ gt11 oligonucleotides were synthesised with terminal *Eco RI* sites to facilitate cloning.

Sequence of primers used for DNA sequencing and PCR:

λgt11 1 5' **GAA TTC** GGT GGC GAC GAC TCC TGG 3'

λgt11 2 5' **GAA TTC** CAG ACC AAC TGG TAA TGG 3'

(bold denotes *Eco*R1 restriction sequences)

2.1.8 Other materials and reagents

- Bacterial growth media were obtained from Difco Laboratories, U.K.
- B.C.A. Protein Assay Kit - Pierce, Chester, U.K.
- CSS/Statistica, computer software was obtained from Statsoft, Letchworth. U.K.
- All deoxyribonucleotides and ribonucleotides were obtained from Pharmacia LKB U.K.
- GeneAmp PCR reagent kit was obtained from Perkin Elmer Cetus.
- Geneclean™II Kit, Mermaid™Kit were obtained from Stratech Scientific, Luton, Beds. U.K.
- 0.22 μm and 0.45 μm Nalgene filters - Millipore, U.K.
- Nylon and nitrocellulose membranes (Hybond™N or C) were obtained from

Amersham International, U.K.

- Polaroid 667 film was obtained from Polaroid, U.K.

- UltraPURE™ LMP agarose was obtained from BRL, U.K.

- Sequagel™concentrate, Sequagel™diluent and Protogel™ acrylamide,

- Protogel™diluent were obtained from National Diagnostics, Bucks. U.K.

-Sequenase™kits,United States Biochemicals, obtained through Cambridge Biosciences U.K.

- Swine anti-rabbit and rabbit peroxidase anti-peroxidase immunoglobulins - Dako Ltd, Bucks, U.K.

- X-ray film (OMAT), X-ray film fixer (FX-40), and developer (LX-24), were obtained from Kodak U.K.

- All other chemicals, solvents and materials were obtained from one of the following: Sigma Chemical Company, Labsystems, Scotlab, Phillips & Harris, Stratagene, British Drug House (BDH) or Fisons Laboratories.

2.1.8.1 Equilibration of phenol

Phenol was equilibrated before use as described by **Sambrook *et al.*, (1989)**. Briefly, phenol was melted at 65°C and an equal volume of 0.5 MTris-HClpH 8.0 added. Hydroquinoline was added to a final concentration of 0.1% (w/v). The two phases were allowed to mix, shaking thoroughly by agitation. When the two phases had been separated, the upper (aqueous) phase was aspirated and discarded. This process was repeated two or three times until the pH of the phenol was greater than 7.8. After the final aqueous phase had been removed, 0.1 volumes of 0.1 MTris-HCl pH 8.0 containing 0.2% v/v β-mercaptoethanol was added to the equilibrated phenol. This was stored at 4°C in the dark (covered in aluminium foil) for up to 4 weeks before use. Equal parts phenol and chloroform:isoamyl alcohol (24:1) were used to extract nucleic acid preparations.

2.1.8.2 Salt and ethanol precipitation of DNA

The DNA isolated was precipitated using 2.5 M ammonium acetate and 50% (v/v) ethanol for 30 minutes at -70°C. The precipitate was pelleted at 13,000 xg in a microfuge and resuspended in 7.5 mM sodium acetate and 75% (v/v) ethanol and incubated at -70°C for a further 30 minutes. The resulting DNA was pelleted, dried for 10 minutes at room temperature after which the pellet was resuspended in 15 µl TE. The amount and size of DNA generated was determined by resolving 5 µl of this preparation on 0.8% TBE agarose gel against known molecular weight markers.

2.2 METHODS

2.2.1 Isolation of hamster sperm acrosomes

Syrian hamsters were killed with an intraperitoneal injection of Euthatal (May and Baker) the cauda epididymides placed in RPMI 1640 (Gibco/BRL) media pre-warmed to 37°C. The tissue was incubated at 37°C, in 5% CO_2 in air for 30 minutes to allow sperm to swim into the medium. The sperm supernatant was then removed and made hypotonic by dilution 3:1 with pre-chilled (4°C) sterile double distilled water and allowed to stand on ice for 15 minutes. Under these conditions, the acrosomes on the hamster sperm head loosen and become detached. The suspension was vortexed vigorously and the degree of acrosome removal monitored under the microscope. The final suspension in 4 ml (acrosomes, sperm without acrosomes and intact sperm with loose acrosomes), was carefully layered on top of a 20 ml discontinuous Percoll gradient consisting of (10%, 20%, 30% and 40% v/v) in RPMI medium. The gradient was centrifuged at 2000 g (Beckman TJ6 centrifuge) for 10 minutes at room temperature after which the bands resolved at the interface were aspirated into fresh tubes and the contents examined for the presence of acrosomes

by light microscope. The acrosome rich fraction was selected and further diluted 1:12 in RPMI 1640 and pelleted at 8,000 g for 10 minutes (Beckman J2-21 centrifuge with JA 20 rotor) in order to harvest pure acrosomes (>99%). The pellet was resuspended in 1/10 of volume (400 µl) RPMI 1640 and stored at -20°C until required.

2.2.2 Extraction of protein from testis, epididymal tissue and spermatozoa

2.2.2.1 Testis and epididymal tissues

Human, chimpanzee, baboon and hamster epididymides were dissected from the testes and spermatozoa flushed out. Each of the testes and epididymides were weighed and 10 volumes of lysis buffer (134 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2.6H_2O$, 5 mM glucose, 5 mM Hepes, 1% SDS) added. The tissues were first cut into pieces and then homogenised in a pestle and mortar homogeniser. The tissue homogenate was incubated for 30 minutes at 37°C and then sonicated using a laboratory mixer emulsifier (Silverson Mechanics Ltd. Bucks, U.K) for 1-2 minutes at medium power. Cellular debris was removed by centrifugation for 10 minutes at 4,000 g at room temperature. The protein concentration of the supernatant was determined as given in Section 2.2.3. The concentration was found to be in the range of 2.4-5.8 mg of protein/ml for the various tissues and all the aliquots were adjusted to 2.0 mg/ml. 200 µl aliquots were stored at -20°C until required.

2.2.2.2 Spermatozoa proteins

Hamster epididymal spermatozoa was obtained from the excised cauda epididymidis as described above. Human spermatozoa was obtained from adult donors by ejaculation. The spermatozoa were allowed to swim up into BWW medium overlayed on the semen for 30 minutes at 37°C and recovered by centrifugation at 2,000 g for

10 minutes. The pellet was resuspended in lysis buffer (see Section 2.2.2..1). Purified hamster sperm acrosomes (100 µl) in RPMI 1640, prepared as given in Section 2.2.1 were mixed with (1 µl) lysis buffer. The spermatozoa and acrosome suspension in lysis buffer were sonicated as described in the preceding section and freeze-thawed 3x in liquid nitrogen. The suspension was centrifuged at 4000 g for 10 minutes at room temperature. The supernatant was recovered and protein concentration determined using a BCA protein assay as described in Section 2.2.3. The protein concentration was found to be in the range 3.5-5.0 mg/ml and was adjusted to 3.0 mg/l. Aliquots (250 µl) were stored at -20°C until required.

2.2.3 Determination of protein concentration in tissue, spermatozoa and acrosomal protein extracts

Protein extracts from tissues, spermatozoa and sperm-acrosomes was diluted 1:10, 1:50 and 1:100 in distilled water and duplicates (20 µl) from each dilution was used in protein assay. The protein concentration was assessed using BCA protein assay kit (Pierce Chemical Ltd), a modification of **Lowry *et al.*, (1951)**, according to manufacturer's instructions.

2.2.4 Deglycosylation of hamster acrosomes

The carbohydrate moiety was cleaved from hamster sperm acrosomes by using the enzyme Endoglycosidase F/N-Glycosidase F (Boehringer Mannheim, W. Germany). Samples, containing 40 µg of acrosomal membrane proteins were microfuged at 13,000 g for 10 minutes The pellet was resuspended in 50 µl Na/K buffer (134 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2.6H_2O$, 5 mM Glucose, 5 mM Hepes), and digested with 100 mU of Endoglycosidase F for 16 h at 37°C. The digested

acrosomes were pelleted at 13,000 g and resuspended in 20 µl Na/K buffer and stored at -20°C before being subjected to reducing 10% SDS-polyacrylamide gel electrophoresis.

2.2.5 Production of polyclonal antisera

Rabbit anti-hamster sperm acrosome polyclonal sera R7, R8, R9, R10 and R11 were kindly given to me by Dr Alison Moore of the Institute of Zoology. The antisera were obtained by immunizing rabbits with highly enriched hamster sperm acrosome fractions treated with various detergents and other deglycosylating agents. This was done to provide a broad spectrum of epitope recognition.

Rabbits were immunised as follows:

Rabbit	Acrosome treatment
7.	1 % NP40 + 8 M urea and 1 % β-mercaptoethanol
8.	1 % SDS + 1 % β-mercaptoethanol, boiling.
9.	Native acrosome (in water to form isotonic lysate).
10 /11.	trifluoromethyl sulphonic acid (TFMS) treatment to remove O-linked sugars, followed by a combination of TFMS and endoglysosidase-F, to remove both the O- and N-linked sugars.

2.2.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated on vertical SDS polyacrylamide slab gels employing the discontinuous buffer system based on the method of **Laemmli, 1970**. The gels were made according to the modification described by **Sambrook *et al.*, 1989** using resolving and stacking phases of 10% and 3% respectively (7.5% and 3% for fusion

proteins). The gels (17 cm wide x 12.7 cm long) were run using Gibco/BRL apparatus in Tris-glycine buffer pH 8.3 (25 mM Tris-HCL, 192 mM glycine). Typically 20-80 μg of total cellular protein was mixed with sample loading buffer (50 mM Tris-HCl pH 6.8, containing 50 mM β-mercaptoethanol, 1% (w/v) SDS, 5% (w/v) glycerol and 0.05% bromophenol blue), heated at 100°C for 4-5 minutes prior to loading onto the gel. Electrophoresis was carried out initially at 8 v/cm constant voltage until the bromophenol blue (BPB) front reached the resolving gel and then at 15 v/cm until the BPB front was approximately 1 cm from the bottom of the resolving gel. The gel was either stained with coomassie blue or transferred to nitrocellulose by electro-blotting.

2.2.7 Western blotting (Immunoblotting)

Proteins were transferred from polyacrylamide gels onto nitrocellulose (Hybond®C) membranes using a Biorad Transblot apparatus according to **Towbin *et al.*,** (1979). After transfer at 150 mA applied for 16-18 h., membranes were probed with appropriate antibody essentially as described in Amersham protocol for biotinylated antibody-strepavidin alkaline phosphatase conjugate immunodetection kit with the following modifications. The filters were incubated at room temperature for 1-2 h in blocking buffer, TBS pH 7.6 (30 mM Tris-HCl, 137 mM Sodium Chloride), containing 1% v/v Tween 20 and 5% w/v dried milk powder to block unbound sites. Blocking buffer was removed and filters washed twice for 5 minutes each in TBS/T (30 mM Tris-HCl, 137 mM Sodium Chloride containing 1% v/v Tween 20) at room temperature. The filters were then incubated for 16-18 h with primary antibody (diluted 1:100 in TBS/T., dilution required assessed empirically) at room temperature. The unbound antibody was removed by rinsing once and three times washing of the membranes in TBS. Membranes were then incubated in horseradish

peroxidase conjugate (Swine anti-Rabbit total immunoglobulins [Dako] diluted at 1:400 in TBS/T) for 1 h at room temperature. For fusion proteins, the membranes were probed with mouse anti-β-galactosidase monoclonal antibody (Sigma) at 1:1000 dilution followed by peroxidase conjugated anti-mouse antibody (Dako) at 1:400 dilution in TBS. The filters were washed for 20 minutes with 2 changes of TBS after which they were developed in 200 ml TBS containing 120 mg 4-chloro-1-naphthol (substrate for horseradish peroxidase) first dissolved in 40 ml ice-cold methanol and containing 240 µl H_2O_2 as a catalyst. The membranes were allowed to develop in this solution until positive signals were observed. The reaction was terminated by adding tap water. The membranes were then dried at room temperature and photographed and/or wrapped in aluminium foil to prevent fading.

2.2.8 Immunocytochemical localisation of germ cell antigens in testis and epididymal sections

Tissues were processed following the method of **Bunch and Saling (1991)** with the following modifications. Frozen sections were cut (14 µm thick) on a Reichert cryomicrotome (2800-FRIGOCUT, Cambridge Instruments, Germany). The slices were collected on glass slides pre-cleaned to avoid loosening of the sections during further manipulations. Three serial sections were mounted on separate slides. One slide from each tissue, was briefly air-dried, and stained in haematoxylin for 15 minutes The slides were washed by dipping them in water and incubated progressively in the following solutions:- once in $LiCO_3$ and 70% ethanol., twice in 100% ethanol and Xylene respectively. DPX mounting medium (modified, BDH), was applied on each tissue, cover-slipped and the tissue observed under microscope. A further 2 slides from each tissue were prepared and subjected to immunostaining in duplicate as described by **Bunch and Saling (1991)** with some modifications. Prior to

immunostaining, tissue sections were fixed in 2% formaldehyde (TEM grade) for 15 minutes at room temperature. Free formaldehyde groups were blocked by treatment with TBS pH 7.6 containing 50 mM glycine, for 30 min at room temperature. Washed tissues were then incubated in corresponding antisera (diluted 1:10 in TBS) for 4 h at 37°C in a moist chamber. For controls, pre-immune rabbit serum (negative control), diluted 1:10., and 18.6 monoclonal hybridoma culture supernatant (positive control, undiluted) were used. Sections were washed and incubated in FITC-conjugated anti rabbit whole immunoglobulin (IgG+A+M), (for rabbit antisera) and anti-mouse whole immunoglobulin (for the monoclonal antibody). Slides were washed to remove unbound FITC, and mounted in Citifluor (Citifluor, University of Kent., UK.). Slides were observed by UV microscope (Nikon Diaphot), fitted with epi-illumination and filters for FITC. Photographs were taken at a range of magnifications using Kodak Ektar or HP5 Ilford films.

2.2.9 Hamster *in vitro* fertilisation assay technique

2.2.9.1 Recovery of spermatozoa

Adult male hamsters (80-120 g body weight), were anaesthetized with 0.15 ml pentobarbitone sodium (Nembutal: May and Baker, Dagenham, U.K.: 60 mg/ml) administered intraperitoneally. The testes and epididymides were removed and cauda epididymides cut carefully with scissors. Hamster spermatozoa from cauda epididymides were diluted in BWW medium previously gassed with 5% CO_2 in air and supplemented with 0.04% (w/v) BSA. The concentration of spermatozoa was adjusted to 1.0×10^6/ml and 400µl drops incubated at 37°C under mineral oil.

2.2.9.2 Recovery of the oocytes

Adult female hamsters (80-120 g), were injected intraperitoneally with 40 i.u. PMSG

(Folligon, Intervet laboratories Ltd., Cambridge., U.K.) on the day of post-oestrous vaginal discharge and 56 h later with 40 i.u. of hCG intraperitoneally (Chorulon, Intervet). The cumulus mass was recovered from each oviduct 15 hours after hCG injection and placed in MEM Eagles medium, (GIBCO laboratories, Uxbridge, U.K.) and incubated at 37°C for 30 minutes in 5% CO_2 in air. The cumulus masses were transferred into BWW medium (**Biggers *et al.*, 1971**) and ova recovered by digesting them with 0.1% hyaluronidase. The ova were washed 4 times in fresh BWW before being placed in 100 µl of BWW under mineral oil. 20- 40 eggs were recovered from each super-ovulated hamster.

2.2.9.3 Effects of antisera on fertilisation outcome in a hamster IVF assay

The effect of anti-acrosome rabbit antisera on sperm-egg binding and fertilisation was tested on a hamster *in vitro* fertilisation assay was carried out following the method of **Moore and Hartman (1984)**.

2.2.9.4 Incubation of hamster oocytes with antisera or β-galactosidase fusion proteins.

Prior to incubation, the sera were heated at 56°C for 10 minutes to inactivate complement activity. A minimum of 9 ova were incubated in each 100µl BWW medium drop under mineral oil containing 5µl hamster epididymal sperm, 5µl of diluted rabbit antiserum. For controls, 5µl pre-immune rabbit serum was used. The drops were incubated at 37°C for 16-18 hours and assessment for binding and signs of fertilisation (sperm tail and decondensed sperm head in ooplasm), fusion of spermatozoa with vitellus and sperm attachment to the vitellus or zona-pellucida.

2.2.10 Screening human testis λgt11 cDNA library

2.2.10.1 Propagation of λgt11 bacteriophages in *E. coli* Y1090 host cells

E. coli strain Y1090 were employed for the growth and propagation of λgt11 bacteriophages. *E. coli* Y1090 cultures were grown as described by **Sambrook *et al.*, (1990)**. These cells are referred to as stationary phase or saturation cells.

2.2.10.2 Determination of plaque forming units per ml (pfu/ml) of the cDNA library

200 µl of stationary phase *E. coli* Y1090 cells and 3.0 ml of top agar containing 600 µM Ca/Mg, were poured on 90 mm LB-agar plates and allowed to set for 30 minutes (**Young and Davis, 1983b**). Serial dilutions of human testis λgt11 cDNA library were made from 10^{-1} to 10^{-6} in a total volume of 100µl of SM buffer (100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl pH 7.5 supplemented with 0.01% gelatin). Aliquots (5 µl) of each dilution were applied to the surface of LB-agar plates (containing a lawn of *E.coli* Y1090 cells), in a regular array. The plates were again left to set on the bench top for 30 minutes to allow bacteriophages to adsorb onto their hosts and were subsequently transferred to 42°C for overnight incubation. The plaque forming units per ml (pfu/ml) of λgt11 bacteriophage library was determined using the following formula:

$$\text{pfu/ml} = N \times 200 \times \text{dilution factor}$$

where, N is number of plaques counted for a particular dilution, 200 is the factor that converts 5 µl into 1 ml (=1000 µl divided by 5 µl) and the dilution factor = 10^{-1}, 10^{-2} up to 10^{-6}.

Using this procedure, the pfu/ml of human testis library was determined to be 8.0×10^{10}.

2.2.10.3 Primary and secondary screening (initial selection, enrichment and confirmation of positive clones)

Human testis cDNA clones in λgt11 library were plated in *E. coli* Y1090 (see Section 2.1.2.) stationary cells and incubated for 3-4 h at 42°C for lytic growth and treated as described by **Huynh *et al.* (1985)** with a few modifications. Nitrocellulose filters (Hybond® C) saturated with 10 mM IPTG (Isopropyl-β, D-thiogalactopyranoside) were then placed onto the Y1090 lawn and incubated for 3-4 h at 37°C for lysogenic growth. IPTG is a gratuitous inducer of lacZ transcription that in turn directs the expression of foreign DNA insert in λgt11. Orientation marks were made on plates by stabbing the nitrocellulose at three asymmetric points. The filter was removed, blocked for 1 h in TBS/T containing 3 % w/v BSA, rinsed twice in TBS/T, wrapped in saran wrap and stored at 4°C overnight. The duplicate nitrocellulose filter was placed on the same plate and incubated at 37°C for a further 16-18 h, blocked and rinsed as for the previous one. The two filters were incubated in primary, secondary antibody and colour developer as in the procedure for Western blotting described in Section 2.2.7. In secondary screening, filters A and B were matched, positive plaques common to both filters marked and a corresponding plug of agarose removed from the plate. This was achieved by punching off the agar corresponding to the position of positive plaques using a sterile pasteur pipette and placing in a 1.5 ml microfuge tube containing 500μl SM buffer. A few drops of chloroform were added to lyse the bacteria and then the tube was incubated for 3-4 hours at 4°C to allow recombinant λ bacteriophages to diffuse into the SM buffer. The phage from each putative positive clone was titrated on *E. coli* Y1090 host cells and pfu/ml determined as described in the preceding section. Phages were then plated at a density of 150-200 phages per 90 mm plate. All the subsequent steps were followed as in primary screen. The phages were diluted, titrated and pfu/ml determined ready for high titre lysate preparation.

2.2.11 High titre lysate

High titre lysate (amplified plaque purified λgt11 recombinant clones), were prepared by a modification of the method of **Short *et al.* (1988)**. A plug of agarose corresponding to a plaque on the plate of tertiary screen was obtained and treated as described in Section 2.2.10.3. Each positive clone of the cDNA library was amplified by plating the phages with Y1090 overnight cells at a confluence 10^5 phages/140 mm LB-agar plates containing 10 mM Mg^{+2} . After an incubation at 42°C overnight, the phages were eluted from the plates by 5 ml SM buffer/plate on a flat level for 3-4 hours to allow phage diffusion. After, the phage suspension was collected and the plates rinsed with an additional 1 ml SM buffer. The two suspensions were combined and transferred to sterile 50 ml falcon tubes and 100 µl of chloroform added to lyse the bacteria. The phages were then recovered in the supernatant by centrifuging at 4000 g for 10 minutes at room temperature. The phage titre was then assessed (see Section 2.2.10.2). An aliquot, 1 ml in cryotube, was prepared and the remainder as 1 ml aliquots in microfuge tubes. Glycerol 30% v/v was added to the cryotube which was then stored at -70°C. A drop of chloroform and DMSO 7% v/v was added to the microtubes which were stored at 4°C. The high titre lysate, ready for a series of subsequent experiments were found to be at a concentration ranging from 10^{12} to 10^{14} pfu/ml.

2.2.12 Isolation of recombinant DNA

2.2.12.1 Isolation of cloned DNA by method of λDNA preparation.

This is a method of isolating phage DNA. Phage lysate (1.5 ml) in SM buffer was made to 50 mM with respect to EDTA., 50 µg/ml proteinase-K was added and the mixture incubated at 56°C for 1 hour. Cetyltrimethylammonium bromide (CTAB), 22

µl of 5% w/v stock solution, was added to a 1:1 ml suspension to give a final concentration of 0.02% v/v which was incubated at 65°C for a further 3 minutes. The suspension was chilled on ice for 10 minutes and then centrifuged at 13,000 g in a Beckman microfuge for 10 minutes at room temperature. The pellet was resuspended in 100 µl sterile distilled water. Sodium acetate was added to a final concentration of 100 mM followed by 250 µl of absolute ethanol. The DNA was recovered from ethanol and extracted twice in phenol/chloroform/isoamylalcohol. The DNA was precipitated at -20°C for 2 h or -70°C for 30 minutes, recovered by centrifugation at 13,000 g for 10 minutes at room temperature and the pellet washed in 70% ethanol. The final pellet was resuspended in 50 µl TE. The purity of DNA product was determined by analysis on TBE-Agarose gel. λDNA was digested by 2 units of *Eco*RI at 37°C for 90 minutes to release the cDNA insert and analyzed (for size) on TBE-Agarose gel.

2.2.12.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR), is an *in vitro* biochemical reaction producing large amounts of a specific DNA fragment of a defined length and sequence from a small amount of complex template (**Saiki *et al.*, 1988**). It is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers that hybridise to the two antiparallel strands of the target DNA (template). It is necessary that the ends of the sequence be known in sufficient detail so that the oligonucleotides (primers) can be synthesised which will hybridise to them and that a small amount of the sequence (template) be available to initiate the reaction. A source of DNA including desired sequence is denatured at elevated temperature in presence of large molar excess of two primers and four deoxyribonucleoside triphosphates. The primers are complementary to different strands of the desired sequence and at relative positions along the sequence such that

the enzyme, DNA *Taq polymerase* extension product of one, when denatured, can serve as a template for the other (**Mullis and Follona, 1987**).

2.2.13 Production of double stranded DNA of cloned inserts from positive clones

Transcripts were amplified in the Techne programmable thermal cycler model number ORI Block® PHC-1 (Techne Cambridge Ltd. U.K), using Perkin Elmer protocol for DNA amplification, with slight modifications. The reaction conditions were altered depending on the target sequence. Complementary (cDNA) inserts in phage λgt11 cloning vector were amplified from crude phage suspensions catalysed by *Taq* polymerase using primers that flank the *Eco*RI insert site for the vector.

The following components were assembled in a final volume of 100 µl; 1x PCR buffer [10x buffer= 100 mMTris-HClpH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ 1% (w/v) gelatin], 200 µM each dNTP, 0.1 µ mol each of upstream and downstream λgt1 specific oligonucleotide primers and 2.0 units of AmpliTaq® DNA polymerase enzyme (Perkin Elmer Cetus. Norwalk. CT. USA), was then added. High titre phage stock (3 µl) was used as template. The entire tube contents were microfuged for 3 minutes at room temperature (21°C). The volume was brought to 100 µl by adding autoclaved ultra-filtered dH_2O and the mixture overlaid with mineral oil (Sigma Chemical Co. St Louis, MO. USA) to reduce evaporation or refluxing. The tubes were transferred to a PCR machine and amplification of the cDNA inserts achieved by using the following programme. Denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and primer extension at 72°C for 2 minutes. The cycle was repeated 29 times followed by a final reaction at 72°C for 5 minutes After amplification, the PCR products were cooled and the DNA extracted by 100µl of

high purity chloroform/isoamylalcohol (24:1 v/v). The aqueous phase (100 µl), was recovered and the size of DNA inserts from various clones determined on 0.8% agarose-TBE gel using *Hind* III cut λDNA and *Hinf* I cut pAT as size markers.

2.2.14 Expression of β-galactosidase fusion proteins

β-galactosidase fusion proteins were generated from the clones according to **Villareal Ramos (1989)**, and tested for their ability to block sperm-egg binding *in vitro* as follows:- *E. coli* Y1090 bacteria (host cells), were grown overnight (16-18 h) in L-broth containing 25 µg/ml ampicillin and 0.2% (w/v) maltose for the induction of the phage receptor. Aliquots (15µl) of bacteria in L-broth containing 10 mM Mg^{2+}, were infected with phage at a ratio of 10:1, 1:10, 1:100 and incubated at 37°C for 20 minutes to allow for phage attachment. The cultures were transferred to 42°C and incubated for 1 hour before adding isopropyl-β-D-thiogalactopyranoside (IPTG), a gratuitous inducer of lac z gene to a final concentration of 10 mM. Incubation was continued at 42°C for a further 1 hour and the cultures then transferred to 37°C for 4 hours. An aliquot (15µl) of each culture was prepared for SDS-polyacrylamide gel electrophoresis and western blotting and the remainder stored at -20°C for sperm-egg binding assay. Protease inhibitor was not included because it would interfere with the egg binding assay.

2.2.15 Plasmid DNA preparations

2.2.15.1. Small alkaline lysis plasmid preparation (Mini-prep)

Bacterial cultures, 10 ml were grown to saturation overnight according to the method of **Grosveld *et al.* (1981)**. The cells were recovered by centrifugation at 1200 g for 10 minutes in a Beckman TJ6 rotor. These were resuspended in 200 µl GTE buffer

(25 mM Tris-HCl pH 8.0 containing 50 mM glucose and 10 mM EDTA) and transferred to sterile eppendorf tubes followed by hydrolysis with 400 µl of freshly prepared 0.2 M NaOH/1% (w/v) SDS. After addition of 3 M potassium acetate, pH 4.8 (200 µl), propan-2-ol (0.6 vols.) was added to supernatant (approx 800 µl) to remove bacterial DNA and cell debris. The DNA was recovered by centrifugation at 13,000 g for 10 minutes in a microfuge. The pellet was washed in 70% v/v ethanol, dried and then resuspended in 200 µl TE. DNAse free RNAse A was prepared by heating at 90°C for 10 minutes. DNAse was added to a concentration of 20 µg/ml and incubated at 37°C for 30 minutes, followed by phenol:chloroform extractions as described in Section 2.2.1.8.1. The final aqueous phase was transferred into fresh eppendorf tubes and 1/10 vol 3 M sodium acetate, 2.5 vol. ice cold absolute ethanol added and precipitated at -70°C for 30 minutes. The DNA precipitate was collected by centrifugation in a Beckman microfuge at 13,000 g for 5 minutes at room temperature. The pellet was washed in 70% ethanol, drained, air-dried and resuspended in 50 µl TE.

2.2.15.2 Large scale PEG DNA Preparation

Bacteria were grown to saturation overnight in an orbital shaker in 500 ml L-broth containing ampicillin prepared as described by **Sambrook *et al.*, (1989)**. The bacteria (in 2 x 250 rotor buckets), were sedimented at 3000 g for 10 minutes at 4°C in a Beckman JA10 rotor and the pellet resuspended in 4 ml ice cold 50 mM Tris-HCl (pH 8.0) containing 25% (w/v) sucrose. Freshly prepared lysozyme was added to a final concentration of 1 mg/ml and incubated on ice for 15 minutes in order to compromise the cell wall of gram negative bacteria. The solution was made 10 mM with respect to EDTA and after a further 15 minute incubation on ice, 0.5 volumes of Triton lysis buffer (150 mM Tris-HCl pH 8.0 containing 375 mM EDTA,3% (v/v) Triton X-100) was added to make the bacterial cell membrane leaky and allow release of plasmid

DNA whilst retaining the bacterial genomic DNA. After incubation for a further 30 minutes on ice, the bacterial DNA was pelleted by centrifugation at 18,000 rpm for 60 minutes in a Beckman JA20 rotor. The plasmid DNA in the supernatant was recovered, transferred to 50 ml falcon tube and NaCl added to a final concentration of 0.5 M before phenol:chloroform and chloroform extractions were performed to extract the DNA (Section 2.2.1.8.1). The plasmid DNA was then precipitated overnight at 4°C with 10% (w/v) PEG 6000. DNA was collected by centrifugation at 13,000 rpm for 20 minutes (4°C) in a Beckman JA20 rotor and resuspended in 500 μl l 0.1 M Tris-HCl (pH 8.0). Bacterial RNA was removed by treatment with 0.2 mg/ml RNAse A (previously heated to 100°C for 10 minutes to inactivate DNAse) for 30 minutes at 37°C. DNA was re-precipitated with an equal volume PEG buffer (10 mM Tris-hcl pH8.0 containing 1 mM EDTA, 1 M NaCl and 20% (w/v) PEG 6000) for 1 hour on ice and then collected by centrifugation in a microfuge at 13,000 g for 10 minutes. The pellet was dissolved in 400 µl 10 mMTris-HClpH 8.0 containing 0.5 M NaCl. DNA was then sequentially extracted with phenol, phenol:chloroform and chloroform (as described in Section 2.2.1.8.1) prior to ethanol precipitation. On recovery, the DNA pellet was drained, dried and resuspended in 200 µl TE. Purity and amount of DNA was determined 0.8% TBE agarose gel.

2.2.16 Restriction endonuclease digestion

Restriction endonuclease digestion of DNA were carried out in the buffers provided by manufacturer and according to their instructions. DNA was analyzed on 0.8% agarose-TBE gel containing 1 µg/ml of ethidium bromide.

2.2.17 Non-denaturing agarose gel electrophoresis

Agarose gels were prepared by melting (0.8% w/v)(BRL, electrophoresis grade) in (hot solution) of TBE (0.09 mM Tris pH 8.35, 0.09 mM Boric acid, 1.25 mM EDTA). After cooling the homogeneous solution to 55°C, ethidium bromide solution (10 mg/ml) was added to give a final dye concentration of 1 μg/ml. The melted agarose was mixed and poured on slab (10.6 cm long x 7.7 cm wide and approximately 1.0 cm deep) and allowed to set at room temperature Samples were adjusted to 8% sucrose-0.025 % bromophenol blue and loaded on corresponding wells on the gel. Electrophoresis was carried out for 1 hour 50 minutes at 40 mA constant current at room temperature. Gels were examined by direct shortwave UV illumination and photographed using Polaroid Type 667 film (Polaroid Corp. Cambridge, Mass., USA).

2.2.18 Denaturing polyacrylamide gel electrophoresis (sequencing gel)

DNA sequence reactions were analyzed on 6% denaturing polyacrylamide gels. These were prepared from pre-made sequagel acrylamide solutions (see Section 2.1.8.) using 33 ml sequagel diluent, 12 ml sequagel concentrate and 5 ml 10x TBE polymerised with 220 μl 25% ammonium persulphate (APS) and 12 μl N,N,N',N'-tetramethyl-ethylene diamine (TEMED). The gel was pre run at 1100 volts for 15-30 minutes before loading the samples. The samples were electrophoresed at 1100 volts for appropriate time intervals depending on the length and the region of DNA fragment to be sequenced. Gels were removed, transferred onto 3 MM™ paper and dried for 1-2 h. The dried gel and film was assembled in a cassete with an intensifying screen in a dark room and exposed for various time intervals at room temperature. The sequence was then read and information sought from a database.

2.2.19 Southern blotting

The transfer of DNA from agarose gels to nylon membranes was carried out as described by **Sambrook *et al.*, (1989)**. DNA was fixed to Hybond®-N membranes by UV illumination (254 nm) for 3-5 minutes at room temperature.

2.2.19.1 Hybridization of Southern blots

The membranes were pre-hybridised (in Hybaid™ bottles in a Hybaid™ oven) for a minimum of 2 hours in pre-hybridisation buffer (Section 2.1.6)at 65°C. Filters were transferred to hybridisation buffer (as pre-hybridisation buffer omitting the EDTA) containing sufficient heat denatured probe to give a final activity of approximately 10^6 cpm/ml and were hybridised as above. The stringency and number of subsequent washes were determined by the nature of cDNA probe used. The progress of DNA left on the filters was monitored by a Geiger Muller counter. The stringencies of the washes were increased by reducing the concentration of SSC: a typical high stringency wash was 0.1x SSC, 0.1% SDS at 65°C for 30 minutes. The filters were then wrapped in Saran Wrap whilst still damp and exposed to film at -70°C for varying lengths of time.

2.2.20 Isolation of DNA fragments from low melting point (LMP) gel

Low melting point (LMP) agarose gel was made by heating 0.5 g LMP agarose in 50 ml TAE electrophoresis buffer. After cooling ethidium bromide was added to it to a final concentration of 1 μg/ml. The gel was poured and allowed to set at room temperature. Digested DNA (10 μl), in sample loading buffer were loaded and electrophoresis carried out at 45 mA constant current for 1-2 hours depending on bands or fragments being separated. The gel band corresponding to the required fragments were excised, put in pre-weighed 1.5 microfuge tubes, and "gene cleaned" using Geneclean®II kit (Stratech Scientific, Luton, U.K) according to manufacturer's instructions. The amount of DNA recovered was determined on 0.8% TBE agarose gel and 200 ng of DNA was used in the ligation reaction.

2.2.21 Construction and selection of recombinant plasmids

2.2.21.1 Preparation of vector DNA

Bluescript SK®+ plasmid DNA was digested with the appropriate restriction enzymes as described in Section 2.1.16. The degree of digestion was monitored by running an aliquot on an agarose gel (Section 2.1.17). The remaining DNA was extracted with phenol:chloroform and DNA recovered by ethanol precipitation. Where necessary DNA was dephosphorylated after digestion to increase the efficiency of ligation using calf intestinal alkaline phosphatase according to the method of **Sambrook *et al.* (1989).**

2.2.21.2 Ligation of DNA fragments and plasmid vectors

"Gene cleaned" plasmid DNA fragments were combined with vector DNA in a 2:1 (insert:vector) molar ratio so that the total mass of DNA did not exceed 200 ng in a

final reaction volume of 10 µl. Ligase buffer (5 mM Tris-HCl pH 7.8, 1 mM $MgCl_2$ and 2 mM DTT), ATP at a final concentration of 1 mM and 10 units of T4 DNA ligase were added and the reaction incubated for 12-16 h at 8-10°C.

2.2.22 Preparation of *E.coli* competent cells and their transformation with plasmid DNA

2.2.22.1 Competent cells

E. coli JM101 overnight cultures were diluted 1:100 in 2TY medium in a total volume of 50 ml and grown at 37°C in a shaking incubator to log phase (when the O.D at 600 nm was between 0.3-0.5). This period usually took 2 hours after which the cells were transferred into sterile falcon tubes and pelleted at 2000 g for 10 minutes at 4°C. The pellets were gently resuspended in 25 ml of ice cold 50 mM Calcium chloride and incubated on ice for 20 minutes. The cells were pelleted again, taken up in 5 ml of ice cold 50 mM Calcium chloride and incubated on ice for 2-4 hours for subsequent use in transformation.

2.2.22.2 Transformation

E. coli JM101 (500 µl) competent cells prepared as described above were transformed with half of the ligation reaction mix (5 µl of ligation reaction mix). The controls included, 1) *EcoR*I digested Bluescript without T4 DNA ligase enzyme to measure the amount of uncut bluescript, 2) *EcoR*I digested bluescript with T4 DNA ligase enzyme to test the amount of non-phosphatased bluescript vector and 3) Undigested bluescript vector at 20 ng to measure the transformation efficiency. Transformation was done as described by **Sambrook *et al.*, (1989).**

2.2.23 Grunstein-Hogness method of colony hybridization (Selection of recombinant plasmids)

Since the Bluescript® plasmid used in this work conferred ampicillin resistance to the host cells, the transformed bacteria were plated onto LB agar plates containing ampicillin (50 μg/ml). Bluescript was constructed such that a fragment of foreign DNA inserted into the multiple cloning site would disrupt the protein coding region of the 5' end of the lac Z gene. This resulted in the failure of the plasmid to display α-complementation activity of the lac Z gene product, β-galactosidase, with the host bacterium. Lac+ bacteria form blue colonies in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) but bacterial colonies containing recombinant plasmids form white colonies. Thus, the plates contained X-gal (800 ng) and an inducer of the lac Z gene, isopropyl-thio-β-D-galactopyranoside, IPTG (1 mM) to facilitate the selection of recombinant plasmids. In order to ensure that the putative positive represented recombinants rather than re-ligated vector DNA which had lost their β-galactosidase complementation activity by a deletion, putative positive white colonies were subjected to *in situ* hybridisation by protocol II described by **Sambrook *et al.*, (1989).**, an adaption of the method by **Grunstein and Hogness (1975)**. Briefly, white (positive) and a small number of blue (negative) colonies were transferred to wells in a 96-well plate each containing approximately 160 μl LB with 50 μg/ml ampicillin. The bacteria carrying plasmids were grown to log phase (approx. 4-5 h) in a 37°C incubator. Hybond N nylon filter was placed on a LB-amp plate and 2 μl of bacteria culture transferred to the filter in a regular array. Plates were inverted and incubated at 37°C for 12-16 hours.

2.2.23.1 Lysis, DNA denaturation and fixation

The filters were lifted from the agar plate and sequentially placed on 500 μl pools 0.5

M NaOH (bacterial side up) and 1 M Tris-HCl pH 7.2 twice each 3 minutes. Filters were then washed in 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl for 20 minutes and the bacterial debris wiped from the filter in 2x SSC (Section 2.1.7). The DNA was fixed to the filters by UV irradiation (254 nm) for 2-5 minutes (with the DNA nearest the light source).

2.2.23.2 Hybridisation and ^{32}P-Autoradiography

The dry membranes were pre-hybridised and hybridised in Hybaid™ bottles in a hybaid oven at 65°C for 1 hr in 2x SSC and 1x Denhardt's reagent as detailed in southern blots (Section 2.2.19). Subsequent stringency and number of washes were determined by monitoring with a Geiger Muller counter. Generally the filters would further be washed with 0.1x SSC/ 0.1% SDS at 65°C and repeated if necessary. The filters were then wrapped in Saran Wrap, and placed under Kodak X-OMAT x-ray film for autoradiography with intensifying screens between 3-24 hours at -70°C.

2.2.24 Preparation of recombinant M13 bacteriophage and the production of single stranded DNA

Digestion and ligation of DNA fragments into double stranded M13 mp18 and mp19 bacteriophage was carried out exactly as described for Bluescript (Section 2.2.21.2). Transformation and selection of recombinant phage was carried out according to the Amersham sequencing manufacturers instructions. Single stranded M13 DNA was subsequently prepared also according to these instructions and half of the product used in sequencing reactions as described.

2.2.25 Radiolabelling of double stranded DNA fragments (Oligo-labelling of DNA fragments)

Linear, double-stranded whole DNA and (*EcoR*I-, *EcoR*I-*Pst*I and *EcoR*I-*Xba*I) fragments purified by "gene clean" containing the 300 bp HA6-2 and 1800 bp HB4-1 long, were radiolabelled according to the oligo-labelling technique of **Feinberg and Volgestein (1984)** using [α-^{32}P]dCTP 3000 Ci/mmol. (Amersham Corp.) to high specific activity as follows: In a screw cap microfuge tube, 5 µl of cDNA fragment was added to 4.5 µl of sterile water and boiled at 100°C for 5 minutes. Oligolabelling buffer (3 µl) Section 2.1.6, containing random hexanucleotides and unlabelled dNTPs (5mM)), BSA (0.6 µl of 10 mg/ml), α-^{32}PdCTP (1.5 µl or 10 µCi) and Klenow DNA polymerase (1 µl) in a total volume of 15 µl, were added to the denatured template and incubated at room temperature for 12-16 hours in a lead pot. 85 µl of STOP solution (20 mM Tris-HCl pH 7.5, containing 20 mM Sodium chloride, 2 mM EDTA, and 25% w/v SDS) was then added and this mixture was used as a probe to select for positive M13 clones. The specific activity of the radiolabelled DNA was determined by spotting 1 µl of probe directly onto a glass fibre disc and counting in a liquid scintillation counter.

2.2.26 DNA sequencing

The dideoxy chain termination method of **Sanger *et al.*, 1977** was used for DNA sequencing analysis. "Gene cleaned" DNA was subcloned into bacteriophage M13 mp18 and M13 mp19 for sequencing in both strands. The enzyme reactions for sequencing, including those of template-primer annealing and labelling extension were done as described in the Sequenase® Version 2.0 sequencing kit (United States Biochemical Corp., Cleveland OH). Sequencing reaction products were separated on

a 6% acrylamide sequencing gel (National diagnostics) and BRL model S2 apparatus at 1,100 V, transferred to 3 MM paper, dried and exposed to the film for 16-18 hours at -70°C.

2.2.27 Sequence analysis

The sequence was read manually and the result analysed using Wisconsin software package (computer program) at Daresbury. The EMBL data bank was used for the DNA sequence homology searches.

CHAPTER 3

SELECTION OF ANTISERA FOR SCREENING THE TESTIS cDNA LIBRARY: CHARACTERISATION USING PRIMATE AND RODENT TESTIS AND SPERM ANTIGENS

3.1 INTRODUCTION

Antibodies to germ cells, spermatozoa, or components of spermatozoa have been used to identify more distinct surface regions on spermatozoa than any other methods (**Eddy, 1988**). Antibodies can be conjugated directly with various labels or indirectly with secondary antibodies that carry labels that are visible by microscopy (**Sternberger, 1979**). Antibodies can also be used to isolate and identify specific molecules and to test their roles in bioassays (**Moore *et al.*, 1985**).

In order to identify cDNA clones expressing polypeptides corresponding to sperm acrosomal and peri-acrosomal plasma membrane components, it was necessary to generate antibodies that recognised sperm antigens. Therefore, a series of rabbit antisera were raised against purified hamster sperm acrosomes (Fig. 3.1) as described previously (**Moore *et al.*, 1987**). It was essential to characterise these sera to select the most appropriate for isolating the cDNA. So that a variety of antibodies would be generated, the acrosomal fractions were subjected to a variety of treatments with various denaturing and deglycosylating agents (e.g. NP-40, β-mercaptoethanol, SDS, TFMS) to expose a wide range of epitope moieties. Following on from previous antisera numbering (as part of a more extensive series of antisera) the current batch was designated R7-11.

The aim of investigations in this chapter was to identify germ cell and spermatozoa antigenic determinants recognised by these antisera and to determine the domains on spermatozoa localised by the antisera. The antisera were to be tested further in a hamster *in vitro* fertilization bioassay and those with desired characteristics would be selected for identification and selection of cDNA clones.

FIGURE 3.1

FIGURE 3.1 HAMSTER SPERM ACROSOMES

Phase contrast microscope picture of hamster sperm acrosomes prepared by isotonic treatment and percol gradient centrifugation. x500

These purified fractions were used to immunise rabbits as well as characterisation of polyclonal sera.

Antisera were characterised using the following methods: 1) Polyacrylamide gel electrophoresis and western blotting; 2) immunofluorescent localisation; 3) hamster *in vitro* fertilisation assay.

3.2 RESULTS

3.2.1 Characterisation of antisera by SDS-PAGE and immunoblotting with reproductive tissue

3.2.1.1 Hamster sperm protein

The antigen used for immunisation of rabbits consisted purified hamster acrosomal membrane as prepared in Section 2.2.1 Figure 3.1 shows a typical acrosomal preparation. The identity of these membranes was confirmed by immunolocalisation with mAb 18.6 which recognised a membrane component of the hamster acrosome **(Moore *et al.*, 1987)**.

To identify antigens recognised by antisera R7-11, purified hamster sperm acrosome preparations were used for SDS-PAGE, immunoblotting and were subsequently probed with antisera. A portion of the gel was stained with coomassie blue (Section 2.2.6) while the remainder was blotted on to nitrocellulose membranes (Section 2.2.7) and strips were probed with each rabbit polyclonal antisera. R8 and R11 antisera displayed poor immunoreactivity compared with R7, R9 or R10 antisera (data not shown) and therefore were not used in subsequent analysis.

The protein profile of hamster sperm acrosome (native and deglycosylated) and the subsequent recognition by antisera R7, R9, and R10 is shown in Fig. 3.2.

Purified hamster sperm acrosomal protein was revealed by coomassie blue staining to

be comprised a complex mixture of polypeptides ranging from 13-90 kDa in apparent molecular weight (M_r). The antigenic peptides reactive to polyclonal sera were shown to be a group of five major protein bands with apparent M_r 29-44 kDa (left arrows on Lane 1, Panels B, C and D). The broad band of apparent M_r 40-44 kDa (which may be a group of polypeptides) stained prominently with coomassie blue in native acrosome preparation (Fig. 3.2, Panel A, Lane 1), but upon digestion with endoglycosidase-F this band disappeared and a more discrete band at 29 kDa appeared (Fig. 3.2, Panel A, Lane 2). All the antisera apparently recognised the native and endoglycosidase treated bands at 40-44 and 29 kDa respectively (Fig. 3.2 Panels B, C, D). In addition all antisera reacted in a similar manner to the fast moving polypeptides of M_r 13-15 kDa. On native acrosomes, R7 and R10, reacted to the entire group of 29-44 kDa epitope (Lane 1, Panel B and D), R9 reacted only to the restricted upper 38-44 kDa moiety (Lane 1, Panel C). Upon deglycosylation, the 29 kDa cleavage product was recognised by R7 instead of the 29-44 kDa native polypeptide (Lane 2, Panel B). R9 reacted to both 29 kDa and the restricted upper band epitope 38-44 kDa polypeptide, while R10 reacted to the 29 kDa product, the largest 44 kDa polypeptide in the broad band, and three other intermediate polypeptides of M_rs 36, 38 and 39 kDa (Panel D, Lane 2). Antiserum R7 reacted further and specifically to a polypeptide of M_r 18 kDa (Panel B, Lanes 1 and 2). In terms of polypeptide recognition, R10 reacted more specifically to a series of polypeptide bands between the broad upper band and the lower 29 kDa band than R9. However, R7 recognised more specific peptides with lesser background than either R9 or R10. As can be seen from the immunoblots the reactivity of each antisera was different probably due to the treatment of the initial antigen.

FIGURE 3.2

FIGURE 3.2 IDENTIFICATION OF HAMSTER SPERM ANTIGENS BEARING EPITOPES REACTIVE TO R7, R9 AND R10 POLYCLONAL ANTISERA

Polyacrylamide gel and Western blots of purified hamster acrosomes probed with R7, R9 and R10. 40 μg of protein was loaded on each lane and resolved on 10% polyacrylamide SDS gel.

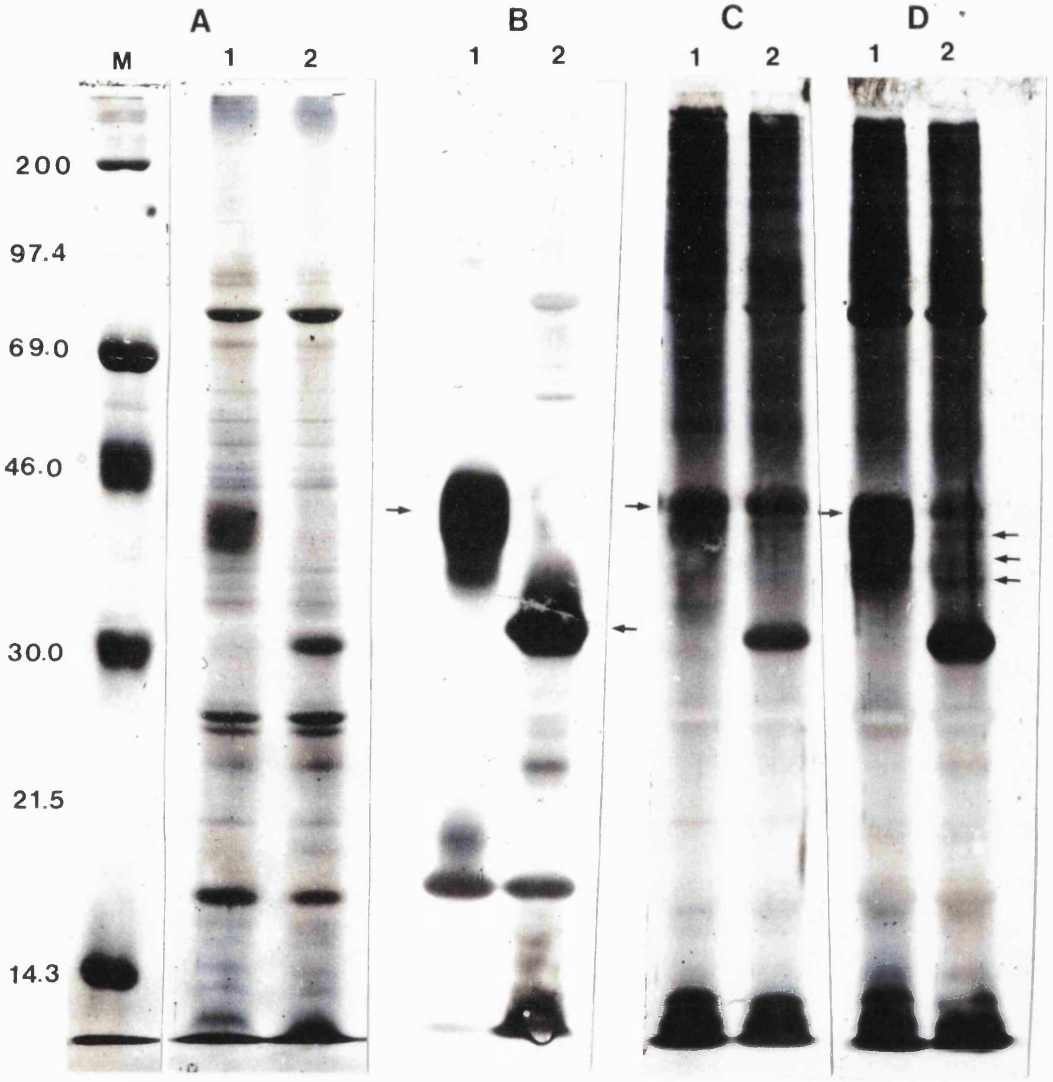
Panel A: Coomassie blue stained acrylamide gel.

Panels B, C, and D: Western blots of corresponding hamster sperm acrosome probed with R7, R9 and R10 respectively, in each case diluted at 1:100 (Section 2.2.7).

Lane 1: Native acrosomes

Lane 2: Acrosome proteins pre-treated with Endoglycosidase-F

Lane M: Rainbow protein™ molecular weight markers containing, Myosin (200 kDa), phosphorylase b (97.4 kDa), Bovine serum albumin (69 kDa), Ovalbumin (60 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (21.5 kDa) and Lysozyme (14.3 kDa).


A
B
C
D
M
1
2
1
2
1
2
1
2
200
97.4
69.0
46.0
30.0
21.5
14.3

3.2.1.2 Comparison of immunoreactivity of mixed antisera R7 and R10 to the antigens of human, chimpanzee, baboon testis and hamster spermatozoa

Ability of antisera raised against hamster acrosomes to recognise comparable antigenic determinants of human, chimpanzee and baboon testis tissue, was determined by running preparations on SDS-polyacrylamide gel and probing with a mixture of R7 and R10 antisera. The results are shown in Fig. 3.3. The samples were prepared and protein concentration determined as described in Sections 2.2.1 and 2.2.3 respectively. The antisera displayed broadly similar reactivity to testis preparations from each primate species (Fig 3.3, Panel B, Lanes 1, 3, 4) but not all bands were shared. Many more bands were visualised on the lane containing hamster testis (Panel B, Lane 5). Antisera reacted strongly to polypeptide bands of apparent molecular mass 34 and 43 kDa in human, chimpanzee and baboon testis extracts. Furthermore the 43 kDa polypeptide was also detected in human and chimpanzee epididymis (data not shown). Human testis, and sperm protein extracts reacted specifically with a high molecular weight polypeptide of molecular mass of 56 kDa. This band was not seen in chimpanzee testis, epididymis and baboon testis, however it was present in hamster spermatozoa. A broad spectrum of polypeptides were recognised in hamster sperm, notably 56, 43, 37, 35, 34, 30 and 22 kDa.

3.2.3 Characterisation of antisera using immunofluorescent localisation

The specificity of polyclonal antisera to germ cells, spermatozoa, and epididymis was demonstrated by immunocytochemical staining of frozen tissue sections. The tissues were prepared as described in Section 2.2.8. Immunofluorescence with antisera R8, R9 and R11 was weaker than with antisera R7 and R10 although essentially the same pattern of immunolocalisation was observed for all the sera. R8 and R11 however showed a patchy fluorescence over the sperm surface. Immunolocalisation of antisera on frozen sections of hamster and baboon testis indicated that fluorescent staining

was confined to the acrosomal component of spermatids (Fig. 3.4). This restricted localisation was also observed on sperm from the epididymis where spermatozoa within the lumen were stained but the epididymal epithelium showed no staining or non-specific staining when compared with pre-immune controls (Fig. 3.5).

The immunolocalisation of antisera on human spermatozoa was determined with methanol-fixed smears of ejaculated spermatozoa. Fluorescent staining was confined to the acrosomal region only for all the antisera (Fig. 3.6).

3.2.3 Effects of rabbit antisera on hamster *in vitro* fertilisation

The effect of antisera on *in vitro* fertilisation (IVF) in a hamster was investigated using the protocol of **Moore and Hartman (1984)**. This involved the pre-incubation of hamster oocytes (free of cumulus mass) or hamster spermatozoa in BWW culture medium in the presence of pre-immune sera or antisera at various dilutions. Gametes were then subjected to standard IVF protocols as described in Section 2.2.9. The eggs were examined and scored for fertilisation. Results were analysed statistically using Fisher exact probability computer program (CSS/Statistica, Section 2.1.8) based on Chi- square method. The values were significant when probability was less than 0.05 otherwise it was rejected. The results are shown in Tables 3.1 and 3.2

Pre-incubating spermatozoa with antisera (at various dilutions), prior to exposure to zona-intact eggs resulted in reduction of fertilisation outcome. For control pre-immune serum the overall fertilization rate was 95% (21/22). In contrast, the reduction in fertilisation rate after spermatozoa were pre-incubated with antisera ranged from 14% (3/22 for R10) to 29% (6/21 for R11). Although only R7 caused significant reduction in fertilisation rate (P= 0.049) at 1/400 dilution, there was marked reduction in fertilisation by R8, R9 and R10 with P values of 0.084 at 1/200

FIGURE 3.3

FIGURE 3.3 PROTEIN COMPOSITION AND IMMUNODETECTION OF CROSS-REACTING HUMAN, CHIMPANZEE, BABOON AND HAMSTER TESTIS POLYPEPTIDES

Lanes **1, 2, 3, 4 and 5** were loaded with human testis, human sperm, chimpanzee, baboon and hamster testis proteins respectively.

Panel A: Coomassie blue stained polyacrylamide gel.

Panel B: Nitrocellulose blot of corresponding antigens probed with immune mixed antiserum R7 and R10, and (C) Nitrocellulose replica probed with pre-immune serum.
Also included in the gel were mixture of pre-stained high and low molecular weight markers (BRL).

Details of antibody incubations and colour developments are given in Section 2.2.7

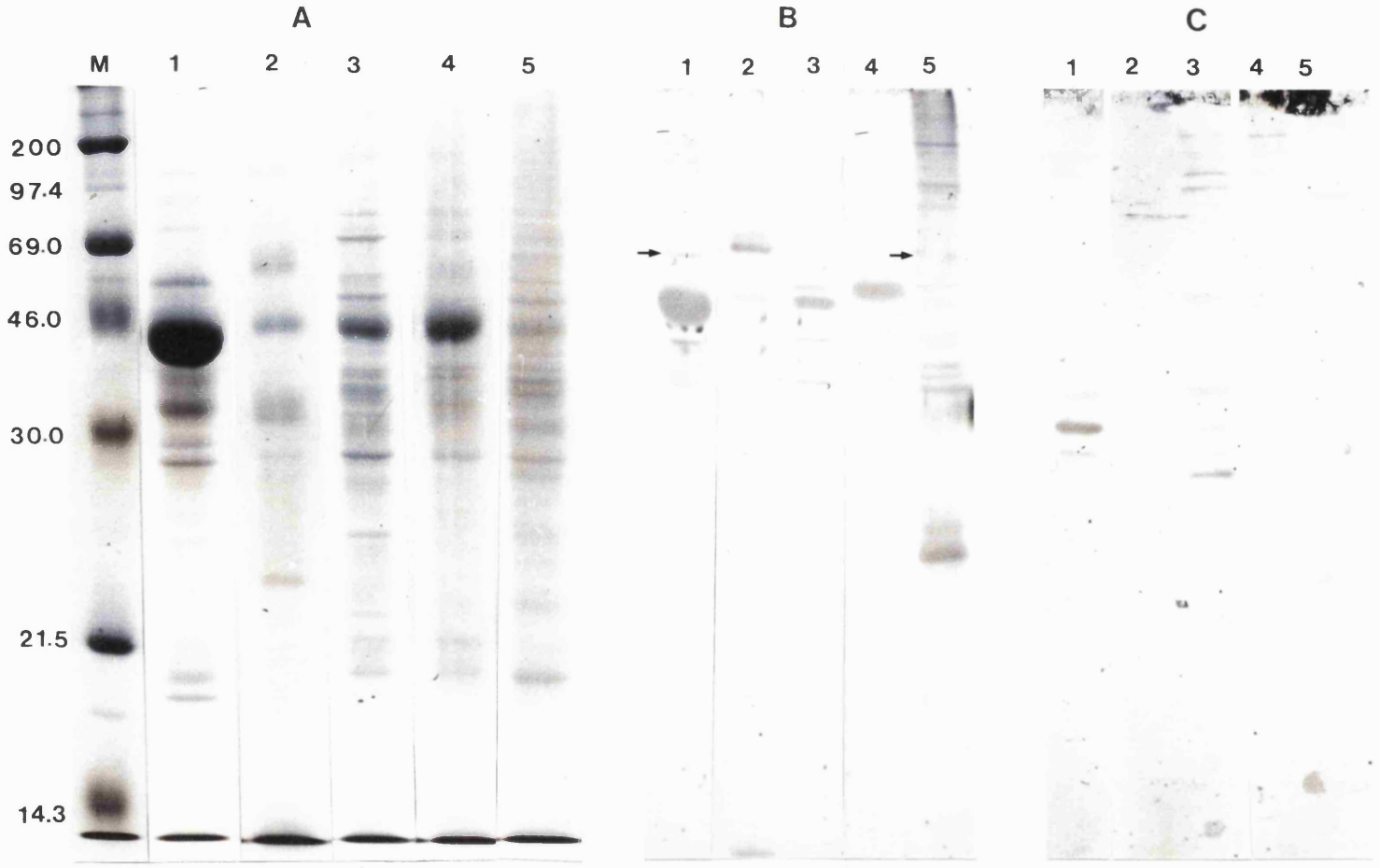

A
M
1
2
3
4
5
200
97.4
69.0
46.0
30.0
21.5
14.3
B
1
2
3
4
5
C
1
2
3
4
5

FIGURE 3.4

FIGURE 3.4 IMMUNOFLUORESCENT LOCALISATION OF HAMSTER AND BABOON GERM CELLS WITHIN THE TESTIS

Immunofluorescent localisation of germ cells and spermatozoa within the seminiferous tubules of mature testis. Frozen tissue sections were fixed in 4% v/v formaldehyde solution, probed with appropriate antiserum and FITC-conjugated secondary antibody.

A. Antiserum R7 immunolabelling of hamster testis seminiferous epithelium showing specific localization of acrosomal cap of mature spermatozoa and late spermatids in the seminiferous tubule.

B.Corresponding phase contrast image x 250.

C. Localisation of baboon seminiferous epithelium by antiserum R7, showing cross-reactivity to mature baboon sperm acrosomes.

D. Corresponding phase contrast image x 250.

E. Antiserum R10 cross-reacting to similar cell type to R7 in baboon seminiferous tubule.

F. Phase contrast image of **(E)** x 250.

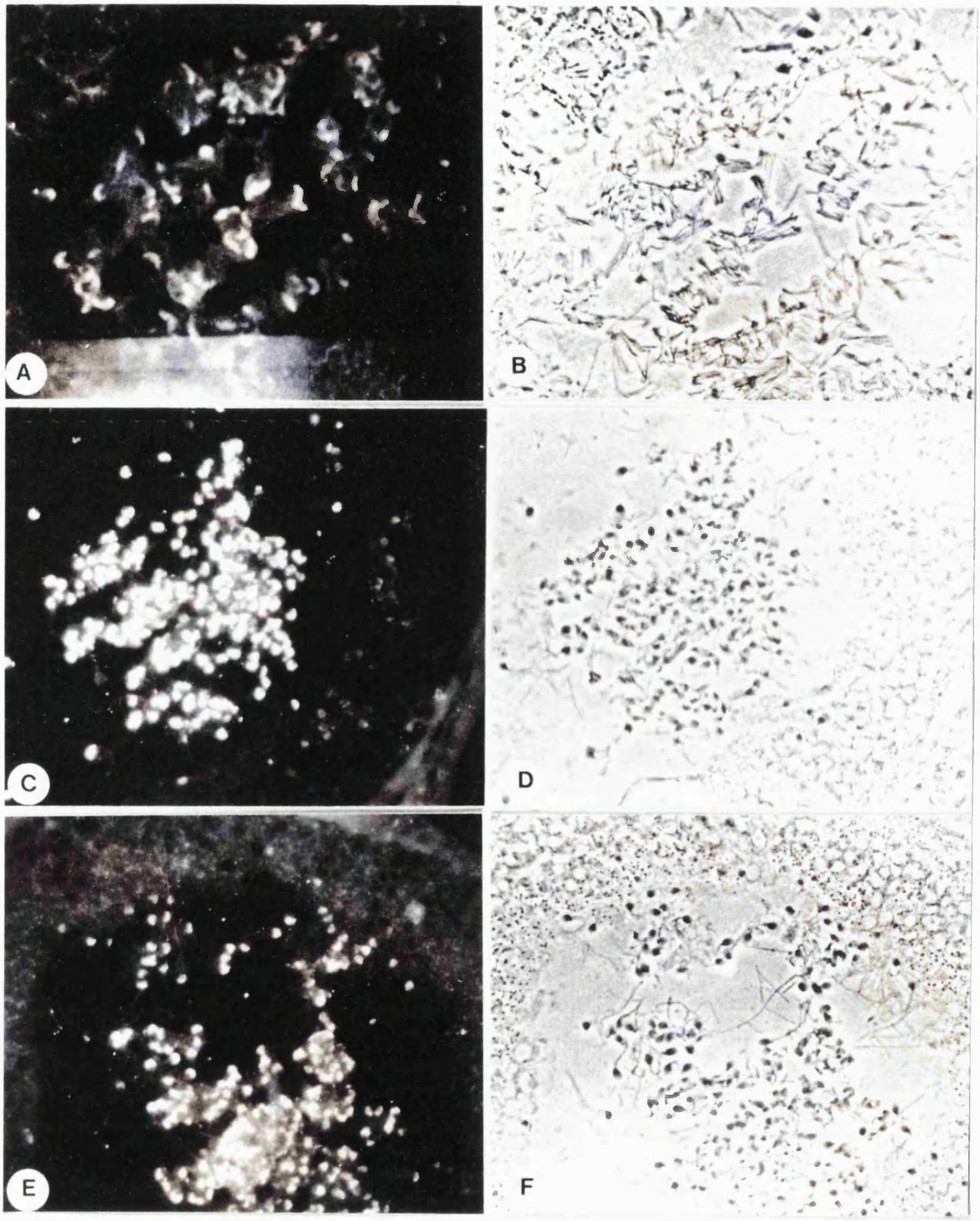
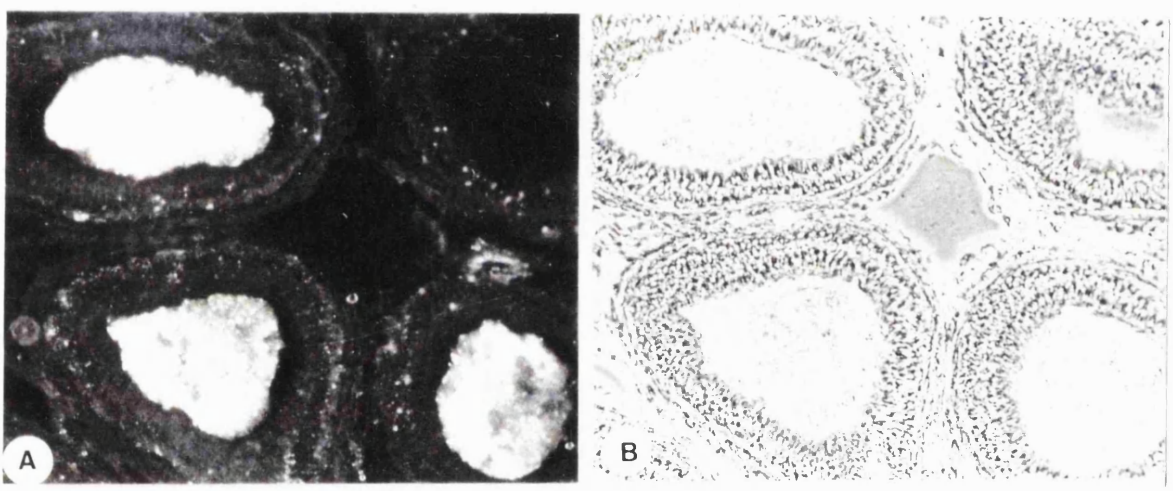

A
B
C
D
E
F

FIGURE 3.5

FIGURE 3.5 IMMUNOFLUORESCENT LOCALISATION OF R10 ANTIGENIC EPITOPES ON BABOON EPIDIDYMAL SPERM

A. Immunofluorescent picture of baboon epididymal section after staining with R10 antiserum.

B. Corresponding phase contrast picture of the epididymal section. x125


A
B

dilution. Considerable reduction of fertilisation was also observed in R8 at 1/100, and 1/400 dilution, R9 and R10 at 1/400 dilution and at 1/1000 dilution all of which had P values ≈ 0.1. At titres of 1/100 antisera usually caused agglutination of the spermatozoa in the fertilisation medium (Table 3.1 and Fig. 3.7). At lower titres, sperm agglutination was not observed but fertilisation was still inhibited. At 1/1000 dilution, the inhibitory effect of antisera was less than at the other dilutions. When diluted to 1/100, antisera caused agglutination in head-head in the presence of antisera R8 and R10 and head-tail in the presence of R7 and R9.

No agglutination was observed in the pre-immune serum and anti-serum R11 at this dilution (Table 3.1 and Fig. 3.7). Dispersion of agglutinated spermatozoa usually occurred after 4-5 hours.

The mechanism of action of various antisera at 1:100 dilution was investigated by pre-incubating the sera with zona-intact eggs (Table 3.2). The eggs were rinsed 3 times in BWW and subsequently incubated with spermatozoa. There was no significant inhibition of fertilisation even when the sera were used at the highest concentration (1:100 dilution). Thus the inhibition of fertilisation was most probably due to the action of antibody on sperm and not on the egg.

Since R8, R9 and R11 displayed poor immunocytochemical localisation it was decided that only R7 and R10 antisera would be used for subsequent cDNA screening procedures and therefore these antisera were subjected to additional analysis as described below.

TABLE 3.1 THE EFFECT OF INCUBATING HAMSTER SPERM WITH ANTISERA ON SUBSEQUENT FERTILISATION OUTCOME IN HAMSTER IVF

	NUMBER OF HAMSTER OOCYTES FERTILISED				
DILUTION	1:100	1:200	1:400	1:1000	TOTAL RATE
PRE-IMMUNE SERUM	5/6	5/5	6/6	5/5	21/22 (95%)
R7	1/4 (0.346) T	1/3 (0.406)	0/6 (0.049)*	2/5 (0.391)	3/8 (16%)
R8	0/5 (0.106)‡H	0/5 (0.084)†	1/5 (0.199)‡	2/5 (0.391)	3/20 (15%)
R9	0/5 (0.261) T	0/5 (0.084)†	0/4 (0.115)‡	3/5 (0.479)	3/19 (16%)
R10	1/5 (0.261) H	0/5 (0.084)†	1/6 (0.144)‡	2/6 (0.160)‡	3/22 (14%)
R11	1/4 (0.346)	1/10 (0.215)	2/7 (0.201)	2/5 (0.391)	6/21 (29%)

Values in brackets are Fisher exact probability obtained from the observed fertilisation outcome using pre-immune values as control.

* Significant reduction in fertilisation rate ($P < 0.05$).

† Marked reduction in fertilisation rate ($P < 0.1$).

‡ Considerable reduction in fertilisation outcome ($P < 0.2$).

T Antiserum and at dilution which caused tail-head agglutination.

H Antiserum and at dilution which caused head-head agglutination.

TABLE 3.2 THE EFFECT OF INCUBATING HAMSTER OOCYTES WITH ANTISERA ON SUBSEQUENT FERTILISATION OUTCOME IN HAMSTER IVF

ANTIBODIES	OOCYTES EXAMINED (n)	OOCYTES FERTILISED (n)	FERTILISATION RATE (%)
PRE-IMMUNE	11	10/11	91
R7	6	6/6	100
		(0.590)	
R8	10	9/10	90
		(0.618)	
R9	12	10/12	83
		(0.565)	
R10	10	8/10	80
		(0.549)	
R11	12	12/12	100
		(0.555)	

There was no significant reduction of fertilisation by any of the sera. ($P>0.05$)

FIGURE 3.6

FIGURE 3.6 IMMUNOFLUORESCENT LOCALISATION OF HUMAN SPERMATOZOA BY R10 ANTISERUM

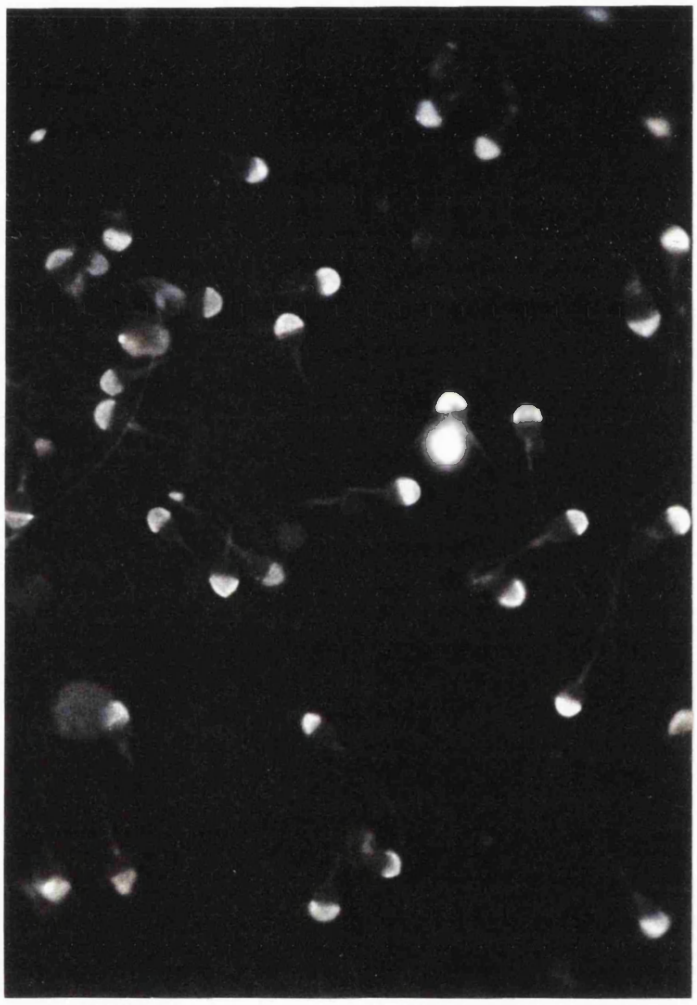


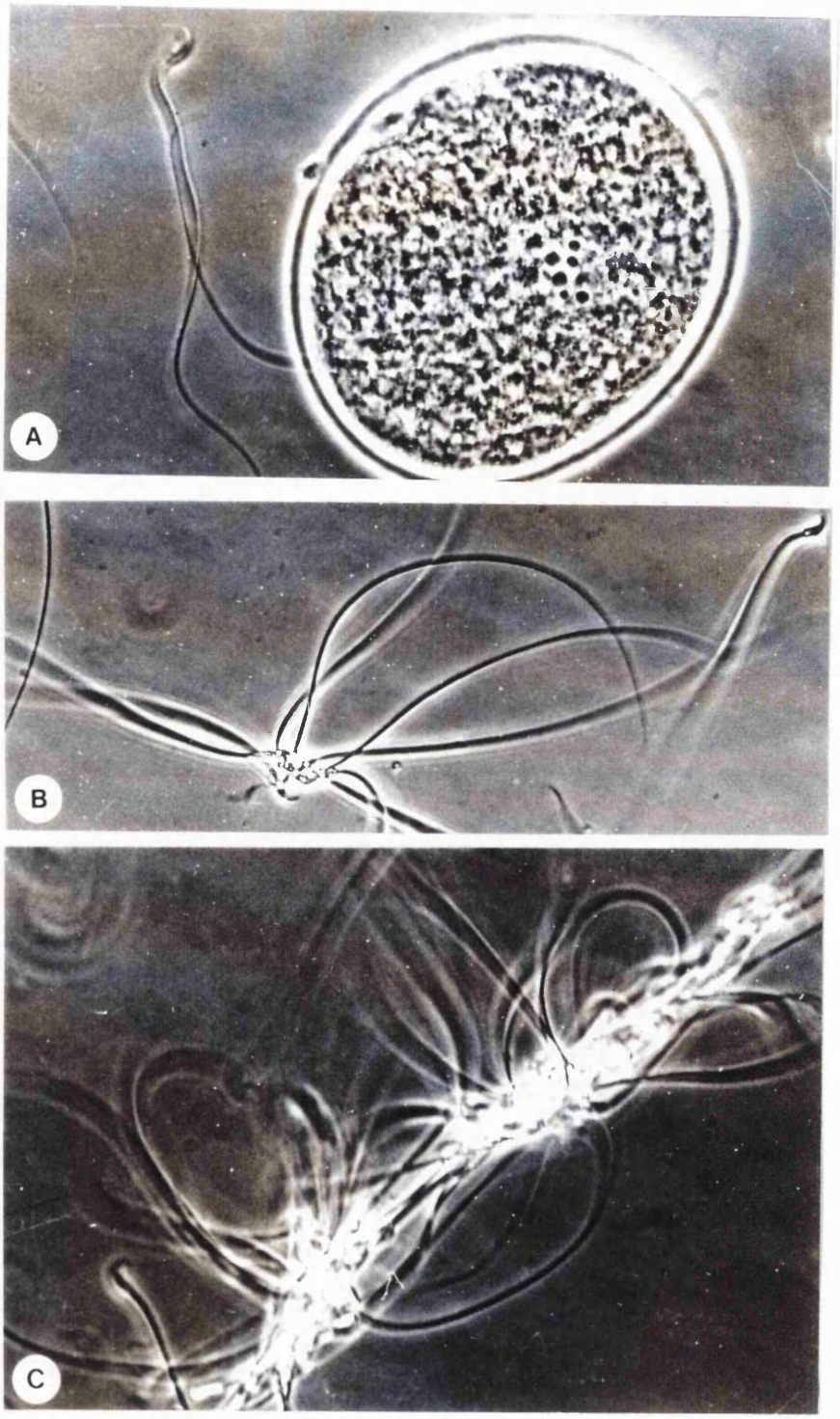
FIGURE 3.7

FIGURE 3.7 ***In vitro*** **ASSAY CHARACTERISTICS OF SPERMATOZOA IN RELATION TO THE OOCYTES. BINDING OF SPERMATOZOA TO HAMSTER OOCYTES AND PATTERN OF AGGLUTINATION**

A) Fewer sperm clusters around the oocyte.

B) Head to head agglutination.

C) Head to tail agglutination.


A
B
C

3.3 DISCUSSION

The aim of work in this chapter was to characterise and select antisera which would be suitable for screening a cDNA library. It was necessary that the antiserum should meet several criteria if it was to be specific probe. 1) It should inhibit sperm-egg interaction and therefore hopefully contain antibodies that interfere with fertilisation 2) It should show a high degree of specificity for spermatozoa or at least germ cells 3) It should possess cross-reactivity for spermatozoa of human and those of baboon if the latter was to become a model for contraceptive vaccine development. Of particular interest was the characterisation of common epitopes between species since these might represent conserved determinants and therefore could be important for mammalian fertilisation events.

To test the effect of antiserum on fertilisation, hamster IVF was used. Pre-incubation of spermatozoa but not oocytes with diluted antiserum (up to a titre of 1/400) led to a complete inhibition of fertilisation in most cases indicating that antibodies were present that interfered with sperm function. This homologous approach (i.e. antibodies against hamster acrosome tested with hamster IVF) was considered valid since in previous studies it was shown that antibodies that inhibited hamster IVF were also effective in inhibiting human sperm-egg interactions (**Moore *et al.*, 1987; Moore *et al.*, 1993**).

The immunofluorescent localisation with the various antisera indicated that antibodies specifically recognised the acrosomal region of spermatozoa in both the testis and epididymis. There was no detectable immunofluorescence on other cell types of the reproductive tract. However, there was good cross-reactivity between species with respect to the localisation to the acrosome.

Polyacrylamide gel electrophoresis and immunoblotting further characterised the antisera. The main conclusions to be drawn from the results were that immunisation of rabbits with native or deglycosylated acrosomal preparations resulted in the production of antisera with varying specificities however some common epitopes were identified in all the sera. Perhaps of more importance was the finding that antisera cross-reacted strongly with just a few human, chimpanzee and baboon testis proteins with corresponding positions on the blot. In particular there was intense reactivity with a determinant with a relative molecular weight of 43 kDa and specific (but less intense) reactivity with a band of 38 kDa. This finding was good evidence that if a selected antisera was used to screen a human cDNA testis library it could indeed recognise human polypeptides.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF cDNA CLONES SELECTED WITH ANTI-ACROSOMAL SERA

4.1 INTRODUCTION

Investigation with antisera directed against acrosomal membrane preparations established that antibodies were present that recognised specific acrosomal components expressed during germ cell development (Chapter 3). Since the immunoblots of antigen treated with endoglycosidase indicated that core polypeptides as well as carbohydrates were recognised by the antisera, it was considered feasible to screen a human testis cDNA expression library to isolate cloned genes. As a means of cloning gene sequences efficiently when antibodies are used as probes, it was thought appropriate to screen the library using the technique developed by **Young and Davis (1983a)**. This procedure utilises the unique characteristics of the expression vector, λgt11 (*lac*5 *nin*5 *c*I857 *S*100) which allows the expression of proteins. The gene map is shown in Fig. 4.1. It is 43.7 kb and contain the *lacZ* gene within its trancriptional unit. It has sequences that contain sufficient unique restriction enzyme sites (multiple cloning site) to facilitate the construction of appropriate recombinant DNAs. The site for the insertion of foreign DNA in this vector is *Eco*RI located within the *lacZ* gene, 53 base pairs upstream of the β-galactosidase termination codon. Phage containing inserts generate an inactive β-galactosidase fusion protein and these phage can be distinguished from non-recombinant phage by their ability to produce blue plaques on a *lacZ-* host on X-Gal plates. Phage containing inserts generate β-galactosidase fusion proteins upon induction by isopropylthio-β-D-galactopyranoside (IPTG), its gratuitous inducer. Antigens produced in λ-phage plaques rather than in λ-lysogen colonies are immobilised on nitrocellulose membranes. The antigens when recognised by antibody enable specific cDNA clones to be isolated. Important genetic markers include: **cI857**, a temperature sensitive repressor, inactive at 42°C., **S100** amber mutation that renders it lysis defective, hence lysogens can be induced by

FIGURE 4.1

FIGURE 4.1 MAP OF λgt11 EXPRESSION VECTOR

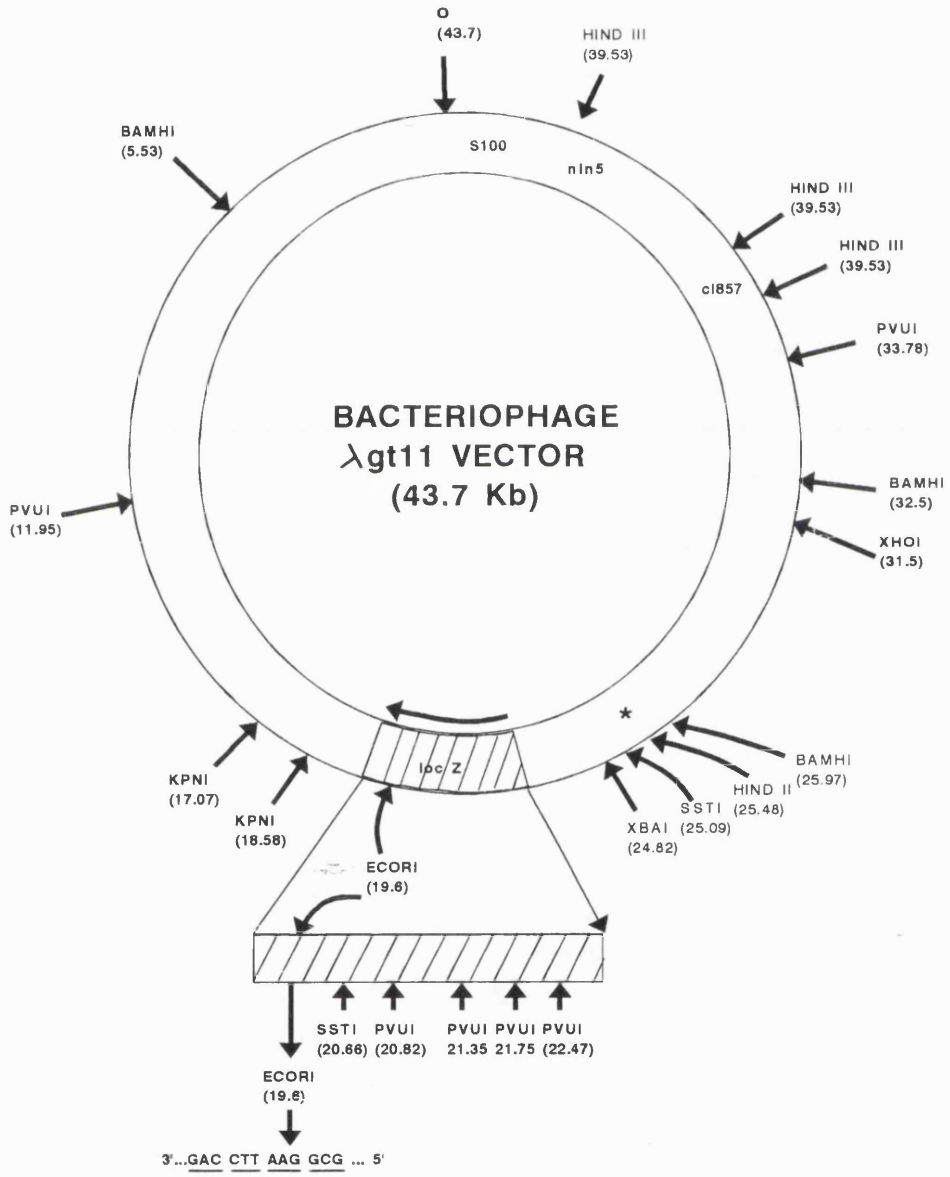
* = λ attachment site.

→ = The transcriptional orientation of *lacZ* in hatched box.

GAC **CTT AAG** GCG = Sequence of *EcoRI* (**CTT AAG**) and the nuleotides that immediately surrounds it.

Important genetic mutations of λ:

*c*I857	temperature sensitive mutation that renders the *c*I gene product thermolabile.
nin5	deletion that removes the transcription termination site t_{R2} from the wild type bacteriophage and thereby renders delayed early transcription independent of the *N* gene product.
S 100	Amber mutations in a gene involved in lysis of the bacterial cell membrane. The mutations are suppressed in *supF* (but not *supE*). The absence of the wild type *S* gene product leads to an intracellular accumulation of infectious bacteriophage particles.


O
(43.7)
HIND III
(39.53)
BAMHI
(5.53)
S100
nIn5
HIND III
(39.53)
HIND III
(39.53)
cI857
PVUI
(33.78)
BACTERIOPHAGE
λgt11 VECTOR
(43.7 Kb)
BAMHI
(32.5)
XHOI
(31.5)
PVUI
(11.95)
*
lac Z
KPNI
(17.07)
KPNI
(18.58)
BAMHI
(25.97)
HIND II
SSTI (25.48)
XBAI (25.09)
(24.82)
ECORI
(19.6)
SSTI PVUI PVUI PVUI PVUI
(20.66) (20.82) 21.35 21.75 (22.47)
ECORI
(19.6)
3'...GAC CTT AAG GCG ... 5'

temperature shift to accumulate large quantities of phage products in the absence of lysis. λ-gt11 primers (sequence shown in Section 2.1.7) used in PCR were also designed to anneal to regions just outside the *Eco*RI site into which the cDNAs were ligated originally. In addition, the primers had an *Eco*RI site, hence digestion with this enzyme would yield in most cases a single piece of double strand DNA with *Eco*RI site at each end suitable to ligate into *Eco*RI/pBluescript SK+ site.

The aim of investigations in this Chapter was to: 1) use the antisera for screening the human testis cDNA library constructed in λ-gt11 expression vector 2) isolate the cloned gene(s) that encode sperm acrosomal polypeptides and further characterise the cDNA clones using molecular biological techniques and a hamster IVF bioassay 3) select the appropriate clones, determine the sequence and analyse information encoded by the gene.

A human testis λ-gt11 cDNA library was therefore obtained and screened as detailed in Section 2.2.10. Prior to screening the library, an attempt was made to remove non-specific anti-coliform antibodies from the antisera. Rabbit polyclonal sera are known to contain antibodies to several bacterial strains due to the existence of such bacteria in their gut. These antibodies might react to the antigens expressed by the *E. coli* Y1090 host cells and result in false positive clones. Generally, affinity purified antibodies are most suitable for recognising λgt11 recombinant proteins (**Young and Davis, 1983b**). However, affinity purification limits the range of antigen recognition at an early stage. In the absence of this purification, polyclonal sera has been used successfully when first pre-absorbed to remove antibodies which react with coliform proteins. This can sometimes be achieved by pre-incubating the antisera with λgt11 lysogen lysate bound to nitrocellulose (**Young and Davis, 1983b**). Therefore an attempt was made to absorb the anti-coliform antibodies by pre-incubating antisera with lysed *E. coli* Y1090 bound on nitrocellulose filters. The result of relative

absorptions were analysed by western blot. Considerable reduction of background non-specific antibody binding was achieved with this pre-absorption technique, however, a reduction in specific antibody reactivity occurred also. Since the latter would be detrimental for screening (as potentially important antibodies might be removed), pre-absorption of antisera was not pursued.

4.2 RESULTS

4.2.1 Screening of a human testis λgt11 cDNA phage plaques for specific antigens

Based on: 1) recognition of polypeptide bands in spermatozoa, epididymis and testis protein extracts; 2) specific localisation on acrosomal region of developing germ cells and 3) reduction of fertilisation in hamster IVF, antisera R7 and R10 were pooled in the ratio 1:1 and used as a probe. To screen the library for specific antigen-producing clones, λ-gt11 recombinant phage was plated on a lawn of *E. coli* Y1090 (a host deficient in the *lon* protease), thereby reducing the degradation of expressed antigens. 4-5 x 10^5 plaques were screened in 4 x 140 mm square plates from a total of 1.6 x 10^6 individual recombinant λgt11 bacteriophages in an amplified human testis cDNA library. Screening details are given in Section 2.2.10.3 and results of screening are shown in Table 4.1 and Fig 4.2. Out of approximately 200 putative positive plaques (Fig 4.2A), 38 plaques (11 from plate A designated HA1, HA2, up to HA11, and 27 plaques from plate B designated HB1, HB2, up to HB27), were chosen on the basis of antibody reactivity. After secondary immuno-screening (enrichment and confirmation) using a further aliquot of the diluted antisera, 36 plaques were confirmed plaque pure (Fig.4.2B). For each of these 36 antibody positive plaques, two were selected at random, phage recovered, titred and re-plated at such a density that would be picked as described in Section 2.2.10.2. All the 72 plaques were found

TABLE 4.1 PUTATIVE POSITIVE PLAQUES OBTAINED FROM SCREENING λ-gt11 cDNA LIBRARY

	Number of positive plaques	Determined plaque density in (pfu/ml)
Primary Screen		
HA1 to HA11	10	4×10^6
HB1 to HB27	27	8×10^6
Secondary screen		
HA1-1 to HA11-2	20	10^6-10^7
HB1-1 to HB27-2	52	10^7-10^8
Tertiary screen		
HA1-1 to HA11-2	20	1×10^7
HB1-1 to HB27-2	52	1×10^7
High titre lysates		
HA1-1 to HA11-2	20	10^{12}-10^{14}
HB1-1 to HB27-2	52	10^{13}-10^{15}

FIGURE 4.2

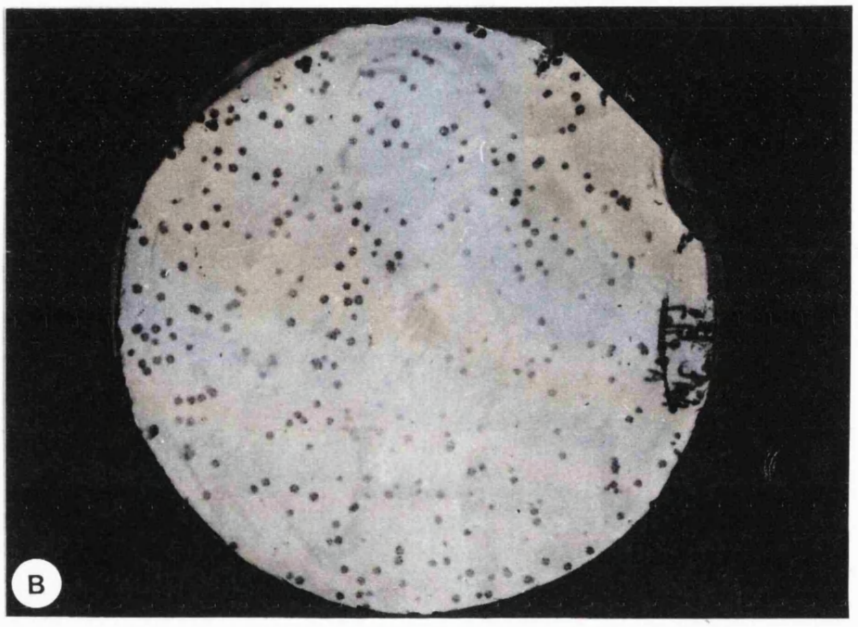
FIGURE 4.2 SCREENING OF HUMAN TESTIS λgt11 cDNA LIBRARY FOR SEQUENCES THAT ENCODE SPERM PROTEINS USING R7 AND R10 ANTISERA

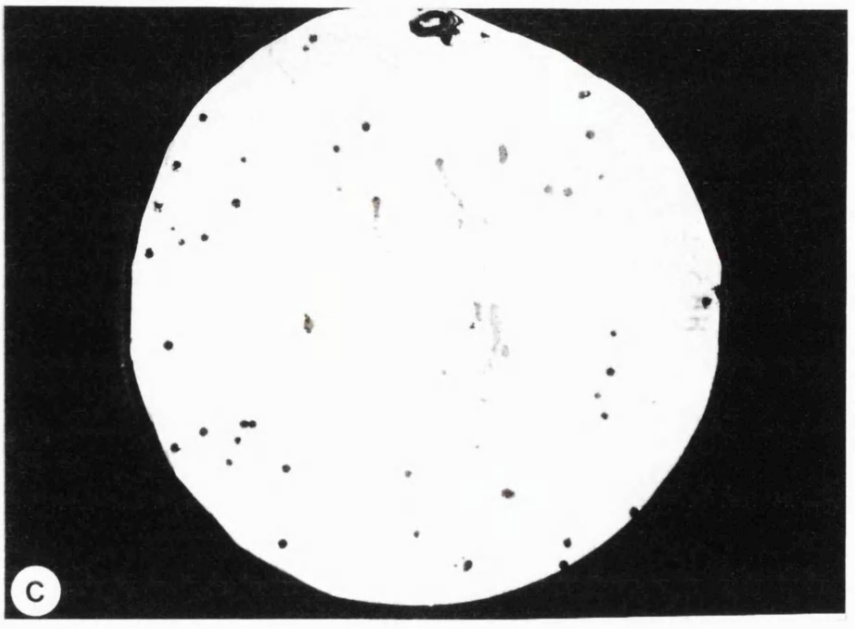
A: Nitrocellulose plaque lifts of primary screen.

B: Nitrocellulose plaque lifts of secondary screen.

C: Nitrocellulose plaque lifts of one of the 72 plaque purified clones.


A


B


C

to be positive. The B series plaques exhibited more efficient growth than the A series as determined by the plaque forming units (pfu) at the end of each stage of screening (Table 4.1). One positive plaque was selected from each plate, pfu determined and used for preparation of high titre λgt11 phage lysate as described in Section 2.2.11.

4.2.2 Expression of β-galactosidase fusion proteins: confirmation of clone identity

An undesired clone from the library might be selected because the protein it encodes could either share an epitope with bacterial (host cell) antigens, or because other non-specific antibodies were generated during the original immunization procedure. Thus it was necessary to confirm the identity of these recombinant clones with an additional experiment. A hamster IVF bioassay was designed to measure the ability of antigen expressed from different clones to prevent sperm-egg binding and fertilisation. β-galactosidase fusion proteins were generated from different clones and analysed on 7.5% acrylamide gel as described in Section 2.2.6. Fig. 4.3 shows the results of some of the clones that were induced to generate fusion proteins.

Clones HA1-2, HA2-2, and HA3-2 (Panel A, Lanes 1, 3, and 5) generated fusion proteins with strong signals comparable to those of clone 134 positive control (Panel A, Lane 7). However, in panel B, although experimental conditions were identical, clones HA2-1 and HA4-1 (Lanes 1 and 3), generated proteins comparable to those of positive control (Lane 7), clone HA6-1 (Lane 5) responded very poorly. In panel C, however, there was a general reduced fusion protein expression and very small quantities were expressed by clones HA5-2 and HA6-2 (Lanes 3 and 5). Therefore, signal strength varied from one preparation to the other depending on experimental conditions. However it was apparent that some clones expressed fusion proteins more efficiently than others.

FIGURE 4.3

FIGURE 4.3 WESTERN BLOT OF λgt11 β-GALACTOSIDASE FUSION PROTEINS STAINED WITH ANTI-β-GALACTOSIDASE MONOCLONAL ANTIBODY

Standard β-galactosidase protein, molecular mass 116 kDa was also resolved on this gel to facilitate estimation of size and the amount of fusion protein generated.

Also included in the gel was fusion protein generated from clone 134 (a clone that encodes for 125 kDa fusion protein), as positive control.

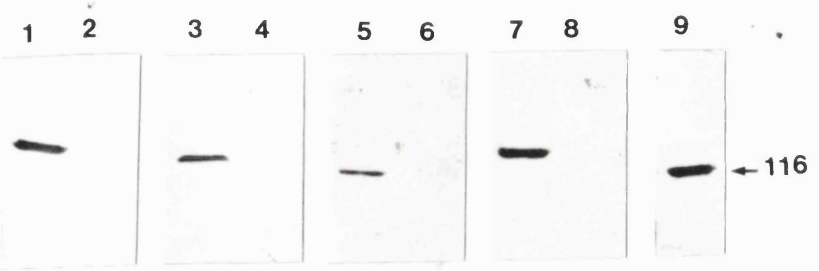
A: Fusion proteins generated from clones HA1-2, HA2-2, HA3-2 and clone 134, a positive control.

Lanes 1, 3, 5, and 7: loaded with 15 μl of bacterial and fusion proteins generated from clones HA1-2, HA2-2, HA3-2, and clone 134, induced with 10 mM IPTG respectively. **Lanes 2, 4, 6,** and **8** are proteins from corresponding clones generated without IPTG.

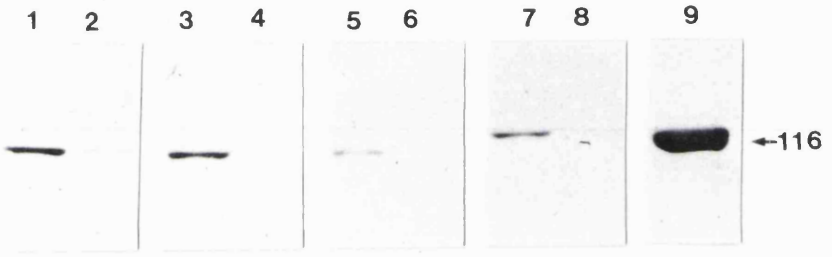
Lane 9: 5 μg of standard 116 kDa β-galactosidase protein.

B: Fusion proteins expressed by clones HA2-1, HA4-1, HA6-1 and clone 134, positive control. All the lanes were loaded with proteins as described in (A) above except **Lane 9** which was loaded with 30 μg of standard β-galactosidase protein.

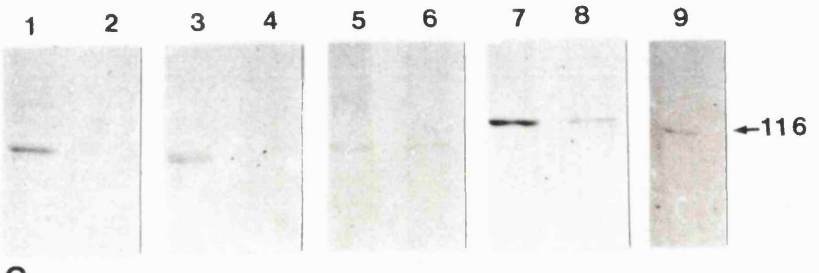
C: Fusion proteins expressed from clones HA4-2, HA5-2, HA6-2 and 134 respectively. All the lanes were loaded with proteins as described in (A) above.



A



B



C

TABLE 4.2 THE EFFECTS OF FUSION PROTEIN GENERATED BY λgt11 cDNA CLONES ON HAMSTER SPERM-OOCYTE BINDING AND FERTILISATION

Clone	Eggs fertilised	Probability of eggs fertilised	Sperm bound per egg	Percent fertilised
HA1-2	6/9	(0.587)	10-20++	67
HA2-1	7/9	(0.626)	15-20	78
HA2-2	6/9	(0.587)	8-9++	67
HA3-2	7/9	(0.626)	20-30	78
HA4-1	8/10	(0.607)	30-40	80
HA4-2	7/9	(0.561)	20-30	78
HA5-2	0/9	(0.030)*	2-5+	0
HA6-1	6/9	(0.587)	30-50	67
HA6-2	3/12	(0.177)**	5-10+	25
HB2-1	7/11	(0.614)	20-30	64
HB4-1	3/12	(0.177)**	0-10+	25
HB6-1	6/8	(0.648)	0-10+	75
Y1090	6/8		20-50	75
134	0/10	(0.022)*	2-3+	0

The effect of fusion proteins (in LB-medium, and host bacteria, *E. coli* Y1090) on zona intact hamster fertilisation assay *in vitro* is shown on Tables 4.2.

Hamster zona intact ova, 5 μl BWW containing 1 x 10^5 hamster epididymal spermatozoa and 5 μg of fusion proteins in 100 μl BWW drop were incubated for 12-16 hours.

E. coli Y1090 bacterial lysate containing 5 μg of fusion proteins were used as negative control.

134 Fusion proteins generated by a clone encoding protease inhibitor was included as positive control (**Moore *et al.*, 1993**).

* significant inhibition of fertilisation ($P < 0.05$).
** marked reduction in fertilisation ($P < 0.2$).
\+ marked reduction in sperm bound per egg.
++ considerably lower levels of sperm number bound per egg.

It was also possible in some cases to demonstrate that the expressed fusion protein was of a greater size than β-galactosidase alone. However, given the resolution of the gels this was not always possible. From the band intensity of the β-galactosidase antigen control, it was estimated that the amount of expressed protein was in the range of 0.1-1 μg.

4.2.3 Effect of fusion proteins on sperm-egg binding and fertilisation during hamster IVF

Fusion protein was generated as given in Section 2.2.14 and analysed on SDS - polyacrylamide gel as described in Section 2.2.6. It was then necessary to investigate the effect of fusion proteins on sperm-egg binding and fertilisation in order to select clones that generated proteins with blocking activity. This was carried out using a hamster *in vitro* fertilisation (IVF) assay as described in Section 2.2.9. Table 4.2 shows the result of the effect of recombinant fusion proteins expressed from different clones on hamster IVF. Where the positive (control) clone 134 fusion protein was included, no oocytes were fertilised and only 2-3 spermatozoa bound per oocyte. This compared with 75% eggs fertilised and 30-40 sperm bound per egg when Y1090 bacterial lysate (negative control) was included in the assay. Recombinant fusion proteins from clones HA5-2, HA6-2, and HB4-1 inhibited the *in vitro* fertilisation of hamster oocytes (fertilisation rate of 0 %, 25 % and 25 %) even though these clones were very poor in expressing protein (Section 4.2.2). Only clone HA5-2 generated fusion proteins with a statistically significant reduction of fertilization (P=0.03). Fusion proteins to clone HA6-2 and HB4-1 had marked reduction of fertilisation with P values of 0.177 each, compared to 0.022 for positive control (134). Although fusion proteins to clones HA6-2 and HB4-1 had a similar effect in terms of spem numbers bound per egg as HA5-2, it was observed that they had relatively high levels of fertilisation rate. Clones HA1-2 and HA2-2 generated

proteins that resulted in reduced levels of sperm bound per oocyte although higher levels of fertilisation rate. Other fusion proteins like those from clones HA2-1, HA3-2, HA4-1, HA4,2, HA6-1, and HB2-1 showed results similar to those of Y1090 (negative control). Thus fusion proteins expressed from some clones inhibited fertilisation and resulted in reduced sperm numbers bound to oocyte. Clones HA5-2, HA6-2, and HB4-1 were considered therefore the best candidates for further characterisation, followed by HA1-2 and HA2-2 .

4.2.4 Isolation of DNA from the selected clones

4.2.4.1 λDNA Preparation and polymerase chain reaction

The recombinant DNA in λphage as high titre lysates were isolated by two methods. 1) λDNA preparation followed by cleavage using *Eco*RI enzyme or 2) by direct amplification using polymerase chain reaction (PCR). Details of each of these methods are given in Sections 2.2.12.1. and 2.2.12.2 respectively. Results are shown in Fig. 4.4. Cloned DNA were isolated by the two methods and 5 µl digested with either *Eco*RI alone, or with *Eco*RI in a solution containing RNAse (method A). In method B, 5 µl of PCR product from each clone were loaded onto corresponding wells. The amount and purity of DNA generated were analyzed on 0.8% TBE agarose gel as described in Section 2.2.17. The DNA isolated were further analyzed by digestion using additional restriction enzymes. DNA and their corresponding restriction enzyme fragments were also analysed on agarose gel as described previously.

4.2.5 A comparison of the amount and purity of DNA isolated by PCR and λ DNA preparation methods

It was possible to obtain λDNA by the λDNA method however, such DNA were mostly contaminated with RNA (Fig 4.4., Lanes 1, 3, 5 and 7). When RNAse A (2 units) was included in the reaction mixture during digestion with *Eco*RI enzyme (Lanes 2, 4, 6 and 8), the DNA that resulted was relatively clean and the inserts cleaved could also be easily visualised. The procedure of λDNA method was laborious and given the number of cDNA clones to be analysed, the alternative PCR method was adopted. Using this procedure, multiple samples could be processed at a time, and with small inserts one could avoid the problem of residual RNA obscuring the band. Hence the need to load large amount of digested λDNA on a gel in order to visualise smaller inserts could also be avoided. However, in both methods, there was still a need to digest the DNA before subcloning and in many cases before use as a probe.

4.2.6 Analysis of DNA produced by polymerase chain reaction: Restriction enzyme digestion.

DNA was amplified from high titre phage lysates as described in Section 2.2.11 and analysed on TBE-agarose gel as described in Section 2.2.17. The result is shown in Fig. 4.5A.

The PCR amplified DNA was digested by *Eco*RI (an enzyme for which the recognition site was incorporated at the 5′ ends of gt11 primers 1 and 2), in order to remove the primers from the amplified DNA. The *Eco*RI cleavage products were analysed on TBE agarose gel as described in Section 2.2.17. The result is shown in Fig. 4.5B. The DNA samples generated by PCR could then be classified into three

categories. i) Clones that generated large amounts of clearly visible DNA such as clones HB7-2, HB17-2, and HB20-1 (Fig. 4.5A: Lanes 1, 2, and 3). ii) Clones which generated very small DNA or even no product at all, such as HB8-1, HB8-2 and HB9-2 (Fig. 4.5B: Lanes 4, 5, and 6), and iii) Those clones having multiple bands such as clone HB13-2 (Fig. 4.5B: Lanes 7). A total of 21 clones had either no detectable PCR product, or the products were to small to be differentiated from primer dimers or there was no product at all. Only two of the clones resulted in multiple DNA bands. Most clones generated PCR products in category (i) that varied in size from 350 bp to 2500 bp. Therefore using PCR, it was possible to generate DNA products of different sizes from different clones. Some clones amplified more efficiently than others.

Sometimes the DNA from different clones was similar in size, and produced a similar pattern of fragments when digested with *Eco*RI. Clones HB2-1 was similar to HB3-1 in generating a DNA insert of about 2.0 kb pairs (Fig. 4.6A: Lanes 1 and 3), and had internal *Eco*RI sites generating five fragments of similar sizes upon digestion with *Eco*RI (Fig. 4.6A: Lanes 2 and 4) respectively. Similar patterns were observed for clones HB4-1 and HB4-2 (Fig 4.6A: Lanes 5 and 6), and (Fig 4.6B: Lanes 1 and 2) respectively. They appeared to be of the same size, even generated multiple fragments of similar size upon digestion with *Eco*RI. In order to distinguish such clones, they were subjected to a further digestion by additional enzymes using *Kpn*I and *Sac*I. Using these enzymes it was then possible to distinguish clones HB2-1 from HB3-1 and HB4-1 from HB4-2 respectively. The differential digestion patterns for HB4-1 with *Kpn*I and with *Sac*I are shown in Fig. 4.6C and 4.6D.

FIGURE 4.4

FIGURE 4.4 ANALYSIS OF DNA ISOLATED FROM λBACTERIOPHAGE BY METHODS OF λDNA AND POLYMERASE CHAIN REACTION

TBE agarose gel of DNA isolated by λDNA method followed by restriction enzyme cleavage (A), and PCR method (B):

A: λDNA method

Lanes 1 and **2** HA2-1; HA2-1/RI
Lanes 3 and **4** HA4-1; HA4-1/RI
Lanes 5 and **6** HA5-1; HA5-1/RI
Lanes 7 and **8** HA6-1; HA6-1/RI
Lane 9 negative control

B: Polymerase chain reaction method.

Lanes 1 and **2** PCR positive control
Lanes 3 and **4** HA2-1; HA2-1/RI
Lanes 5 and **6** HA4-1; HA4-1/RI
Lanes 7 and **8** HA6-1; HA6-1/RI

Markers:

*Hinf*I cut pAT markers (75, 154, 221, 298, 396, 517, and 1631 bp)
*Hind*III cut λDNA markers (2.0, 2.3, 4.4, 6.7, 9.4, and 23.1 kbp).

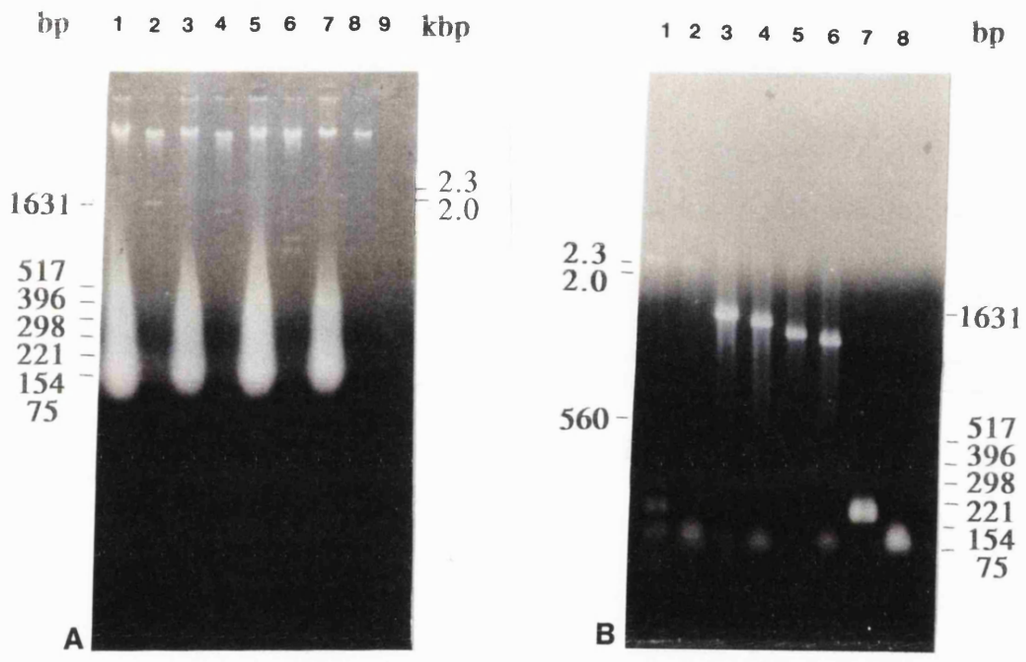

bp
1 2 3 4 5 6 7 8 9
kbp
1631
517
396
298
221
154
75
2.3
2.0
A
1 2 3 4 5 6 7 8
bp
2.3
2.0
560
1631
517
396
298
221
154
75
B

FIGURE 4.5

FIGURE 4.5 ANALYSIS OF THE SIZE AND AMOUNT OF DNA INSERTS GENERATED BY PCR

A: Clones that amplified DNA more efficiently than others.

Lane 1. HB7-2

Lane 2. HB17-2

Lane 3. HB20-1

Lane 4. PCR positive control

Lanes 5 and **6.** PCR negative control

B: *Eco*RI DNA cleavage products.

Lane 1. HB5-2

Lane 2. HB6-2

Lane 3. HB7-2

Lane 4. HB8-1

Lane 5. HB8-2

Lane 6. HB9-2

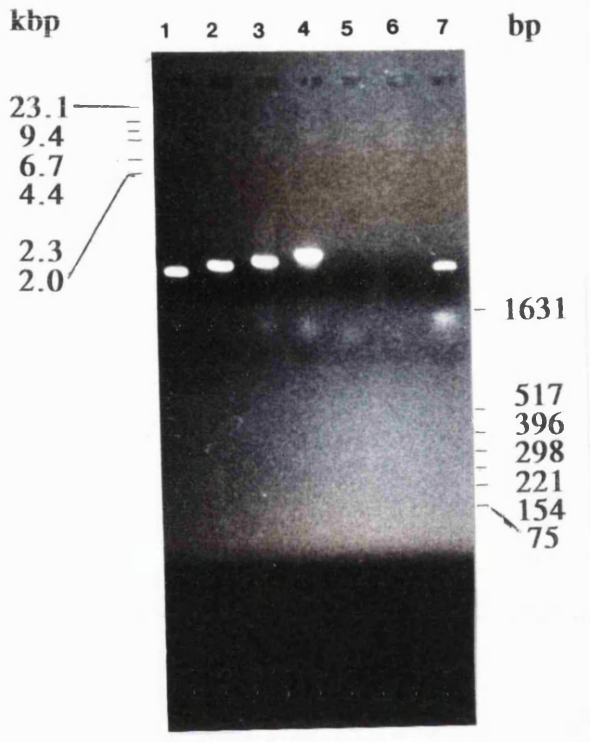
Lane 7. HB13-2

Lane 8. HB14-2

Lane 9. negative control

Markers:

*Hind*III cut λDNA and *Hinf*I cut pAT markers as for Fig. 4.4.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bp
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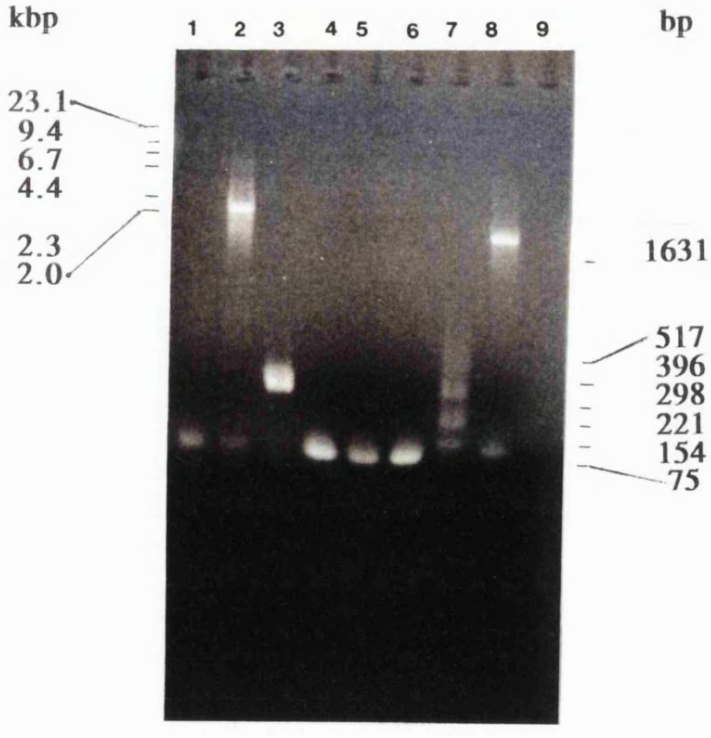
9.4
6.7
4.4
2.3
2.0
1631
517
396
298
221
154
75

A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bp
23.1
9.4
6.7
4.4
2.3
2.0
1631
517
396
298
221
154
75

B

FIGURE 4.6

FIGURE 4.6 DIFFERENT CLONES WITH SIMILAR DNA DISTINGUISHED BY RESTRICTION ENZYME DIGESTION

A: **Lanes 1** and **2** HB2-1; HB2-1/*Eco*RI

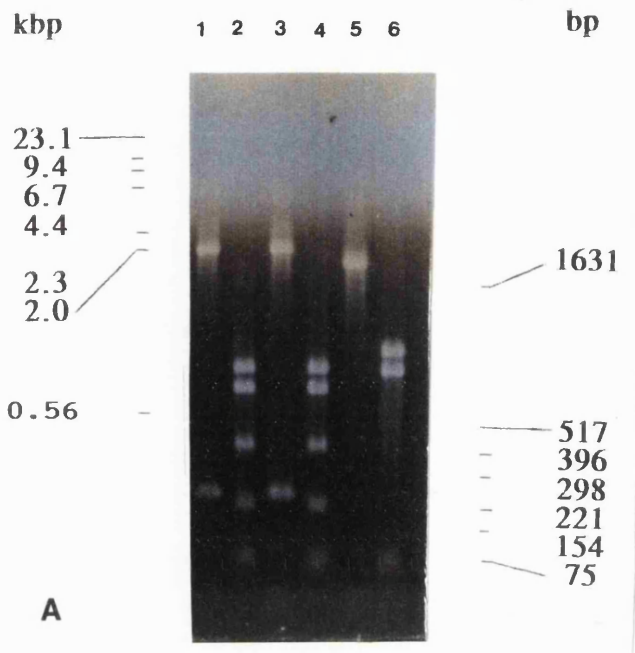
Lanes 3 and **4** HB3-1; HB3-1/*Eco*RI

Lanes 5 and **6** HB4-1; HB4-1/*Eco*RI

B: **Lanes 1** and **2** HB4-2; HB4-2/*Eco*RI

C: **Lanes 1, 2 and 3** HB4-1; HB4-1/*Kpn*I; HB4-1/*Sac*I

D: **Lanes 1, 2 and 3** HB4-2; HB4-2/*Kpn*I; HB4-2*Sac*I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bp
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0.56
1631
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396
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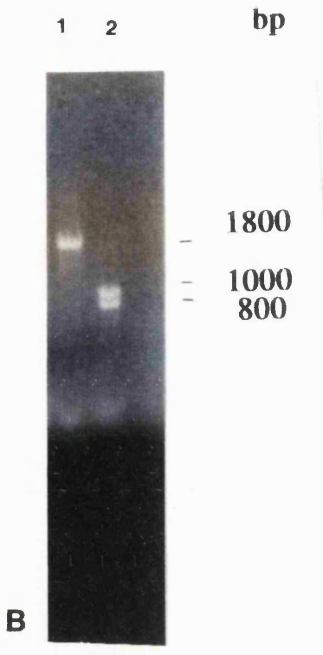
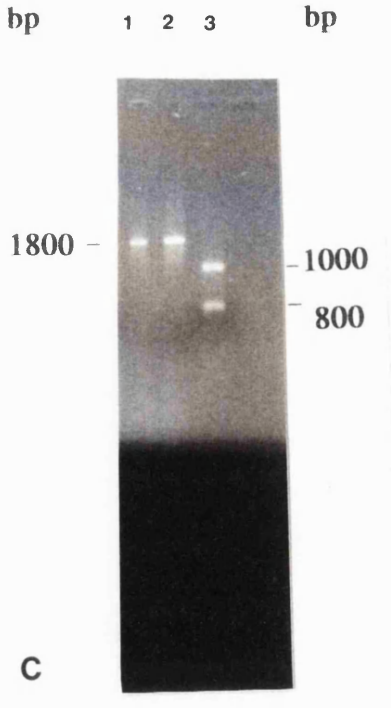
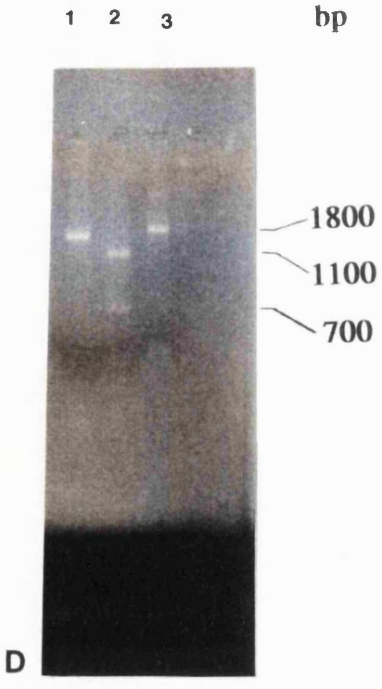
221
154
75
A


1 2
bp
1800
1000
800
B


bp
1 2 3
bp
1800
1000
800
C


1 2 3
bp
1800
1100
700
D

4.3 DISCUSSION

Although antibody quality plays a critical role in a successful screen, our attempt to pre-absorb cross-reacting *E. coli* antibodies from the antisera proved inconclusive. Signals from cross-reacting antibodies recognising *E. coli* components could be reduced but only at the expense of reducing specific antibody signal. Thus we were compelled to use the non-absorbed antisera in screening the testis cDNA library. Non-absorbed rabbit anti-sera R7 and R10 was successfully used to identify over 70 expression cDNA clones from a human testis λgt11 library.

Proper expression of foreign DNA in λgt11 depends on the orientation and reading frame of the insert with respect to those of *lacZ*. The amount of antigen generated from the foreign DNA sequences by the vector, is therefore mainly dependent on these two parameters in relation to the β-galactosidase transcriptional unit (**Young and Davis, 1983a**). The observation that different cDNA clones generated varying amounts of β-galactosidase fusion proteins was therefore not uncommon. Inappropriate orientation or incorrect reading frame may have contributed to the poor expression of fusion proteins in some clones e.g HA5-2, HA6-1 and HA6-2. Whereas inappropriate orientations may be unlikely, incorrect reading frames are more often encountered in cases where the restriction enzymes sites are compromised. Bacterial host cells are also known to be very unstable with eukaryotic proteins especially those generated from large cDNA inserts. Although *E. coli* Y1090 strain is *lon-* (engineered to be deficient in eukaryotic protein degradative activity), degradation of generated fusion proteins is sometimes inevitable. **Moore *et al.*** (1993) encountered degradation by clone 222 using host cells with similar strains. Thus some clones may have generated enough fusion proteins but were degraded prior to their use in the hamster IVF assay since no protease inhibitor was used. A protease inhibitor like phenyl methyl sulphonic fluoride (PMSF) would enhance the stability of

fusion proteins. However, it was felt inappropriate to use protease inhibitor as the fusion protein lysate would be used eventually in a hamster IVF assay and a protease inhibitor in the lysate would block the acrosome reaction and fertilisation (**Saling, 1986**). The other factor, and most crucial, is the nature of proteins encoded by the foreign DNA insert. These may vary from proteases to protease inhibitors. Whereas the latter may be beneficial because it will help to further inhibit bacterial and other proteolytic activities, the former would be detrimental as it would tend to auto-degrade as soon as it is formed.

Hamster IVF has been used successfully to select clones on the basis that the expressed fusion proteins interfere with fertilisation (**Moore *et al.*, 1993**). When fusion proteins generated from different cDNA clones were tested in the hamster IVF, different characteristics were observed for each clone. Fusion proteins from clones HA5-2, HA6-2 and HB4-1 were quite effective at inhibiting fertilisation. Also observed was their ability to interfere with sperm-egg binding as compared to negative control. It was noted that some fusion proteins that inhibited fertilisation also had some effect in reducing the number of spermatozoa bound per egg. Clones HA5-2, HA6-2 and HB4-1 had values in the range 3-4, 0-10, and 0-10 respectively compared to 0-3 for clone 134 (positive control). It may be suggested that other clones such as HA1-2 with value of 10-20 and relatively higher levels of fertilisation rate may also have produced important proteins to warrant further investigation. investigation.

Recombinant DNA in bacteriophages as high titer lysates are usually prepared by λ DNA method and the insert subsequently recovered by digestion using restriction enzymes. Currently the PCR procedure is more frequently used for direct amplification and isolation of, and even sequencing the DNA insert, using λ-gt11 specific primers. Although PCR has become state of the art procedure in molecular

cloning, shortcomings such as mis-incorporation of nucleotides are often encountered. Observations such as being able to recover a piece of DNA insert by λ-DNA method and not by PCR (clone HA5-2), may not be that unusual since in PCR compromising a restriction enzyme cloning site may lead to difficulties in amplifying out the insert. λ-DNA procedure still remain an invaluable method for isolation of λ-DNA and the subsequent recovery of the insert.

In conclusion, the results from the work in this chapter strongly suggested that cDNA clones HA5-2, HA6-2 and HB4-1, contained relevant recombinant DNA for further analysis.

CHAPTER 5

SEQUENCING AND ANALYSIS OF THE SELECTED CLONES

5.1. INTRODUCTION

The human testis λgt11 cDNA library was screened with mixed rabbit anti-acrosomal antisera R7 and R10. Positive clones were identified, cDNA inserts amplified by PCR and preliminary restriction enzyme sites determined by digesting with selected enzymes. Three clones (HA5-2, HA6-2 and HB4-1) generated β-galactosidase fusion peptides which interfered with sperm binding and fertilisation during hamster IVF. The clones were to be characterised further by: 1) digestion with restriction enzymes in order to determine the restriction map; 2) subcloning the restriction fragments into plasmid, amplifying and transfering the insert into bacteriophage vector for sequencing and 3) sequencing and analysing the sequence information of the cDNA insert.

The map of pBluescript® SK (+/-) phagemid is shown in Fig. 5.1. It is a 2.96 kb phagemid derived from pUC19. Features that were important for this work are: **lacZ**, a portion of lacZ gene that provides α-complementation for blue/white colour selection of recombinant phagemids. This is an inducer of lac promoter upstream from lacZ gene that permits fusion protein expression with the β-galactosidase gene product. Multiple cloning site (**MCS**), contain sequences for unique restriction enzymes (including *Eco*RI) that allows for directional cloning of short DNA fragments. pBluescript® also has an ampicillin resistance gene for antibiotic selection of the phagemid vector.

It was important to obtain sufficient amount of clean DNA inserts with corresponding restriction enzyme sequences at the ends to facilitate ligations. PCR amplified DNA from high titre lysates of these clones were therefore used in restriction enzyme digestion.

FIGURE 5.1

FIGURE 5.1 MAP OF pBLUESCRIPT®SK(+/-) PHAGEMID

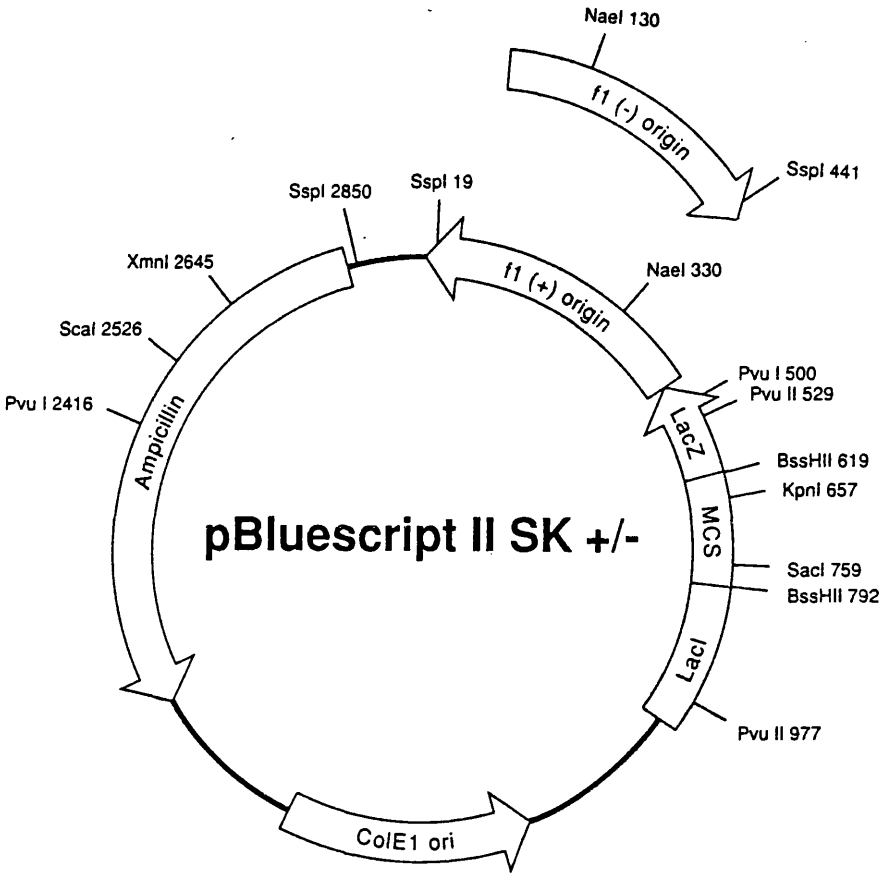
pBluescript® SK (+/-) is a 2.96 kb cloning vector with the polycloning site (containing *Eco*RI restriction site) located within a lacZ gene. (Adapted from **Sambrook *et al* 1989**).

A: The SK designation indicates the polylinker is orientated such that the lacZ transciption proceeds form *Sac*I to *Kpn*I

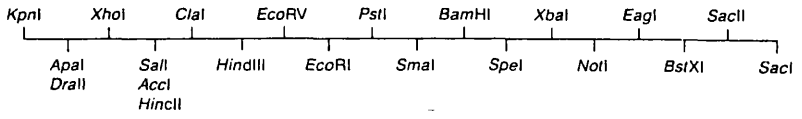
The upper strand is the (+) strand and the lower strand is the (-) strand

B: Restriction enzymes and sites on the multiple cloning site

A


NaeI 130
f1 (-) origin
SspI 441
SspI 2850
SspI 19
XmnI 2645
f1 (+) origin
NaeI 330
ScaI 2526
PvuI 500
PvuII 529
PvuI 2416
Ampicillin
LacZ
BssHII 619
KpnI 657
pBluescript II SK +/-
MCS
SacI 759
BssHII 792
LacI
PvuII 977
ColE1 ori

B


KpnI
XhoI
ClaI
EcoRV
PstI
BamHI
XbaI
EagI
SacII
ApaI
DraII
SalI
AccI
HincII
HindIII
EcoRI
SmaI
SpeI
NotI
BstXI
SacI

The PCR DNA insert was therefore to be digested using *Eco*RI enzyme for subsequent cloning onto these vectors, and using *Eco*RI either alone or in combination with other appropriate restriction enzymes to generate probes for radiolabelling. Determination of restriction sites for other enzymes would also facilitate rapid sequencing of long clones in short manageable fragments.

To obtain a reasonable amount of clean DNA for sequencing, it was necessary to digest and subclone the DNA into pBluescript® for amplification and then into M13 mp18 and M13 mp19 for final sequencing.

5.2 RESULTS

5.2.1 Restriction mapping of clone HB4-1

Clone HB4-1 was digested with a set of restriction enzymes chosen on the basis that they were: 1) located only in the multicloning site of pBluescript®, as well as that in the M13 mp18 and mp19 cloning vectors and 2) have 6 bp and not 4 bp recognition sequences. Restriction endonucleases with 4 bp recognition sequences would cut too frequently to be of use in initial mapping and sequencing, there would also be a risk of digesting the vector. These recognition sequences were as follows: *Eco*RI (G↓AATTC), *Bam*HI (G↓GATCC), *Hind*III (A↓AGCTT), *Pst*I (CTGCA↓G), *Xba*I (T↓CTAGA), *Kpn*I (GGTAC↓C), and *Sac*I(*Sst*I) (GAGCT↓C). Clone HB4-1 was digested with a single, or combinations of two (selected) restriction enzymes. Fig. 5.2 shows the results of these restriction enzyme digestion. On single digest, no internal site for the enzymes *Sph*I and *Sma*I was found. There was internal site for each of *Xba*I and *Bam*HI, on which upon digestion, fragments of sizes 600 + 1200 and 700 + 1100 bp were generated respectively (Fig. 5.2A). Double digests using a mixture of

*Eco*RI + *Bam*HI, revealed that *Bam*HI cut the 1000 bp fragment into 200 and 800 bp fragments., + *Pst*I cut the 800 fragment into 300 and 500 bps, + *Xba*I cut the 1000 fragment into 400 and 600 bp fragments. However, double digests using either *Pst*I alone, *Pst*I + *Kpn*I or *Pst*I + *Sac*I, just reduced the size of DNA by about 300 bp only, but *Pst*I + *Xba*I resulted into three fragments 250, 600 and 1050 suggesting that *Pst*I fragment is on the 800 bp portion while *Xba*I site is along the 1000 bp portion (Fig. 5.2 B). A restriction map was therefore drawn (Fig. 5.6)

5.2.2 Digestion of clones HA5-2, HA6-2 and HB4-1 using *Eco*RI

PCR amplification of clones HA5-2, HA6-2 and HB4-1 bacteriophages was carried out as described in Section 2.2.12.2. DNA produced was analysed on TBE agarose gel as detailed in Section 2.2.17 and was determined to be 150, 300, and 1800 base pairs respectively as shown in Fig. 5.3. Upon digestion with *Eco*RI restriction endonuclease, clones HA5-2 and HA6-2 had even smaller DNA inserts. However, the 1800 bp long HB4-1 was found to contain an internal *Eco*RI site resulting in the generation of 800 and 1000 bp fragments. All the three clones showed reduction of molecular sizes upon restriction enzyme cleavage signifying the removal of primers at each ends.

5.2.3 Subcloning the inserts into pBluescript® SK+

pBluescript®SK+ was digested with *Eco*RI to expose the cohesive sites. The resulting linear DNA was treated with calf intestinal alkaline phosphatase (CIAP) to prevent re-ligation as described in Section 2.2.21.1.

FIGURE 5.2

FIGURE 5.2 ANALYSIS OF RESTRICTION DNA FRAGMENTS FROM CLONE HB4-1 ON ETHIDIUM BROMIDE STAINED AGAROSE GEL AFTER DIGESTION WITH SINGLE OR MORE RESTRICTION ENZYMES

A. Digestion using single restriction enzymes.

Lane 1: Undigested HB4-1
Lane 2: HB4-1 digested by *Sph*I
Lane 3: HB4-1 digested by *Xba*I
Lane 4: HB4-1 digested by *Sma*I
Lane 5: HB4-1 digested by *Bam*HI

B. Digestion using single and combination of two restriction enzymes.

Lane 1: HB4-1 uncut (control)
Lane 2: Cut by *Eco*RI alone
Lane 3: " *Eco*RI + *Bam*HI
Lane 4: " *Eco*RI + *Pst*I
Lane 5: " *Eco*RI + *Xba*I
Lane 6: " *Eco*RI + *Kpn*I
Lane 7: " *Eco*RI + *Sac*I
Lane 8: " *Pst*I alone
Lane 9: " *Pst*I + *Kpn*I
Lane 10: " *Pst*I + *Xba*I
Lane 11: " *Pst*I + *Sac*I

Also included in the gel were *Hind*III cut λDNA and *Hinf*I cut pAT markers as shown in Fig. 4.4.

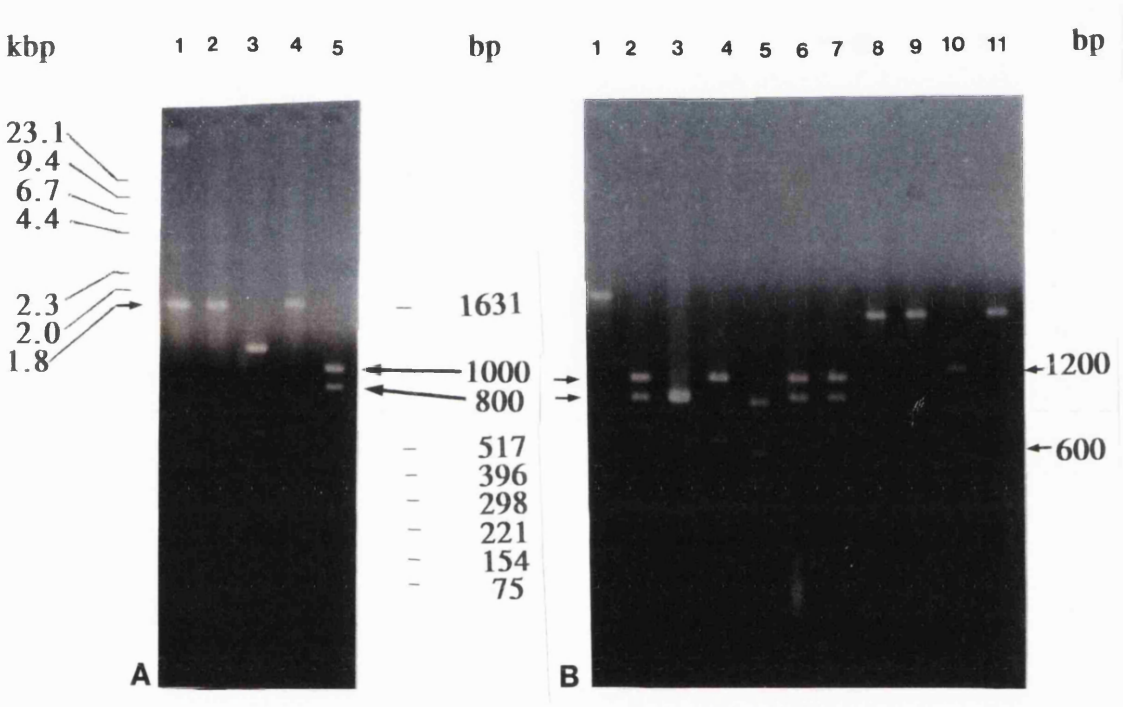

kbp
1 2 3 4 5
23.1
9.4
6.7
4.4
2.3
2.0
1.8
A
bp
1631
1000
800
517
396
298
221
154
75
1 2 3 4 5 6 7 8 9 10 11
bp
B
1200
600

FIGURE 5.3

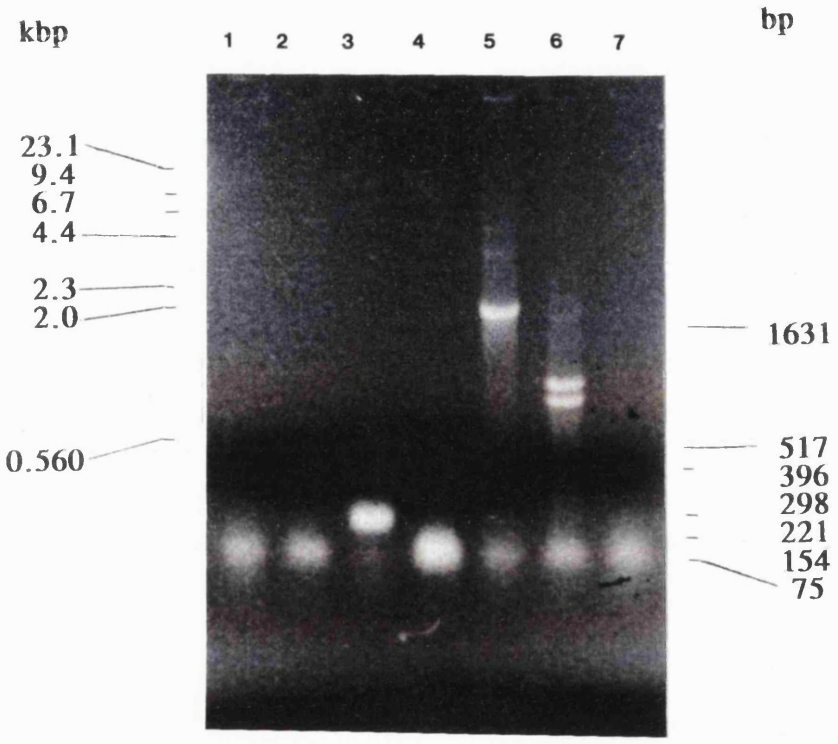
FIGURE 5.3 ETHIDIUM BROMIDE STAINED AGAROSE GEL OF *Eco*RI RESTRICTION DIGESTION DNA FRAGMENTS GENERATED FROM CLONES HA5-2, HA6-2 AND HB4-1

Following amplification, 5µl of the products was digested with *Eco*RI and analysed on 0.8% TBE agarose gel.

Lane 1: HA5-2 uncut
Lane 2: HA5-2 cut by *Eco*RI
Lane 3: HA6-2 uncut
Lane 4: HA6-2 cut by *Eco*RI
Lane 5: HB4-1 uncut
Lane 6: HB4-1 cut by *Eco*RI
Lane 7: Negative control sample.

Negative control contained all PCR reactants (including primers) except the DNA template.

Markers: *Hind* III cut DNA and *Hinf*I cut pAT markers were as given in Fig. 4.4


kbp
1 2 3 4 5 6 7
bp
23.1
9.4
6.7
4.4
2.3
2.0
0.560
1631
517
396
298
221
154
75

The linear DNA was resolved on LMP agarose and purified by gene cleaning. The concentration was estimated against standard DNA markers and adjusted to 20 ng/ml. Both gene cleaned insert and vector were ligated in excess molar ratio of insert to vector (mole ratio 2:1, Insert : Vector) in order to force the ligation of the insert. Appropriate volumes of clones HA5-2 and HA6-2 *Eco*RI digests were heat inactivated (to denature any trace of *Eco*RI) in a water bath at 75°C for 10 minutes. Ligation reactions were carried out as described in Section 2.2.21.2. The insert in pBluescript® was isolated by method of "mini prep" as described in Section 2.2.15.1. The results are shown in Fig. 5.4. The DNA prepared was further digested by *Eco*RI restriction enzyme to determine the existence of the insert. Two extreme categories of recombinant plasmids were observed as judged by blue and white selection procedure. In the first category were some white (recombinant) plasmids from which the insert was easily recovered upon digestion with *Eco*RI (HB4-1_{1000}F, Panel A, Lane 2, HB4-1_{800}D, Panel B, Lane 6). The second category consisted of numerous white (presumably recombinant) plasmids in which even after digestion with restriction enzyme, it was still impossible to linearise the plasmid, let alone recover the insert. These exhibited characteristics depicted by HB4-1_{1000}G (Panel A, Lanes 3 and 4) and HB4-1_{1000}F (Panel B, lanes 3 and 4). There was an intermediate category which comprised recombinant plasmids which were easily linearised but the insert was not easy to recover (HB4-1_{800}G, Panel A, Lanes 7 and 8).

5.2.3.1 Identification of plasmids carrying DNA insert.

In the experiment shown in Fig. 5.5, a large number of colonies of *E.coli* JM101 carrying different recombinant plasmids(pBluescript^R)were screened to determine which recombinant plasmids contained specified DNA sequence. This was carried out using the method of **Grunstein and Hogness (1975)**, Section 2.2.23. DNA probes

were generated and prepared by two main methods. (A): Clone HB4-1 was fairly large with several restriction enzyme sites, including an internal *Eco*RI site. This clone was digested using *Eco*RI, and a combination of *Eco*RI and other enzymes which had been determined to cut some site (s) within the HB4-1 clone. These included *Pst*I and *Xba*I, *Bam*HI, and *Kpn*I. (B): Clone HA5-2 and HA6-2 were fairly small and required no additional restriction mapping. However, the size presented a problem. The DNA was not always recovered at the purification stage in required amount for subsequent use as probes. After *Eco*RI digestion to remove the primers, they were either (i), heated at 65°C for 10 minutes before use to inactivate the restriction enzyme., or (ii) "gene cleaned" using Mermaid™ kit (Section 2.1.8). DNA fragments derived from (A) and (B,ii) above were separated on low melting point agarose and purified by either "gene clean™" kit of Mermaid™Kit and labelled with ^{32}P-αdCTP to high specific activity as described in Section 2.2.25. As was expected, background signal was observed when non-recombinant plasmids (blue), were probed with corresponding sequences prepared as in A above (Fig.5.5, plates 2 and 3., spots H1, H2, H3, and H4)., or as in B (ii) above (Fig. 5.5, plate 1, spots H1, H2, H3, and H4). There was also background signal from recombinant pBluescript® carrying HA6-2 DNA insert regardless of the source of probe either from source B (i) spots A4 to A8., or from source B (ii) spots A1 to A3. Six strong signals were produced when recombinant pBluescript plasmid carrying the 1000 bp fragment was probed with corresponding sequences prepared as in A above was used. All of the positive signals derived from the "gene cleaned" probe (spots A10, B5, C5, and C7., no signal was derived from the "Mermaid" cleaned probe source(spots F1 toF12 and G1 to G6). Also shown is lack of signal on spots G7 to G8, recombinant plasmids probed using the 800 bp fragment of clone HB4-1 cleaned by "Mermaid". It was very disappointing

FIGURE 5.4

FIGURE 5.4 AGAROSE GEL OF NON- AND RECOMBINANT PLASMID DNA ISOLATED FROM BLUESCRIPT BY "MINI PREP".

DNA was isolated and a portion digested by *Eco*RI. 5µl of uncut and *Eco*RI plasmid digestion product were resolved on 0.8% TBE agarose gel as described in the methods section. Subscripts designate fragment size and capitalised letters after the subscripts designate the plasmid colony from which the DNA was extracted.

A.

Lane 1: HB4-1_{1000}F uncut
Lane 2: HB4-1_{1000}F cut with *Eco*RI
Lane 3: HB4-1_{1000}G uncut
Lane 4: HB4-1_{1000}G cut with *Eco*RI
Lane 5: HB4-1_{800}F uncut
Lane 6: HB4-1_{800}F cut with *Eco*RI
Lane 7: HB4-1_{800}G uncut
Lane 8: HB4-1_{800}G cut with *Eco*RI

B.

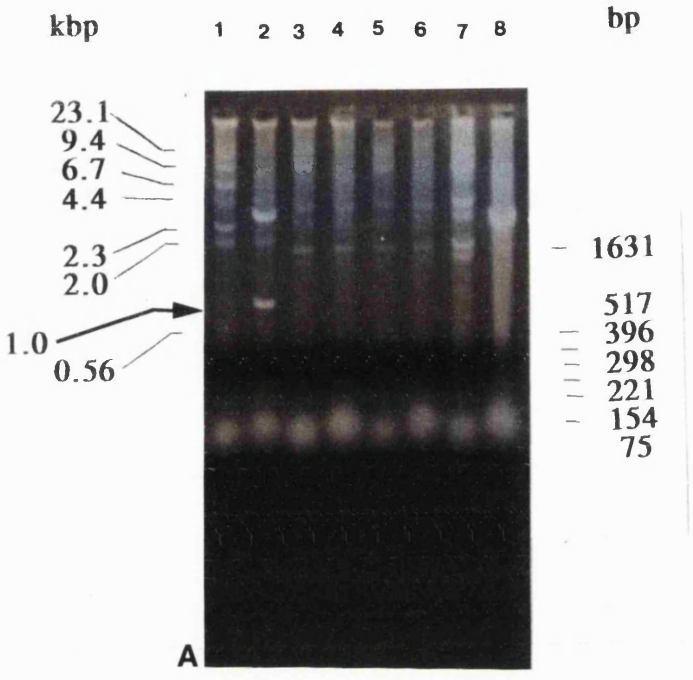
Lane 1: HB4-1 Whole PCR DNA.
Lane 2: HB4-1 PCR restriction products of *Eco*RI.
Lane 3: HB4-1_{1000}F uncut
Lane 4: HB4-1_{1000}F *cut with Eco*RI
Lane 5: HB4-1_{800}D uncut
Lane 6: HB4-1_{800}D *cut with Eco*RI
Lane 7: Non-recombinant bluescript (blue), uncut
Lane 8: Non-recombinant bluescript (blue), cut with *Eco*RI
Lane 9: Bluescript (SK+) uncut

Also included in the gel were *Hind*III cut λ-DNA and *Hinf*I cut pAT markers as shown in Fig. 4.4.

The position of fragment 1000 and 800 bp insert from one of the positive recombinant bluescript is shown by arrow.
Controls included were:

Non-recombinant transformed plasmid (blue), uncut and cut with *Eco*RI, panel B, lanes 8 and 9 respectively.

Non-recombinant bluescript DNA (uncut) sample from the batch used for ligation. PCR amplified full length and *Eco*RI DNA fragments from clone HB4-1, were also included as additional size controls.


kbp
1 2 3 4 5 6 7 8
bp
23.1
9.4
6.7
4.4
2.3
2.0
1.0
0.56
1631
517
396
298
221
154
75
A

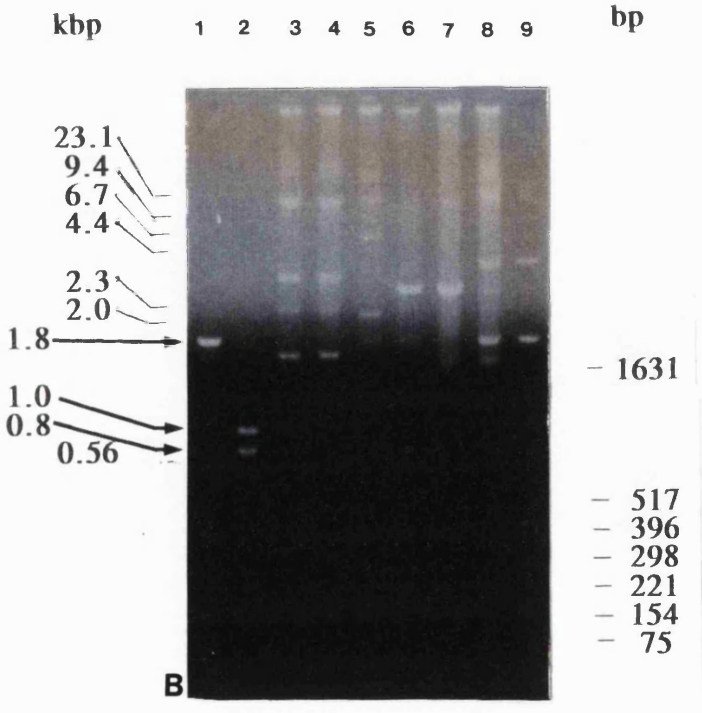

kbp
1 2 3 4 5 6 7 8 9
bp
23.1
9.4
6.7
4.4
2.3
2.0
1.8
1.0
0.8
0.56
1631
517
396
298
221
154
75
B

FIGURE 5.5

FIGURE 5.5 DETECTION OF BACTERIAL COLONIES CARRYING RECOMBINANT PLASMID DNA BY COLONY HYBRIDISATION

Plate 1: Was probed using DNA prepared from Clone HA6-2, cleaned by "Mermaid" kit as follows.

(i) Spots A1 to A3 contained DNA lysed from bacteria carrying recombinant plasmids ligated to "Mermaid" cleaned HA6-2 insert.

(ii) Spots A4 to A6, as (i) above but the recombinant plasmid was ligated to Heat inactivated DNA from clone HA6-2.

Plate 2: Was probed using the 1000 bp fragment of HB4-1 "gene cleaned" DNA. Spots A9 to A12, B1 to B12, and C1 to C12, contained DNA from lysed bacteria carrying recombinant plasmids ligated to "gene cleaned" HB4-1_{1000} insert.

Plate 3: Was probed using the 1000 bp fragment of HB4-1 "gene cleaned" DNA. Spots D1 to D12, and E1 to E12, contained recombinant plasmid DNA ligated as in plate 2.

Plate 4: Was probed using the 1000 bp fragment of HB4-1 "gene cleaned" DNA.

(i) Spots F1 to F12 and G1 to G6, the recombinant plasmid was ligated to "Mermaid" cleaned HB4-1_{1000} insert.

Plate 5: Was probed using the 800 bp fragment of HB4-1 "gene cleaned" DNA.

(i) Spots G7 and G8 the recombinant plasmid was ligated to "Mermaid" cleaned HB4-1_{800} insert.

The controls included were: Two blue (spots H1 and H2) and two white colonies (spots H3 and H4) carrying non-recombinant pBluescript, digested by *Eco*RI and alkaline phosphatased subjected through the same experimental conditions.

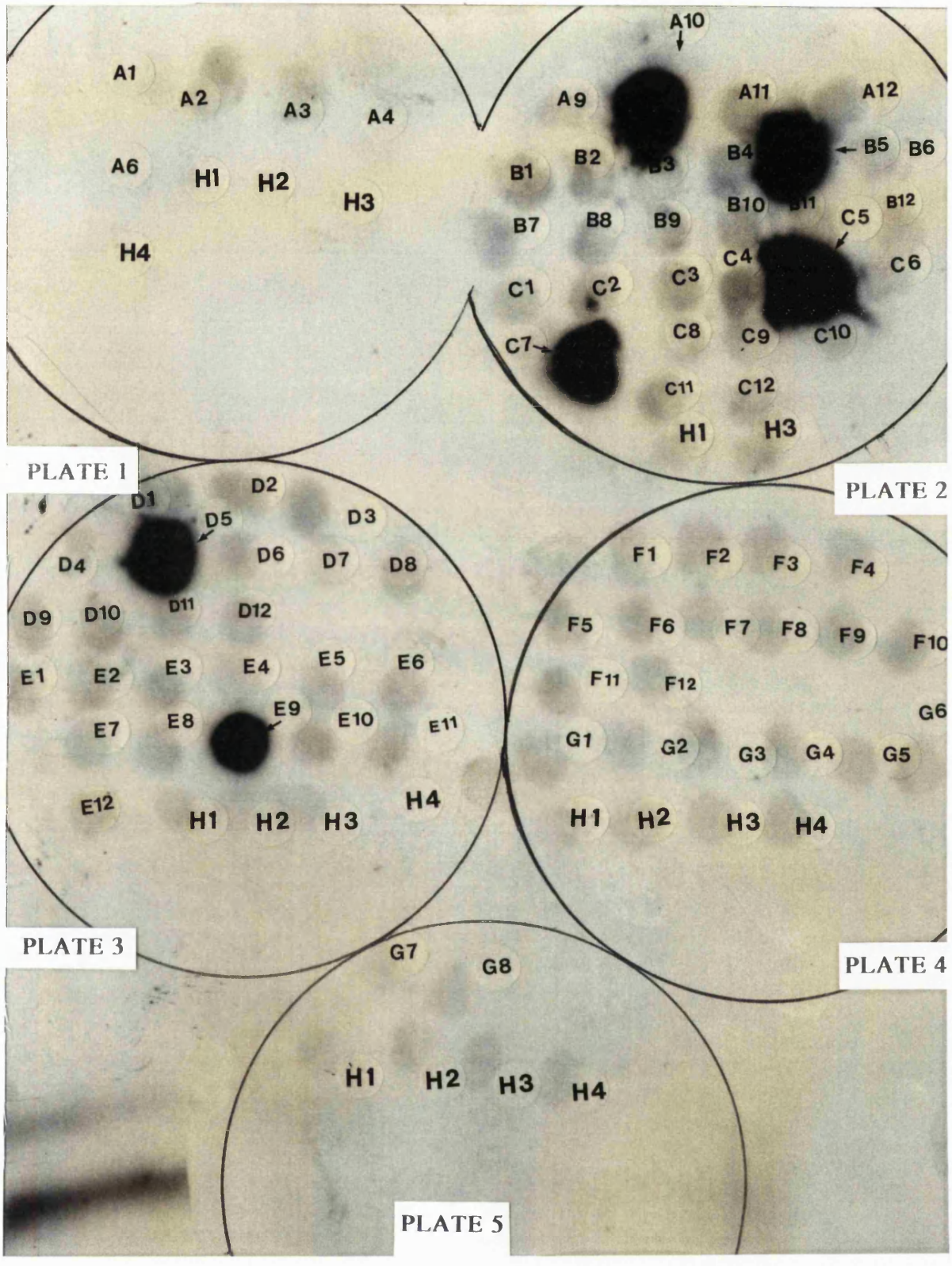

A1
A2
A3
A4
A6
H1
H2
H3
H4
PLATE 1
A10
A9
A11
A12
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B12
C5
C4
C6
C1
C2
C3
C7
C8
C9
C10
C11
C12
H1
H3
PLATE 2
D1
D2
D5
D3
D4
D6
D7
D8
D9
D10
D11
D12
E1
E2
E3
E4
E5
E6
E9
E7
E8
E10
E11
E12
H1
H2
H3
H4
PLATE 3
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
G6
G1
G2
G3
G4
G5
H1
H2
H3
H4
PLATE 4
G7
G8
H1
H2
H3
H4
PLATE 5

FIGURE 5.6

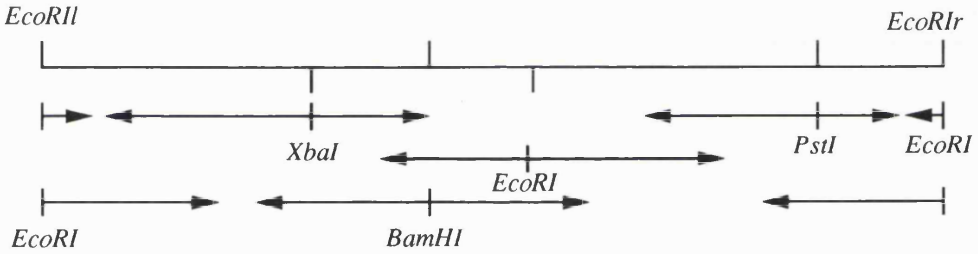
FIGURE 5.6 RESTRICTION MAPPING AND SEQUENCING STRATEGY OF CLONE HB4-1.

(A) The names and positions of selected restriction enzymes used to characterise clone HB4-1 are shown. The *Eco*RI sites that lies at each ends of the clone are designated *Eco*RI$_l$ and *Eco*RI$_r$ respectively. Also shown is the internal (*Eco*RI$_m$) site. Each fragment was cloned into Bluescript and plasmid M13 mp18 or M13 mp19 for directional sequencing.

(B) Sequencing strategy. The fragments generated by restriction enzymes were cloned into pBluescript^R and M13 mp18 or M13 mp19 for directional sequencing. The direction and length of each sequencing run are indicated by the arrows. The sequence of complementary strands of the cDNA insert was determined completely.


EcoRIl
XbaI
BamHI
EcoRIm
PstI
EcoRIr

A


EcoRIl
EcoRIr
XbaI
PstI
EcoRI
EcoRI
EcoRI
BamHI

B


0
400
800
1200
1600
1750 bp

not to have been able to subclone HA5-2 and HA6-2 at this stage. However, all the fragments from clone HB4-1 were successfully subcloned in subsequent attempts. One of each of the positive fragments from clone HB4-1_{1000}F and HB4-1_{800}D were selected for subsequent subcloning sequencing vector. An attempt was made to subclone clones HA5-2 and HA6-2 into M13 sequencing vector with difficulties. However HB4-1 *Eco*RI restriction fragments were subcloned into pBluescript^R and M13 sequencing vector with less problems. Several white recombinant plasmids (positive for inserts) were picked and the DNA was extracted by "mini plasmid prep" (Section 2.2.15.1). All the DNA from white colonies were digested with *Eco*RI enzyme from which one clone from each fragment (HB4-1_{1000}F and HB4-1_{800}D) was selected as true positive for further subcloning into M13 mp18 and M13 mp19 for sequencing.

5.2.4 Sequencing and sequence analysis

The cDNA insert contained in the λ-gt11 HB4-1 was mapped using restriction enzymes. The fragments were isolated cloned into pBluescript^R plasmid and subsequently into M13 bacteriophages. Both strands of each fragment were sequenced completely. Sequenced restriction fragments and sequencing strategy is shown in Fig. 5.6 and complete nucleotide sequence is shown in Fig. 5.7. The HB4-1 cDNA was shown to be 1749 bp in size and contain an internal *Eco*RI (*Eco*RI_m) site at position 943. Restriction enzymes used to digest and remove the fragments for subcloning were: *Xba*I, *Bam*HI, and *Pst*I, located at positions 512, 798, and 1511 respectively. The nucleotide sequence of HB4-1 was compared to the Genbank database and showed >96% homology to human testis specific LDH-C4 (**Milan *et al* 1987**), from nucleotide number 12 to 1182 of the HB4-1 cDNA sequences (see Fig.5.8A and Fig. 5.9). This homology extended throughout the whole length of the

LDH-C4 gene. It also showed >71% homology to internal sequences of *Chlamydomonas* mRNA for caltractin gene (**Lee et al 1991**), a basal-body associated Ca^{2+}-binding protein. This homology extended from nucleotide number 1166 to 1601 of the HB4-1 cDNA sequences. There was no overlap in homology between these two genes (B). Further analysis of the non-overlapping region of the HB4-1 cDNA sequences (nucleotide number 1000 to 1300) for restriction enzymes revealed one *SstI/Sac*I and three *Taq*I sites. No other restriction sites were revealed in this region (C).

5.3 DISCUSSION

As pointed out earlier in chapter 4, the primers used to amplify DNA insert from each clone had *Eco*RI restriction enzyme site at each end. Digestion of PCR products with this enzyme would yield in most cases a single piece of double strand DNA with *Eco*RI site at each end, and primer or primer dimers. These would easily be recognised when analysed on agarose gels. Analysis of clone HB4-1 *Eco*RI fragments on agarose gels revealed two fragments instead of one. Two fragments would only result if a clone has an internal *Eco*RI site and clone HB4-1 was indeed confirmed after sequencing to contain one internal *Eco*RI site. In PCR, the whole process is purely a synthetic reaction, unlike in living systems where any potential *Eco*RI cleavage site is methylated and therefore not recognised. Methylation is used during library preparation to block the susceptible site for *Eco*RI restriction enzyme. Finding an *Eco*RI site in the PCR amplified product of clone HB4-1 was therefore not unusual although not anticipated considering that HB4-1 is about 1.8 kb long and *Eco*RI recognises 6 bp sequences. Suitable short sites for other restriction enzymes

FIGURE 5.7

Fig. 5.7 Complete nucleotide sequence of human testis cDNA clone HB4-1.

5′	GAATTCAGGGCAAAGGTCCTCCAAATGTCAACTGTCAGGA	40
	GCAGCTAATTGAGAAGCTAATTGAGGATGATAAAAACTCC	80
	CAGTGTAAATTACTATTGTTGGAACTGGTG CCGTCAGTGG	120
	CTTGTGCTATTAGTATCTTACTGAAGGATTTGGCTGATGA	160
	ACTTGCCCTTGTGATGTTGCCATGGACTTTCTGAAGGGAG	200
	AATGATGGATCTTCAGCATGGCAGTCTTTTCTTTAGTACT	240
	TCAAAGATTACTTCTGGAAAAGATTACAGTGTATCTGCAA	280
	ACTTCCAGAATAGTTATTGTCACAGCAGGTGCAAAGCAGC	320
	AGGAGGAAGAAACTCGCCTTGCCCTGGTCCAACGTAATGT	360
	GGCTATAATGAAATCAATCATTCCTGCAATAGTCCATATA	400
	GTCCTGATTGTAAAATTCTTGTTGTTTCAAATCCAGTGAT	440
	ATTTTGACATATATAGTCTGGGAAGATAAGTGGCTTACCT	480
	GTAACTCGTGTAATTGGAAGTGGTTGTAATCTAGACTCTA	520
	AAGTTTCCGTTACCTAATTGGAGAAAAGTTGGGTGTCACC	560
	CACAAGCTGCCATGGTTGGATTATTGGAGAACATGGTGAT	600
	TCTAGTGTGCCCTTATGGAGTGGGGTGAATGTTGCTGGTG	640
	TGGCTCTGAAGAGTCTGGACCCTAAATTAGGAACGGATTC	680
	AGATAAGGAACACTGGAAAAATATCCATAAACAAGTTATT	720
	CAAAGTGCCTATGAAATTATCAAGCTGAAGGGGTATACCT	760
	CTTGGCTATTGGTCTGTCTGTGATGGATCTGGTAGGATTC	800
	ATTTTGAAAATCTTAGGAGAGTGCACCCAGTTTCCACCAT	840
	GGTTAAGGGAAATTATATGGAATAAAAGAAGAACTCTTTC	880
	TCAGTATCCCTTGTGGTCTGGGGCGCAATGGTGTCTCAGA	920
	TGTTGTGAAAATTAACTTGAATTCTGAGGAGGAGGCCTTT	960
	TCAAGAAGAGTGCAGAAACACTTTGGAATATTCAAAAGGA	1020
	TCTAATATTTTAAATTAAAGCCTTCTAATGTTCCACTGTT	1040
	TGGAGAACAGAAGATAGCAGGCTGTGTATTTTAAATTTTT	1080
	GAAAGTATTTGATTTGATCTTTAAAAAATAAAAACAAATT	1120
	GGGAGACCTGAAAAAAAGAGAAAGGTGGCACCTAAGCCCG	1160
	AGCTCACTGATTATCAGAAGCAAGAGTTCGGGAAGCATTT	1200
	GACCTCTTCGACGTGACGAGTGGACCATCGACGCGAAGGA	1240
	GCTGAAGGTGGCATGAGAGCGCTGGCTTCGACCAGAAGGA	1280
	AGAGATGAGAAATGATCTCCGAGGTGGACAGGGGAGGCAC	1320
	GGGGAAGATCAGCTTCAATGACTTCCTGGCCGTGATGACG	1360
	CAGAAGATGTCCGAGGAAGGCACCAAAGAAGAAAATCCTG	1400
	AAGGCCTTCAGGCTCTTTGATGACGATGAGACCGGGAAGA	1440
	TCTCGTTCAAAAACCTGAAGCGTGTGACCAACGAGCTGGG	1480
	GGAGATACCCACGGATGAGGAGCTGCAGGAGATGATCGAC	1520
	GAAGCTGATCGGGATGGGGACGGCGAAGTGAACGAGGAGG	1560
	AGTCTTTCGGATCATGAAGAAGACCAGCCTTTACTGAAGT	1600
	CGGTTCAGAAGCTAAAGTGACTCTCTGGGTTGCCTGCTTC	1640
	CATTTTGTGAAACCTTAGAGACAGCGGCTGCCTGTCCCTT	1680
	CTTCACCCCCCTTCACCCCCATAATTTGTCTAGATCTATT	1720
	TCCATATCTCTAGTTCCCCGAATTC 3′	1760

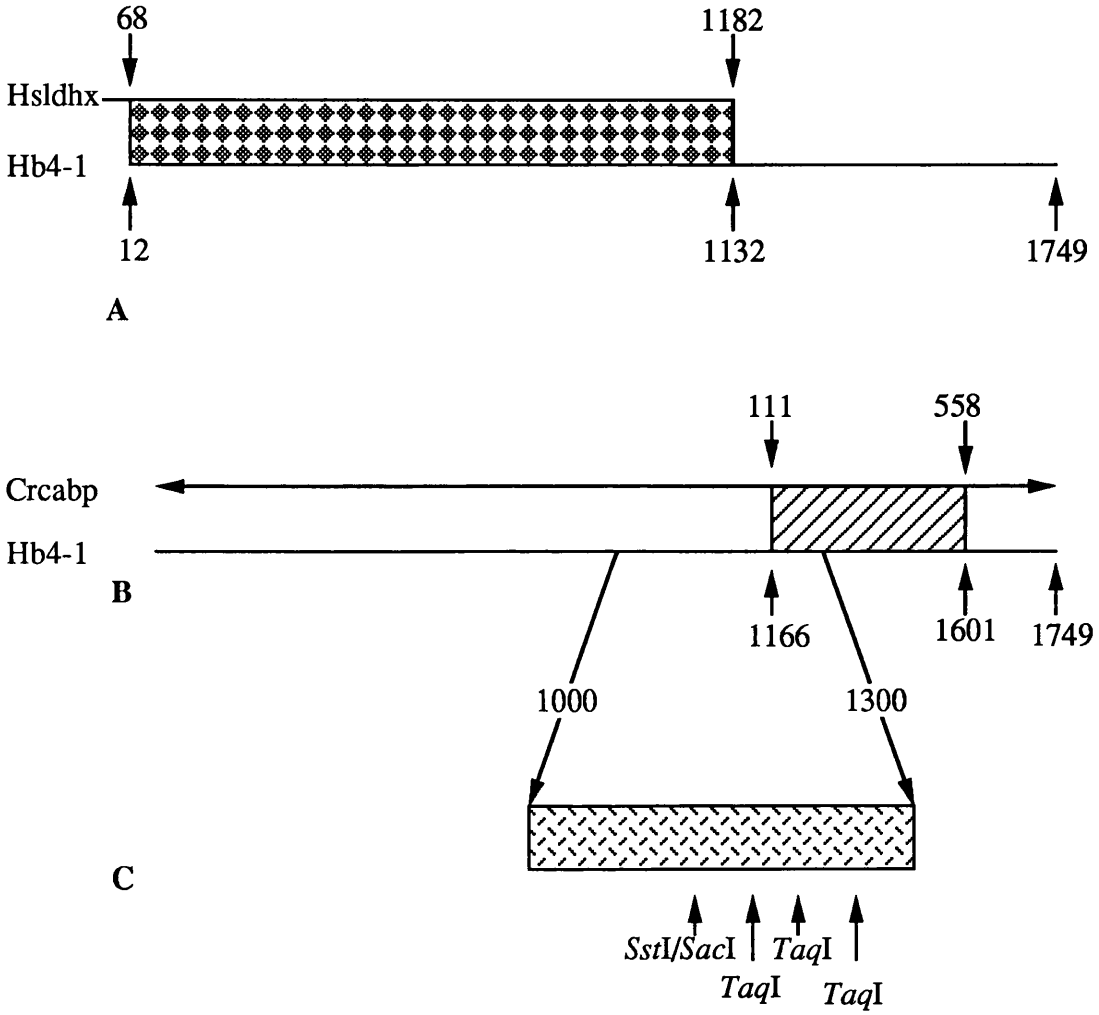
FIGURE 5.8

FIGURE 5.8 COMPARISON OF CLONE HB4-1 SEQUENCES TO THOSE OF LDH-C4 AND *CHLAMYDOMONAS* CATRACTIN GENES

Regions of clone Hb4-1 sequences with homology to human testis specific lactate dehydrogenase-C4(Hsldhx) sequences **(A)**

Regions of clone Hb4-1 sequences with homology to *Chlamydomonas* caltractin gene (Crcabp) **(B)**

Enlarged 1000-1300 bp region of clone HB4-1 and the restriction enzyme sites **(C)**


68
1182
Hsldhx
Hb4-1
12
1132
1749
A
111
558
Crcabp
Hb4-1
B
1166
1601
1749
1000
1300
C
SstI/SacI
TaqI
TaqI
TaqI
Scale in bp
0
400
800
1200
1600
1750

were also found. These fragments were then subcloned into pBluescript® and M13 mp18 and mp19 bacteriophages.

An attempt to recover the insert from clone HA5-2 proved a difficult experience. When analysed on agarose gel, the fragments were too small and the bands formed were not different in size from those of primers. However it should be remembered that clone HA5-2 generated fusion proteins that inhibited fertilisation in a hamster IVF (Chapter 4). Why it was not possible to recover the DNA insert from this clone remains unclear.

Recombinant fragments were cloned into pBluescript identified by means of blue and white selection and later confirmed by radiolabelling using the Grunstein and Hogness method. Confirmed recombinant plasmids were then subcloned into M13 bacteriophages and sequenced. Clone HB4-1 was found to be 1749 bp in size with >96% homology to human testis specific LDH-C4 gene in its first 1118 bases. It also showed >71% homology to *Chalmydomonas* gene for caltractin in its sequences further away from those of LDH-C4. Analysis of nucleotide sequences between these two homologies for possible restriction enzyme site revealed three sites for *Taq*I and one for the *SstI/Sac*I. Since homology of clone HB4-1 to LDH-C4 gene spans from start codon through stop codon to the consensus polyadenylation site, it may be concluded that the homology of HB4-1 to *Chlamydomonas* gene may have come due to a cloning artefact. Otherwise it is not understood what might have resulted in the chimeric nature of clone HB4-1.

FIGURE 5.9

FIGURE 5.9 DATA BASE ANALYSIS OF SEQUENCE HOMOLOGIES BETWEEN CLONE HB4-1, LDH-C4 AND *CHLAMYDOMONAS* CALTRACTIN GENE

HB4-1	Sequences for clone HB4-1
Hsldhx	Sequences for human testis specific lactate dehydrogenase-X
Crcabp	Sequence for *Chlamydomonas* caltractrin gene
ATG	Transcription start codon
TAA	Transcription stop codon
AATAA	Polyadenylation sequences
****	Sequences corresponding to the internal *Eco*RI site in clone HB4-1 and LDH-C4 gene


Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Hsldhx
Hb4-1
Crcabp
Hb4-1
Crcabp
Hb4-1
Crcabp
Hb4-1
Crcabp
Hb4-1
Crcabp

CHAPTER 6

DISCUSSION

Background: There are three major approaches currently being pursued with the objective of developing a contraceptive vaccine. Immunogens based on human chorionic gonadotrophin (hCG), have reached the most advanced stage of development, phase 1 clinical trials completed with phase 2 studies about to commence (**Aitken *et al*** 1993). The advantage of this approach is that the molecule involved is extremely well characterised and its relationship with the pituitary is accurately defined. The major problem with the hCG approach remain those of specificity and efficacy. Furthermore, it is a form of abortion and this will render it unacceptable in many areas of the world.

In contrast, the remaining two approaches to vaccine development aim to prevent conception by interfering with intricate cascade of interactive events that characterise the union of male and female gametes at fertilisation. The zona glycoprotein, ZP3, is a prime candidate for engineering such a vaccine, in view of its important role in the recognition and activation of spermatozoa and its unique antigenic composition. A major problem with this approach involves the loss of primordial follicles and the disruption of normal ovarian cyclicity observed in the many *in vivo* studies in which active immunity against this protein has been induced (**Aitken *et al*** 1990). Disruption of fertilisation through the induction of immunity against sperm antigens is a third approach, for there is good clinical evidence in the form of anti-sperm antibodies induced in experimental animals as well as patients exhibiting infertility associated with the appearance of spontaneous immunity against sperm antigens (**Hjort, 1990**). Unfortunately, this potential has been very slow to translate into practical reality. Most sperm antigen contraceptive vaccine candidates identified so far have been reported to cause reduction rather than complete block of fertility. The only antigen that has provided evidence of full and reversible contraception on immunisation of female animals is the guinea pig sperm protein PH-20 (**Primakoff *et al* 1988**). Therefore, the need to identify and isolate more sperm specific antigens which are

relevant to fertility can not be overstated.

Antibodies: The obvious route is to pursue and characterise the epitopes targeted by antibodies against spermatozoa. Much of the work in this thesis has been to devise a practical strategy for identifying sperm components which might be candidates for contraceptive vaccine development. A major drawback, often not appreciated by molecular biologists outside the immediate field is the fact that the mature spermatozoon has ceased all gene transcription and translation and therefore cannot be used directly for recombinant techniques. Screening for particular genes must be attempted indirectly using cDNA prepared from testicular tissue. It is crucial then that a screening strategy takes into account that germ cells or testicular spermatozoa are not capable of recognising or fertilising an oocyte. For this reason a combination of a protein and gene based strategy was used that also assessed sperm function.

An early decision was to use polyclonal antiserum rather than more specific monoclonal antibodies. There were several reasons for using polyclonal antisera. Firstly, antiserum will recognise multiple epitopes on a particular protein and therefore are more likely to recognise the protein fragments produced by the expression vector since these may not have undergone their normal conformational changes. Moreover, antiserum is more likely to recognise core polypeptide epitopes of glycoproteins rather than just carbohydrate moieties. This is an important consideration when using expression vectors such as λgt11 which will not express oligosaccharide. Many monoclonal antibodies generated against spermatozoa are only to carbohydrate moieties (H.D.M. Moore, unpublished observation) and this clearly limits their usefulness as probes for gene cloning purposes.

Antiserum will seldom be as specific as monoclonal antibodies but to minimise the amount of non-specific antibodies and to target highly conserved sperm epitopes (important for sperm function and fertilisation) a heterologous antibody-antigen

system was adopted. At first site, generating antibodies against hamster sperm components that will be used to detect human protein does not seem logical but in fact this approach tends to generate highly specific antibody when used with the heterologous antigen. The results of the immunofluorescence clearly demonstrated that R7, R9 and R10 polyclonal sera recognised sperm acrosome-specific components across species. This was particularly important with regard to the baboon because in the long term this animal would be used as a non-humate primate model to test the efficacy of any vaccine.

Further experiments on the cross-reactivity of these sera with a broad range of tissue was not undertaken as the objectives of the project was to explore the strategy of screening. Moreover, absolute specificity of antisera was not necessary for the isolation of germ cell specific genes. However for future studies it might be appropriate to undertake a preliminary screen with non-reproductive tissue (by immunofluorescence or immunoassay) to eliminate sera with high somatic cell cross-reactivity.

In addition to immunocytochemistry, the hamster IVF assay also played a central role in the selection of antisera since an important consideration was whether antibodies were present that interfered with fertilisation. Previous studies in this laboratory had demonstrated the usefulness of IVF for identifying antibodies to functional sperm components (**Moore and Hartman, 1984, Moore *et al.*, 1987**) and this was borne out in this investigation. Initially, it was hoped that baboon and human oocytes (**Moore, *et al.*, 1987**) could also be used for antisera screening. Efforts to obtain baboon oocytes were thwarted however due to importation restrictions and insufficient human oocytes (failed to fertilise eggs) precluded the use of a strictly controlled assay. The production of recombinant proteins of human oocytes (i.e. ZP proteins) would be of great practical value in this respect since reproducible assays of

sperm binding to zona receptors could be developed.

λgt 11 Screening: No serious problem was encountered with the screening of the cDNA testis library. A potential problem in using rabbit serum is the presence of low titres of antibodies (in the serum of pre-immune and immune animals) that react against *E. coli* and are produced as part of a natural defence mechanism. Attempts to remove these antibodies were only partially successful and therefore were not used. In the event, appropriate titration of the antiserum was enough to prevent non-specific background.

Perhaps the greatest drawback with the overall approach with the λgt11 vector was the inability to generate much β-galactosidase fusion protein for further testing and much valuable time was wasted in trying to obtain fusion protein from a number of the clones. The reasons for the inhibition of protein expression (small insert, compromised *Eco*RI site) are discussed in chapter 4 and will not be considered further here, but any future study should find a more practical solution to this problem. Perhaps one possibility is to move directly to synthetic production of short polypetides for testing using consensus sequence of cDNA. This approach has been used by **Goldberg (1990)** to study LDH-C4 epitopes and was used in this laboratory by **Moore *et al.* (1993)** to isolate a sperm specific protease inhibitor.

The hamster IVF assay was used also to test fusion proteins that were generated. It is important to realise that the mode of action of fusion proteins in this assay may differ to that of antiserum. With the latter, inhibition of fertilisation probably results from antibodies interfering with sperm surface antigens either directly or indirectly by steric hindrance (see chapter 3). On the other hand fusion proteins, if they are germ cell expressed components should be similar to sperm surface antigens. In this case, interference of fertilisation must be due to a direct competition for putative zona

binding sites or due to an interference of capacitation and/or acrosome reaction as has been shown previously (**Moore *et al.*, 1993**). Due to the small quantity of fusion protein that was produced the exact reason for inhibition of fertilisation in the present investigation could not be readily ascertained.

HB4-1/LDH-C4: Because of the limitations of time only the selected clone HB4-1 was sequenced. This was found to be 1749 bp in size with >96% homology to human testis specific LDH-C4 gene in its first 1132 bases. This testis specific gene has been extensively studied and is already the subject of investigations as a immunocontraceptive model since antibodies to this enzyme inhibit fertilisation (**Goldberg,1990**). With respect to this project, the main question to consider is - was LDH-C4 cDNA isolated as a result of the search strategy adopted (LDH-C4 clones had never been isolated previously from this laboratory with this library) or was its selection merely a fortuitous coincidence? Several characteristics of LDH-C4 strongly suggest that the former premise is correct. Firstly LDH-C4 is a germ cell specific isoenzyme whose expression in the testis increases as spermatogenesis continues. There is evidence that post-meiotic gene expression occurs (**Millan *et al.*, 1987**). The protein is highly conserved across mammalian species (**Goldberg, 1977**) and therefore it is likely that antisera raised against hamster antigen would cross-react with human protein. Although the isozyme is of cytoplasmic origin it readily leaks from damaged cells (at all stages of development) and then is preferentially located over the acrosomal membrane (**Hintz and Goldberg, 1977**). Antibodies to LDH-C4 are thought to interfere with fertilisation by binding to the protein over the sperm head (**Goldberg, 1977**) and the fertility of baboons is inhibited when immunised with specific LDH-C4 epitopes (**Millan *et al.*, 1987**). Why fusion protein from HB4-1 inhibited fertilisation is less clear. The functional role of LDH-C4 as a sperm enzyme has never been clearly elucidated but its specific germ cell expression along with its close association with the sperm head may indicate that it has a role in a fertilisation

or an associated process (capacitation, AR) not as yet elucidated. In this case exogenous fusion protein might interfere with fertilisation. It is unlikely that the segment of the HB4-1 insert encoding for part of a caltractin gene would produce any fusion protein (see chapter 5) although this possibility cannot be completely discounted. Why a chimaeric insert was produced is unknown but cloning artifacts of this sort do occur occasionally (Dr Alison Moore, personal communication).

Future Work. Given that HB4-1 encodes a prime immunocontraceptive antigen and its isolation and characterisation was most likely due to the protein and gene strategy adopted, there will be considerable merit in sequencing the remaining clones HA5-2 and HA6-2. Initial attempts to subclone failed with these two clones but it may be feasible to use a different approach. This would entail isolating DNA from high titre lysates and then cleaving the insert using alternative enzymes to *Eco*RI. PCR could also be used by employing different primers.

Another important objective would be to make or obtain a baboon cDNA testes library for screening with antiserum. This would enable candidate vaccine molecules to be isolated and characterised in a primate that could be used for further contraceptive vaccine testing. This would be an important development because the efficacy and safety of immunocontraception needs to be established in a non-human primate before its use in humans.

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