DNA BINDING AND DIMERISATION PROPERTIES OF THE TRANSCRIPTION FACTOR COUP-TF II

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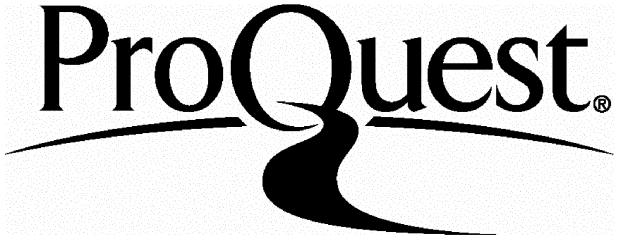

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ABSTRACT

The chicken ovalbumin upstream transcription factors (COUP-TF I and II) are members of the nuclear receptor family. These proteins have no identified ligand and can repress the transcriptional activity of a number of other nuclear receptors including the receptors for retinoic acid (RAR and RXR), thyroid hormone (TR) and oestrogen (ER). Analysis of COUP-TF protein revealed that its expression can be regulated by both retinoic acid and oestrogen indicating that COUP-TF mediated repression *in vivo* may be subject to hormonal controls.

COUP-TF II was shown to bind DNA *in vitro* either as a homodimer or as heterodimer with RXRα or TRβ. To establish whether COUP-TF II could form homodimers and heterodimers *in vivo* the dimerisation properties of this orphan receptor were investigated in transfected cells using a two hybrid assay. In conditions where COUP-TF II homodimers and RXRα/RARα heterodimers were formed heterodimers between COUP-TF II and RXRα were not detected. Moreover, no interaction between COUP-TF II and RXRα could be detected on DNA in intact cells. Similarly, COUP-TF II homodimers and RXRα/TRβ heterodimers were favoured over COUP-TF II/TRβ heterodimers. These results suggest that the formation of functionally inactive heterodimers is unlikely to represent a general mechanism by which COUP-TF represses the transcriptional activity of nuclear receptors and a model is favoured in which repression is mediated by COUP-TF homodimers competing for DNA binding. In support of this model COUP-TF II homodimers recognised a variety of nuclear receptor response elements that differed in the spacing and orientation of the half site motifs in contrast to the more restricted binding of other members of the family. Multiple and independent dimerisation interfaces were identified within COUP-TF II which may impart the flexibility of binding required for the degenerate response element specificity displayed by the homodimer.

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ABBREVIATIONS

AF1/AF2	activation function 1/activation function 2
ARP-1	apolipoprotein AI regulatory protein 1
ATP	adenosine 5' triphosphate
AR	androgen receptor
BES	N, N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CEF	chicken embryo fibroblast
COUP-TF	chicken ovalbumin upstream promoter transcription factor
C-terminal	carboxyl terminal
CTD	carboxyl terminal domain
CTP	cytidine 5'-triphosphate
CMV	cytomegalovirus
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DCC	dextran coated charcoal
DEAE	diethylaminoethylamine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DR+X	direct repeat with X nucleotide spacing
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
ER	oestrogen receptor
ER+X	everted repeat with X nucleotide spacing
ERE	oestrogen response element
FCS	foetal calf serum
FRE	SF-1 response element
GR	glucocorticoid receptor

GRE	glucocorticoid response element
GST	glutathione-S-transferase
GTP	guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine N'-2-ethansulphonic acid
hsp	heat shock protein
HRE	hormone response element
IR+X	inverted repeat with X nucleotide spacing
Kb	kilobase
kD	kilodalton
K_d	dissociation constant
lacZ	β-galactosidase gene
MMTV	mouse mammary tumor virus
MR	mineralocorticoid receptor
mRNA	messenger RNA
NBRE	NGFI-B response element
NGFI-B	nerve growth factor inducible protein-B
NMR	nuclear magnetic resonance
NP40	nonidet p40
N-terminal	amino terminal
ODx	optical density at wavelength of x nm
PAGE	polyacrylamide gel electrophoresis
PBSA	phosphate buffered saline A
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
RAR	retinoic acid receptor
RARE	retinoic acid response element
RNA	ribonucleic acid
RNase	ribonuclease
RXR	retinoid X receptor
S	Svedberg units
SDS	sodium dodecyl sulphate
SF-1	steriodogenic factor-1
SV40	simian virus 40
T3	3, 5, 3'-triiodo-L-thyronine
TAF	TBP associated factor

TBP	TATA binding protein
TEMED	*N'N'N'N'*-tetramethylethylenediamine
TK	thymidine kinase
TR	thyroid hormone receptor
TRE	thyroid hormone response element
TREpal	palindromic thyroid hormone response element
Tris	tris(hydroxymethyl)aminoethane
Triton X-100	octyl phenoxy polyethoxyethanol
Tween-20	polyoxyethylenesorbitan monoluarate
UAS	upstream activating sequence
UTP	uridine 5'-triphosphate
UV	ultra violet
VDR	vitamin D receptor

CHAPTER 1
INTRODUCTION

CHAPTER 1

Introduction

The cellular processes required for the growth, development and homeostasis of an organism involve highly complex patterns of gene expression. A multitude of mechanisms are involved in providing the required spatial and temporal regulation and one layer of this control is provided by the action of transcription factors which bind to specific cis acting sequences and modulate gene transcription. This thesis describes a study of the chicken ovalbumin upstream promoter transcription factor (COUP-TF), which belongs to a family of transcription factors known as nuclear receptors. Initial studies on this family focused on the properties of the hormone receptors. More recently, related proteins that have no identified ligand, of which COUP-TF is one example, have also been investigated. The aim of this chapter is to review our current understanding of the molecular mechanisms by which nuclear receptors function within the cell.

The identification of intracellular hormone receptors

Hormone receptors mediate the action of many important cell signalling molecules that include the steroid hormones, thyroid hormone, retinoids and vitamin D. These regulatory molecules play important roles in growth, development and homoeostasis. The sex steroid hormones, androgens, progestins and oestrogen are required for the normal development and function of reproductive systems in mammals (King 1974, Cunha *et al* 1991). The adrenal steroid hormones, glucocorticoids and mineralocorticoids, are involved in the maintenance of homeostasis in particular carbohydrate and mineral metabolism respectively (Jensen 1991). Vitamin D is required for proper bone formation and calcium metabolism (Haussler *et al* 1988) and the retinoids and thyroid hormones are involved in growth and development in mammals and in addition, metamorphogenesis in amphibians (Chin 1991, Kastner *et al* 1994, Ragsdale and Brockes 1991).

These hormones can pass readily into the cell and act via intracellular receptors in contrast, peptide hormones do not enter the cell and their receptors are located in the cell membrane. The existence of intracellular receptors was first suggested by the observation that labelled oestradiol was retained, against a concentration gradient, by oestrogen responsive tissues (Jensen and Jacobsen 1962). Cell fractionation studies, using uterine cells

treated with labelled oestradiol, demonstrated that there were two sites of high affinity hormone binding. Most of the oestradiol bound to the nucleus and a lesser amount was retained in the cytoplasm. Prior to hormone treatment most of the receptor was found in the more readily extracted cytoplasmic form. This cytoplasmic receptor complex had a sedminentation coefficient of 9.5 S while the receptor complex extracted from nuclei sedimented at 5 S (Jensen *et al* 1968, Toft and Gorski 1966). A 'two step' model was proposed to account for these observations in which upon binding hormone the cytoplasmic 9.5 S form of the oestrogen receptor (ER) was translocated to the nucleus and transformed into the 5 S form (Jensen *et al* 1968). The conversion of receptor from a 8-10 S to a 4 S form upon ligand binding has since been demonstrated for the progesterone, androgen and glucocorticoid receptors (PR, AR and GR respectively) (reviewed in Jensen 1991). This ligand dependent transformation process could be mimicked *in vitro* by heat treatment and inhibited by some metal ions such as molybdate (Dahmer *et al* 1984).

The ligand induced alteration in the receptor complex involves the dissociation of associated proteins and also seems to involve receptor dimerisation (DeMarzo *et al* 1991, Miller *et al* 1985). A number of proteins have been identified associated with steroid hormone receptors in cell extracts including the heat shock proteins (hsp) 70 and 90, p23, p50, p54, p59 and p60 (for review see Smith and Toft 1993). Hsp 90 is the best characterised of these proteins and dissociates from the receptor upon ligand binding or heat treatment (reviewed in Pratt 1993). The ligand sensitive interaction between the GR and hsp 90 has also been demonstrated to occur in intact cells suggesting that the interaction observed *in vitro* is not an artefact generated during the extraction of the receptor. In pulse chase experiments labelled hsp 90 could be coimmunoprecipitated with the glucocorticoid receptor from untreated cells but not from cells treated with the synthetic glucocorticoid dexamethasone (Howard and Distelhorst 1988). In addition, unliganded GR could be crosslinked to hsp 90 in intact cells (Rexin *et al* 1988). The hormone binding domains of the GR and PR are sufficient for the interaction with hsp 90, however, binding to the ER requires other regions of the receptor in addition to the hormone binding domain (reviewed in Pratt 1993).

Hsp 90 is required for high affinity ligand binding by the GR although not for the ER, AR or PR (reviewed in Smith and Toft 1993). A positive role for this heat shock protein in GR function is supported by the report that GR

expressed in a mutant yeast strain, expressing reduced levels of hsp 90, exhibited reduced transcriptional activity (Picard *et al* 1990a). Hsp 90 may act as a chaperone in protein folding, promoting a structure of the receptor required for receptor function or transport across membranes (reviewed in Smith and Toft 1993). Hsp 90 also appears to maintain unliganded steroid hormone receptors in an inactive conformation although the specific activity it blocks is not clear and may differ between receptors. It has been suggested that hsp 90 might mask the nuclear localisation signal of the GR (Ylikomi *et al* 1992), however, as this receptor displays a different subcellular distribution to the other steroid receptors it is unlikely that retention in the cytoplasm is a commmon property of hsp 90. A second inhibitory role for hsp 90 was suggested by the report from Sanchez and coworkers (Sanchez *et al* 1987) that hsp 90 can inhibit the DNA binding activity of the GR *in vitro* . It is possible that *in vivo* the DNA binding activity of the steroid hormone receptor is blocked until ligand dissociates the hsp 90 or, alternatively, hsp 90 may block protein interactions required for transactivation. The retinoic acid, thyroid hormone and vitamin D receptors (RAR, TR and VDR respectively) do not form stable complexes with hsp 90 and, in contrast to the steroid hormone receptors, are 'tightly' bound to the nucleus in the absence of hormone (Dalman *et al* 1990, Dalman *et al* 1991). It remains to be determined whether hsp 90 interacts with any of the orphan receptors.

Nuclear localisation

On the basis of cell fractionation experiments the original two step model proposed that in the absence of ligand the steroid hormone receptors were retained in the cytoplasm. Using immunocytochemistry, however, the ER (King and Greene 1984, Welshons *et al* 1985), PR (Gasc *et al* 1989, Perrot-Applanat *et al* 1985) and GR (Govindan 1980, Papamichail *et al* 1980, Picard and Yamamoto 1987, Wickström *et al* 1987) have been demonstrated to enter the nucleus in the absence of hormone. The ER and PR are predominately nuclear in the absence of ligand while the GR is almost exclusively cytoplasmic. More recently, it has been demonstrated that these receptors are actually shuttling between the cytoplasm and nucleus (Dauvois *et al* 1992, Guiochon-Mantel *et al* 1991, Madan and Defranco 1993). The discrepancy with the cell fractionation results may be due to 'tighter' nuclear binding of the ligand bound receptor. Nuclear localisation signals have been identified for all three receptors and contain at least two karyophilic signals, a constitutive NLS is present within or adjacent to the DNA binding domain

and a hormone inducible NLS is also present within the receptor (Guiochon-Mantel *et al* 1989, Picard *et al* 1990b, Picard and Yamamoto 1987, Ylikomi *et al* 1992). The constitutive NLS is sufficient for nuclear targeting of the ER and PR whereas in the GR this NLS is masked by the hormone binding domain and ligand is therefore required both for the hormone inducible NLS and for revealing the 'constitutive' NLS (Picard *et al* 1990b, Ylikomi *et al* 1992). This difference may explain the altered cellular localisation of the unliganded GR as compared with the ER and PR.

The nuclear receptor superfamily

The first hormone receptors for which complementary DNAs (cDNAs) were obtained were the receptors for glucocorticoid and oestrogen. Comparison of the predicted protein sequence of these receptors identified regions of sequence homology suggesting that they might belong to a family of ligand activated transcription factors (Green *et al* 1986, Hollenberg *et al* 1985). This was confirmed with the isolation of cDNA clones for the receptors of the other major steroid hormones and thyroid hormone, retinoic acid, vitamin D (reviewed in Beato 1989, Evans 1988, Green and Chambon 1988, Ham and Parker 1989, Parker 1993) and most recently for farnesol (Forman *et al* 1995). The clones for the TR were shown to be the cellular homologues of the viral oncogene v-*erb*A found in the avian erythroblastosis virus (Sap *et al* 1986, Weinberger *et al* 1986). Other members have been isolated that do not have an identified ligand, the so-called orphan receptors. To date there are approximately 30 different members of this family identified in vertebrates, two thirds of which are orphan receptors (Table 1.1) and a number of nuclear receptor homologues have also been identified in *Drosophila melanogaster* (Table 1.1) (reviewed in Oro *et al* 1992, Segraves 1994).

Construction of phylogentic trees illustrates that the nuclear receptor family evolved from a single progenitor protein (Amero *et al* 1992, Laudet *et al* 1992). The only exceptions being the three *Drosophila* proteins *knirps*, *knirps-related*, and *embryonic gonad* that have acquired a different C-terminal domain during evolution. Analysis of genomic clones for several nuclear receptors revealed a similar exon/intron arrangement also suggesting that these receptors originated from a common precursor. In general, the N-terminus is encoded by a single exon and is seperate from the DNA binding domain which is encoded by two exons, with the intron positioned between the zinc fingers (see later). There are exceptions, COUP-

Hormone Receptors		Orphan Receptors	Drosophila Proteins	
Glucocorticoid	GR	COUP-TF I (Ear-3)/COUP-TF II (ARP1)	Seven-up	(svp)
Progesterone	PR	Ear-2		
Mineralocorticoid	MR	HNF-4	HNF-4(D)	
Androgen	AR	Tlx (tll)	Tailless	(tll)
Oestrogen	ER	TR2		
		TR4		
		ERR 1/2		
Retinoic acid	RAR α/β/γ	LXR	Ultraspiracle	(usp)
Retinoid X	RXRα/β/γ	PPARα/β/γ	Ecdysone Receptor	(EcR)
Thyroid hormone	TRα/β	RLD1		
Vitamin D	VDR	MB67		
Farnesoid X	FXR	RIP 15		
		Rev-erbα (Ear-1)/Rev-erbβ (BD73/RVR)	E75	
		RZR/RORα/β	E78	
		NGFI-B (Nurr 77)	DHR3	
		NURR1	DHR39	
		RNR-1		
		LRHI		
		SF-1 (Ad4BP/ELP)	Fushi-tarazu F1α/β	(FTZ-F1α/β)
		GCNF		
			Knirps*	(kni)
			Knirps-related*	(knrl)
			Embryonic gonad*	(egon)

Table 1.1 The Nuclear Receptor Superfamily

Compilation of members of the nuclear receptor family (Amero *et al* 1992 , Laudet and Adelmant 1995 , Laudet *et al* 1992).

TF lacks the intron between the two zinc fingers (Ritchie *et al* 1990) and another orphan receptor, NGFI-B, lacks the intron between the N-terminus and the first zinc finger (Ryseck *et al* 1989).

A number of receptors, including the hormone receptors RAR, TR and retinoid X receptor (RXR), are encoded by multiple genes. The complexity is increased further by the observation that different isoforms of the gene products can be produced by alternative promoter usage and differential splicing (reviewed in Chin 1991, Leid *et al* 1992a). The receptors for retinoic acid display the greatest diversity with two main types of receptor, the RARs which bind both 9-*cis* and all-*trans* retinoic acid and the RXRs which bind 9-*cis* retinoic acid only. Both of these receptors are encoded by multiple genes (α, β, γ) and different isoforms of these receptors are also observed (see Leid *et al* 1992a). Some orphan receptors also appear to have undergone gene duplication, for example COUP-TF has two identified loci (I and II) (Ritchie *et al* 1990). In general, a specific receptor isoform shows higher sequence conservation between species than between different receptor isoforms from the same species.

COUP-TF

COUP-TF was originally characterised by its ability to bind the COUP element in the promoter of the chicken ovalbumin gene (Bagchi *et al* 1987, Pastorcic *et al* 1986, Wang *et al* 1987). The proteins that bound this element were purified from HeLa nuclear extracts using column chromatography. Multiple polypeptides were purified, four low molecular weight COUP-TFs in the range of 43-47 kD and four higher molecular weight proteins of 66-74 kD. Antisera raised to these proteins were used with concatenated COUP elements to screen a HeLa cDNA library. The cDNA clone isolated from this screen encoded a protein with a predicted molecular weight of 46.2 kD. This clone, COUP-TF I (Wang *et al* 1989), contained identical sequence to the orphan receptor ear-3, previously identified by its homology to v-*erb*A (Miyajima *et al* 1988). A second low molecular weight COUP-TF, COUP-TF II, was identified by its binding to the promoter of the apolipoprotein AI gene and is also known as apolipoprotein regulatory protein 1 (ARP1) (Ladias and Karathanasis 1991). The high molecular weight COUP binding proteins have not been cloned and will not be discussed further. The term COUP-TF is used throughout this thesis to refer to the low molecular weight proteins.

COUP-TF I and II are highly homologous, with the DNA binding domains differing by a single conservative amino acid change (threonine/ serine) and the C-terminal region sharing 97% amino acid identity. Within the nuclear receptor family the COUP-TFs are most highly related to the orphan receptor ear-2 (Miyajima *et al* 1988). Interestingly, COUP-TF also shares regions of strong homology with RXR, indeed RXR is more highly related to COUP-TF than to RAR (Leid *et al* 1992a).

The gene *seven-up* is the *Drosophila* homologue of COUP-TF (Mlodzik *et al* 1990) and the sequence conservation between these proteins is particularly high, with greater than 85 % identity between the DNA binding domains and the C-termini. The overall conservation between the two proteins is striking (>75%) and is higher than for any other known set of *Drosophila* and vertebrate homologues (Fjose *et al* 1993). COUP-TF homologues have also been identified in *Xenopus laevis*, zebrafish (*Brachydanio rerio*) and sea urchin (*Strongylocentrotus purpuratus*) (Chan *et al* 1992, Fjose *et al* 1993, Matharu and Sweeney 1992) demonstrating that this protein has been highly conserved during evolution. In *Drosophila*, *seven-up* is involved in the determination of photoreceptor neurons during eye development. A role for COUP-TF in early neurogenesis is supported by the predominant expression of the zebrafish homologues [*44*] and [*46*] in the developing central nervous system and eye (Fjose *et al* 1993). Furthermore, murine COUP-TF homologues are expressed at high levels in the central nervous system during development (Qiu *et al* 1994) and a chicken COUP-TF homologue is expressed in developing motor neurons and brain (Lutz *et al* 1994). At present the mechanism of COUP-TF function during development is unclear although in *Drosophila* it appears to require an active ras signalling pathway (Begemann *et al* 1995). In transfected cells COUP-TFs can repress the activity of other nuclear receptors including RXR. Intriguingly the *Drosophila* protein *usp*, which is the homologue of vertebrate RXR, is also required for normal eye development and it is tempting to predict that *seven-up* restricts the activity of this protein.

Other orphan receptors

Other orphan receptors have been identified through a number of different screening protocols, although the majority of the members of this group were identified through sequence homology to hormone receptors some were isolated on the basis of functional properties. Proteins such as HNF-4 and SF-1 were identified as DNA binding factors (Lala *et al* 1992,

Sladek *et al* 1990). NGFI-B was cloned by several groups as an early response gene which is rapidly and transiently induced by growth factors such as nerve growth factor (Hazel *et al* 1988, Watson and Milbrandt 1989) and another related immeadiate early gene, RNR1, has since been cloned (Scearce *et al* 1993). A protein interaction screen in yeast identified RIP 15 as an orphan receptor that interacts with the ligand binding domain of RXR (Seol *et al* 1995) and the gene for Rev-erbα (ear-1) was discovered by virtue of it being encoded on the noncoding strand of the TRα gene (Lazar *et al* 1989b).

Nuclear receptor response elements

Members of the nuclear receptors family can be divided into three general classes on the basis of their DNA binding activity. Class I contains the steroid hormone receptors which bind as homodimers to palindromic sequences (Figure 1.1). Steroid response elements were initially identified through deletion analysis of target gene promoters and alignment of glucocorticoid response elements (GREs) and oestrogen response elements (EREs) revealed that they contained similar palindromic sequences. The replacement of just two base pairs in the palindrome converted the ERE of the *Xenopus* vitellogenin A2 gene into a GRE (Klock *et al* 1987, Martinez *et al* 1987). From point mutagenesis the nucleotides required for receptor recognition were discriminated and consensus GRE and ERE sequences identified (Figure 1.1) (see Martinez and Wahli 1991). The symmetry of the sites suggested that these receptors bound to DNA as dimers and this was confirmed using gel shift analysis (Kumar and Chambon 1988). Surprisingly, the PR, AR and the mineralocorticoid receptor (MR) could also stimulate transcription from a GRE (Cato *et al* 1986, Chalepakis *et al* 1988, Darbre *et al* 1986, Ham *et al* 1988, Strahle *et al* 1987). Although these receptors bind common response elements it seems that their DNA binding domains make different contacts, producing distinct DNase I footprinting and methylation interference patterns (Chalepakis *et al* 1988, von *et al* 1985). The receptors also displayed different sensitivites to alterations in the response element sequence (Cato *et al* 1988, Chalepakis *et al* 1988).

The class II receptors bind to response elements composed of direct, inverted and everted repeats of a hexamer sequence similar to that of an ERE half site (see Figure 1.1). This group of receptors includes the TR, RAR, VDR and RXR as well as many of the orphan receptors. Mutagenesis of the thyroid hormone repsonse element (TRE) from the rat growth hormone gene identified a consensus binding site for the TR that consisted of a palindromic

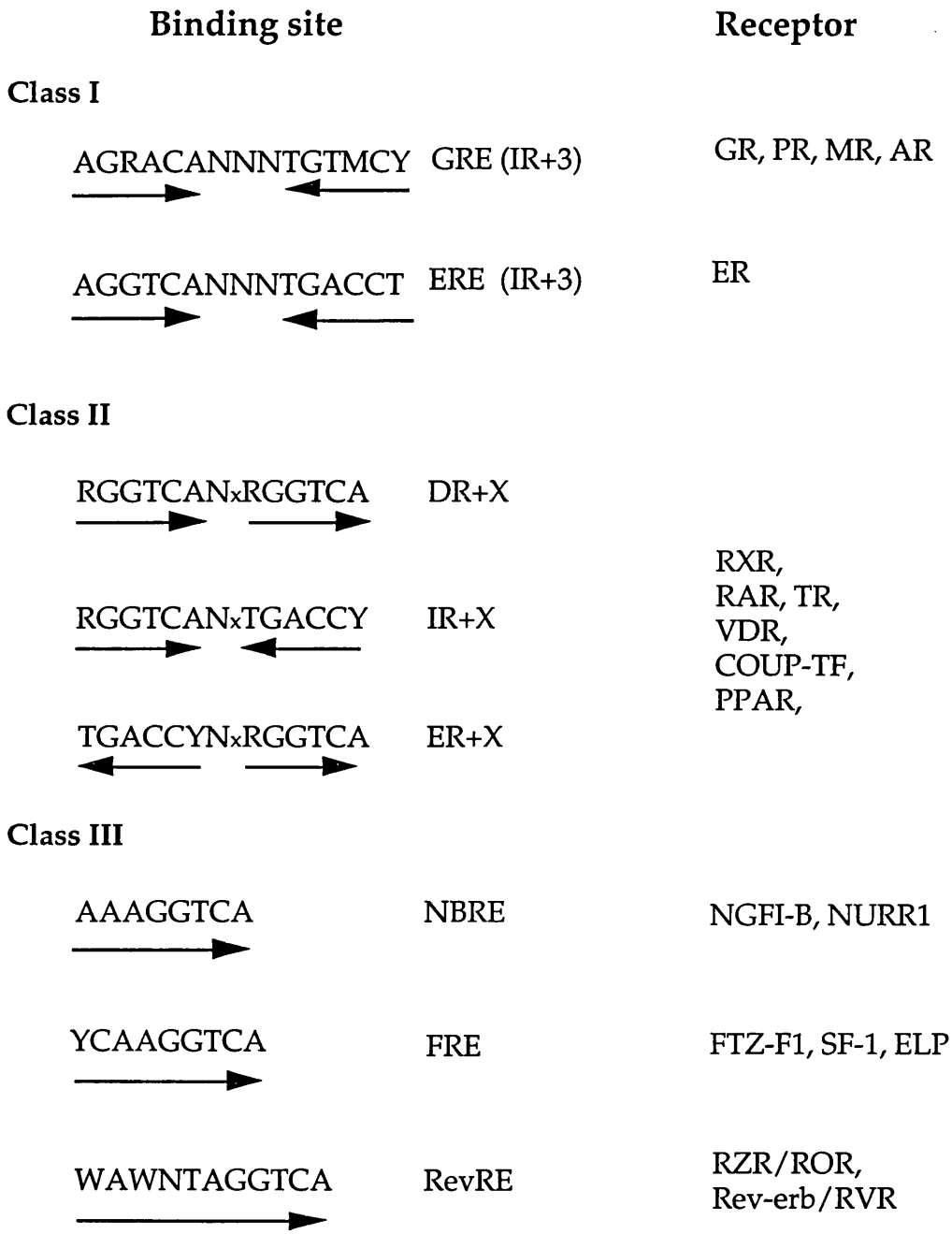


Figure 1.1 Nuclear receptor response elements.
Class I, II and III consensus binding sites are shown (Laudet and Adelmant 1995 , Martinez and Wahli 1991). The inverted (IR), direct (DR) or everted (ER) repeats are indicated by the arrows. N indicates any nucleotide and x the variable spacing between the repeats. R indicates A/G, M indicates A/C, Y indicates C/T and W indicates A/T. Examples of the receptors that bind to the different classes of binding site are listed.

arrangement of the sequence AGGTCA and is referred to as a TREpal (Brent *et al* 1989, Glass *et al* 1988). This element was similar to the consensus ERE with the exception that the three base pairs separating the repeats were missing in the TREpal. This element was also shown to be retinoic acid inducible (Glass *et al* 1989, Umesono *et al* 1988) and subsequent reports have similarly demonstrated that the response element specifcity of the thyroid hormone and retinoic acid receptors can overlap (Graupner *et al* 1989, Mangelsdorf *et al* 1990).

The observation that the TRE identified in the rat myosin heavy chain promoter conferred T3 but not retinoic acid inducibility led to the discovery of another class of binding site. The AGGTCA related half sites of this element are arranged in a direct repeat arrangement with a four nucleotide spacing between the repeats. The use of natural and idealised sites led to the observation that direct repeats with different spacings were selective for different receptors (Naar *et al* 1991, Umesono *et al* 1991) The VDR bound preferentially to a direct repeat with a three base pair spacing (DR+3), TR to a four (DR+4) and RAR to a five nucleotide spacing (DR+5). This selectivity was also observed in transient transfection experiments with a DR+3 element conferring the strongest response to VDR, DR+4 element to TR and DR+5 element to RAR. This lead to the proposal of the 3-4-5 rule in which the selectivity for a particular hormonal response was dictated by the spacing between the half sites (Umesono *et al* 1991). In addition it was also proposed that direct repeats with a one base pair spacing were specific for RXR as this receptor binds to a DR+1 element with high affinity and was shown to mediate preferential transactivation of the rat celullar retinol-binding protein II promoter which contains five directly repeated half sites with a one nucleotide spacing (Mangelsdorf *et al* 1991). However, it now appears that such a code is too simplistic and receptor discrimination of the spacing is rather more degenerate than first appreciated. In particular, a number of natural retinoic acid response elements (RAREs) composed of direct repeats with nucleotide spacings other than 5 nucleotides have been reported (Durand *et al* 1992, Rottman *et al* 1991, Vasios *et al* 1991). The sequence of the half sites and of flanking nucleotides have also been shown to be important factors which influence RAR and TR recognition of response elements (Mader *et al* 1993b, Mader *et al* 1993, Vivanco *et al* 1991) illustrating that sequence, as well as spacing, dictate the binding site selectivity.

From *in vitro* DNA binding studies it was apparent that a cofactor present in nuclear extracts enhanced the binding activity of RAR, TR and VDR (Burnside *et al* 1990, Glass *et al* 1990, Murray and Towle 1989). These factor(s) formed novel heterodimeric complexes with RAR or TR that had higher binding affinity than the homodimeric receptors (Glass *et al* 1990, Lazar *et al* 1991, Naar *et al* 1991). Intriguingly, the TR had been shown to form a heterodimer with RAR *in vitro* that bound to various TREs with ten fold greater affinity than the TR homodimer (Glass *et al* 1989) although as this heterodimer did not bind with increased affinity to an RARE it was unlikely that TR was the RAR cofactor present in nuclear extracts. This observation did suggest, however, that the stimulatory factor might be another nuclear receptor and conformation came when the RAR coregulator was isolated and shown to be identical to RXR (Leid *et al* 1992b, Marks *et al* 1992, Yu *et al* 1991).

RXRα was originally cloned by Mangelsdorf et al (Mangelsdorf *et al* 1990) by virtue of its homology to RARα and Hamada et al (Hamada *et al* 1989) had cloned RXRβ, also known as H-2RIIBP, by its interaction with region II of the major histocompatibility complex class I genes. Although originally classified as an orphan receptor it has since been shown to be bound and activated by 9-*cis* retinoic acid (Heyman *et al* 1992, Levin *et al* 1992). RXR enhances the binding of RAR, TR and VDR to inverted and direct repeats response elements (Kliewer *et al* 1992b, Leid *et al* 1992b, Marks *et al* 1992, Yu *et al* 1991, Zhang *et al* 1992a) and similarly increases the DNA binding activities of some orphan receptors including PPAR, LXR, RLD-1 and MB67 (Apfel *et al* 1994, Baes *et al* 1994, Gearing *et al* 1993, Kliewer *et al* 1992c, Willy *et al* 1995). The majority of the receptors of the class II group tested so far bind preferentially as a heterodimer to direct and inverted repeat response elements. The formation of heterodimers has been conserved during evolution as the DNA binding and transactivation of the *Drosophila* ecdysone receptor is enhanced through the formation of heterodimers with *ultraspiracle* the *Drosophila* homologue of RXR (Thomas *et al* 1993, Yao *et al* 1992). Strikingly, *Ultraspiracle* can substitute for RXR in forming heterodimers with RAR, TR, VDR and PPAR while RXR can form heterodimers with the ecdysone receptor (Yao *et al* 1992).

Although TR homodimers only bind weakly to direct and inverted repeats they can bind with high affinity to everted repeats spaced by 4, 5 or 6 base pairs (Kurokawa *et al* 1993, Naar *et al* 1991, Piedrafita *et al* 1995). Heterodimers also bind to everted repeats, for example, an RARE in the γF-

crystallin gene, composed of an everted repeat with an 8 base pair spacing, is bound preferentially by RXR/RAR or TR/RAR heterodimers (Tini *et al* 1994). A TR/VDR heterodimer has also been detected on everted repeats with 7 and 9 base pairs (Schrader *et al* 1994b).

Response element selectivity for the class II group of receptors is therefore rather complex and is dictated by a variety of factors including whether the receptor binds as a homodimer or heterodimer, the orientation, spacing and sequence of the half sites and in addition, the sequence of the flanking nucleotides. When the work described in this thesis was initiated COUP-TF had only been shown to bind DNA as a homodimer. However, during the course of this thesis it become apparent that COUP-TF may also form heterodimers and this question is addressed in the work described in Chapter 4.

The third class of receptors consists of orphan receptors that bind DNA as monomers and includes NGFI-B, SF-1, Rev-erb and the *Drosophila FTZ-F1*. When the group of Milbrandt and coworkers (Wilson *et al* 1991) originally identified a response element for NGFI-B they made the surprising discovery that it contained a single half site motif. The failure to detect a heterodimer between wild type and truncated protein on this site in gel shift analysis supported the prediction that NGFI-B bound as a monomer (cited in Wilson *et al* 1992). Mutational analysis of the element identified nucleotides important for receptor bindng *in vitro* and the sequence AAAGGTCA was proposed as a consensus NGFI-B response element (NBRE) (Wilson *et al* 1992). The other members of this group also bind as monomers to response elements that consist of an extended half site motif with the nucleotides immeadiately 5' of the half site involved in specifying receptor selectivity (Figure 1.1) (Ueda *et al* 1992, Wilson *et al* 1993). In addition, RZR (also known as ROR) can also bind as a homodimer to certain sites that contain two half site motifs (Carlberg *et al* 1994). More recently, NGFI-B and NURR1 have been reported to form heterodimers with RXR (Forman *et al* 1995, Perlmann and Jansson 1995) and it is possible that receptors from this class may bind DNA either as a monomer, homodimer or heterodimer depending on the type of response element.

Nuclear receptor response elements are found within genes in either orientation and at various distances from the initiation site (see Martinez and Wahli 1991). In many cases the promoter/enhancer contains more than one receptor binding site and often there are binding sites for other

transcription factors as well (Carter *et al* 1994, Chalepakis *et al* 1988, Martinez *et al* 1987, reviewed in Martinez and Wahli 1991). On such sites synergistic transcriptional activation can be observed. This occurs when the transcriptional activation due to two or more transcription factors is greater than the sum of the activation observed when each is bound individually (Ptashne 1988) and this synergy is due to cooperation of the factors in binding DNA or stimulating transcription (reviewed in Martinez and Wahli 1991).

Nuclear receptor structure

On the basis of sequence similarity between the predicted protein sequences of the ER and GR several regions (A-F) were defined (Krust *et al* 1986). These regions display different degrees of evolutionary conservation amongst the members of the nuclear receptor family. Region C, the DNA binding domain, is highly conserved between the different receptor types whereas, in contrast, the N-terminal regions A/B are highly variable both in sequence and size, varying from 24 amino acids in the VDR to 603 in the (MR). Similarly, region D shows poor sequence conservation between receptor types. Region E is the largest domain and is involved in ligand binding, dimerisation and transactivation. This region is only moderately well conserved presumably reflecting the different ligand specificties of the receptors. Region F, the sequence C-terminal to the ligand binding domain, is poorly conserved and is lacking in some receptors altogether. Between different species each receptor type is well conserved with regions C and E containing the highest homology. In particular, region C shows over 90% conservation.

The modular structure of nuclear receptors has been a significant advantage in the analysis of their functional properties. The aim of the following section is to provide an overview of the DNA binding, dimerisation, ligand binding and transactivation properties of nuclear receptors.

DNA binding

The first experimental evidence that region C contained the DNA binding domain came from 'domain swap' experiments. Green and Chambon (Green and Chambon 1987) demonstrated that the DNA binding specificity of the ER *in vivo* could be converted to that of the GR by the replacement of region C with that of the GR. A cysteine rich sequence within this region showed similarity to the zinc finger DNA binding motif of the

transcription factor IIIA (TFIIIA). In the TFIIIA motif each zinc ion was tetrahedrally coordinate by two cysteines and two hystidines (Miller *et al* 1985) and by analogy it was suggested that region C contained two zinc finger DNA binding motifs, with the zinc ions tetrahedrally coordinated by cysteine residues. Evidence supporting the existence of a zinc finger DNA binding motif was provided by mutagenesis of the GR. Significantly, whilst replacement of eight of the conserved cysteine residues of region C to alanine or serine residues abolished DNA binding, replacement with histidines retained the activity (Severne *et al* 1988). Furthermore, measurements made using extended X-ray absorption fine structure suggested that the DNA binding domain contained two zinc ions with each ion coordinated by four cysteines (Freedman *et al* 1988).

Additional mutagenesis studies of the ER and GR identified individual amino acid residues involved in providing the response element specificty. Green et al (Green and Chambon 1988) initially localised this property to the N-terminal zinc finger. The replacement of two amino acids, between the third and fourth cysteine of the N-terminal zinc finger of the GR, with the corresponding ER residues resulted in a mutant that stimulated transcription efficiently from an ERE and poorly from a GRE (Danielsen *et al* 1989, Umesono and Evans 1989). To convert the ER to specifically recognise a GRE a residue located between the zinc fingers was required in addition to the two amino acid residues identified in the GR (Mader *et al* 1989). These three amino acids are referred to as the P box. In the ER the P box residues are glutamine, glycine and alanine while in the GR they are glycine, serine and valine. The GR P box sequence is conserved between the PR, MR and AR, which all recognise a GRE. Those receptors that recognise the same half site sequence as the ER contain the glutamine and glycine residues of the P box but not the third residue, alanine. The identity of the third residue is conserved between receptors of the same sub group, for instance, COUP-TF, *seven-up* and ear-2 contain a serine at the third position and TR, RAR, RXR and VDR contain a glycine. In contrast, only the first residue of the P box of SF-1 and FTZ-FI is conserved with the ER, the second and third residues being replaced by serine and glycine.

The ER and TR recognise response elements that differ only in the spacing of the half sites (Figure 1.1). The TR DNA binding domain binds cooperatively to a TREpal but not an ERE, conversely, the ER DNA binding domain binds an ERE as a dimer and the TREpal as a monomer. Using chimeric receptors residues in the D box were identified as necessary for

recognising the particular spacing and are required for the formation of the dimer interface on their respective response elements (Hirst *et al* 1992, Mader *et al* 1993a, Umesono and Evans 1989).

NMR analysis of the 3D structure of the DNA binding domains of the ER and GR in solution and X-ray crystallographic analysis of their structure on DNA confirmed and extended the results obtained from the mutagenesis studies. In solution the DNA binding domains are monomeric and the two zinc finger motifs are folded to form a single domain, unlike the zinc finger motifs of TFIIIA which form independent units (Hard *et al* 1990b, Schwabe *et al* 1990). The DNA binding domains contain two amphipathic α helices, that start at the C-terminal pair of cysteines of each zinc finger and are arranged at right angles relative to each other. The region between the two fingers is poorly ordered with a number of hydrophobic side chains forming a hydrophobic core between the helices. Resolving of the crystal structure of these receptors on DNA confirmed that two molecules of the DNA binding domain bind cooperatively to adjacent major grooves on the same side of the double helix. As predicted, the N-terminal zinc finger is involved in sequence discrimination with one of the P box residues making contact with a nucleotide from the half site in both the GR/DNA and ER/DNA complexes (Luisi *et al* 1991, Schwabe *et al* 1993). Recognition of response elements by the ER and GR appears to involve not only the use of these discrimatory amino acids that favour interactions with different nucleotides but also a global change in the arrangement of the side chains of common residues (Schwabe *et al* 1993).

Residues in the D box form part of a dimerisation interface between the DNA binding domains (Luisi *et al* 1991, Schwabe *et al* 1993). This region in the ER DNA binding domain is disordered in solution and the domain is monomeric and it seems that binding of the receptor to DNA is required for the correct interface to be formed (Schwabe *et al* 1993). Whether the corresponding region of the GR DNA binding domain is disorderd in solution is less clear (Baumann *et al* 1993, Berglund *et al* 1992, Hard *et al* 1990a, Hard *et al* 1990b, Luisi *et al* 1991).

In addition to specific nucleotide contacts, residues of the receptor stabilise the DNA complex through phosphate contacts with the backbone DNA. Interestingly, residues from the dimer interface are involved in forming these phosphate contacts such that the protein/protein contacts between the monomers enhance the protein/DNA interactions and increase

the stability of the receptor/DNA complex (Luisi *et al* 1991, Schwabe *et al* 1993).

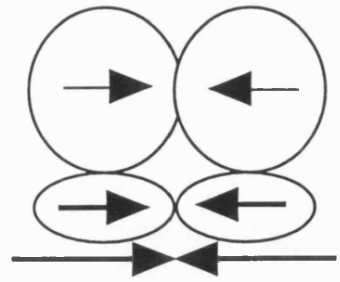
The ten carboxy amino acids of region C, immeadiately following the second amphipathic α helix, are required for stable binding of the ER on DNA (Schwabe *et al* 1993). These residues are unstructured in both the NMR and X-ray crystallographic analysis (Schwabe *et al* 1993, Schwabe *et al* 1993). Mader et al (Mader *et al* 1993a) have shown that amino acids in region D are also involved in stabilising DNA binding of the ER especially on imperfect palindromes. The involvement of residues downstream of the zinc finger motifs in high affinity DNA binding was originally demonstrated for RXR and the orphan receptor NGFI-B (Wilson *et al* 1993) and these additional residues, the T box in RXR and the A box in the TR, have been shown to form α helices (discussed later) (Lee *et al* 1993, Rastinejad *et al* 1995). It is possible that the corresponding residues of the ER also form an additional structure that is disordered by crystal packing or, alternatively, the unstructured residues may provide a number of weak non specific contacts with DNA. Throughout this thesis the conserved zinc finger motif (residues 180-249 of the human ER) (Mader *et al* 1993a) is defined as the 'core DNA binding domain', distinct from the region of the receptor required for high affinity binding.

Reports from several groups indicated that nucleotides flanking the half sites influenced the affinity with which receptors bound DNA (Katz and Koenig 1994a, Katz *et al* 1995, Kurokawa *et al* 1993, Mader *et al* 1993, Wilson *et al* 1992) and this effect was particularly marked for those receptors that bound as monomers, for example, NGFI-B, SF-1 and FTZ-F1 (Lala *et al* 1992, Lavorgna *et al* 1991, Wilson *et al* 1993, Wilson *et al* 1992). In binding site selection both RXR/TR and RXR/RAR exhibited partial sequence selectivity of the nucleotides at the 5' of half sites (Kurokawa *et al* 1993). Supporting evidence for the involvement of flanking nucleotides was provided by DNA methylation experiments which indicated that the heterodimers made minor groove contacts upstream of the half site motifs (Kurokawa *et al* 1993). Of interest was the observation by Wilson et al (Wilson *et al* 1992) that amino acids downstream of the core DNA binding domain of NGFI-B were involved in DNA binding of the monomer. These residues, referred to as the A box, are required by NGFI-B, SF-1 and FTZ-F1 for recognition of nucleotides 5' of the half site sequence (Ueda *et al* 1992, Wilson *et al* 1993). The A box of the TR was also implicated in high affinity DNA binding by Kurokawa et al (Kurokawa *et al* 1993) who predicted that in an RXR/TR

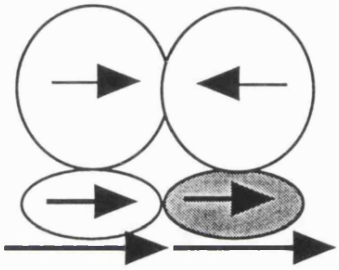
heterodimer residues from the A box of the TR would form DNA contacts with the 5' end of each half site. High affinity DNA binding by RXR homodimers to direct repeats with a one base pair spacing required a region (the T box) that was distinct, but adjacent to, the A box (Wilson *et al* 1992).

On direct repeat elements a novel asymmetric interface between the DNA binding domains of the receptors must be predicted as the symmetrical interface used on inverted repeats would not be properly aligned (Figure 1.2). Especially intriguing is the ability of the DNA binding domain of RXR to bind cooperatively, as a heterodimer, to direct repeats with half site spacings of one to five base pairs. NMR structural analysis confirmed that, like the steroid hormone receptors, the two zinc finger motif of RXRα formed a single domain with two α helices perpendicular to each other. In contrast to the ER and GR, however, residues C-terminal to the zinc finger motif formed a third α helix (Lee *et al* 1993). These residues were located in the T box (Wilson *et al* 1993) and mutagenesis studies indicated that amino acids within the helix were involved in both contacting DNA and in forming protein/protein contacts between monomers (Lee *et al* 1993). Computer modelling suggested that the binding of two RXR DNA binding domains to a DR+1 repeat would promote a dimerisation interface between the third helix of the RXR monomer on the 3' half site and the C-terminal zinc finger of the monomer on the 5'site.

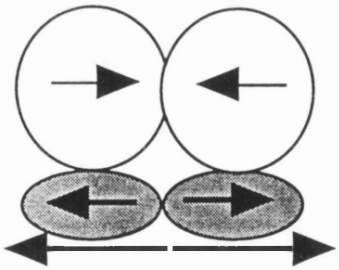
The heterodimers RXR/TR and RXR/RAR preferentially recognise direct repeat elements on which their DNA binding domains bind cooperatively. Studies on these discriminatory interfaces have implicated distinct regions within the different receptor types. Perlmann and coworkers (Perlmann *et al* 1993) demonstrated that on a DR+5 element the cooperative binding of RXR and RAR involved the D box of RXR and the N-terminal zinc finger motif of RAR and, similarly, the binding of an RXR/TR heterodimer on a DR+4 element requires the D box of RXR and the N-terminal zinc finger motif of TR. A more detailed mutagenesis study of these interfaces by Zechel et al (Zechel *et al* 1994a, Zechel *et al* 1994b) allowed discrimination between the region of the N-terminal zinc finger motif involved in the RAR and TR heterodimers. Residues between the second and third cysteine residues of the N-terminal zinc finger, referred to as the 'tip' of the finger were required in RAR while in contrast, the residues involved in the TR mapped to the region N-terminal to the first cysteine, the 'prefinger' region. This group also studied the dimerisation interface formed between the DNA binding domains of RXR and RAR on a DR+2 site



INVERTED REPEAT



DIRECT REPEAT



EVERTED REPEAT

Figure 1.2 Differential orientations of the DNA binding domains on inverted, direct and everted repeats

Model for dimers bound to inverted, direct and everted repeats. The shaded DNA binding domains are predicted to be rotated by 180^{o} with respect to the C-terminal domain of the receptor as compared with the orientation on the inverted repeat.

on which the T box of RAR and a region of the C-terminal zinc finger of RXR distinct from the D box were required. Thus RAR and RXR appear to form distinct interfaces on the different spacings.

The formation of an asymmetric dimerisation interface between the DNA binding domains of RXR and its heterodimer partners RAR and TR implied that these heterodimers bind DNA with polarity. Work from several groups has supported this prediction, an RXR/TR heterodimer on a DR+4 element is orientated with RXR on the 5' half site and TR on the 3' half site. Similarly, an RXR/RAR heterodimer on a DR+2 or DR+5 element binds with RXR on the 5' half site (Kurokawa *et al* 1993, Mader *et al* 1993, Perlmann *et al* 1993, Zechel *et al* 1994b). On a DR+1 element, however, the polarity of the RXR/RAR heterodimer is reversed with RAR placed on the 5' half site (Kurokawa *et al* 1994).

The determination of the crystal structure of the DNA binding domains of a RXR/TR heterodimer bound to a DR+4 response element (Rastinejad *et al* 1995) has refined the models predicted from the mutagenesis studies. As predicted RXR was located on the 5' half site and the dimer interface, which is formed over the minor groove of the DNA, involved different regions of the individual receptors. The C-terminal zinc finger of RXR is involved in the dimer interface, including residues from the D box region whereas three regions of the TR are involved, the 'prefinger' region of the N-terminal finger, the N-terminal finger and the T box. As observed for the ER and GR, residues in the dimer interface are also involved in contacting DNA illustrating the interplay between high affinity DNA binding and dimer formation.

As for the ER and GR, the sequence specificty of the RXR/TR heterodimer is provided by the N-terminal α-helix that makes specific contacts with the half site. Non specifc phosphate contacts are made with the backbone DNA, again reminiscent of the binding of steroid receptors. In addition, residues of the A box of the TR form a third α-helix (A-helix) that forms direct contacts with the nucleotides upstream of the 3' half site. These protein/DNA contacts provide the selectivity displayed by the TR for the nucleotide sequence of the 'spacer' region (Katz and Koenig 1994a, Katz *et al* 1995, Kurokawa *et al* 1993, Mader *et al* 1993, Wilson *et al* 1992). The A-helix also makes non specific phosphate contacts with the backbone DNA, this α-helix is relatively long (24 amino acids) and the large number of DNA contacts formed provide the TR with sufficient stability to bind as a

monomer (Katz and Koenig 1994b, Schrader *et al* 1994a). The orphan receptors that bind to DNA as monomers are predicted to form extensive DNA contacts that obviate the requirement for the additional free energy provided by cooperative binding of molecules. By analalogy to the TR it is likely they contain an A-helix type structure that provides some of the required protein/DNA contacts (Ueda *et al* 1992, Wilson *et al* 1993, Wilson *et al* 1992).

Computer modelling and mutagenesis studies predict two mechanisms by which the RXR heterodimers discriminate the spacing between half sites. The first is that the correct stereochemical alignment of the two monomers required to form a stable interface only occurs on certain spacings. The second is dictated by steric hindrance, for example, RXR/TR heterodimers are predicted to be blocked from binding direct repeats spaced by less than four base pairs due to the long A-helix of the TR (Mader *et al* 1993b, Rastinejad *et al* 1995, Zechel *et al* 1994b). Steric hindrance is similarly thought to prevent two molecules of ER binding to inverted repeats seperated by less than three base pairs (Schwabe *et al* 1993).

The T box of RXR is unstructured in the crystal structure of RXR/TR in contrast to the α-helical structure observed by NMR analysis of the isolated DNA binding domain (Lee *et al* 1993, Rastinejad *et al* 1995). The T box of the TR, bound to the 3' motif of the DR+4 site forms a loop that is involved in the RXR/TR dimer interface. It is possible that the T box of RXR is also required on those sites on which it binds to the 3' half site of the element, such as the DR+1 element either as a RXR homodimer or RAR/RXR heterodimer (Lee *et al* 1993, Zechel *et al* 1994b).

Dimerisation

As discussed in the previous section the majority of nuclear receptors bind to DNA as dimers. Full length ER forms dimers in solution as well as on DNA (Fawell *et al* 1990a, Kumar and Chambon 1988) while in contrast the isolated DNA binding domain requires DNA to dimerise and is monomeric in solution (Schwabe *et al* 1993). Deletion analysis revealed that the region required for dimerisation in solution is contained within region E (Fawell *et al* 1990a). This region also stabilises the dimer on DNA, a mutant ER that lacks region E has an affinity for DNA ten fold lower than that of wild type (Fawell *et al* 1990a). Region E is also important in the dimerisation of other receptors including the TR, RAR, VDR, RXR and COUP-TF (Forman

et al 1989, Glass *et al* 1989, Kurokawa *et al* 1993, Ladias and Karathanasis 1991, Leid *et al* 1992b, Marks *et al* 1992, Rosen *et al* 1993, Selmi and Samuels 1991, Spanjaard *et al* 1991) and is often referred to as the major dimerisation activity.

Sequence alignment of a number of receptors identified a conserved heptad repeat of hydrophobic residues within region E (Fawell *et al* 1990a). Point mutagenesis of the mouse ER suggested that residues critical for dimerisation were located in the N-terminal half but not the C-terminal half of this motif (Fawell *et al* 1990a). Forman et al (Forman *et al* 1989) identified nine heptad repeats within the equivalent region of the TR (the conserved region identified by Fawell et al (Fawell *et al* 1990a) overlaps the ninth heptad). These heptad repeats were reminiscent of the coiled-coil dimerisation motif of the leucine zipper and helix-loop-helix transcription factors although structurally distinct and were suggested to form five α-helices that created a 'regulatory zipper'(Forman and Samuels 1990, Forman *et al* 1989).

Homodimers of RAR, TR and RXR, unlike heterodimers, are not readily detected off DNA (Barettino *et al* 1993, Glass *et al* 1990, Hermann *et al* 1992, Kliewer *et al* 1992b, Leid *et al* 1992b, Zhang *et al* 1992a) implying that the interface formed by region E is weaker in the homodimer than the heterodimer and requires DNA for stabilisation. Furthermore, the formation of RXR/TR heterodimers in solution is favoured over DNA bound TR homodimers (Kurokawa *et al* 1993) thus, for the TR at least, heterodimeric interactions with RXR off DNA seem to be stronger than homodimeric contacts on DNA. The isolated C-terminal domain of RAR forms homodimers as efficiently as heterodimers with RXR (Kurokawa *et al* 1993) indicating that the N-terminus or the DNA binding domain of RAR must restrict the formation of the homodimer interface.

Mutagenesis studies have revealed that the C-terminal homodimer and heterodimer interfaces of TR, RAR and RXR are not superimposable. Point mutagenesis identified mutations in the ninth heptad repeat of TR and RAR which destabilise heterodimers with RXR but do not affect homodimer formation (Au *et al* 1993). Residues within the second and third heptads of the TR and residues N-terminal to the heptad repeats in RAR and TR have also been implicated in heterodimerisation with RXR (Darling *et al* 1991, O'Donnell *et al* 1991, Rosen *et al* 1993, Spanjaard *et al* 1991). Furthermore, the residues of RXR required for the formation of

homodimers and heterodimers are distinguishable, C-terminal deletion analysis of RXR suggests that homodimerisation involves residues C-terminal to those sufficient for heterodimerisation although the ninth heptad repeat is required for both the homodimer and heterodimer functions of RXR (Leng *et al* 1995, Marks *et al* 1992, Zhang *et al* 1994).

The crystal structure of region E of human RXRα has recently been solved (Bourguet *et al* 1995). The domain contains eleven α-helices in a three layer structure that folds to form a novel antiparallel α-helical sandwich. This "sandwich" exists as a dimer with symmetrical contacts provided by two of the helices, helix ten and, to a lesser extent, helix nine. An intervening loop structure between helix seven and eight also forms part of the dimer interface. Interestingly, although residues of the ninth heptad are located in helix 10 their side chains are packed away from the dimer interface. Indeed, the hydrophobic residues of all the heptad repeats seem to to be involved in stabilising the α-helical structure rather than being positioned at the dimer interface. As these hydrophobic residues are conserved it is likely that the overall fold of the domain will be common throughout the family. Differences in the residues at the dimer interface may then specify the dimerisation properties of the receptors, such as whether it forms homodimers exclusively, like the steroid hormone receptors, or can also form heterodimers.

The DNA binding domain of the receptor bound to the 3' half site of a response element is predicted to be rotated by 180° on a direct repeat in comparison to the orientation on a inverted repeat. On an everted repeat both DNA binding domains would be rotated by 180° (see Figure 1.2). To accommodate the different orientations of the DNA binding domain a flexible hinge region connecting the DNA binding domains to the C-terminal domain has been proposed. This hinge region corresponds to region D which contains the A- and T boxes, residues from these boxes have been suggested to be involved in providing the differential orientations of the DNA binding domain (Kurokawa *et al* 1993).

Ligand Binding

Analysis of the hormone binding activity of mutants of the ER, GR and TR revealed that region E of the receptor was sufficient for high affinity binding (Giguere *et al* 1986, Godowski *et al* 1987, Hollenberg *et al* 1987, Horowitz *et al* 1989, Kumar *et al* 1986, Rusconi and Yamamoto 1987). The

integrity of the entire region, of approximately 250 amino acids, appeared to be required for efficient binding suggesting that this region folded as a single domain. This was supported by studies that showed that the hormone binding domain was retained in a 28 kD protease resistant fragment of the ER and GR (Eisen *et al* 1985, Katzenellenbogen *et al* 1987). Region E contains a number of conserved hydrophobic residues and it has been proposed to form a hydrophobic 'pocket' in which the ligand binds. Ligand affinity labelling has identified residues that are part of the ligand binding site and in addition point mutagenesis has identified residues that are required for ligand binding (reviewed in Dauvois and Parker 1993, McPhaul 1993). Region E also contains the major dimerisation interface (see above), point mutagenesis of the mouse ER (Fawell *et al* 1990a) and rat TRα (Spanjaard *et al* 1991) suggests that these two functions overlap but are not superimposable. Analysis of the crystal structure of region E of RXR, determined in the absence of ligand, has lead to the identification of a potential ligand binding site. The putative site consisits of a hydrophobic cavity created by four surrounding α-helices into which a molecule of 9-*cis* retinoic acid can be modelled with little adjustment. This model fits well with the mutagenesis and crosslinking data obtained with other nuclear receptors. It is intriguing that although RAR and RXR both bind 9-*cis* retinoic acid their ligand binding domains display only moderate sequence conservation. This region of mouse RXRα shares approximately 30% amino acid identity with mouse RARα compared with 44% identity with mouse COUP-TF I (Leid *et al* 1992a). This divergence may have been required to provide RAR, unlike RXR, with the ability to bind all-*trans*-retinoic acid.

Transcriptional activation domains

Two autonomous activation functions, AF-1 and AF-2, have been identified in most of the hormone receptors with the relative contribution of these two activities varying between the different receptors (reviewed in Danielsen 1991, Green and Chambon 1991, Gronemeyer 1993). AF-1 is located in the N-terminus (A/B region) and can stimulate transcription in a ligand independent manner when fused to a heterologous DNA binding domain or when the C-terminus of the receptor is deleted (Berry *et al* 1990, Gronemeyer *et al* 1991, Lees *et al* 1989, Meyer *et al* 1990, Nagpal *et al* 1993, Tora *et al* 1989). In the ER, RAR and RXR this activity is highly promoter and cell type specific (Berry *et al* 1990, Bocquel *et al* 1989, Durand *et al* 1994, Nagpal *et al* 1993, Nagpal *et al* 1992, Tora *et al* 1989) presumably reflecting a requirement

for cell type and promoter specific cofactors. AF-2 is located in the C-terminus (region E) and its activity is ligand dependent (Hollenberg *et al* 1987, Lees *et al* 1989, Meyer *et al* 1990, Nagpal *et al* 1993, Webster *et al* 1988). None of the hormone receptors contains a conventional transcriptional activation motif with the possible exception of the GR in which these regions are relatively rich in acidic residues (see later).

Sequences important for AF-2 activity of the mouse ER were identified in the C-terminus of region E (Lees *et al* 1989). Point mutagenesis of this region identified an element composed of conserved hydrophobic and acididc residues that was required for transcriptional activation by the ER and GR (Danielian *et al* 1992). This region has since been implicated in ligand dependent activation by the TR, RAR and RXR (Barettino *et al* 1994, Durand *et al* 1994, Saatcioglu *et al* 1993, Toney *et al* 1993). This conserved region was predicted to form an amphipathic α-helix that might play an important role in ligand dependent transcriptional activation by all hormone receptors (Barettino *et al* 1994, Danielian *et al* 1992, Durand *et al* 1994). From the crystal structure of RXRα it is now known to form part of an α-helix that is orientated away from the dimeric ligand binding domain at an angle of 45^{o} (Bourguet *et al* 1995). The role of ligand in producing the transcriptional activity of this domain is not clear and awaits the resolving of the crystal structure of the RXR ligand binding domain bound to 9-*cis* retinoic acid. It has been proposed, however, that the ligand acts to disrupt intramolecular contacts between the 'activation helix' (containing the conserved AF-2 motif) and other regions of the domain (Bourguet *et al* 1995).

The activation domains of the constitutively active orphan receptors have not been characterised as extensively as those of the hormone receptors. The transcriptional activity observed may depend solely on expression levels or alternatively, be regulated by an endogenous ligand or by another type of posttranslational modification such as phosphorylation (see O'Malley and Conneely 1992). In addition, it remains a possibility that these receptors might also respond to an exogenous ligand. The constitutive activity of NGFI-B has been mapped to the N-terminus which is phosphorylated *in vivo* (Davis *et al* 1993, Fahrner *et al* 1990, Hazel *et al* 1991, Paulsen *et al* 1992). Mutation of a serine and a threonine residue within this region reduced the activity by 65% suggesting that phosphorylation may be involved in the transcriptional activity of NGFI-B (Paulsen *et al* 1992). Phosphorylation of this protein has also been proposed to regulate both its DNA binding activity and nuclear localisation (Davis *et al*

1993). Regulation by phosphorylation may not be exclusive to the orphan receptors, several studies suggest that phosphorylation may also alter the properties of hormone receptors such as PR, ER and RAR (Gaub *et al* 1992, Huggenvik *et al* 1993, Ignar-Trowbridge *et al* 1992, Ignar-Trowbridge *et al* 1993, Power *et al* 1991b, Rochette *et al* 1992, Tahayato *et al* 1993).

Whether orphan receptors stimulate transcription through an AF-2 like activity is unclear. While it is conceivable that the AF-2 'activation helix' could be exposed by an alternative form of posttranslational modification rather than ligand binding sequence comparisons with the conserved AF-2 motif of the hormone receptors show that many of the orphan receptors including, COUP-TF, SF-I and NGFI-B, lack one or more of the conserved residues or ,as in the case of ELP, lack the whole motif (Durand *et al* 1994).

Transcriptional Regulation

RNA polymerase II directed transcription of mRNA coding genes requires the assembly of a functional initiation complex at the promoter. In the absence of factors bound at cis-acting sites the transcription is of low level and termed basal or uninduced. When positive acting factors are bound upstream (or downstream) the transcription is referred to as stimulated or activated. For clarity, the term initiation factor will be used throughout this thesis to refer to proteins that are involved in basal transcription although it should be noted that some of these factors may also be involved in other stages of transcription such as elongation. Those proteins that stimulate (or repress) basal transcription will be referred to as transcription factors. In the following section the components of basal transcription mediated by RNA polymerase II will be briefly discussed. Next, the mechanisms by which transcription factors may promote activated transcription will be detailed with particular emphasis on interactions with the RNA polymerase II initiation complex. Finally, the action of negative transcription factors will be discussed.

Basal transcription mediated by RNA polymerase II

Factors required for initiation have been identified by reconstitution studies using fractionated nuclear extracts. These fractions are named TFII-A, B, D, E, F, G, H and J (Zawel and Reinberg 1992). Some of the polypeptides from these fractions have been cloned and many of the others have been highly purified. The order of initiation complex formation *in vitro* has been

extensively studied using purified and recombinant components (reviewed in Buratowski 1994, Conaway and Conaway 1993, Zawel and Reinberg 1992). The initial step in formation of the pre-initiation complex involves the binding of TFIID to the promoter at a specific sequence, the TATA element which is usually located approximately 30 base pairs upstream of the transcription start site (see Breathnach and Chambon 1981). The TFIID fraction contains TBP, the TATA-binding protein and a number of TBP associated factors (TAFs), TBP alone is sufficient for recognition of the TATA element and for the formation of a complete pre-initiation complex. The TAFs appear to be required for activated transcription, functioning as coactivators that mediate the stimulatory effects of transcription factors bound at cis-acting elements (see later). TFIIA binds to TFIID and may act to stabilise TBP on the TATA element (reviewed in Buratowski 1994, Maldonado and Reinberg 1995). TBP has been cloned from a number of species and is highly conserved (see Pugh and Tjian 1992) on DNA it forms a 'saddle like' structure that contacts the minor groove of the TATA element and induces a bend in the DNA (reviewed in Hernandez 1993).

TFIIB is the next component to join the complex, binding directly to TBP, and is responsible for recruiting RNA polymerase II a process that also seems to involve TFIIF (reviewed in Buratowski 1994). These assembled proteins, TFIID, TFIIA, TFIIB, TFIIF and RNA polymerase II constitute the minimal pre-initiation complex and under certain conditions, this complex is sufficient to initiate transcription (Tyree *et al* 1993). In general, however, TFIIE and TFIIH are also required to form a complete pre-initiation complex (Wang *et al* 1992). Initiation from these complexes involves hydrolysis of ATP and this step is referred to as the activation of the complex (Bunick *et al* 1982, Sawadogo and Roeder 1984).

TFIIF consists of two RNA polmerase associated proteins (RAPs), RAP30 and RAP74 (reviewed in Conaway and Conaway 1993). RAP30 may act to decrease the affinity of the polymerase for non-specifc DNA (Killeen and Greenblatt 1992). Initial studies with purified TFIIF suggested that it contained an ATP dependent helicase activity involved in unwinding the DNA in front of the polymerase (Sopta *et al* 1989). However, more recent studies using highly purified preparations or recombinant protein indicate that TFIIF does not contain a helicase activity (see Conaway and Conaway 1993 for a review of TFIIF function). The function of TFIIE has not been clearly defined but it appears to be required for TFIIH to join the complex. TFIIH contains several activities, including a kinase activity, a DNA

dependent ATPase and a DNA helicase activity and is involved in both the processes of DNA repair and transcription (reviewed in Drapkin and Reinberg 1994, Maldonado and Reinberg 1995).

The C-terminal domain (CTD) of the largest subunit of RNA polymerase II has been implicated in contacting DNA (Suzuki 1990) and TBP (Usheva *et al* 1992). The CTD contains a heptapeptide repeat (YSPTSPS) which has been highly conserved during evolution, although the number of repeats differ, with 26 in yeast and 52 in mammals. The CTD is phosphorylated *in vivo* and the polymerase exists as at least two distinuguishable forms that differ in the extent of this phosphorylation (reviewed in Conaway and Conaway 1993). The hypophosphorylated form of the polymerase is preferentially recruited into the pre-initiation complex whilst the transcribing enzyme is hyperphosphorylated. TFIIH, which can phosphorylate the CTD, has been implicated in the conversion of the initiation complex into an elongation complex (Goodrich and Tjian 1994b). Phosphorylation of the CTD may 'mark' the polymerases that have succesfully initiated transcription, weakening the interaction of the CTD with DNA or TBP and releasing the polymerase from the initation site.

Activated transcription

Transcription factors bound to cis acting sites can alter gene transcription (reviewed in Johnson and McKnight 1989). Studies of the mechanism of action of these proteins have focused primarily on their ability to increase transcriptional initiation. The stimulatory action of these transcription factors is proposed to be mediated via direct or indirect protein/protein contacts between an activation domain of the transcription factor and the pre-initiation complex (see Ptashne and Gann 1990, Roeder 1991, Tjian and Maniatis 1994).

Transcription factors are often modular in arrangement with separable DNA binding, dimerisation and activation functions (reviewed in Frankel and Kim 1991). This has facilitated the identification of the transcriptional activation domains of a variety of factors and motifs have been identified that are rich in acidic, proline or glutamine residues (reviewed in Mitchell and Tjian 1989). For example, the transcriptional activation domain of VP16 is rich in acidic residues (Triezenberg *et al* 1988), NF1 in proline residues (Mermod *et al* 1989) and SP1 in glutamine residues (Courey and Tjian 1988, Kadonaga *et al* 1988). Initial studies on the acidic

activation domain of GAL4 suggested that acidic residues per se rather than a structural motif were important for activation (Gill and Ptashne 1987, Ma and Ptashne 1987a, Ma and Ptashne 1987b). Particularly striking was the observation that when a random pool of peptides fused to the GAL4 DNA binding domain were tested in yeast most of the peptides that supported transcriptional activation contained acidic resdiues and a net negative charge (Ma and Ptashne 1987b). Further work has suggested that the acidic residues might need to be within an α-helical structure (Giniger and Ptashne 1987), however, the structural motifs of the different classes of activation domain (acidic, glutamine or proline rich) remain poorly characterised.

The trancriptional activation domains of the nuclear receptors do not, in general, appear to contain acidic, glutamine or proline rich motifs. Exceptions include the GR which has been reported to contain acidic rich motifs in the N and C-terminal activation domains (Tasset *et al* 1990) and the N-terminal activation domain of NGFI-B which contains a proline rich region (Paulsen *et al* 1992). The conserved AF-2 activation motif of nuclear receptors forms part of an α-helix in RXRα (Bourguet *et al* 1995) and it appears that this structure is required for its function.

The divergence of the activation motifs suggests that they may target distinct proteins of the preinitiation complex. The different steps and the variety of components involved in pre-initiation complex formation provide a variety of potential targets for the action of transcriptional activators. The first initiation factor identified as a target of upstream activators was TFIID. Sawadogo and Roeder (Sawadogo and Roeder 1985) demonstrated that USF (MLTF) contacted TFIID on DNA and subsequently ATF (Horikoshi *et al* 1988b) and GAL4 (Horikoshi *et al* 1988a) were shown to alter the DNA footprint of TFIID. Since then a number of upstream activators have been shown to interact directly with TBP *in vitro* (reviewed in Hernandez 1993). Within the TFIID complex, however, TBP is tightly associated with a number of other proteins, TAFs (Dynlacht *et al* 1991, Tanese *et al* 1991) (reviewed in Hernandez 1993), and it is not clear whether the required interfaces of TBP will be available for the binding of the transcription factors *in vivo*. One of the best studies examples is the interaction of the acidic activation domain of VP16 with TBP (Stringer *et al* 1990). Support for a direct interaction between VP16 and TBP *in vivo* was provided by the observation that point mutations of VP16 that inhibit transcriptional activity *in vivo* also reduce its interaction with TBP *in vitro* (Ingles *et al* 1991). Studies with purified TBP suggested that for other

transcription factors the interaction may not be direct. Pugh and Tjian (Pugh and Tjian 1990) demonstrated that partially purified TFIID but not purified TBP could support the action of SP1 and that the addition of TAFs to TBP *in vitro* restored the activation. This lead to the the coactivator hypothesis in which the TAFs acted as bridging proteins (coactivators) linking the transcription factors to the pre-initiation complex (Dynlacht *et al* 1991, Pugh and Tjian 1990).

Biochemical analysis indicated that some of the TAF proteins were present in substochiometric amounts (Brou *et al* 1993a, Chiang *et al* 1993, Tanese *et al* 1991). Distinct TFIID complexes could be chromatographically separated and these were shown to support transcriptional activation by different transcription factors *in vitro* (Brou *et al* 1993b, Jacq *et al* 1994) leading to the suggestion that different classes of transcription factor may interact with distinct TAFs. The cloning of individual TAFs and characterisation of their ability to interact with activators has supported this prediction.

Eight TAFs have been cloned from *Drosophila*, dTAF$_{II}$- 250, 150, 110, 80, 60, 40, 30α and 30β (see Goodrich and Tjian 1994a). Three of the human homologues have also been cloned, hTAF$_{II}$250, hTAF$_{II}$70 and hTAF$_{II}$30. Drosophila TAF$_{II}$110 interacts with the glutamine rich activation domain of SP1 (Hoey *et al* 1990). In support of the proposal that the interaction is functionally significant, mutations that reduced activation by SP1 *in vivo* also reduced its interaction with dTAF$_{II}$110 *in vitro* (Gill *et al* 1994). Interestingly, TAF$_{II}$110 does not appear to interact with TBP directly but is recruited via TAF$_{II}$250 (Weinzierl *et al* 1993). In contrast, the acidic activation domain of VP16 contacts a different TAF, dTAF$_{II}$40 that makes direct contacts with TBP (Goodrich *et al* 1993). Antibodies against this TAF can specifically block VP16 activation but not basal transcription *in vitro* (VP16 was tethered to the GAL4 DNA binding domain in this study).

The ER can contact TBP directly *in vitro*, however, this interaction is insenstitive to mutations that abolish AF-2 function. Furthermore, the squelching of transcriptional activation by the ER observed when the receptor is overexpressed can only be partially overcome by cotransfection with TBP (Sadovsky *et al* 1995). From *in vitro* studies it seems that TFIID is required for complete rescue suggesting that ER mediated transactivation involves coactivator(s)/TAFs (Brou *et al* 1993a, Brou *et al* 1993b). Recently, hTAF$_{II}$30, which is only present in a distinct class of TFIID, has been shown

to interact directly with the C-terminal domain of the ER (Jacq *et al* 1994). Immunodepletion of whole cell extracts using anti-hTAF$_{II}$30 antibodies inhibited transcription by a GAL4-ER chimera *in vitro* suggesting that this TAF was required for ER mediated stimulation of transcription. The interaction between hTAF$_{II}$30 and the ER is ligand independent and the region of the receptor involved in contacting the TAF is not sufficient for transactivation in mammalian cells (Jacq *et al* 1994, Pierrat *et al* 1994). This suggests that other coactivators might be involved in mediating the ligand dependent transcriptional activity (AF-2) of the ER. The search for such potential coactivators has identified a number of candidate proteins including, TIF1, RIP140, RIP160, ERAP140 and ERAP160 (Cavailles *et al* 1994, Halachmi *et al* 1994, Le Douarin *et al* 1995). Unlike hTAF$_{II}$30, the binding of these proteins to the ER *in vitro* is increased by agonist ligands and requires the integrity of the AF-2 activation domain. TIF1 and ERAP160 can also interact with RXR and RAR (Halachmi *et al* 1994, Le Douarin *et al* 1995) and RIP140 interacts with a number of other nuclear receptors including RAR, RXR, TR, VDR and PPAR (S. Dauvois, Molecular Endocrinology Laboratory, ICRF, personal communication). Expression of TIF1 or RIP140 in transfected cells results in a strong decrease in receptor activity, possibly due to squelching (Cavailles *et al* 1995, Le Douarin *et al* 1995). Although, at low concentrations of its expression vector, RIP140 can increase approximately two fold the ER mediated transactivation.

As neither TIF1 nor RIP140 interacts with TBP (or TFIIB) *in vitro* (Cavailles *et al* 1995, Le Douarin *et al* 1995) it is possible that, like TAF$_{II}$110, they contact TBP indirectly via contacts with other TAFs or, alternatively, they may contact another component of the pre-initiation complex. Intriguingly the TIF1 protein is predicted to contain a bromodomain (Tamkun *et al* 1992). This domain was first identified in the yeast SWI2/SNF2 protein that is part of the SWI/SNF complex involved in activation by a number of transcription factors, including the GR, in yeast (Yoshinaga *et al* 1992, reviewed in Carlson and Laurent 1994). The human homologues of SWI2, hbrm and hBRG1, can potentiate transcription by the GR, RARα and ER (Chiba *et al* 1994, Khavari *et al* 1993, Muchardt and Yaniv 1993). In addition to the bromodomain, SWI2/SNF2 and the human homologues, contain a DNA dependent ATPase motif that is important for their function whereas TIF1 lacks this ATPase domain (Le Douarin *et al* 1995). It seems that many of the coactivators of nuclear receptors may have evolved from the transcriptional regulatory proteins of yeast. The human

homologue of the yeast transcriptional coactivator Sug1 has been identified as a potential coactivator for the TR. Moore and coworkers (Lee *et al* 1995) identified Trip1 (the human homologue of Sug1) as a protein that interacted with TRβ using a protein interaction screen in yeast. Trip interacts with TRβ *in vitro* and the binding is increased by the TR specific ligand, 3, 5, 3'-triiodo-L-thyronine (T3). Sug1 also contains an ATPase activity and has been shown to interact directly with TBP (Swaffield *et al* 1995, reviewed in Maldonado and Reinberg 1995). Another protein proposed to act as a bridging factor to TBP, in this case for RAR, is the viral protein E1a (Berkenstam *et al* 1992). A cellular E1a-like protein has been reported to be expressed in embryonal carcinoma cells which may be involved in transcriptional activation by RAR (Berkenstam *et al* 1992). Thus a variety of potential coactivators for nuclear receptors have been identified, some of which may be shared between the different receptor types.

In addition to TBP and TAFs, TFIIB may also be a target for activators (reviewed in Roberts *et al* 1995). The acidic activation domain of VP16 interacts with TFIIB *in vitro* and mutations in VP16 and TFIIB that reduce activated transcription *in vivo* disrupt the interaction *in vitro* (Lin *et al* 1991)(Roberts *et al* 1993). Intriguingly, the same mutations in VP16 had been previously shown to abolish binding of TBP (Ingles *et al* 1991). Hence VP16 has been shown *in vitro* to interact with TBP, $dTAF_{II}40$ and TFIIB. Although the region of VP16 recognised by TBP and TFIIB appears to overlap the binding sites for TFIIB and $dTAF_{II}40$ do not. As $dTAF_{II}40$ also interacts with TFIIB (Goodrich *et al* 1993) it seems that a number of contacts may be involved in stabilising the association of VP16 with the pre-initiation complex. A number of nuclear receptors have also been reported to bind directly to TFIIB *in vitro*, including the ER, TR, RAR, VDR and COUP-TF (Baniahmad *et al* 1993, Blanco *et al* 1995, Ing *et al* 1992, Sadovsky *et al* 1995). The interaction with COUP-TF may stabilise the binding of the orphan receptor to DNA (Ing *et al* 1992, Tsai *et al* 1987). Mutations in the ER that abolish the activity of the AF-2 activation domain do not effect the interaction of the receptor with TFIIB (Sadovsky *et al* 1995) and the functional significance of the *in vitro* interaction is not clear. Recent work has provided circumstantial evidence that the interaction between the N-terminus of TRβ and TFIIB could be involved in stimulating transcription. These observations were made while comparing the transcriptional activity of the TRβ1 and β2 isoforms that differ only in the sequence of the N-terminus. On a direct repeat or inverted palindrome response element TRβ1

mediates a stronger T3 reponse than TRβ2 and this difference in activity is due to the more potent transactivation domain within the N-terminus of TRβ1. Intriguingly, while the N-terminus of TRβ1 contacts TFIIB the N-terminus of TRβ2 does not interact (Tomura *et al* 1995). The VDR also interacts with the C-terminal domain of TFIIB *in vitro* and has been shown to functionally interact with this basal transcription factor *in vivo*. The cotransfection of VDR with TFIIB in P19 embryonal carcinoma cells results in a vitamin D dependent cooperative activation of transcription. This effect was cell type specific, cotransfection of TFIIB with VDR in NIH 3T3 cells resulted in inhibition of the ligand response, possibly due to the necessary transcription cofactor(s) being limiting in this cell type (Blanco *et al* 1995). In addition it is possible that other components of the pre-initiation complex, distinct from TFIID and TFIIB, may also be the target of activators and some of these contacts may be mediated via the positive cofactors PC1-4 (reviewed in Maldonado and Reinberg 1995). Transcription factors may also act to displace negative cofactors which have been incorporated into the pre-initiation complex (see later) for example, the adenovirus E1a transcription factor can reverse the inhibitory effect of Dr1 (Kim *et al* 1995).

Although reconstitution and protein interaction assays allow identification of the initiation factors that activators target, the mechansim of activation is still unclear. From *in vitro* studies it appears that either the rate of initiation and/or the number of productive complexes formed may be increased by the action of transcription factors (reviewed in Hori and Carey 1994, Kingston and Green 1994). In addition to targeting the formation of a pre-initiation complex there are other models for the mechanism of action of transcription factors and three alternative models will now be briefly outlined. It should be noted that these models are not mutually exclusive and it is possible that an individual transcription factor can function by more than one method.

Firstly, DNA *in vivo* is complexed with proteins and condensed into nucleosomes and higher order structures collectively known as chromatin (see Hager and Archer 1991, Wolffe 1994). Actively transcribed genes, however, often contain nucleosome free regions characterised by the appearance of DNase I hypersensitivity sites (Gross and Garrard 1988). These alterations in chromatin seem to be required for, rather than a consequence of, gene transcription. It has been proposed that transcription factors might promote 'remodelling' of the nucleosome structure that would otherwise restrict the access of the necessary intiation factors to the gene (see Felsenfeld

1992, Grunstein 1990, Wolffe 1994) and support for this model has come from a number of experiments. *In vitro* studies have shown that transcription initiation can be inhibited by the formation of nucleosomes on template DNA and this repression can be relieved by the addition of certain transcription factors (reviewed in Kornberg and Lorch 1992). Additional evidence for an inhibitory function for nucleosomes came from the generation of yeast strains that had one copy of the yeast histone H4 gene deleted. These mutant strains had an impaired chromatin structure and expressed several genes under conditions when they are normally silent (Durrin *et al* 1992, Durrin *et al* 1991, Han and Grunstein 1988, Han *et al* 1988). Genetic studies in yeast have also indicated that the SNF/SWI family of transcriptional activators are involved in chromatin reorganisation. Mutations in members of this family resulted in impaired transcriptional activity which could be compensated for by mutations in genes encoding components of chromatin including *SPT* and *SIN* (reviewed in Carlson and Laurent 1994, Kornberg and Lorch 1995, Winston and Carlson 1992).

There is convincing evidence to suggest that the GR can act to displace nucleosomes positioned over promoters and in so doing activate transcription. For example, addition of glucocorticoid results in the GR mediated disruption of a specific nucleosome within the MMTV promoter the removal of which allows NF1 and TFIID to bind and stimulate transcription (see Hager and Archer 1991).

The second model is based on the action of the transcription factor LEF-1 on the T cell receptor α gene enhancer (reviewed in Grosschedl 1995, Tjian and Maniatis 1994). *In vitro* studies indicate that LEF-1 induces a bend in the DNA that promotes interactions between other proteins at the enhancer (Giese *et al* 1992). Other transcription factors that may function in a similar manner include HMG I(Y) (Thanos and Maniatis 1992) and YY1 (Natesan and Gilman 1993). Specific alterations in the topology of the DNA may be a feature of a number of enhancers and is reminscent of the action of the *Escherichia coli* CAP protein that can bend DNA by 90^{o} (Schultz *et al* 1991).

Finally, certain transcription factors appear to enhance transcriptional elongation by RNA polymerase II, a mechansim of transcriptional control originally reported for prokaryotes. Studies on genes such as c-myc, hsp 70 and HIV-1 suggest that this may also be a common regulatory mechanism in eukaryotes (reviewed in Krumm *et al* 1993, Maldonado and Reinberg 1995).

Transcriptional repression

In general, analysis of the regulation of eukaryotic gene transcription has focused on the action of positive acting factors consequently less is understood about the mechanisms by which negative factors repress transcription. It is likely, however, that selective repression of gene transcription is of importance in generating the required complex patterns of gene expression. In the following section various models will be outlined for the potential mechanisms by which negative factors may act. There are two main types of repression, passive repression which involves inhibition of activated transcription and active represion which involves inhibition of basal transcription.

Passive repression

Passive repression involves the prevention of an upstream activator from stimulating transcription. One mechanism by which this can be achieved is blocking the positive acting factor from binding DNA. This appears to be achieved in two main ways, firstly, the repressor may compete for the same, or an overlapping, binding site for example, in adults the factor NF-E appears to repress transcription of the foetal γ-globin gene by binding to the CCAAT box and displacing the activator CP1 (Superti *et al* 1988). An intriguing aspect of this type of repression is that the inhibitor can often activate transcription when bound to other sites, presumably when bound to 'silent' sites the transcription factor is not in a conformation competent for transactivation. For example, the *Drosophila* protein *ultrabithorax* inhibits transcription from the promoter of the *antennapedia* gene but activates transcription from its own promoter (Krasnow *et al* 1989). Similarly, the TR transactivates from a TRE but is transcriptionally silent when bound to an ERE (Glass *et al* 1988). When the C-terminal domain was deleted from the TR the mutant could constitutively activate transcription from the ERE and a TR chimera, in which the C-terminal domain was replaced with the corresponding sequence from the ER, stimulated transcription in an oestrogen dependent manner (Holloway *et al* 1990). Thus it seems that on the ERE the C-terminal domain of the TR adopts an inappropriate conformation for transactivation. The behaviour of the TR on a direct repeat element with no spacing further emphasises the effect of the response element on the transcriptional properties of this receptor. This element confers constitutive activity on the TR in the absence of T3 and repression in the presence (Naar *et al* 1991) the converse behaviour to that displayed on a

TREpal element and presumably reflects the different conformation of the receptor on these two types of site. An intriguing variation on the theme of competition for DNA binding has been proposed for YB-1. This protein seems to inhibit expression of the human major histocompatability complex class II genes by inducing a local distortion of the DNA and thereby preventing activators from binding to nearby sites (Macdonald *et al* 1995).

An alternative mechanism for blocking DNA binding by activators involves the formation of DNA binding deficient complexes in solution. Many helix loop helix proteins can dimerise with Id another member of the family as Id lacks a functional DNA binding domain these heterodimers are unable to bind stably to DNA (Benezra *et al* 1990). Some nuclear receptors may act in a similar manner to sequester AP-1 off DNA (discussed in Chapter 7). An alternative ploy is used by the inhibitory protein IκB which prevents DNA binding of the activator NFκB by retaining it in the cytoplasm (reviewed in Whiteside and Goodbourn 1993).

Some repressors interfere with the transcriptional activity of a DNA bound activator. This may occur when the repressor binds to an adjacent site, a process sometimes referred to as quenching (see Levine and Manley 1989). Examples include the binding of PEA2 next to the activator PEA1 in the polyoma viral enhancer (Wasylyk *et al* 1988) and similarly, the yeast mating type protein α2 binds next to MCM1 to prevent activation of a-specific genes (see Levine and Manley 1989). This type of intereference may also be mediated by repressors that are not bound directly to the DNA but are instead tethered to the activator. The prototypic example of this type of interference is the action of GAL80, which binds to GAL4 and blocks its transactivation (Ma and Ptashne 1988). The ability of the ER and GR to inhibit the activity of the pituitary specific transcription factor Pit1 in the absence of receptor binding sites may be due to the formation of an inactive Pit-1/receptor complex on the Pit-1 response element (Adler *et al* 1988).

A third model for passive repression involves the concept of squelching, a phenomenon which was originally noted for the activators VP16 and GAL4 (Gill and Ptashne 1988, Triezenberg *et al* 1988). Overexpression of these transcription factors resulted in reduced expression of genes and it was proposed that this was due to the titration of a cofactor required for transcription such as a coactivator or a component of the basal transcription complex (in the latter case basal transcription would also be inhibited). It was initially thought that this type of repression may not be

physiologically relevant, however, it has since been reported to occur between endogenous levels of transcription factors (Meyer *et al* 1989). Squelching has been proposed to explain the ability of E1a to inhibt a wide variety of transcription factors (reviewed in Renkawitz 1990) and the mutual inhibtion observed between members of the nuclear receptor family and members of the AP-1 family could also be due to titration of a common coactivator (see Saatcioglu *et al* 1994). The action of nuclear receptors in passive repression is discussed further in Chapter 7.

Active repression

Active repression (silencing) involves inhibition of basal transcription rather than the blocking of the stimulatory action of an upstream activator. Interference of the basal transcription machinery may be achieved by a variety of mechansims, firstly, the silencer could bind to the promoter and block the binding of the transcription machinery. The Simian Virus 40 (SV40) T antigen appears to down regulate its own gene by binding to the promoter and inhibiting transcription initiation (see Tjian 1981), a common mechanism in prokaryotic repression. Secondly, silencers can act analogously to an activator, binding upstream to elements that are usually position and orientation independent and contacting the pre-initiation complex either directly or indirectly, in the case of the silencer, however, the contacts are inhibitory rather than stimulatory. This mechanism may explain the silencing function of the *Drosophila* protein *even-skipped* (Johnson and Krasnow 1992), the murine factor MSX-1 (Catron *et al* 1995) and certain nuclear receptors (see below). Alternatively, the silencer may be incorporated into the pre-initiation complex, several proteins have been identified that associate with TBP and prevent formation of a complete pre-initiation complex including the yeast protein ADI and the human proteins Dr1, Dr2 and NC1 (see (Auble and Hahn 1993, Kim *et al* 1995) and references therein). Finally, just as activators may stimulate transcription by displacing nucleosomes, factors such as the yeast protein SSN6 appear to repress gene expression by positioning nucleosomes over the genes and blocking transcription (Cooper *et al* 1994).

TRα and *v-erb*A were the first members of the nuclear receptor family shown to actively repress transcription. The C-terminal domains of these proteins are sufficient to mediate silencing when fused to a heterologous DNA binding domain (Baniahmad *et al* 1992a). TRβ has also been shown to contain a transferrable silencing domain within its C-terminus (Baniahmad

et al 1992b). The silencing activity is a property of the unliganded receptor and addition of ligand abolishes the repression. As *v-erbA* does not bind hormone the active repression mediated by this form of TR is not relieved upon addition of T3 and instead this protein acts as a constitutive repressor (Baniahmad *et al* 1992a). Using an *in vitro* transcription assay Fondell and coworkers (Fondell *et al* 1993) showed that unliganded TRα can inhibit an early step in the formation of the preinitiation complex. Baniahmad et al (Baniahmad *et al* 1993) have demonstrated that the C-terminal part of the ligand binding domain of TRβ directly contacts the basal transcription factor TFIIB *in vitro* and this interaction is weakened significantly upon addition of ligand. The authors proposed that in the unliganded state the TR 'locks' TFIIB in an inactive form and that binding of T3 releases TFIIB and hence abolishes silencing. The C-terminus of RARα fused to a heterologous DNA binding domain can also actively repress transcription in the absence of ligand although it displays greater cell type variation than for the TR (Baniahmad *et al* 1992a). In contrast, the unliganded C-terminal domains of the steroid hormone receptors do not appear to silence transcription (Danielian *et al* 1992, Lees *et al* 1989, Webster *et al* 1988) (S. Hoare, Molecular Endocrinology Laboratory, ICRF, personal communication) yet in the absence of ligand the C-terminus of the ER does interact with TFIIB *in vitro.* This indicates that there is not a strict correlation between the interaction of the unliganded C-terminal domain of a receptor with TFIIB and its ability to mediate transcriptional silencing.

In summary, active repressors may prevent gene transcription by a variety of mechansims. These include restricting access to the promoter, either by promoting an inhibitory chromatin structure or physically masking the initiation site. Alternatively, the formation of a functional pre-initiation complex may be blocked by the repressor which can be either bound at a cis-acting site or directly assembled into the complex.

CHAPTER 2
MATERIALS AND METHODS

CHAPTER 2

MATERIALS

Chemicals and solvents

All chemicals and solvents used were of analytical grade and were obtained from either FSA Laboratory Supplies, Loughborough, UK, BDH Chemicals Ltd, Poole, U.K. or Sigma Chemicals Ltd, Poole, U.K. except for the following :

Absolute alcohol	Hayman Ltd, Witham, U.K.
Acrylamide	National Diagnostics, U.S.A. and Scotlab Ltd, Strathclyde, Scotland
Agarose	FMC Bioproducts, U.S.A.
Ammonium persulphate	Bio-Rad, U.K.
Ampicillin	Beechams Research Laboratories,U.K.
Amplify	Amersham International plc, Amersham, U.K.
Bromophenol blue	Bio-Rad
Coomassie Brilliant Blue R-250	Bio-Rad
DEAE-dextran	Pharmacia Biotech, U.K.
Dextran T-70	Pharmacia Biotech
Dithiothreitol	Bio-Rad
ECL western blotting detection reagents	Amersham International plc
Glycogen (molecular biology grade)	Boehringer Mannheim, Lewes, U.K.
Human placental ribonuclease inhibitor (HPRI)	Amersham International plc
Liquid scintillation fluid (Ultima gold)	Amersham International plc
Nucleotide triphosphates	Pharmacia Biotech
Poly (dI-dC)· (dI-dC)	Pharmacia Biotech
Protein A Sepharose	Pharmacia Biotech

TEMED	Bio-Rad
Tween-20	Bio-Rad

Radiochemicals

All radiochemicals used were supplied by Amersham International plc (Amersham, U.K.).

Compound	Specific activity
[1-^{14}C] acetyl-coenzyme A	50-60 mCi/mmol
[^{35}S] dATPαS	400 Ci/mmol, 10mCi/ml
[γ-^{32}P]ATP	3000 Ci/mmol, 10mCi/ml
[α-^{32}P] dCTP	3000 Ci/mmol, 10mCi/ml
L-[^{35}S] methionine	>1000 Ci/mmol

Enzymes

Restriction enzymes were routinely supplied by New England Biolabs, USA.

Calf Intestinal Alkaline Phosphatase (CIP)	Boehringer Mannheim
DNase I (RNase free)	Boehringer Mannheim
DNA polymerase I (Klenow Fragment)	Boehringer Mannheim
Deep Vent polymerase	New England Biolabs
Exonuclease III	Stratagene, Cambridge, U.K.
Mung Bean Nuclease	Stratagene
RNase A	Boehringer Mannheim
T7/T3/SP6 RNA polymerase	Stratagene
T4 DNA ligase	Gibco BRL, UK.
T4 Polynucleotide Kinase	Pharmacia Biotech
T7 Sequenase version 2.0	U.S. Biochemical Corporation, U.S.A.

Miscellaneous

Dried skimmed milk powder	Boots Plc, UK.
Film: RX	Fuji
X-OMAT	Kodak
Filtration units	Nalgene Company, Rochester, U.S.A.
Galacto-light β-gal assay kit	TROPIX Inc, Massachusetts, U.S.A.
Gene Pulser cuvettes (0.4 cm)	Bio-Rad
Glutathione Sepharose 4B	Pharmacia Biotech
Hybond nylon membranes	Amersham International plc
Luminometer cuvettes	Labsystems Group, U.K.
NA-45 DEAE membrane	Schleicher and Schuell, Germany
Nitrocellulose	Schleicher and Schuell, Germany
Peroxidase conjugated goat anti-rabbit immunoglobulins	Dakopatts, Denmark
Prestained SDS-PAGE standards (Low range; 18.5-106 kD)	Bio-Rad
Oligonuleotides	Synthesised by Ian Goldsmith, ICRF
Peptides	Synthesised by N. O'Reilly, ICRF
Protein Dye Reagent Concentrate	Bio-Rad
Sequenase sequencing kit	U.S. Biochemical Corporation, U.S.A.
TNT Coupled Reticulocyte Lysate	Promega
Wizard Maxiprep DNA purification	Promega

Plasmids

pGEM7Z-hTRβ, pGAL4-TR(DE), pGAL4-TRL454A(DE), pVP16RXR pUASx2-TKluc	Dr. K. Chatterjee, Addenbrokes Hospital, University of Cambridge
pSG5-hRXRα, pSG5-hRARα	Dr. A. Dejean, Institut Pasteur, Paris, France
pBSK-hRXRα	Dr. R. Evans, Salk Institute, San Diego, U.S.A.
pGEM7Z-hCOUP-TF I	Dr. B. O'Malley, Baylor College of Medicine, Houston, U.S.A.
pBS-mSF-1	Dr. K. Parker, Duke University Medical Center, U.S.A.

Antibodies and tissues

Polyclonal αCOUP antibody	Dr. M-J. Tsai, Baylor College of Medicine, Houston, U.S.A.
Breast and endometrial tumour cytosols	Professor. H. Rochefort, INSERM, Montpellier, France

Buffers

All solutions were prepared using water that was quartz distilled and deionised. Solutions were stored at room temperature unless otherwise stated.

BBS (2x)	50 mM BES adjusted to pH 6.95 with 1M NaOH, 280 mM NaCl, 1.5 mM Na_2HPO_4. (filter sterilised, stored at 4°C)
Reaction buffer diluent β-gal assays)	0.1 M sodium phosphate pH 8.0, 1 mM $MgCl_2$ (for (stored at 4°C)
CIP buffer (10x)	0.5 M Tris-HCl pH 8.5, 1mM EDTA (stored at 4°C)

Denaturing solution	1.5 M NaCl, 0.5 M NaOH
DCC suspension	0.025% (w/v) dextran, 0.25% (w/v) charcoal suspended in TE, pH 7.4 (stored at 4°C)
DNA loading buffer (5x)	0.25% (w/v) bromophenol blue, 5x TBE, 25% (v/v) glycerol
Exonuclease III buffer (2x)	0.1 M Tris-HCl pH 8.0, 10 mM $MgCl_2$, tRNA 20 µg/ml (stored at -20°C)
Galacto-light reaction buffer diluent	100 mM sodium phosphate pH 8.0, 1 mM MgCl2 (stored at 4°C)
Gel shift buffer (4x)	40 mM HEPES-KOH pH 7.5, 200 mM NaCl,4 mM EDTA, 4 mM DTT, 20% (v/v) glycerol (stored at -20°C)
HBS (2x)	40 mM HEPES, 275 mM NaCl, pH 7.1 (filter sterilised, stored at 4°C)
IP-A	50 mM KCl, 25 mM, HEPES-KOH pH 7.9, 6% (v/v) glycerol, 0.2 mg/ml BSA, 0.002% NP40, 2 µM $ZnSO_4$, 1mM DTT, 0.5 mg/ml bacitracin, 40 µg/ml PMSF, 5 µg/ml pepstatin, 5µg/ml leupeptin. (stored without protease inhibitors or DTT)
IP-B	as for IP-A but with 200 mM KCl
Kinase buffer (10x)	0.5 M Tris-HCl pH7.4, 100 mM $MgCl_2$, 1 mM EDTA pH 8.0 (stored at -20°C)
Ligation buffer (5x)	250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM DTT, 5 mM ATP, 25% PEG^8000 (stored at -20°C).
Klenow buffer (10x)	0.5 M Tris-HCl pH7.4, 70 mM $MgCl_2$, 10 mM DTT (stored at 4°C)
Luciferase/CAT/Bgal lysis buffer	0.65% NP40, 10 mM Tris-HCl pH8.0, 1 mM EDTA pH 8.0, 150 mM NaCl
Luciferase reaction buffer	25 mM glycylglycine pH 7.8, 5 mM ATP pH 8.0, 15 mM $MgSO_4$ (stored at 20°C).

MBN buffer (10x)	0.3 M sodium acetate, 0.5 M NaCl, 10 mM $ZnCl_2$ stored at -20°C)
MBN dilution buffer	10 mM sodium acetate pH 5.0, 0.1 mM zinc acetate, 1 mM cysteine, 0.1% Triton X-100, 50% glycerol (stored at - 20°C)
NETN	100 mM NaCl, 1 mM EDTA, 20 mM Tris-Hcl pH 8.0, 0.5% NP401mM DTT, 0.5 mg/ml bacitracin, 40 µg/ml PMSF, 5 µg/ml pepstatin, 5µg/ml leupeptin (stored without protease inhibitors or DTT)
Neutralising solution	1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA
NTE (1x)	0.1 M NaCl, 10 mM Tris-HCl pH7.5, 1 mM EDTA pH8.0
PBSA	140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.2
Phenol/chloroform	Redistilled phenol, equilibrated in TE pH 8.0, in a 50:50 mix (v/v) with chloroform
Poly (dI-dC)· (dI-dC)	Resuspended at 1 mg/ml in NTE and heated to 45°C for 10 minutes (stored at -20°C)
Ponceau Red stain (10x)	2%(w/v) Ponceau S, 30% trichloroacetic acid, 30% sulfosalicylic acid
Prehybridisation buffer	0.2 M sodium phosphate pH 7.2, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS, 15% (v/v) deionised formamide)
Protein loading buffer (2x)	4% (w/v) SDS, 125 mM Tris-HCl pH 6.8 and 20% (v/v) glycerol, 0.05% bromophenol blue, 2% B-mercaptoethanol
Repair buffer (10x)	0.5 M Tris-HCl pH 7.4, 70 mM $MgCl_2$, 10 mM (stored at -20°C)
Restriction enzyme buffers (stored at -20°C);	
Low salt (10x)	0.1 M Tris-HCl, 0.1 M $MgCl_2$, 10 mM DTT, pH7.5
Medium salt (10x)	As low salt buffer but with 0.5 M NaCl

High salt (10x)	As low salt buffer but with 1 M NaCl
Very high salt (10x)	As low salt buffer but with 1.5 M NaCl
Sequenase buffer (5x)	200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl
Sequencing loading buffer	80% (v/v) deionised formamide, 10 mM NaOH, 1mM EDTA pH 8.0, 0.1% (v/v) xylene cyanol, 0.1% (v/v) bromophenol blue
SDS-PAGE buffer (10x)	250 mM Tris base, 1.9 M glycine, 1% (w/v) SDS
SSC (20x)	3 M NaCl, 0.3 M sodium citrate
STET buffer	8% (w/v) sucrose, 0.1% (v/v) Triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0
SM buffer	0.1 M NaCl, 8 mM $MgSO_4.7H_20$, 50 mM Tris-HCl pH 7.5, 2% (w/v) gelatin
TBE (10x)	0.9 M Tris-borate, 20 mM EDTA pH 8.0
TE buffer (1x)	10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0
Tfb1	30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2.4H_2O$, 15% (v/v) glycerol. Adjust pH to 5.8 with acetic acid and filter sterilise. (stored at 4°C)
Tfb2	10 mM MOPS, 75 mM $MgCl_2$, 10 mM RbCl, 15% (v/v) glycerol. Adjust pH to 6.5 with KOH and filter sterilise. (stored at 4°C)
Transfer buffer	25 mM Tris base, 192 mM glycine, 20% MeOH, pH8.3, 0.01% SDS
Wash buffer	40 mM sodium phosphate pH 7.2, 1mM EDTA, 1% SDS
Whole cell extract buffer	0.4 M KCl, 20 mM HEPES pH 7.4, 20% (v/v) glycerol, 1mM DTT, 0.5 mg/ml bacitracin, 40 µg/ml PMSF, 5 µg/ml pepstatin, 5µg/ml leupeptin. (stored without protease inhibitors or DTT at 4°C)
Wizard cell lysis solution	0.2 M NaOH, 1% SDS

Wizard cell resuspension solution	50 mM Tris-HCl pH7.5, 10 mM EDTA, 100 µg/ml RNase A
Wizard column wash solution	85 mM NaCl, 8 mM Tris-HCl pH 7.5, 2 mM EDTA, 55% ethanol
Wizard neutralisation solution	1.32 M potassium acetate pH 4.8

Bacterial media and agar

All the organic components listed below were supplied by Difco Laboratories, Michigan, U.S.A.

L-broth	1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.1% (w/v) glucose
L-agar	As for L-broth except with 1.5% (w/v) bactoagar
NZY broth	0.5% yeast extract, 1% bacto tryptone, 0.5% NaCl, 0.2% $MgSO_4.7H_2O$. Adjust pH to 7.5 with NaOH
NZY agar	As for NZY broth exceptwith 1.5% bacto agar
Top agar	As for NZY broth except with 0.7% agarose
ψ broth	2% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) MgSO4. Adjust pH to 7.6 with KOH.
ψ agar	As for ψ broth except with 1.4% bacto agar

Cell culture media

Chick serum	Sigma
Dulbecco's modified Eagle's medium	ICRF media supplies
Foetal bovine serum	Gibco BRL, U.K.
PBSA	ICRF media supplies

Trypsin	ICRF media supplies, 0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.01% (w/v) Na_2HPO_4, 0.01% (w/v) U/ml penicillin, phenol red (stored at -20°C).
Versene	ICRF media supplies, 0.02% (w/v) EDTA in PBSA, phenol red

METHODS

Storage of bacteria

The *Escherichia coli* DH5α strain was used for the propagation of all plasmids. These bacteria were stored in L-broth containing 50% (v/v) glycerol at -70°C. All plasmids described in this thesis carry the β-*lactamase* gene (Amp^R) which confers resistance to ampicillin. Bacterial transformants were grown either in media or on agar containing 50-100μg/ml ampicillin.

Preparation of competent bacteria

This is the unpublished method of M. Scott and V. Simanis, derived from Hanahan (1983). All the glassware used was prewashed with ψ broth. Bacteria were streaked out on ψ agar and grown overnight at 37°C until the colonies were approximately 2 mm in diameter. Single colonies were then inoculated into 5 ml ψ broth and incubated with vigorous shaking at 37°C until the OD_{550} reached 0.3 (about 3 hours). This was then subcultured into 100 ml of pre-warmed ψ broth and grown for 2-3 hours with continuous shaking at 37°C until the OD_{550} reached 0.48. The cells were then chilled on ice for 5 minutes and pelleted by centrifugation at 4000 x g for 10 minutes at 4°C. The cell pellet was gently resuspended in 40 ml (2/5 originalvolume) ice cold Tfb1 buffer and incubated for 5 minutes on ice. The cells were then respun at 4000 x g for 10 minutes at 4°C. The pellet was then resuspended in 4 ml (1/25 origianl volume) of chilled Tfb2 buffer. The cells were snap frozen on cardice in aliquots and stored at -70°C.

Transformation of competent bacteria

Competent cells were thawed on ice and typically, 50μl of cells were added to 20μl prechilled DNA solution (less than 1 ng DNA/μl cells). After 20-30 minutes on ice, the cells were heat shocked at 42°C for 1 minute and then incubated on ice for a further 5 minutes. Following the addition of 4 volumes of L-broth the cells were incubated at 37°C for 30-50 minutes. The cells were then spread on L-plates containing 50-100μg/ml ampicillin, inverted and incubated at 37°C overnight. Competent DH5α cells typically gave 10^6-10^8 bacterial colonies per μg of supercoiled DNA.

Preparation of plasmid DNA

Small scale preparation of plasmid DNA (miniprep)

The 'boiling' miniprep method was routinely used to prepare between 2-10 µg of plasmid DNA for diagnostic restriction enzyme digestion and direct double stranded sequencing. Single colonies were inoculated into 5 ml of L-broth containing 50-100 µg/ml ampicillin and grown overnight at 37°C with continuous shaking. 1ml of overnight culture was spun in a microfuge for 20 seconds and the cell pellet resuspended in 350 µl STET buffer. After adding 25 µl of 10mg/ml lysozyme (in 10 mM Tris-HCl pH8.0) and mixing, the suspension was immediately placed in boiling water for 40 seconds. The suspension was then spun for 10 minutes in a microfuge at room temperature. The pellet was removed and the plasmid DNA precipitated by the addition of 40 µl of 3M sodium acetate pH 7.0 and 420 µl of isopropanol, mixing and incubation on cardice for 10 minutes. The DNA was pelleted by centrifugation for 20 minutes at room temperature, dried and resuspended in distilled water.

Large scale preparation of plasmid DNA

The Wizard Maxiprep DNA purification system (Promega) was used to prepare between 0.3-1.5 mg of plasmid DNA. Plasmid DNA prepared by this method was used for cloning, transient transfection, *in vitro* transcription and sequencing. A 5 ml overnight culture was inoculated into 400 ml L-broth containing 50-100 ug/ml ampicillin and grown overnight at 37°C (maximum of 16 hours). The bacteria were harvested in 500 ml Sorvall bottles by centrifugation at 6000 x g for 10 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of cell resuspension solution then 15 ml cell lysis solution was added with thorough mixing. When lysis was complete (up to 20 minutes) 15 ml of neutralisation solution was added and the suspension spun at 6000 x g for 15 minutes at 4°C. The supernatant was then carefully filtered through medical gauze into a clean Sorvall bottle and the plasmid DNA precipitated by the addition of 0.6 volumes isopropanol and pelleted by centrifugation at 6000 x g for 10 minutes at 4°C.

The pellet was then resuspended in 2 ml of TE buffer and 10 ml of Wizard Maxipreps DNA purification resin was added. The resin/DNA mix was transferred to a Wizard Maxicolumn and a vacuum applied. The Sorvall bottle was rinsed with 2 x 12 ml wash solution and poured onto the

Maxicolumn. To rinse the resin 5 ml of 80% ethanol was added to th column and the resin was then dried by centrifugation in a swing out rotor at 1300 x g for 5 minutes. To elute the DNA 1.5 ml of preheated (65-75 ^{o}C) TE was applied to the Maxicolumn for 1 minute and then spun at 1300 x g for 5 minutes. The eluted DNA was reprecipitated by the addition of 150 µl sodium acetate pH 5.2 and 4.5 ml 95% ethanol, mixing and incubation on cardice for 10 minutes. The DNA was pelleted by centrifugation at 13, 000 x g for 5 minutes, washed with ice cold 70% ethanol, dried and resuspended in distilled water (0.5-1 ml). The DNA concentration and purity was determined by measurement of the OD_{260} and OD_{280} (Sambrook *et al* 1989). The DNA was run on an agarose gel and typically found to be 80% supercoiled.

DNA manipulation and subcloning

Restriction endonuclease digestion

Restriction enzyme digests were performed at 37^{o}C in low, medium, high or very high salt buffers unless other conditions were recommended by the supplier. DNA was digested with a 3-5 fold excess of enzyme with the final volume of glycerol not exceeding 5% (v/v). Restriction enzyme digests were stopped by extraction with an equal volume of phenol/chloroform and the aqueous phase transferred to a fresh eppendorf. The DNA was precipitated by the addition of 1/50 volume 5 M NaCl and 2.5 volumes of absolute ethanol. After incubation on cardice for 10 minutes the tube was spun in a microfuge for 10 minutes, washed with ice cold 70% ethanol, dried and resuspended in distilled water. For analysis of digestion products by gel electrophoresis the digestion was stopped by the addition of DNA loading buffer to 20% (v/v).

Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose at 0.8- 2% (w/v) in 1x TBE by heating the suspension in a microwave oven. The solution was allowed to cool, ethidium bromide added to 1µg/ml and the solution poured into a gel mould. Once set the gel was submerged in 1x TBE buffer in a gel tank and the DNA samples, containing 20% DNA loading buffer, loaded into the wells. Electrophoresis was carried out at 7.5 V/cm until the DNA fragments were well resolved. DNA was visualised by illumination on a long wave UV light box and photographed. The size of DNA fragments was

estimated by comparison of their mobility to that of restriction fragments of known size, typically lambda phage DNA digested with Hind III.

Purification of DNA fragments from agarose gels

Restriction fragments were purified from agarose gels by electroelution onto NA-45 DEAE membrane. A cut was made in the gel immeadiately in front of the fragment to be purified and the membrane (presoaked in TE buffer) inserted. If necessary another piece of membrane was inserted behind the fragment to prevent contamination with other fragments. The DNA was then transferred to the membrane by reapplying the voltage to the gel for 5-10 minutes. The membrane was then transferred to an eppendorf, 250 µl 1 M NaCl added and the DNA eluted by heating at 70°C for 20 minutes. The membrane was removed and the DNA recovered by ethanol precipitation, washed with 70% ethanol, dried and resuspended in distilled water.

Oligonucleotide kinasing and annealing

Oligonucleotides were synthesised by I. Goldsmith (ICRF) with hydroxyl groups at both the 5' and 3' termini. In order to ensure efficient ligation the 5' ends of these oligonucleotides were kinased prior to annealing. 100 ng of oligonucleotide were incubated in 1x kinase buffer, 1 mM ATP and 1 µl (approximately 10 Units) of T4 polynucleotide kinase in a final volume of 30 µl at 37°C. for 30 minutes. After the addition of 70 µl NTE buffer the complementary oligonucleotides were mixed and the solution heated at 85°C for 3 minutes. The oligonucleotides were then annealed by allowing the solution to cool slowly to room temperature.

Preparation of vectors

Typically 1-5 µg of plasmid DNA was digested with the appropriate restriction enzyme(s) and then extracted with phenol/chloroform, ethanol precipitated and washed with 70% ethanol. The DNA pellet was resuspended in 26 µl distilled water. To prevent self ligation of the vector the 5' terminal phosphates were removed by treatment with calf intestinal phosphatase (CIP). This was achieved by the addition of 3 µl of 10x CIP buffer and 1 µl CIP (20 units) and incubation at 37°C for 30-60 minutes. The plasmid DNA was then reextracted with phenol/chloroform, ethanol precipitated, 70% ethanol washed, dried and resuspended in distilled water at a final concentration of 10- 20 ng/µl.

Ligations

Ligations were carried out with 10-50 ng of vector DNA and a 1:1, 1:5 and 1:20 molar ratio of vector to insert DNA (either a purified DNA fragment isolated from an agarose gel or a pair of annealed oligonucleotides). Ligations were carried out in a final volume of 20 µl that contained 4 µl of 5x ligase buffer and 1µl T4 DNA ligase (1 unit) and incubated at room temperature for > 2 hours or overnight at 14°C. Blunt end ligations were most commonly carried out at 14°C.

Polymerase chain reaction

Polymerase chain reactions were performed using Deep Vent DNA polymerase in a 100 µl reaction volume that consisted of 1x Vent buffer, 0.25 mM dNTPs, 0.1 mg/ml BSA, 1 µM of each primer, 1-100 ng plasmid DNA and 1 unit of Deep Vent DNA polymerase. Mineral oil was added to cover the mixture which was the subjected to 30 cycles of: 94°C for 60 seconds, the appropriate annealing temperature (typically 5°C below the Tm of the primers) for 60 seconds and 72°C for 90 seconds. For the last cycle an additional step of 72°C for 5 minutes was included. The reaction mixture was phenol/chloroform extracted and an aliquot (typically 10%) analysed by gel electrophoresis. The remainder was ethanol precipitated, washed with 70% ethanol, dried and resuspended in distilled water.

DNA sequencing

1-5 µg of plasmid DNA was denatured by the addition of 2 µl of 2 M NaOH in a final volume of 20 µl and incubation at 68°C for 20 minutes. The single stranded DNA was precipitated by the addition of 8 µl of ammonium acetate pH 5.4 and 100 µl of ethanol and incubation on cardice for 5 minutes. The DNA was pelleted by spinning for 10 minutes in a microfuge at room temperature, washed with 70% ethanol, dried and resuspended in 7 µl of distilled water. The denatured DNA was then annealed to the primer by the addition of 2 µl of 5x Sequenase buffer and 1 µl of oligonucleotide primer (2.5 ng), heated to 65°C for 3 minutes and allowed to cool to room temperature. Double stranded DNA sequencing was carried out exactly according to the USB Sequenase protocol.

Denaturing gel electrophoresis

Sequencing reactions were separated on a 6% denaturing polyacrylamide gel. The gel solution (Easigel) consisted of 6% (w/v) acrylamide, 0.3% bis-acrylamide, 7 M urea and 1x TBE. Gel polymerisation was initiated by the addition of 50 µl TEMED and 500 µl of 10% ammonium persulphate to 75 ml of gel solution. This mixture was poured between glass plates separated by 0.25 mm spacers and the blunt side of a sharks tooth comb inserted. Once the gel was set the comb was removed, the well rinsed with 0.5x TBE and a sharks tooth comb inserted to form the sample wells. The gel was set up with 0.5x TBE in the upper buffer chamber and 1x TBE in the lower and was pre-run for 15-30 minutes at 45 mA. Just before loading on the gel the samples were boiled for 2-5 minutes to ensure denaturation and then placed on ice. Typically 2/3rds of the way through the gel run the lower buffer was made 1 M in sodium acetate by the addition of 1/2 volume of 3 M sodium acetate pH 5.0. This treatment causes slower migration of the molecules at the bottom of the gel and results in a more even distribution of bands from top to bottom of the gel. When the gel had run sufficiently far the plates were separated and the gel fixed for 15 minutes by immersion in 12% methanol, 10% acetic acid. The gel was then transferred to Whatman 3MM paper, under vacuum at 80°C for 40 minutes and autoradiographed.

Exonuclease III and mung bean nuclease deletion of DNA

The enzyme exonuclease III sequentially removes nucleotides from the 3' end of double stranded DNA that contains either a 5' overhang or blunt ends but not from a 3' overhang. The use of suitable restriction enzyme sites therefore allows unidirectional deletion of DNA. Unique 5' (or blunt) and 3' restriction sites are required with the 5' site placed between the sequence to be deleted and the 3' site. 50 µg of plasmid DNA was digested at unique 5' and 3' restriction sites. The digestion was monitored by analysing an aliquot by agarose gel electrophoresis to ensure the digests had gone to completion. The DNA was phenol/chlorofom extracted, ethanol precipitated, dried and resuspended in 50 µl of distilled water. The DNA was then incubated at 30°C for 10 minutes in a solution that contained, 125 µl 2x exonuclease III buffer, 25 µl 100 mM 2-mercaptoethanol and 40 µl distilled water. Following the addition of 10 µl of exonuclease III (1000 units), 25 µl aliquots were taken at 1 minute intervals and added to ice cold 25 µl 10X MBN buffer and 155 µl distilled water and placed on dry ice. After the final

time point the tubes were heated at 68°C for 15 minutes and then placed on ice. The DNA was then treated with mung bean nuclease to digest the single stranded DNA created by the exonuclease III digest. To each aliquot 15 µl of mung bean nuclease, diluted to 1U/µl in 1x MBN dilution buffer just prior to use, was added and incubated at 30°C for 30 minutes. Following the incubation each aliquot was extracted with the following mix: 10 µl 1 M Tris-HCl pH 9.5, 20 µl 8 M LiCl, 8µl 10% SDS and 250 µl phenol/chloroform. The DNA was then precipitated by the addition of 25 µl of 3 M sodium acetate pH 5.4, tRNA at a final concentration of 10 ng/µl and 650 µl ethanol and incubated on cardice for 15 minutes. The DNA was recovered by spinning for 20 minutes at room temperature, washed in 70% ethanol, dried and resuspended in 15 µl distilled water. An aliquot of the treated DNA (5 µl) was analysed by gel electrophoresis to check the extent of digestion. The remaining 10 µl of DNA was treated with DNA polymerase I Klenow fragment to ensure the DNA contained blunt ends that could be ligated. The DNA was incubated at room temperature for 15 minutes in a solution (final volume 30 µl) that contained, 5 µl of distilled water, 3 µl of 10X Klenow buffer, 10 µl dNTP mix and 2 µl Klenow fragment (4 units). 10 µl of this mixture was then ligated using, 5 µl of distilled water, 4 µl of 5x T4 DNA ligase buffer and 1 µl T 4 DNA ligase (1 unit) in a final volume of 20 µl and incubated at 14°C overnight. An aliquot of the ligation reaction (10 µl) was then used to transform 25 µl of competent *E.coli*.

DNA screening of cDNA library

The cDNA library used in this thesis was a λZAPII ZR-75-1 cDNA library (oligo (dT) primed) constructed by R. White (Molecular Endocrinology Laboratory, ICRF). The library was screened essentially as per manufacturers instructions (Stratagene). The XL1-Blue host cells used were prepared as follows, a colony from a fresh (1- 2 day) L-agar/tetracycline plate was used to inoculate an L-broth culture (50 ml) supplemented with 0.2% maltose and 10 mM $MgSO_4$. The culture was grown overnight at 30°C or for 5- 6 hours at 37°C with vigorous shaking. The cells were then spun at 1, 000 x g for 10 minutes, gently resuspended in 0.5 volumes of 10 mM $MgSO_4$ and stored at 4°C. For infection the cells were diluted with $MgSO_4$ to an OD_{600} of 0.5 and incubated with phage at 37°C with shaking for 15 minutes. To titer the library, 400 µl XL1-Blue cells (infected with the phage) and 3 ml top agar were plated out on 10 cm NZY agar plates. Once the titre had been ascertained the library was plated out with 2 ml host cells and 30 ml top

agar/plate on 15 cm x 15 cm NZY agar plates to approximately 150, 000 plaques/plate, 2x 10^6 plaques were screened in total. The plates were incubated at 37°C for 5- 7 hours and then chilled for 1 hour at 4°C to prevent the top agar sticking to the filters .

A hybond-N+ membrane was carefully placed on the agar surface for 1 minute and a sterile needle used to orientate the filter. The membrane was then carefully peeled off and placed, plaque side up, on Whatman 3MM paper. A duplicate filter was also used as above except that it was left on the plate for 4 minutes to ensure sufficient transfer of plaques. The filters were then placed, plaque side up, on Whatman 3MM paper soaked in denaturing solution for 7 minutes and then transferred to Whatman 3MM paper soaked in neutralising solution for 3 minutes. The filters were then placed on fresh paper soaked in neutralising solution for a further 3 minutes. The filters were then washed in 2x SSC and transferred to Whatman 3MM paper soaked in 0.4 M NaOH for 20 minutes. The filters were then rinsed in 5x SSC for less than 1 minute and transferred to prehybridisation solution.

The filters were prehybridised for at least an hour at 65°C with gentle shaking. The labelled oligonucleotide probe was then added (10^5 cpm/ml) and the incubation continued overnight. Following hybridisation, the filters were incubated with wash solution at 65°C for 3 periods of 30 minutes. The filters were then removed, wrapped in saran wrap and autoradiographed. Plaques of interest (those that gave signals on both duplicate filters) were identified, plugged from the agar plate and eluted into 3 ml of SM buffer, containing 120 µl chloroform, at 4°C overnight. The eluted phage were then rescreened, as above except on a smaller scale, typically 400 µl XL1-Blue cells (infected with the phage) and 3 ml top agar were plated out on 10 cm NZY agar plates. Plaques that made it through the second and a tertiary screen were then isolated and the pBluescript double stranded phagemid with the cloned DNA insert excised. For the *in vivo* excision 200 µl XL1-Blue cells, 200 µl UniZAP XR phage (> 1x 10^5 phage) and 1 µl of R408 helper phage (1x 10^6 pfu/ml) were incubated at 37°C for 15 minutes. 1- 100 µl of the mixture was plated out on L-agar/ampicillin, incubated overnight at 37°C. The next day colonies containing the pBluescript plasmid (with cDNA insert) were selected.

End labelling of oligonucleotides

Complementary pairs of oligonucleotides, synthesised by I. Goldsmith (ICRF) with hydroxyl groups at both the 5' and 3' termini, were annealed and end labelled using T4 polynucleotide kinase. The oligonucleotide (100 ng) was incubated at 37°C for 30 minutes in a 50 µl reaction mixture that contained 5 µl 10x kinase buffer, 5 µl [γ^{32}P]-ATP (10 mCi/ml) and 1 µl T4 polynucleotide kinase (10 units). The solution was then precipitated using an equal volume of 5 M ammonium acetate pH 5.4 to remove unincorporated labelled nucleotide and 1 µl glycogen to ensure efficient precipitation of the oligonucleotide. The precipitate was washed 3 times with 70% ethanol, dried and resuspended in distilled water.

In vitro protein analysis

In vitro protein synthesis

A coupled rabbit reticulocyte lysate system was used to synthesise and translate cRNA. A typical reaction contained 37.5 µl of TNT lysate, 3 µl of TNT reaction buffer, 1.5 µl of 1 mM amino acid mixture lacking methionine, 1.5 µl HPRI, 1.5 µg plasmid DNA and 1.5 µl TNT RNA polymerase (SP6, T3 or T7), made up to 60 µl with distilled water. Of this reaction mix, 20 µl was transferred to a second eppendorf containing 2 µl of [^{35}S]-methionine (10 mCi/ml) and 3 µl of distilled water, to the remining reaction mixture 1 µl of 1 mM amino acid mixture minus leucine was added and the final volume adjusted to 50 µl with distilled water. The translation reactions were incubated in parallel at 30°C for 60- 90 minutes, glycerol added to 15% (v/v) and stored at -70°C. The radiolabelled translation reactions were used to assess the size and yield of receptor proteins, typically 3 µl of the radiolabelled reactions were analysed by gel electrophoresis. The relative amounts of the translated receptors were determined by quantification of the amount of incorporated [^{35}S]-methionine by phosphoimage analysis of the gel. The values were normalised relative to the methionine content of each receptor. [^{35}S]-methionine labelled proteins were also used in immunoprecipitation analysis and unlabelled proteins were used for gel shift analysis.

SDS polyacrylamide gel electrophoresis

Proteins were analysed on discontinuous polyacrylamide gels using the Atto Corporation AE-6220 dual slab chamber. Gels were prepared from 2

solutions to form the resolving and stacking gels. The resolving gel contained 10% acrylamide (30% acrylamide, 0.8% bis-acrylamide stock), 375 mM Tris-HCl pH 8.8 and 1 % SDS in a final volume of 50 ml. The stacking gel contained 4% acrylamide (30% acrylamide, 0.8% bis-acrylamide stock), 125 mM Tris-HCl pH 6.8 and 1 % SDS in a final volume of 12.5 ml. Polymerisation of the resolving gel was initiated by the addition of 20 µl TEMED and 300 µl of 10% ammonium persulphate. The gel was then poured between the glass plates to within 3 cm of the top and overlaid with water saturated isobutanol. Once the gel had set (approximately 30 minutes) the isobutanol was poured off and the top of the gel rinsed with distilled water. Polymerisation of the stacking gel was initiated by the addition of 10 µl of TEMED and 200 µl of 10% ammonium persulphate, the gel solution was poured on top of the resolving gel and a comb inserted. Once the stacking gel had set (approximately 40 minutes) the comb was removed and the wells rinsed with 1x SDS-PAGE buffer. Samples in protein loading buffer were boiled for 2- 5 minutes and loaded into the 6 mm wells as were prestained molecular weight markers. The gel was run in 1x SDS-PAGE buffer at 250 V. The plates were then separated and the gel fixed in 10% acetic acid, 30% methanol for 15 minutes and then incubated in Amplify for 15 minutes. The gel was dried under vacuum at 80°C for 60- 90 minutes and the radiolabelled bands visualised by flurography using Fuji RX film. For western blotting the gels were not fixed but processed as described below.

Western blotting

The protein samples were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane using a wet blotting method (Sambrook *et al* 1989). The gel was placed in a 'sandwich' of a fibrous pad, a piece of Whatman 3MM paper, the gel, a piece of nitrocellulose, another piece of Whatman 3MM paper and another pad. The paper, pad, nitrocellulose and gel had all been pre-equilibrated in transfer buffer and during the assembly of the 'sandwich' care was taken to make sure no air bubbles were present. The 'sandwich' was then enclosed in a basket and placed in transfer buffer in a blotting tank (Bio-Rad) with the nitrocellulose towards the anode and the gel towards the cathode. The transfer was performed at 30 V overnight and then 70 V for 1 hour at 4°C.

The blotting apparatus was then disassembled and the nitrocellulose membrane rinsed with distilled water, incubated brieflywith 1x Ponceau Red stain and the excess stain rinsed off with distilled water. Ponceau S stains the

proteins on the membrane, allowing the efficiency of transfer to be assessed and the dye can then be washed off by rinsing with PBSA. The membrane was then incubated, with gentle shaking, in blocking solution (0.1% (v/v) Triton X-100, 1 % (w/v) non-fat milk in PBSA) for 1 hour at room temperature. The membrane was then incubated for 1-2 hours with 2-5 ml of the primary antibody or pre-immune sera diluted 1:1000 with blocking solution in a sealed bag. The membrane was removed, washed three times with blocking solution (5 minutes each wash) and then incubated for 1-2 hours with 2-5 ml of blocking solution containing peroxidase conjugated antibody against the primary antibody diluted 1:1000. The membrane was then washed five times in PBSA containing 0.1% (v/v) Triton X-100 (three washes of 5 minutes followed by 2 washes of 10 minutes). The membrane was then processed for enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham International) using Fuji RX film.

If the membrane was to be reprobed with a different primary antibody the membrane was 'stripped' of the first set of antibodies by treatment with 0.1 M glycine pH 2.5 for 5- 10 minutes. The membrane was then washed twice in 1 M Tris-HCl pH 7.5 (5- 10 minutes each wash), once with PBSA and reequilibrated in blocking solution. The membrane was then incubated with the antibodies as above.

Gel shift assay

Equal amounts of *in vitro* synthesised proteins (with unprogrammed reticulocyte lysate used to maintain an equal protein concentration in each reaction) or whole cell extract were pre incubated with or without pre- or immune sera, ligand or additional DNA in 1x gel shift buffer and 0.5% BSA in the presence of 1 μg of poly (dI-dC)-(dI-dC) for 5 minutes at room temperature. 0.5 ng of the appropriate ^{32}P-labeled oligonucleotide probe was added and the reactions incubated for a further 25 minutes. A 6% polyacrylamide (30% acrylamide, 0.8% bis-acrylamide stock) 0.5 X TBE non denaturing gel was pre-run for 30 minutes at 100 V. The protein-DNA complexes were loaded on the gel and run in 0.5x TBE at 220 V for 60-90 minutes. Gels were fixed for 15 minutes in 10% acetic acid, 30% methanol, dried under vacuum at 80^{o}C for 40-60 minutes and subjected to autoradiography.

Probes were prepared by annealing complementary pairs of oligonucleotides to form the binding site and 5' overhanging ends. The

oligonucleotides were labelled by filling in the overhanging ends in the presence of [α-^{32}P]-dCTP. 200 ng of annealed oligonucleotide was incubated in a final volume of 20 µl containing 1x repair buffer, 0.1 mM dATP, dGTP, dTTP and 8 µl [α-^{32}P]-dCTP (10 mCi/ml, 3000 Ci/mmol) and 1 µl Klenow enzyme (labelling grade, 2 units) at room temperature for 30 minutes. The labelled oligonucleotide was then extracted twice with phenol/chloroform, an equal volume of ammonium acetate pH 5.4 and 1 µl of glycogen added and the probe ethanol precipitated. The pellet was resuspended in distilled water and reprecipitated as above then washed three times with 70 % ethanol, dried and resuspended in 20 µl distilled water.

Determination of protein concentration

A dye binding assay was used to determine the protein concentration of cell extracts Bradford (1976). The dye concentrate was supplied by Bio-Rad and was used according to the manufacturers protocol. Typically, duplicate aliquots of each extract (for the blank an aliquot of extract buffer alone was used) were diluted to 800 µl in distilled water and 200 µl dye concentrate added. After mixing the samples were transferred to polystyrene spectrophotometer cuvettes and incubated for 5-30 minutes at room temperature prior to reading the absorbance at OD595. The protein concentrations were determined by extrapolation from a standard curve prepared from the absorbance at OD595 of a series of dilutions of a BSA standard. In this assay BSA binds twice as much dye compared to other proteins and this was taken into account when constructing the standard curve.

Generation of polyclonal antibodies

The peptide MP31: P-G-S-D-K-Q-Q-Q-Q-Q-H-I and MP32: S-T-W-R-D-P-Q-D-E-V-P-G corresponding to the residues 66- 77 and 6- 17 of COUP-TF II respectively were synthesised by N. O'Reilly (ICRF) on a model 430A Applied Biosystems Solid Phase Synthesiser and analysed by reverse phase HPLC and mass spectroscopy. The petides were coupled to the carrier protein, Keyhole Limpet haemocyanin (KLH) using the unpublished method of G. Evan (ICRF). An equal weight (6.25 mg) of peptide and KLH were dissolved in 0.1 M $NaHCO_3$ at a concentration of 2 carrier mg/ml. Freshly thawed glutaraldehyde was added to the solution to a final concentartion of 0.05% and mixed overnight at room temperature. The pH was adjusted to 8.4 with NaOH and 1 M glycine ethyl ester pH 8.0 to a final concentration of 0.1 M and

the solution incubated for 30 minutes at room temperature with continuous stirring. The coupled carrier was precipitated by the addition of 4-5 volumes of ice cold acetone and incubation on cardice for 30 minutes. The protein was pelleted by centrifugation at 10, 000 x g for 10 minutes and resuspended in saline at a carrier concentration of 1 mg/ml. The coupled peptides were used to immunise rabbits (see later).

The fusion protein GST-COUP-TF II that comprises glutathione-S-transferase (GST) fused to full length COUP-TF II was purified from *Escherichia coli*. Briefly, a culture of bacteria transformed with the vector GST-COUP-TFII was grown overnight at 37°C in L-broth/ampicillin with continuous shaking. The culture was then diluted 1:10 in 400 ml fresh media and grown until the OD_{600} reached 0.7-0.8. IPTG was then added to a final concentration of 0.1 mM and the culture grown for a further 3 hours with shaking at 37°C. The bacteria were then harvested in 500 ml Sorvall bottles by centrifugation at 5, 000 x g for 5 minutes at 4°C. The pellet was resuspended in 1/10 volume of NETN, (containing protease inhibitors), transferred to 15 ml corex tubes (10 ml/tube) and sonicated at 14 microns for three bursts of 20 seconds at 4°C using a Soniprep 150 Ultrasonic Disintegrator using a 3 mm probe. The sonicates were then centrifuged at 10, 000 x g for 5 minutes at 4°C and the supernatants rocked with 1 ml of glutathione sepharose slurry (1:1 in NETN and 0.5% (w/v) non-fat milk) for 1 hour at 4°C with continuous mixing. The beads were then washed five times with NETN buffer and then 1ml of freshly prepared 20 mM glutathione in 100 mM Tris-HCl pH 8.0, 120 mM NaCl was added. The suspension was rocked at 4°C for 30 minutes. The beads were pelleted by centrifugation, the supernatant carefully removed and made to 10 % (v/v) glycerol and stored at -70°C.

The coupled peptides and the GST-COUP-TF II fusion protein were used to immunise rabbits (D. Watling, ICRF Central Services Animal Unit) according to the unpublished procedure of G. Evan (Biochemistry of the Cell Nucleus Laboratory, ICRF). The antisera obtained were tested for specific antibodies to COUP-TF by immunoprecipitation and western blotting analysis.

Immunoprecipitation

Immunoprecipitation of [^{35}S]-methionine labelled *in vitro* translated receptors was used to assess the specificity of the polyclonal antisera MP31-3.

Equal amounts of the [^{35}S]-methionine labelled receptors (with unprogrammed reticulocyte lysate used to maintain an equal protein concentration in each reaction) and 1 µl of pre- or immune sera were diluted to 100 µl with IP-A buffer in an eppendorf and incubated with gentle agitation for 2 hours at 4°C. Protein A sepharose (equilibrated in IP-A buffer at 4°C) was used to precipitate the immunocomplexes, 100 µl of protein A sepharose 1:1 suspension with IP-A and 400 µl of IP-A buffer were added to each eppendorf and incubated for 1 hour at 4°C with gentle agitation. The mix was then spun in a microfuge for 5 minutes at 4°C and the supernatant removed by aspiration. The beads were washed twice with 500 µl IP-A buffer and then three times with 500 µl IP-B buffer. The beads were then resuspended in 50 µl 2x protein loading buffer, boiled for 5 minutes and analysed by SDS-PAGE.

Cell culture methods

Maintenance of cell stocks

Cells were grown as monolayer cultures on 175 cm^2 tissue culture flasks (Nunc) at 37°C in a humidified atmosphere maintained at 10% (v/v) CO_2. Cells were maintained in DMEM with 10% (v/v) foetal calf serum (FCS). Chicken embryo fibroblast (CEF) cells (primary cell cultures provided by ICRF Central Services, Cell Production) were further supplemented with 1% chick serum. Cell cultures were subcultured once or twice a week, depending on their rate of growth. The media was removed and the cell layer washed with 25 ml of PBSA. The cells were then incubated at room temperature for approximately 5 minutes with 5 ml of prewarmed trypsin/versene mix (1:5). The flask was gently agitated until the cells detached and 5 ml of growth media containing serum was added to inhibit the trypsin. The 10 ml mix was transferred to a sterile universal bottle and spun at 1, 200 rpm in an MSE bench top centrifuge for 5 minutes. The pellet was gently resuspended in fresh growth media and subcultured at a suitable dilution.

Storage of cells

Subconfluent monolayer cultures were trypsinised and pelleted as above. The cell pellet was then resuspended in 4.5 ml of DMEM containing 10% FCS and 0.5 ml DMSO added. 1 ml aliquots were transferred to 2.5 ml freezing vials (Nunc). The vials were wrapped in tissue and frozen at -20 °C

for 2 hours, -70 °C overnight and then transferred to liquid nitrogen for long term storage. Cells recovered from liquid nitrogen were thawed rapidly at 37°C and plated out in growth medium. Once the cells had attached to the flask the medium was changed to remove the residual DMSO.

Charcoal treatment of serum

Foetal calf serum contains endogenous steroids, retinoic acid and T3 that might mask the effect of exogenously added ligands in the transient transfection experiments. Serum used for these experiments was therefore treated with dextran coated charcoal (DCC). This treatment removes small molecules including the aforementioned ligands. The protocol used was modified from Page and Parker (1983). A dextran-coated charcoal suspension consisting of 5 g activated charcoal, 0.5 g dextran T70 and 5 ml Tris-HCl pH 7.4 made up to 500 ml with distilled water was divided equally between four 250 ml disposable centrifuge bottles and spun at 2, 000 g for 15 minutes at 4 °C. The supernatant was removed and each pellet was resuspended in 250 ml of serum. The suspension was shaken vigorously at 55°C for 30 minutes and then respun. The serum was then decanted into centrifuge bottles containing a fresh dextran-coated charcoal pellet, re-incubated and respun. The treated serum was filter sterilised using 0.22 µm Nalgene filter units, aliquoted and stored at -20°C.

Transient transfection

Calcium phosphate precipitation- HBS method

Unless otherwise stated CEF cells were transiently transfected by the HBS -calcium phosphate/DNA coprecipitation method, essentially that described by Graham and Van der Erb (1973). Cells were seeded at a density of 2x 10^5/6 cm dish in 4 ml of DMEM containing 10% (v/v) DCC treated FCS and 1% (v/v) DCC treated chick serum. The cells were refed with fresh medium 20- 24 hours later and treated with the calcium phosphate/DNA precipitate with a total of 10 µg of DNA per dish. The precipitate was prepared by mixing the freshly prepared solutions A and B (per 2 dishes):

Solution A	500 µl 2x HBS pH 7.1,
	5 µl 70 mM NaH_2PO_4, 5 µl 70 mM Na_2HPO_4

Solution B	500 µl distilled water, 60 µl $CaCl_2$, 20 µg supercolied plasmid DNA

Solution B was added at a rate of 1 drop per second to solution A while air was continuously bubbled through solution A to aid the mixing. The mixture was incubated at room temperature for 20- 30 minutes to allow the precipitate to form. 500 µl of the mixture was then added dropwise to the each of the duplicate dishes. After 12-16 hours incubation at 37°C, 5% (v/v) CO_2, the medium was removed and the cells washed three times with 4 ml of prewarmed DMEM to remove the residual precipitate. The cells were then fed with DMEM containing 10% (v/v) DCC treated FCS and 1% (v/v) DCC treated chick serum and ligand or ethanol carrier and incubated at 37°C, 10% (v/v) CO_2. Cells were routinely harvested 24- 28 hours post transfection. The ligands were dissolved in ethanol and stored at -20°C.

Calcium phosphate precipitation- BBS method

HeLa and JEG-3 cells were routinely transfected by an alternative calcium phosphate/ DNA coprecipitation method modified from the method of Chen and Okayama., (1987). Cells were plated at 50-60 % confluency in 24-well microtitre plates (Falcon) by diluting the required number of cells in DMEM containing 5% (v/v) DCC treated FCS and adding 1 ml of the cell suspension per well. The cells were refed with fresh media 24 hours later. The DNA to be transfected (3.2 µg supercoiled plasmid DNA) was diluted to 90 µl with distilled water and 10 µl of 2.5 M $CaCl_2$ added. To this solution 100 µl 2x BBS was added, mixed gently and left at room temperature for 15 minutes. The precipitate was then mixed gently and 100 µl added dropwise to duplicate wells (with a total of 1.6 µg of DNA per well). The cells were then incubated for 12-16 hours at 37°C, 5% CO_2. The media was then removed and the cells carefully washed three times with 1 ml of DMEM to remove residual precipitate. The cells were refed with fresh DMEM containing 5% (v/v) DCC treated FCS and ligand (stored as above) or ethanol carrier and incubated at 37°C, 10% CO_2. Cells were routinely harvested 24- 28 hours after transfection.

Electroporation

COS-1 cells were transfected by electroporation. Cells were grown to 70- 80% confluency in 175 cm^2 flasks, trypsinised (as described for

maintenance of cell stocks), recovered by centrifugation and the pellet resuspended in PBSA at a concentration of 1x 10^6/ml. 0.9 ml of the cell suspension was added to 15 µg of plasmid DNA in a 0.4 cm electroporation cuvette, mixed and incubated on ice for 10 minutes. The cells were then electroporated using a Bio-rad gene pulser, at 450 V and 250 µF giving time constants of 4.6-5.2 milliseconds. The cuvettes were then incubated on ice for a further 10 minutes, the cell suspension removed and diluted in DMEM containing 10% FCS and plated as required. After the cells had plated down the media containing dead cells was replaced and the cells harvested 40-48 hours after transfection.

Harvesting of transiently transfected cell monolayers

The method used for harvesting cells is based on that of S. Goodbourn and P. King (Gene Expression Laboratory, ICRF). The media was removed from the cells, the monolayers washed twice with PBSA and any excess liquid aspirated. Lysis buffer was added to each dish or well (100 µl and 50 µl respectively) and incubated until only the nuclei were visible under a microscope (approximately 2 minutes). The lysate was recovered, transferred to an eppendorf on ice and spun for 2 minutes to remove cell debris. The supernatant was then transferred to a fresh eppendorf and kept on ice until the luciferase assay had been performed. Due to the short half life of luciferase in cell extracts its activity was assayed as soon as possible. Both CAT and β-galactosidase are stable in cell extracts for a couple of months when stored at -20°C.

Luciferase activity assay

This assay is based on the method of de Wet et al., (1987), typically 20 µl of extract was added to 0.35 ml luciferase reaction buffer in a luminometer cuvette. The samples were then loaded into an LKB luminometer and 33 µl of 3 mM luciferin injected, mixed and the peak light emission recorded. The assay was linear up to 4000 units (personal observations) and extracts that gave higher values were diluted in lysis buffer and reassayed. Mock extracts gave background values of approximately 0.6- 0.9 units. The β-galactosidase activity of each of the samples was used to correct for transfection efficiency.

CAT activity assay

The extracts to be assayed were heated to 65°C for 5 minutes to denature endogenous deacetylases. The method used is based on that of Sleigh et al., (1986) and measures the transfer of the [1-^{14}C]-acetyl group from [1-^{14}C]-acetyl coenzyme A to chloramphenicol. Chloramphenicol and its acetylated derivatives are soluble in organic solvents whereas acetyl coenzyme A is insoluble. The [1-^{14}C]-acetylated chloramphenicol products can therefore be separated from the labelled substrate by extraction with ethyl acetate. The [1-^{14}C]-acetylated chloramphenicol products that are extracted in the organic phase can be quantitated by scintillation counting. Routinely, 20 µl of extract was added to 80 µl of mix that contained, 20 µl 8 mM chloramphenicol, 30 µl 0.25 M Tris-HCl pH 7.8, 10 µl lysis buffer and 20 µl of acetyl coenzyme A mix (0.1 µCi [1-^{14}C]-acetyl coenzyme A in 0.5 mM unlabelled acetyl coenzyme A). This mixture was incubated for 1 hour at 37°C and then stopped by the addition of 110 µl of ice cold ethyl acetate. The samples were vortexed, spun for 2 minutes in a microfuge and 80 µl of the upper organic phase transferred to a scintillation vial. This extraction was repeated using 100 µl of ethyl acetate and 100 µl of the upper phase transferred to the scintillation vial. Scintillation fluid (5 ml) was added, the samples loaded into a scintillation counter and the number of counts per minute recorded for each sample. This assay was linear up to 55, 000 cpm (personal observation) and extracts that gave higher values were diluted in lysis buffer and reassayed. Mock extracts were always assayed and gave background values of approximately 150-300 cpm. The mean background value was subtracted from the counts obtained from transfected cell extracts prior to correction for transfection efficiency with β-galactosidase activity.

β-galactosidase activity assay

β-galactosidase activity was measured using a Galacto-light kit supplied by TROPIX Inc. The Galacton substrate was diluted 1:100 with Galato-light reaction buffer diluent to make the reaction buffer. Typically 10 µl of extract was added to 100 µl of the reaction buffer in a luminometer cuvette and incubated at room temperature for 1 hour. The samples were then loaded into an LKB luminometer, 165 µl of accelerator injected (5x 33 µl) and the peak light emission recorded. Mock extracts were always assayed and gave background values of approximately 10-100 units. The mean background value was subtracted from the values obtained for the

transfected cell extracts and these values were then used to correct for transfection efficiency. Transfections that included the expression vector pSG-VP16CII were not corrected for β-galactosidase activity because VP16CII repressed transcription of the β-galactosidase reporter plasmid, pJ7lacZ. This plasmid contained the cytomegalovirus promoter linked to the lacZ gene. Extracts from cells transfected with pSG-VP16CII were normalised instead for protein concentration. Similarly transfections using 24 well plates and 500ng of the expression vector pSG5-COUP-TF II were normalised for protein concentration.

Preparation of whole cell extracts

This method is essentialy that described by Fawell et al., (1990b). Cells on 10 or 15 cm plates were washed three times with PBSA and scraped off the dish with a rubber policeman into 5 ml of ice cold PBSA and transferred to a 10 ml universal bottle. The dish was rinsed with a further 5 ml of ice cold PBSA and then spun at 1200 rpm for 5 minutes in an MSE bench top centrifuge. The supernatant was removed and the pellet frozen at -70°C. The cell pellets were thawed on ice into approximately 10 volumes of whole cell extract buffer (containing DTT and protease inhibitors) and passed through a 25 gauge needle 5 times. The lysates were transferred to eppendorfs (kept on ice) and then spun at 50, 000 x g for 15 minutes at 4°C to remove the insoluble material. Aliquots of the supernatant were then transferred to eppendorfs (kept on ice) and stored at -70°C.

CHAPTER 3
ANALYSIS OF COUP-TF EXPRESSION

CHAPTER 3

Introduction

COUP-TF I and II have been shown to repress the transcriptional activity of a number of nuclear receptors including RXR, RAR and ER that are involved in regulating via complex networks a variety of growth and developmental pathways. Retinoids have been shown to have antiproliferative and differentiating effects on a number of mammalian cell types and are essential for normal embryonic development (Lippman *et al* 1987, Moon and Mehta 1990, Peto *et al* 1981, Thaller and Eichele 1990). Oestrogen is essential for the normal development and function of reproductive systems in mammals and has been shown to stimulate the proliferation of ER containing breast cancer cell lines. Furthermore, prolonged exposure to this steroid hormone has been implicated in a high incidence of several forms of human cancer (Dickson and Lippman 1986, Henderson *et al* 1982). The proposed ability of COUP-TF to down regulate the signalling pathways of these molecules has significant implications for the differentiation, developmental and proliferative processes they control.

This chapter describes the identification of a full length COUP-TF II cDNA clone from the human breast cancer cell line ZR-75-1 and studies on the expression of COUP-TF protein in cell lines and tumour samples.

Sequencing of a full length COUP-TF II cDNA

We had previously isolated a COUP-TF II cDNA from a λ-ZAP ZR-75-1 cDNA library (provided by R.White, Molecular Endocrinology Laboratory, ICRF) (see methods). From preliminary sequence information the 5' region of the insert of clone 22.2 was known to be identical to a region of the 5' untranslated region of COUP-TF II (from nucleotide position 176). Diagnostic restriction enzyme digests indicated that the clone contained the entire coding region of human COUP-TF II. This clone was modified by the introduction of an oligonucleotide that allowed the removal of the 5' untranslated region of this clone and the introduction of a Kozak consensus translation initiating sequence (Kozak 1984) generating the clone pBSKCOUP-TF II (see appendix A1). This manipulation was carried out to improve the efficiency of *in vitro* transcription and translation of the cDNA. The modified cDNA insert was then transferred to the prokaryotic expression vector pDel (appendix A2) to create pDel-COUP-TF II (appendix A1). A series of nested 5' and 3' deletion mutants were generated using exonuclease III (Figure 3.1) and the cDNA inserts of these mutants were

The λ-ZAP ZR-75-1 cDNA library was screened with a [^{32}P] labelled oligonucleotide probe (see methods) encoding a region of the highly conserved DNA binding domain of human NGFI-B (TR3/ NAK1) (Chang *et al* 1989). The probe contained the nucleotide sequence 959-988 (corresponding to amino acids 284-293);
5'- TGTGAGGGCTGCAAGGGCTTCTTCAAGCGC-3'
Ten clones were isolated, 8 of the cDNAs corresponded to COUP-TF II and two were TRα cDNAs.

Figure 3.1 Construction of a series of 5' and 3' deletion mutants of COUP-TF II.

A schematic representation of the exonuclease III/mung bean nuclease deletion system used to create the deletion mutants. The enzyme exonuclease III sequentially removes nucleotides from the 3' end of double stranded DNA that contains either a 5' overhang or blunt ends but not from a 3' overhang. By the use of appropriate unique restriction enzyme sites exonuclease III can be used to obtain unidirectional deletion of DNA.

For the 3' deletion mutants the vector pBSKCOUP-TF II was digested with the restriction enzymes Xho I (to generate a 5' overhang) and Kpn I (to generate a 3' overhang). For the 5' deletion mutants the vector was digested with Eco RI (to generate a 5' overhang) and Bst XI (to generate a 3' overhang). The vectors were then treated with exonuclease III for different periods of time to allow progressive deletion of the DNA. The resultant single stranded DNA was treated with mung bean nuclease and then DNA polymerase I Klenow fragment to generate blunt ends that could be religated.

Clones were screened by restriction enzyme analysis of mini-preparations of DNA. The 5' deletion series were digested with Sac I and Xho I and the 3' deletion series were digested with Eco RI and Bgl II. These digestions cut out the COUP-TF II insert and its size was visualised on an agarose gel. Suitable deletion mutants were selected and sequenced. The pDel vector contains stop codons in all three frames between the Kpn I and Bgl II restriction sites ensuring that all the 3' deletion mutants contained an in frame termination codon.

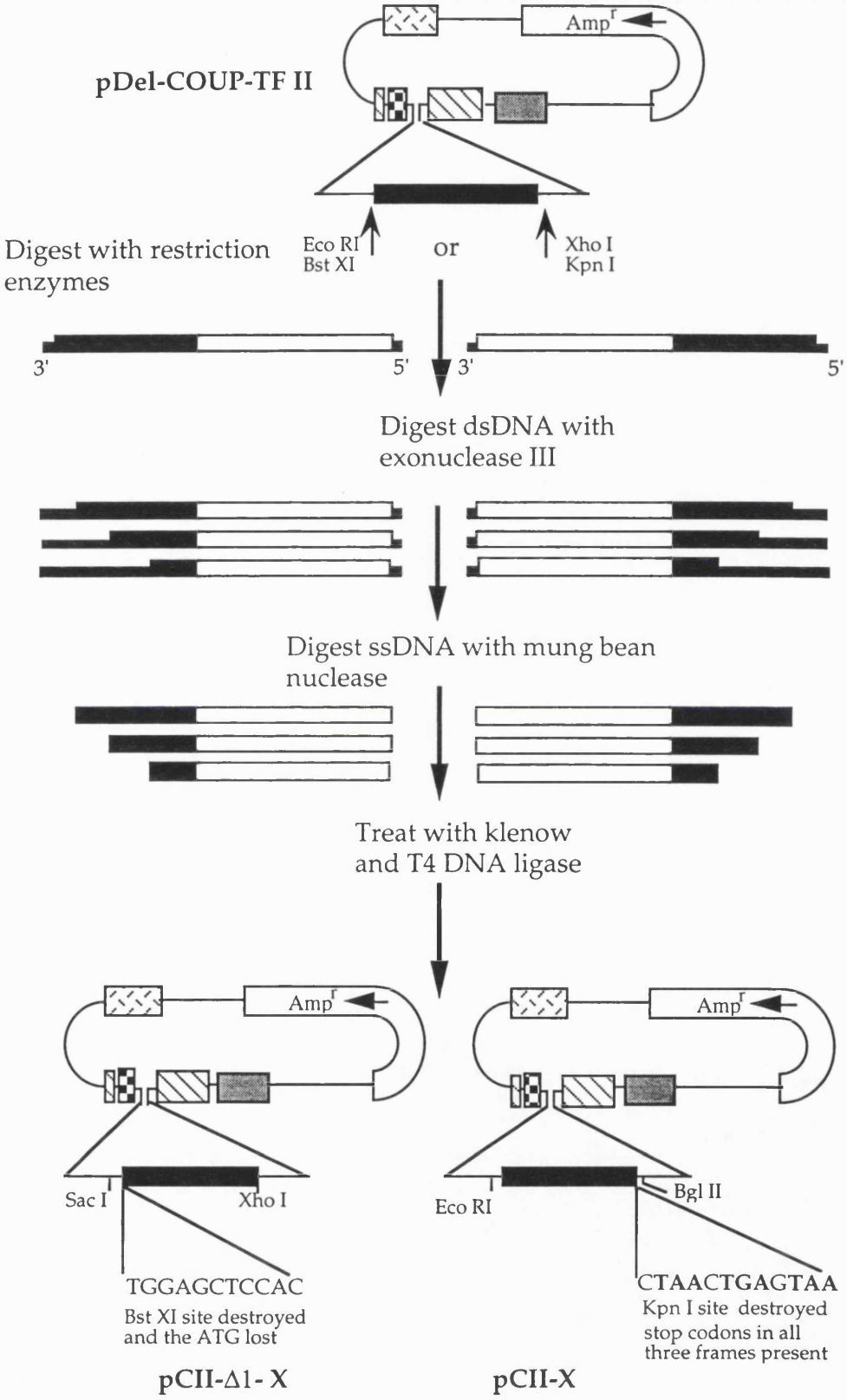


Figure 3.1 Construction of a series of 5' and 3' deletion mutants of COUP-TF II .

sequenced. The sequence data revealed that pDelCOUP-TF II contained COUP-TF II sequence from nucleotide 343 to 1740 (with the coding sequence from nucleotide 343 to 1587). This sequence was identical to that reported for the original clone isolated from human placenta (Ladias and Karathanasis 1991).

Generation of polyclonal antibodies to COUP-TF

To examine the expression of COUP-TF protein in cell lines and tumour samples polyclonal rabbit antibodies specific for COUP-TF I and II were generated. Antisera MP31 and MP32 were raised against peptides corresponding to amino acids 66-77 and 6-17 of COUP-TF II respectively and antiserum MP33 was raised against purified GST-COUP-TF II (see methods). The antisera were tested for specific antibodies to COUP-TF using immunoprecipitation, western blot analysis and gel shift analysis.

Immunoprecipitation using MP31, MP32 and MP33 antisera

The ability of the antisera MP31, MP32 and MP33 to immunoprecipitate COUP-TF proteins was tested. COUP-TF I, COUP-TF II and RXRα were synthesised *in vitro* in the presence of [^{35}S]-methionine and the labelled proteins analysed by SDS-PAGE (Figure 3.2a). It was noticed that the *in vitro* synthesised COUP-TF II had a slower mobility than COUP-TF I (compare tracks 2 and 3) which was surprising since COUP-TF II is composed of 414 amino acids (predicted molecular weight of 45.6 kilodaltons) and COUP-TF I is composed of 423 amino acids (predicted molecular weight of 46.2 kilodaltons). The greater apparent molecular weight of COUP-TF II may be due to differences in the posttranslational modification of the two proteins.

Equal amounts of the labelled *in vitro* synthesised proteins were incubated with the antisera and subjected to immunoprecipitation analysis. Antiserum MP31 immunoprecipitated COUP-TF II only, whereas MP32 and MP33 immunoprecipitated both COUP-TF I and II, although COUP-TF II was precipitated more efficiently (Figure 3.2b, c and d). The recognition of COUP-TF by the antisera was specific as the highly related protein RXRα was not immunoprecipitated and the pre-immune sera had no effect. In adddition immunoprecipitation of COUP-TF II by the anti-peptide sera MP31 and MP32 could be blocked by coincubation with the immunising peptide but not by an unrelated peptide. In immunoprecipitation analysis therefore, MP31

Figure 3.2 (a) Determination of the relative amounts of the *in vitro* synthesised COUP-TF I, COUP-TF II and RXRα.
(b, c and d) Immunoprecipitation of COUP-TF II using the antisera MP31, MP32 and MP33.

Immunoprecipitation of [^{35}S]-methionine labelled receptors was used to assess the specificity of the polyclonal antisera MP31-3. The immunoprecipitated proteins were analysed by SDS-PAGE.
(a) The relative amounts of [^{35}S] labelled *in vitro* translated COUP-TF I, COUP-TF II and RXRα protein were assessed by phosphoimage analysis and equal amounts used in further experiments. Control (unprogrammed) reticulocyte lysate was used to maintain an equal protein concentration in each reaction (see methods). Tracks 1-4 represent 1/5th of the input used in the immunoprecipitation reactions.
(b) The antiserum MP31 was incubated with the control lysate (track 1), COUP-TF II (tracks 2, 4 and 5), COUP-TF I (tracks 6 and 7) and RXRα (track 8). The incubations with COUP-TF II were carried out in the absence (track 2) or presence of 2 μg specific peptide (the immunising peptide) (track 4) or a non specific peptide (amino acid residues 5-17 of hRXRβ) (track 5). As a control the pre-immune serum was also incubated with COUP-TF II (track 3) and COUP-TF I (track 7).
(c) As above except the antiserum MP32 was used.
(d) The antiserum MP33 was incubated with the control lysate (track 1), COUP-TF II (track 2), COUP-TF I (track 4) and RXRα (track 6). As a control the pre-immune serum was also incubated with COUP-TF II (track 3) and COUP-TF I (track 5).

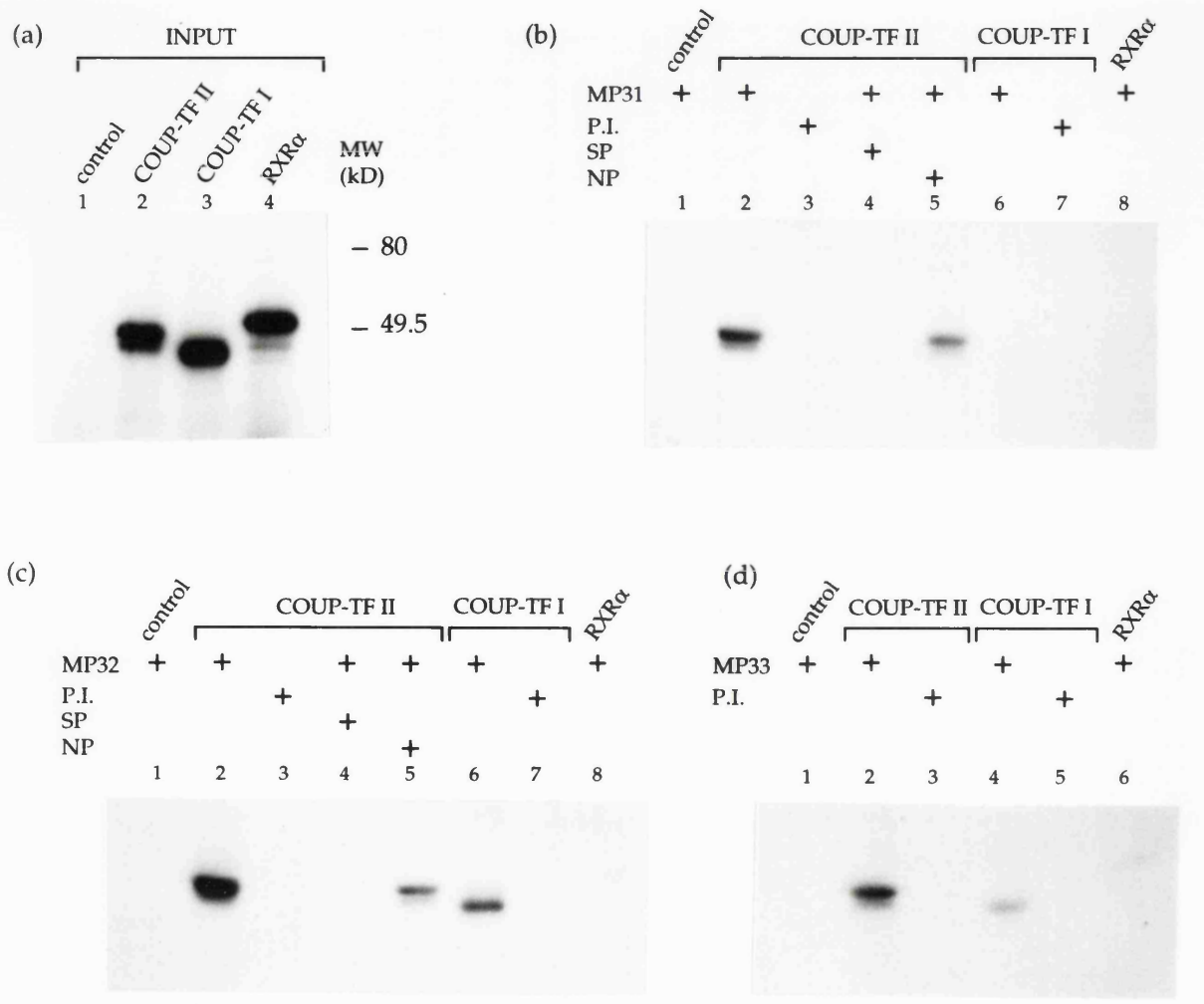


Figure 3.2 Immunoprecipitation using the antisera MP31, MP32 and MP33

specifically recognises COUP-TF II whereas MP32 and MP33 specifically recognise both COUP-TF I and II.

Western blot analysis of the antisera MP31, MP32 and MP33

The ability of the antisera MP31, MP32 and MP33 to specifically detect COUP-TF I and II in western blot analysis was tested. Whole cell extracts were prepared from COS-1 cells transfected with either an expression vector for COUP-TF I (pSG5-COUP-TF I, see appendix A3), COUP-TF II (pSG5-COUP-TF II, see appendix A4 and A5), RXRα (pSG5-hRXRα, provided by Dr. Dejean) or parental vector (pSG5). To ensure that COUP-TF I, COUP-TF-II and RXRα were expressed at similar levels in the COS-1 cells the DNA binding activity of the extracts was assessed. Gel shift analysis was used with an oligonucleotide probe containing a DR+1 response element (COUP-TF I, COUP-TF II and RXRα bind to this site with similar affinity, personal observations). The DNA binding activities of the COS-1 expressed receptors were similar and were therefore assumed to be expressed at approximately equivalent levels (Figure 3.3d).

In western blot analysis the antiserum MP31 specifically detected COUP-TF II (Figure 3.3a) and the antisera MP32 and MP33 specifically detected both COUP-TF I and II although COUP-TF II was recognised with greater affinity (Figure 3.3b and c respectively). MP31 and MP32 could be blocked by coincubation with the specific immunising peptide but not an unrelated peptide and none of the pre-immune sera cross reacted with the receptor bands. thus, using two different assays the antiserum MP31 was shown to specifically recognise COUP-TF II and the antisera MP32 and MP33 to specifically recognise both COUP-TF I and II.

It was noted that the COS-1 cell expressed COUP-TF II detected by western blot analysis migrated with slower mobility than the *in vitro* synthesised protein analysed by SDS-PAGE (compare figure 3.2a track 2 with Figure 3.3a, b and c track 2). Endogenous COUP-TF protein was also observed to migrate with a slower mobility than either *in vitro* translated COUP-TF I or II (see Figure 3.7 and data not shown). This apparent increase in molecular weight might be due to additional posttranslational modification of the protein in the cell extracts.

The ability of the antisera MP31, MP32 and MP33 to detect endogenous levels of COUP-TF was tested by western blot analysis of whole cell extracts of the breast cancer cell lines ZR-75-1 and MCF-7 (data not shown). Both the

Figure 3.3 (a, b and c) Western blot analysis of the antisera MP31, MP32 and MP33. (d) DNA binding activity of COS-1 cell expressed COUP-TF I, COUP-TF II and RXRα

Equal amounts of whole cell extracts (10 μg) prepared from COS-1 cells transiently transfected with pSG5-COUP-TF II, pSG5-COUP-TF I, pSG5-RXRα or the expression vector alone (lacking a cDNA) were resolved by SDS-PAGE and analysed by western blotting. The ability of the antisera to detect COUP-TF I and II was then assessed (see methods). The positions of the molecular weight markers of the indicated size (kilodaltons) are marked.

(a) Detection using the antiserum MP31. The antiserum MP31 was incubated alone (tracks 1-4) or in the presence of a specific peptide (the immunising peptide) (5 μg/ml) (tracks 7 and 8) or a non specific peptide (amino acid residues 5-17 of hRXRβ) (5 μg/ml) (tracks 9 and 10). As a control the pre-immune serum was also tested (tracks 5 and 6).

(b) Detection using the antiserum MP32. As above except the antiserum MP32 replaced MP31.

(c) Detection using the antiserum MP33. The antiserum MP33 (tracks 1-4) or pre-immune sera was used.

(d) Gel shift analysis of the whole cell extracts. To assess the relative levels of expression of COUP-TF -I and -II and RXRα in the COS-1 cell extracts the DNA binding activities of these extracts were examined by gel shift analysis. A labelled oligonucleotide containing an ~~ApoAI-RARE~~ was used in excess as the probe (free probe not shown).

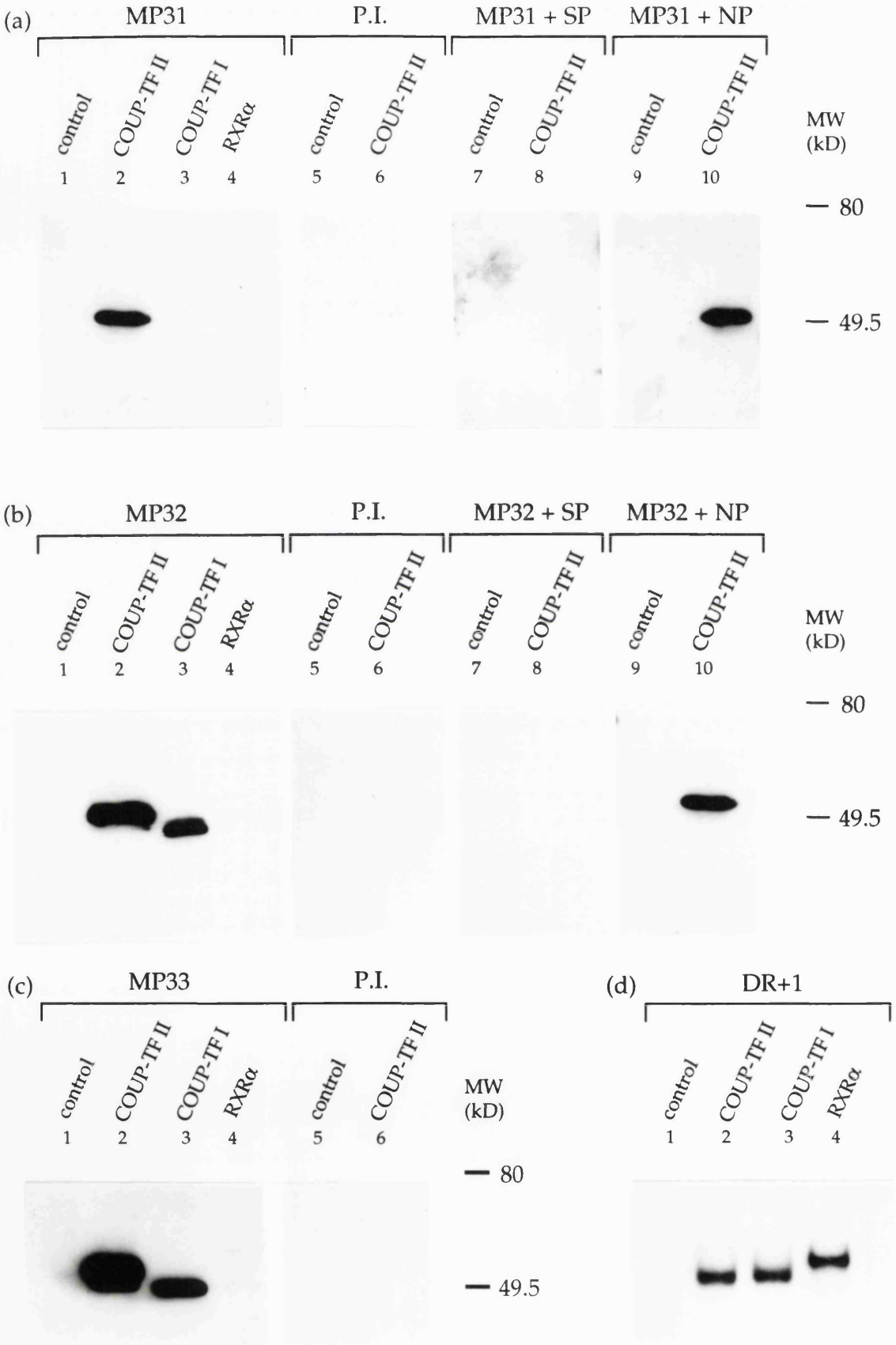


Figure 3.3 Western blot analysis of the antisera MP31, 32 and 33

antiserum MP32 and MP33 could detect endogenous COUP-TF, as MP33 gave the strongest signal it was routinely used in western blot analysis. Despite detecting COUP-TF II overexpressed in COS-1 cells, the MP31 antiserum was unable to detect COUP-TF at endogenous levels. COS-1 cells contain an origin defective mutant SV40 T antigen that allows plasmids containing an SV40 origin of replication to be maintained at high copy numbers (Mellon *et al* 1981). The plasmid pSG5, the expression vector used in the work of this thesis to overexpress proteins in COS-1 cells, contains an SV40 origin of replication. It would be expected that the levels of COUP-TF proteins expressed in the transfected COS-1 cells would be significantly higher than the endogenous level in the cell lines. The inability of MP31 to detect endogenous COUP-TF may therefore be due to lack of sensitivity. However, as MP31 was raised against a peptide it cannot be ruled out that this region in the endogenous protein (residues 66-77) is modified in some way such as to prevent recognition by the antibody. Alternatively, as MP31 is specific for COUP-TF II another explanation would be that in ZR-75-1 cells COUP-TF I is the predominate protein.

Gel shift analysis of the antisera MP31, MP32 and MP33

Using gel shift analysis the antisera were further characterised to determine if they could effect the migration of the COUP-TF II/DNA complex. *In vitro* translated COUP-TF II was preincubated with the antiserum MP31, MP32 or MP33 prior to addition of the oligonucleotide probe containing a DR+1 binding site. Addition of MP31 resulted in two retarded COUP-TF II/DNA complexes but had no effect on COUP-TF I (Figure 3.4a tracks 3 and 7). The COUP-TF II/DNA complex was also retarded by addition of MP32 antiserum (Figure 3.4b track 3). The effect of MP31 and MP32 on the COUP-TF II/DNA complex could be prevented by coincubation with the specific immunising peptide but not an unrelated peptide (Figure 3.4a, b and c tracks 5 and 6 respectively). Addition of MP33 abolished the COUP-TF II/DNA complex altogether (Figure 3.4c). The pre-immune serum for MP31, MP32 and MP33 had no effect (Figure 3.4a, b and c track 4).

Expression of COUP-TF protein in cancer cell lines

The expression of COUP-TF protein in a panel of human cancer cell lines was analysed by western blotting (Figure 3.5a). COUP-TF was expressed at comparable levels in Hela (cervical carcinoma), JEG-3 (chorioncarcinoma), MCF-7 (breast adenocarcinoma), MDA-MB-468 (breast adenocarcinoma) and

Figure 3.4 Gel shift analysis of antisera MP31, MP32 and MP33.
The effect of the antisera MP31, MP32 and MP33 on the DNA binding activity of COUP-TF II was analysed by gel shift analysis. Equal amounts of *in vitro* synthesised proteins were preincubated for 5 minutes at room temperature with or without antibodies or pre immune serum (1 µl) and peptides (2 µg) as indicated prior to the addition of a [^{32}P] labelled DNA probe containing a DR+1 response element. The position of the COUP-TF I and II homodimers are indicated by an arrow, the retarded complex by an open arrowhead and the free probe by a closed arrowhead.
(a) COUP-TF II was preincubated alone (track 2) or with MP31 (track 3), preimmune serum (track 4), MP31 and specific peptide (track 5) or MP31 and nonspecific peptide (track 6). Unprogrammed reticulocyte lysate (track1) did not shift the probe. COUP-TF I was preincubated alone (track 7) or with MP31 (track 8).
(b) As above for tracks 1-6.
(c) COUP-TF II was preincubated alone (track 2) or with MP33 (track3) or preimmune serum (track 4). Unprogrammed reticulocyte lysate did not shift the probe (track 1).

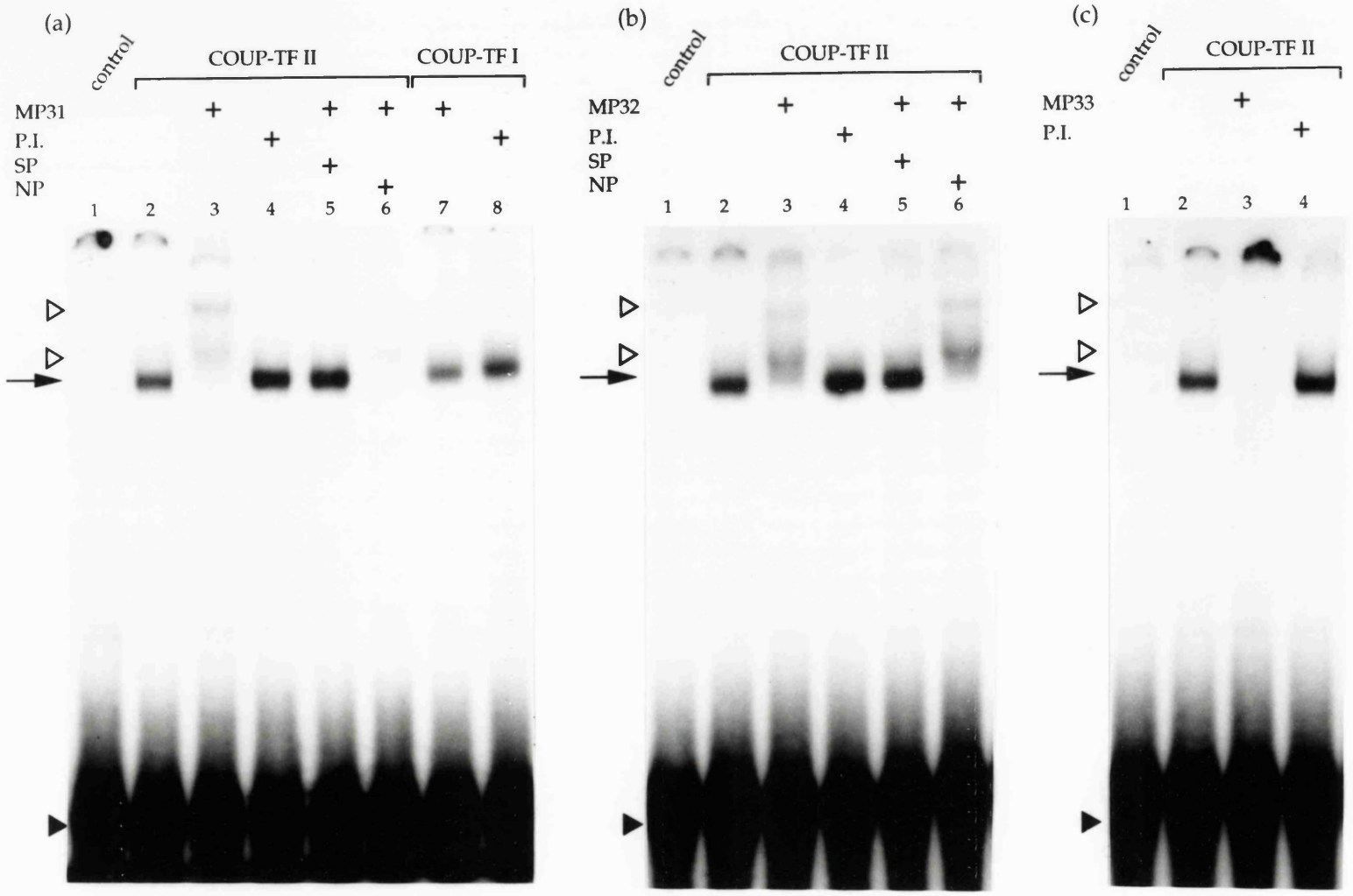


Figure 3.4 Gel shift analysis of the antisera MP31, MP32 and MP33

RL95-2 (endometrial carcinoma) with lower levels in ZR-75-1 (breast carcinoma) and was undetectable in the Ishikawa cell line (endometrial carcinoma). To confirm that the difference in levels of COUP-TF expression between the two endometrial carcinoma cell lines (Ishikawa and RL95-2) was not an artefact of the antiserum used another COUP-TF specific antibody was also tested (provided by M-J. Tsai). As shown in (Figure 3.5b), although a specific complex was detected in the Ishikawa extract (track 2) the level of COUP-TF in this cell line was significantly lower than in either the MCF-7 or RL95-2 cell line (tracks 1 and 3 respectively).

Expression of COUP-TF protein in breast and endometrial tumour samples

Breast and endometrial tumour cytosols (provided by H.Rochefort) were also analysed by western blotting (Figure 3.6). There was a correlation between tumour type and the amount of COUP-TF protein detected. The breast tumour cytosols displayed low levels and the endometrial tumour cytosols significantly higher levels of COUP-TF. This pattern of COUP-TF expression was not observed between the breast and endometrial cancer cell lines tested (Figure 3.5). The discrepancy between the pattern of COUP-TF expression in primary tumour tissue and the expression in cell lines, commonly used in laboratories as models of these cancers, is not clear. The screening of more tumour samples is required to eliminate the possibilty that the difference in COUP-TF expression between the tumour types was a fortuitous observation due to the small sample size.

Regulation of COUP-TF expression by oestradiol and retinoic acid

The effects of oestradiol and retinoic acid on the expression of COUP-TF protein in two ER positive breast cancer cell lines, MCF-7 and ZR-75-1 and the ER negative cell line MDA-MB-468 were investigated. Cells were grown for 5 days in the absence of exogenously added steroids or retinoids in phenol red free DMEM (as phenol red has been shown to be a weak oestrogen (Berthois *et al* 1986) containing 10% FCS that had been treated to remove endogenous steroids and retinoids (see methods). The cells were then treated with either 1x 10^{-7}M oestradiol, 1x 10^{-7}M all-*trans* retinoic acid, 1x 10^{-7}M 9-*cis* retinoic acid or the ethanol carrier alone for a further 72 hours before harvesting for whole cell extracts. Western blot analysis of these extracts (Figure 3.7) showed that oestradiol down-regulated expression of the COUP-TF protein in the cell lines that contained ER. The effect was most marked in the MCF-7 cells and to a lesser extent in the ZR-75-1 cells. As expected

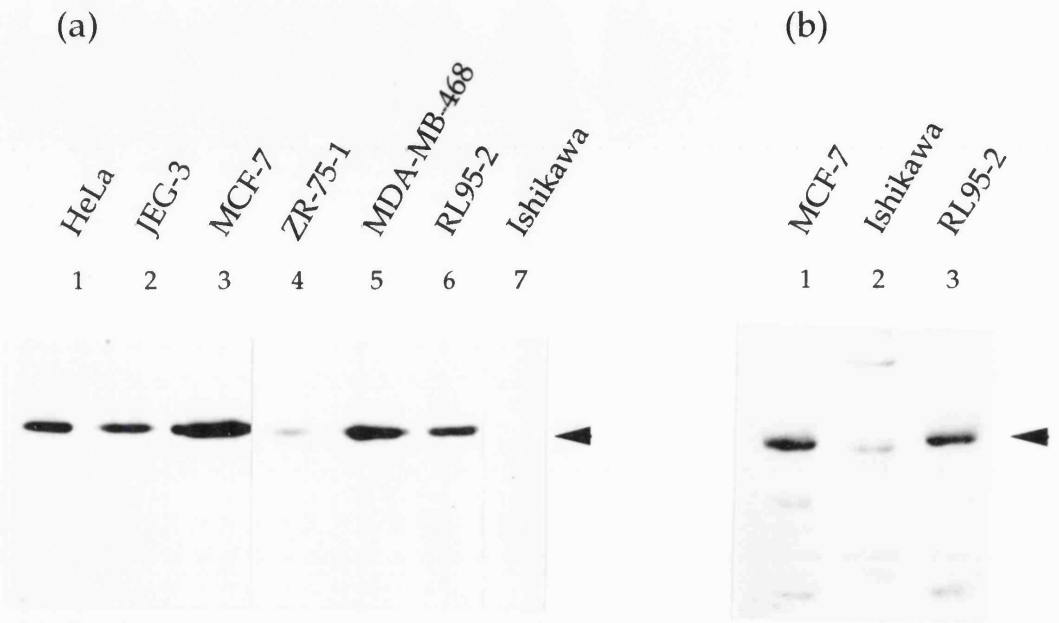


Figure 3.5 Expression of COUP-TF protein in cell lines.
Cells were grown in DMEM containing 10% FCS, harvested when 80-90% confluent and whole cell extracts prepared. Equal amounts of the extracts (100 μg) were resolved by SDS-PAGE and analysed by western blotting and COUP-TF protein detected using polyclonal rabbit antiserum (a) MP33 (b) αCOUP. The arrowhead indicates the COUP-TF specific complex.

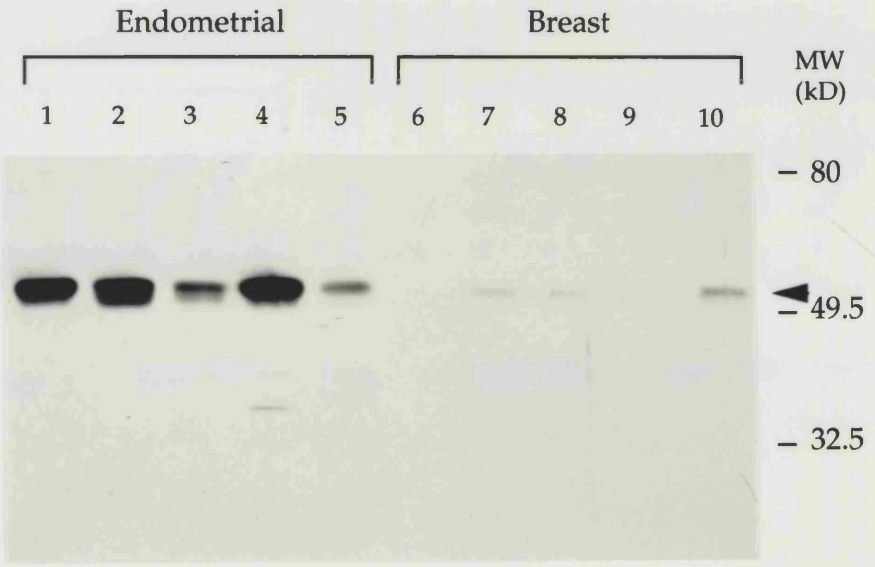


Figure 3.6 Expression of COUP-TF protein in breast and endometrial tumour samples.
Equal amounts (100 μg) of endometrial (tracks 1-5) and breast (tracks 6-10) tumour cytosols (provided by H. Rochefort) were resolved by SDS-PAGE and analysed by western blotting using the antiserum MP33 to detect the COUP-TF protein. The arrowhead indicates the COUP-TF specific complex. The position of the molecular weight markers of the indicated size (kilodaltons) are marked.

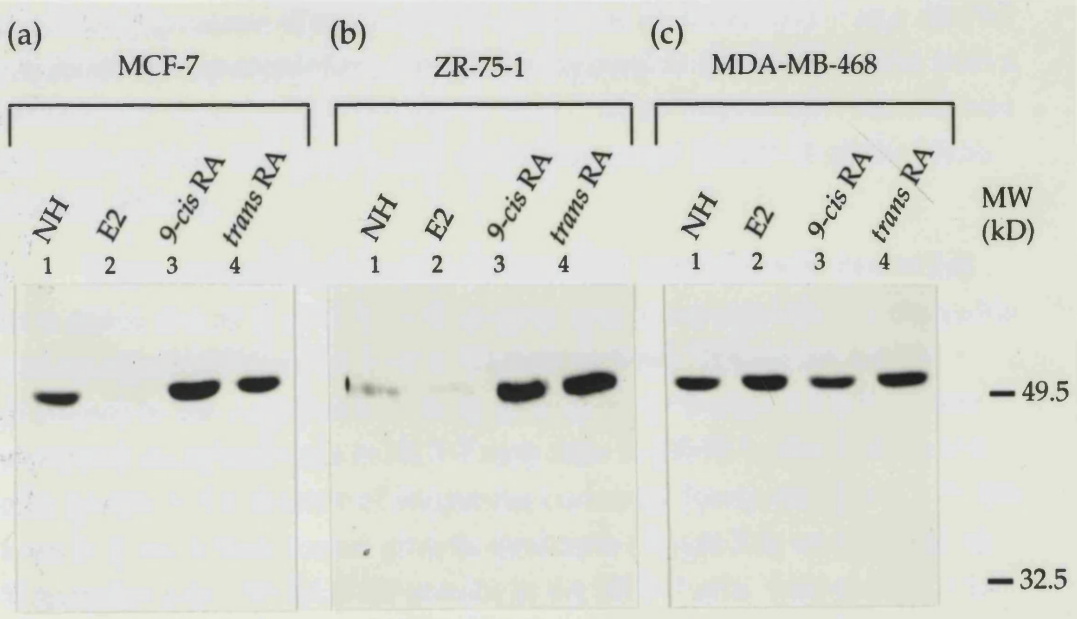


Figure 3.7 Regulation of COUP-TF II expression by oestradiol and retinoic acid. The breast cancer cell lines (a) MCF-7, (b) ZR-75-1 and (c) MDA-MB-468 were grown in phenol red free DMEM containing 10% (v/v) DCC treated FCS (see methods) for 5 days and then treated with ethanol carrier (no hormone, NH) (track 1), 100 nM oestradiol (E2) (track 2), 100 nM 9-*cis* retinoic acid (9-*cis* RA) (track 3) or 100 nM all-*trans* retinoic acid (*trans* RA) (track 4). The cells were harvested after 72 hours stimulation and equal amounts of whole cell extracts (100 μg) resolved by SDS-PAGE and analysed by western blotting using the antiserum MP33 for detection of the COUP-TF proteins. The positions of the molecular weight markers of the indicated size (kilodaltons) are marked.

oestradiol treatment had no effect on the expression of COUP-TF in the MDA-MB-468 cell line, which does not express the ER.

Treatment with all-*trans* retinoic acid or 9-*cis* retinoic acid up-regulated expression of the COUP-TF protein in both the MCF-7 and ZR-75-1 cell lines. The up-regulation was most prominent in the ZR-75-1 cells with a modest (but reproducible) increase in MCF-7 cells. Surprisingly, retinoic acid (all-*trans* or 9-*cis*) had no effect on the expression of COUP-TF in the MDA-MB-468 cell line.

These results showed that, in the two ER positive breast cancer cell lines tested, the level of COUP-TF receptor was down-regulated by oestradiol and up-regulated by retinoic acid (all-*trans* or 9-*cis*). The reason for the difference in the magnitude of the responses is not known. COUP-TF was expressed at higher levels in MCF-7 cells than in ZR-75-1 cells, both in the cells grown in the absence of exogenous hormones (compare Figure 3.7a and b track 1) and under normal growth conditions (Figure 3.5a tracks 3 and 4). This higher level of COUP-TF protein in the MCF-7 cells may explain why in this cell line a more dramatic down regulation was observed upon oestradiol treatment and conversely why all-*trans* or 9-*cis* retinoic acid treatment resulted in a more marked up-regulation of COUP-TF in ZR-75-1 cells. As expected there was no regulation of COUP-TF expression by oestradiol in the ER negative cell line MDA-MB-468 and retinoic acid (all-*trans* or 9-*cis* retinoic acid) similarly had no effect.

Time course analysis of the oestradiol induced down regulation of COUP-TF protein

MCF-7 cells were grown in the absence of exogenously added steroids or retinoids for 5 days as above. The cells were then treated with 1x 10^{-7}M oestradiol for various time intervals before harvesting to prepare whole cell extracts. Western blot analysis of these extracts showed that the steady state level of COUP-TF started to decrease after 24 hours treatment (Figure 3.8) reaching the lowest level by 48 hours and remaining at this repressed level after 120 hours treatment.

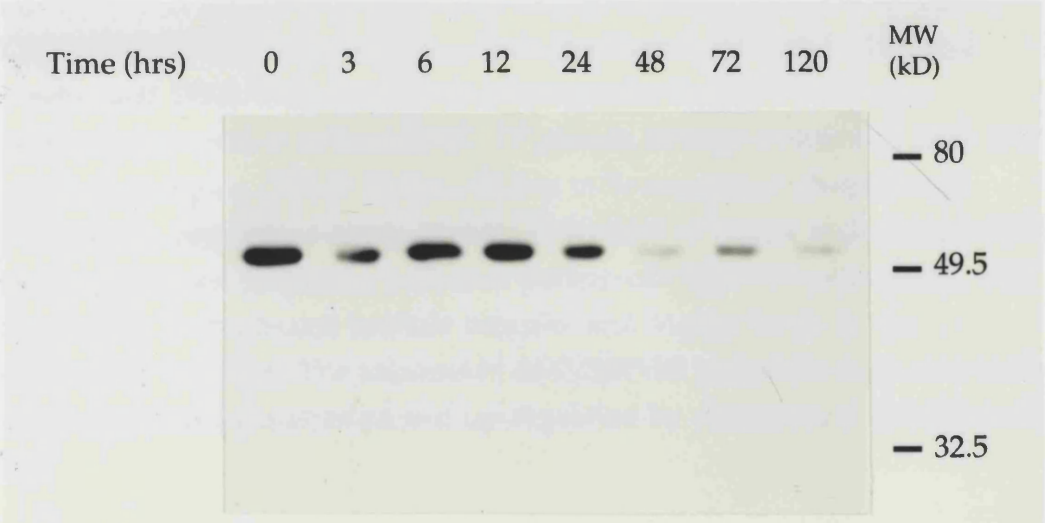


Figure 3.8 Time course of the down regulation of COUP-TF induced by oestradiol.

MCF-7 cells were maintained in phenol red free DMEM containing 10% (v/v) DCC treated FCS (see methods) for 5 days. The cells were then treated with 100 nM oestradiol (E2) and harvested at the times (in hours) indicated and whole cell extracts prepared. Equal amounts of the extracts (100 μg) were resolved by SDS-PAGE and analysed by western blotting using the antiserum MP33 for detection of the COUP-TF proteins. The position of the molecular weight markers of the indicated size (kilodaltons) are marked.

Summary and conclusions

A cDNA clone isolated from the human breast cancer cell line ZR-75-1 was sequenced and shown to contain coding sequence identical to that of COUP-TF II (ARP1) isolated from a human placenta library (Ladias and Karathanasis 1991). Polyclonal antibodies specific for COUP-TF were generated and used to detect COUP-TF protein, both in cancer cell lines and in tumour samples. COUP-TF was shown to be expressed ubiquitously although the relative amounts varied. The panel of breast and endometrial tumours analysed displayed a distinct pattern of COUP-TF expression, with lower levels in the breast tumour samples and higher levels in the endometrial tumours. The expression of COUP-TF protein was shown to be down-regulated by oestradiol and up-regulated by retinoic acid (all-*trans* or 9-*cis*).

CHAPTER 4

DIMERISATION PROPERTIES OF COUP-TF II

Introduction

COUP-TF I and II have been shown to repress the transcriptional activity of a number of nuclear receptors. Two types of model have been proposed to account for the mechanism by which this repression occurs, the first involves the formation of inactive COUP-TF heterodimers (Figure 4.1a and b) and the second the formation of COUP-TF homodimers that compete for binding to DNA (Figure 4.1c). Inactive heterodimers could either be formed between COUP-TF and a number of different receptors such as the TR and RAR (Figure 4.1a) or alternatively with the common partner RXR (Figure 4.1b), in both cases such dimerisation might take place on or off DNA. This chapter describes an analysis of the ability of COUP-TF II to form homodimers and heterodimers *in vitro* and in intact cells. The ability of COUP-TF II to mediate active repression of transcription (silencing) was also investigated.

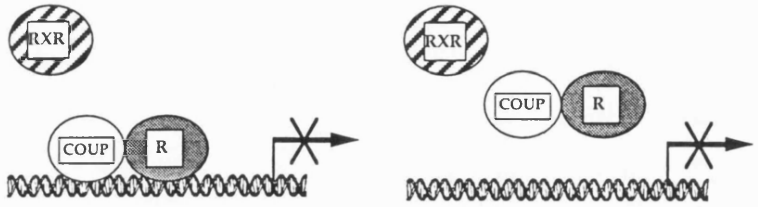
COUP-TF II forms both homodimers and heterodimers *in vitro*

To investigate the ability of COUP-TF II to bind to DNA *in vitro* as a homodimer and as a heterodimer *in vitro* translated proteins were produced. The receptors were synthesised using the plasmids pSG5-COUP-TF II, -hRXRα, -hRARα, pGEM7Z-hTRβ and pBS-mSF-1 in a coupled *in vitro* transcription and translation reaction (see methods). Parallel reactions were carried out in the presence of [^{35}S]-methionine, the labelled proteins were separated by SDS-PAGE and used to assess the size and yield of the translated proteins. The results indicated that the synthesised products were of the expected size, a representative gel is shown in Figure 4.2. The relative amounts of the products varied and were quantitated by determining the amount of incorporated radioactivity for each protein by phosphoimage analysis and correcting for the number of methionine residues in the protein (see methods). Equal amounts of the proteins were incubated with the appropriate [^{32}P]-labelled oligonucleotide and the complexes that bound detected by gel shift analysis (see Table 5.1 for the nucleotide sequences of the probes used).

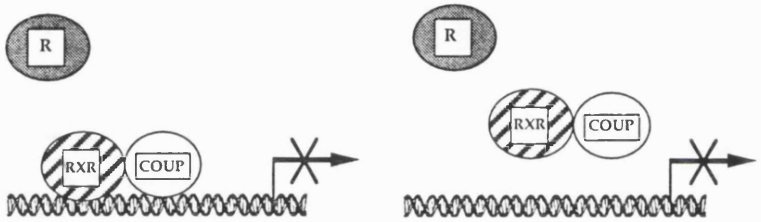
DR+1 response element

COUP-TF II and RXRα each generated a single complex when incubated alone with an oligonucleotide probe that contained a DR+1 response element (Figure 4.3a tracks 3 and 5) and an additional complex of

(a) Formation of inactive COUP-TF heterodimers



(b) Sequestration of the common partner RXR



(c) Competition by COUP-TF homodimers

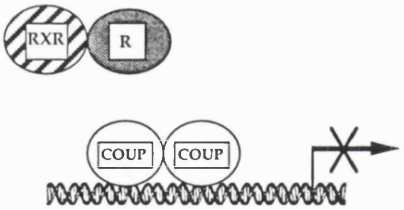


Figure 4.1 Models for the mechanism by which COUP-TF represses transactivation mediated by nuclear receptors.

(a) Formation of inactive heterodimers between the receptor and COUP-TF, either on or off DNA.

(b) Sequestration of RXR, the DNA binding partner of a number of nuclear receptors, either on or off DNA.

(c) Competition for DNA binding to response elements by COUP-TF homodimers.

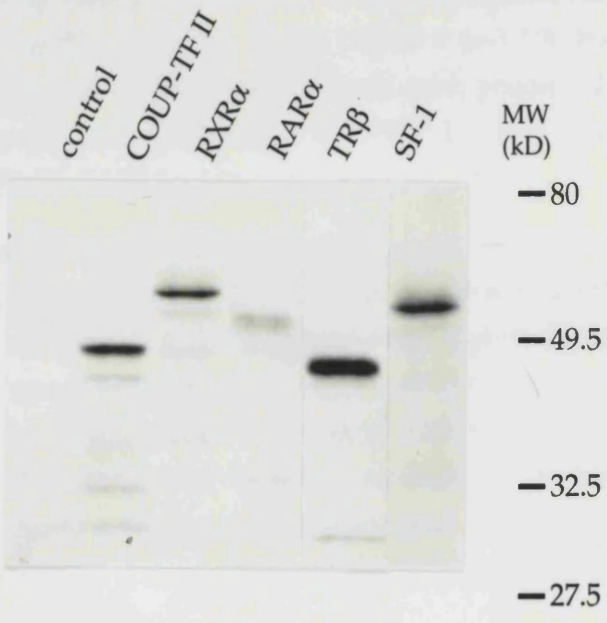


Figure 4.2 Determination of the relative amounts of the nuclear receptor proteins synthesised *in vitro* .
Proteins were synthesised from the corresponding expression vector using a coupled *in vitro* transcription and translation system containing [35S]-methionine. Aliquots (3 µl) of unprogrammed (control) or programmed lysates were analysed by SDS-PAGE and the resultant bands visualised by flurography and quantitated using phosphoimage analysis. The position of the molecular weight markers of the indicated size (kilodaltons) are marked.

intermediate mobility was formed when the two proteins were coincubated (track 7). The ability of antibodies specific for either COUP-TF or RXRα (provided by R. Evans) to retard the mobility of the intermediate complex indicated that it comprised a heterodimer of these receptors confirming the work of Kliewer et al (Kliewer *et al* 1992a) (tracks 9 and 10). Furthermore, the addition of the ligand for RXR, 9-*cis* retinoic acid, promoted the binding of RXRα homodimers (track 6) (Zhang *et al* 1992b) but had no effect on the formation of COUP-TF II/RXRα heterodimers (track 8).

RARα alone did not bind to a DR+1 response element when incubated alone (Figure 4.3b track 2) but when mixed with RXRα a novel complex was formed. This complex migrated only slightly faster than the RXRα homodimer complex (compare tracks 5 and 6) but its binding was greater, presumably reflecting the formation of RXRα/RARα heterodimers. Coincubation of RARα and COUP-TF II had no effect on the binding of the COUP-TF II homodimer complex and no additional complexes were formed suggesting that COUP-TF II does not form heterodimers with RARα on this site (compare tracks 3 and 5).

DR+2, DR+4 and DR+5 response elements

Next the DNA binding activity of COUP-TF II was examined on oligonucleotide probes containing the DR+2, DR+4 or DR+5 response elements. Homodimers of COUP-TF II were detected on all of the sites tested (Figure 4.4 tracks 2, 9 and 16). The ability of COUP-TF II to bind with high affinity to response elements containing different spacings between the half sites is investigated further in Chapter 5. When incubated alone, RXRα did not generate a complex on any of the oligonucleotides indicating that RXRα homodimers did not recognise these sites (tracks 3, 10 and 17). Coincubation of COUP-TF II and RXRα on the DR+4 and DR+5 elements resulted in the formation of an additional complex that migrated only slightly slower than the COUP-TF II homodimer (tracks 11 and 18) presumably representing a COUP-TF II/RXRα heterodimer. On the DR+2 element mixing of COUP-TF II and RXRα also resulted in the formation of a novel complex but on this site COUP-TF II homodimer binding was lost (track 4).

No complexes were detected on either the DR+2, DR+4 or DR+5 elements when RARα was incubated alone (tracks 5, 12 and 19). When COUP-TF II was coincubated with RARα, COUP-TF II homodimer binding was unaffected and no additional complex was formed (compare tracks 2 and

Figure 4.3 Formation of COUP-TF II homodimers and heterodimers on the DR+1 response element *in vitro*.

The DNA binding activities of COUP-TF II, RXRα and RARα on the DR+1 response element were analysed by gel shift analysis. Equal amounts of the *in vitro* synthesised nuclear receptors alone or together were preincubated in the presence or absence of retinoic acid (100 nM, 9-*cis* or all-*trans*) as indicated prior to the addition of [^{32}P] labelled DNA. Where indicated antibodies specific for either COUP-TF or RXRα were added prior to the addition of probe. To allow better resolution of the complexes the free probe was run off the gel.

(a) The position of the COUP-TF II homodimer is indicated by a solid arrowhead, the COUP-TF II/RXRα heterodimer by an open arrowhead and RXRα homodimer by the arrow.

(b) The position of the COUP-TF II homodimer is indicated by a solid arrowhead, the RXRα/RARα heterodimer by an open arrowhead and the RXRα homodimer by the arrow.

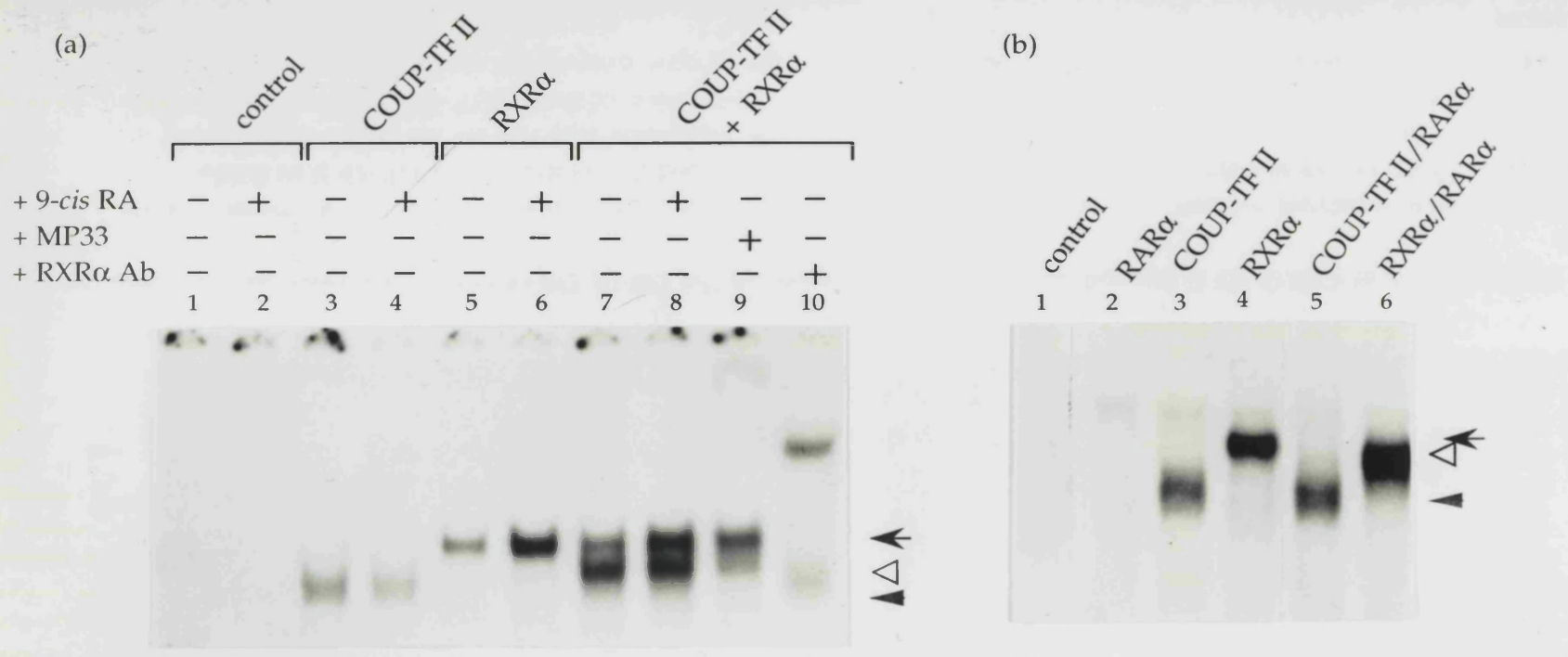


Figure 4.3 Formation of COUP-TF II homodimers and heterodimers on the DR+1 response element *in vitro*. The DNA binding activities of COUP-TF II, RXRα and RARα on the DR+1 response element were analysed by gel shift analysis. Equal amounts of the *in vitro* synthesised nuclear receptors alone or together were preincubated in the presence or absence of retinoic acid (100 nM, 9-*cis* or all-*trans*) as indicated prior to the addition of labelled DNA. Where indicated antibodies specific for either COUP-TF or RXRα were added prior to the addition of probe. To allow better resolution of the complexes the free probe was run off the gel. The position of the COUP-TF II homodimer is indicated by a solid arrowhead, the heterodimer by an open arrowhead and RXRα homodimer by the arrow.

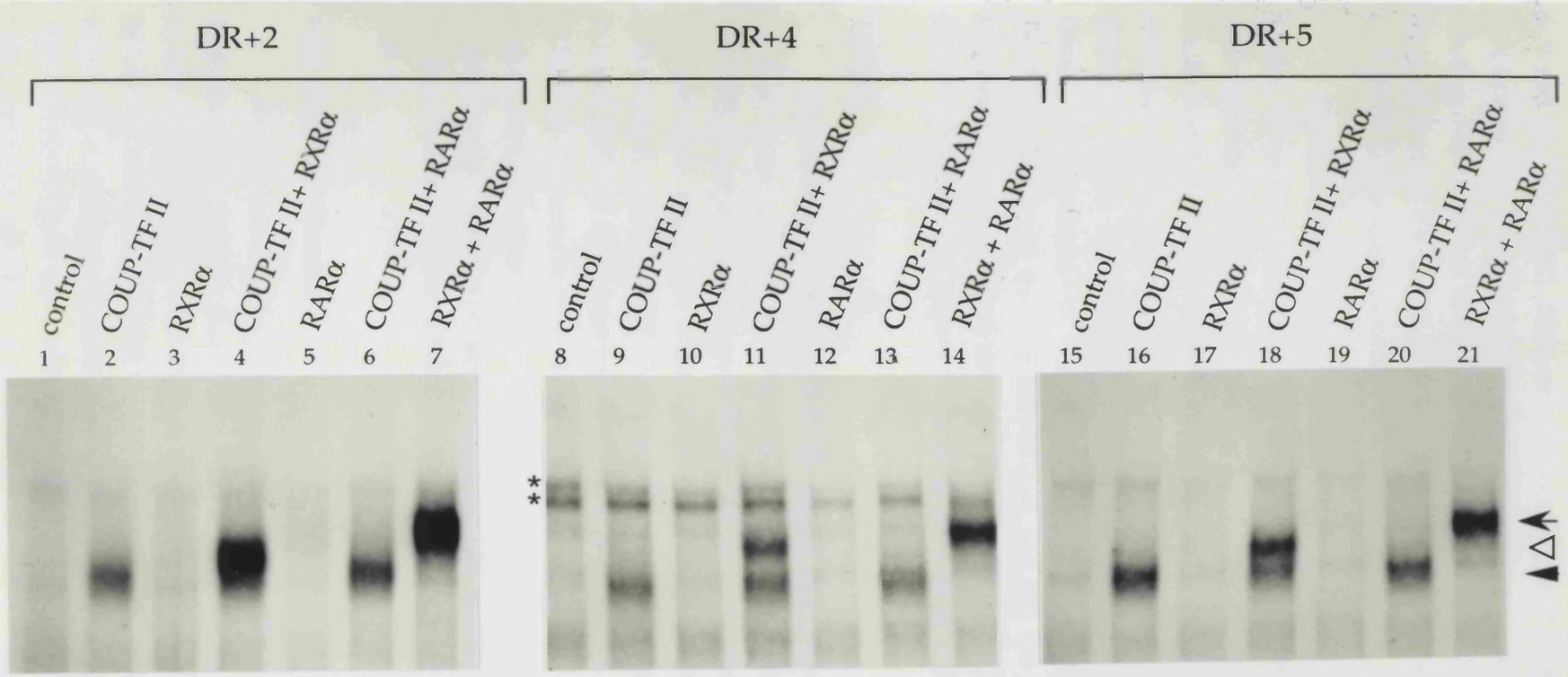


Figure 4.4 Formation of COUP-TF II homodimers and heterodimers on the DR+2, DR+4 and DR+5 response elements *in vitro*.

Gel shift analysis was carried out using an oligonucleotide containing either a DR+2 (tracks 1-7), a DR+4 (tracks 8-14) or a DR+5 (tracks 15-21) response element. Equal amounts of *in vitro* synthesised RARα and/or COUP-TF II or RXRα receptors were preincubated prior to the addition of labelled DNA. The free probe was run off the gel to allow better resolution of the complexes. The asterix denotes non-specific bands. The position of the COUP-TF II homodimer is indicated by a solid arrowhead, the COUP-TF II/RXRα heterodimer by an open arrowhead and RXRα/RARα homodimer by the arrow.

6, 9 and 13, 16 and 20). Although neither RARα or RXRα could bind as homodimers to the oligonucleotides, when they were mixed together strong binding was observed to all three elements (tracks 7, 14 and 21). These data suggest that on the DR+2, DR+4 and DR+5 response elements RXRα but not COUP-TF II can form heterodimers with RARα.

The binding of TRβ to the DR+4 containing oligonucleotide was also tested. When incubated alone a weak TRβ specific complex was observed that was abolished upon addition of the TR specific ligand T3 (Figure 4.5a tracks 3 and 4). The destabilisation of TR homodimers by T3 on DR+4 elements confirms previous reports (Andersson *et al* 1992, Yen *et al* 1992a, Yen *et al* 1992b). As before, COUP-TF II homodimer binding was observed (track 2). Coincubation of TRβ with COUP-TF II had no effect on the COUP-TF II homodimer or the TRβ homodimer and did not result in the formation of an additional complex (tracks 5 and 6). Thus on this TRE COUP-TF II does not appear to form heterodimers with TRβ.

TREpal

To examine whether COUP-TF II and TRβ could form heterodimers on aTRE containing an inverted repeat an oligonucleotide probe containing the TREpal sequence was used. When TRβ was incubated alone no complex was detected, regardless of the addition of the TR specific ligand T3 (Figure 4.5b tracks 3 and 4). COUP-TF II homodimer binding was observed (track 2). When COUP-TF II was coincubated with TRβ the complex corresponding to COUP-TF II homodimer binding was maintained but an additional band of higher mobility, consistent with the formation of COUP/TF II-TRβ heterodimers, was also observed (track 5). The addition of T3 had no effect on the binding of either TR homodimers or heterodimers to DNA (tracks 4 and 6).

SF-1 response element

An oligonucleotide probe containing the -210 element of the steroid 21-hydroxylase gene, a characterised SF-1 response element (FRE), was used to test the ability of COUP-TF II to form heterodimers with SF-1. When incubated individually, both COUP-TF II and SF-1 formed complexes that migrated at positions consistent with binding as a dimer and monomer respectively (Figure 4.6 tracks 2 and 3). When the two orphan receptors were coincubated no complex of intermediate mobility was formed and the

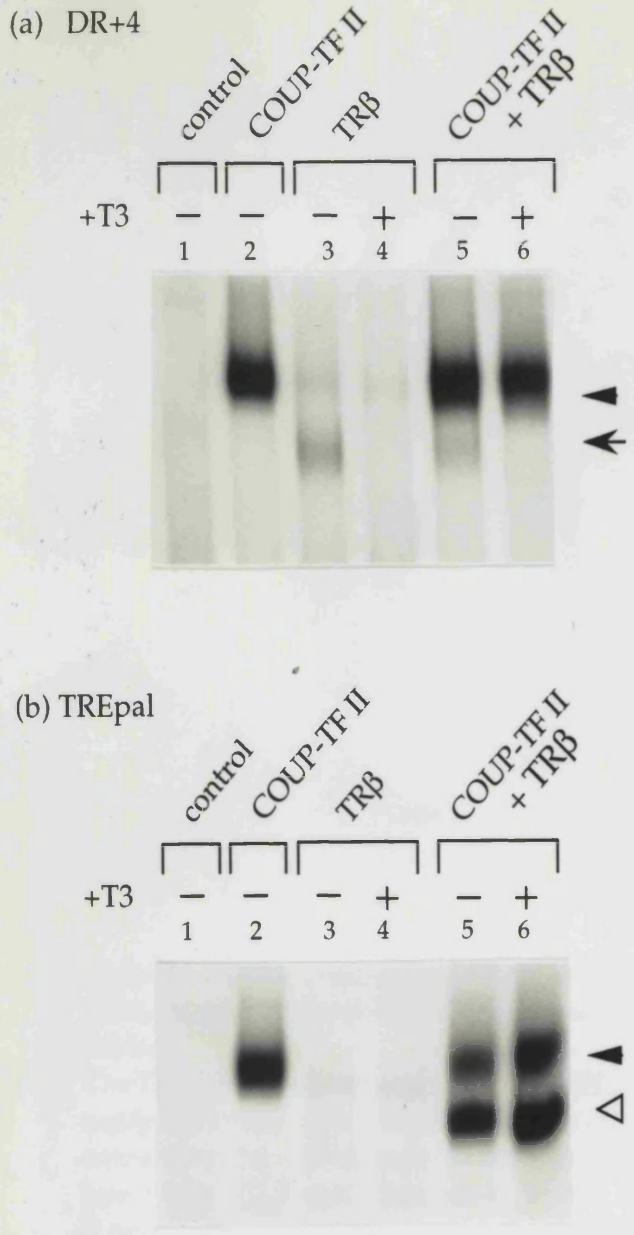


Figure 4.5 DNA binding of COUP-TF II and TRβ on the DR+4 and TREpal response elements *in vitro*.
The DNA binding activities of COUP-TF II and TRβ were analysed by gel shift analysis using an oligonucleotide containing (a) DR+4 or (b) TREpal response element. Equal amounts of *in vitro* synthesised COUP-TF II and/or TRβ were preincubated in the presence or absence of T3 (200 nM) prior to the addition of labelled DNA.
The position of the COUP-TF II homodimer is indicated by the solid arrowhead, the TRβ homodimer by the arrow and the COUP-TF II/TRβ heterodimer by the open arrowhead. The free probe was run off the gel to allow better resolution of the complexes.

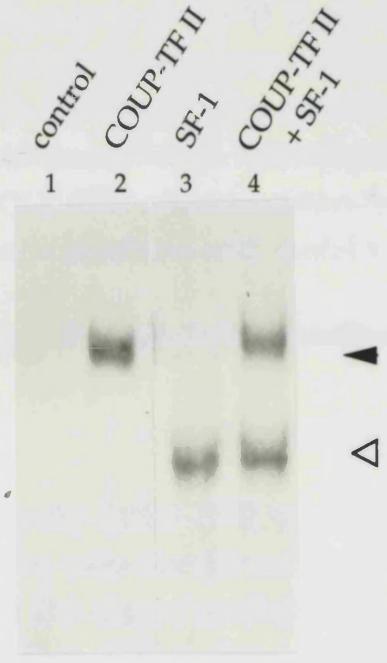


Figure 4.6 DNA binding of COUP-TF II and SF-1 to the -210 element of the steroid 21-hydroxylase gene (-210 21OH) *in vitro*.

The DNA binding activities of COUP-TF II and SF-1 were analysed by gel shift analysis using an oligonucleotide containing the -210 element of the steroid 21 hydroxylase gene (see Table 5.1). Equal amounts of *in vitro* synthesised COUP-TF II and/or SF-1 were preincubated prior to the addition of labelled DNA. The position of the COUP-TF II homodimer is indicated by a solid arrowhead and SF-1 monomer by an open arrowhead. The free probe was run off the gel to allow better resolution of the complexes.

binding of SF-1 and COUP-TF II was unaffected (track 4) consistent with non cooperative binding to this response element by the two orphan receptors.

These results demonstrate that *in vitro* COUP-TF II is able to bind as a homodimer to response elements that it has been shown to repress *in vivo*, consistent with model c in Figure 4.1c. COUP-TF II can also bind as a heterodimer with RXRα to response elements on which it represses the transcriptional activity of this retinoid receptor and this is consistent with model b (Figure 4.1b). In addition, although heterodimers with SF-1 or RARα were not detected COUP-TF II could be shown to form heterodimers with TRβ on a TREpal element consistent with model a (Figure 4.1a).

Examination of the formation of COUP-TF II homodimers and heterodimers *in vivo*

Two hybrid assay

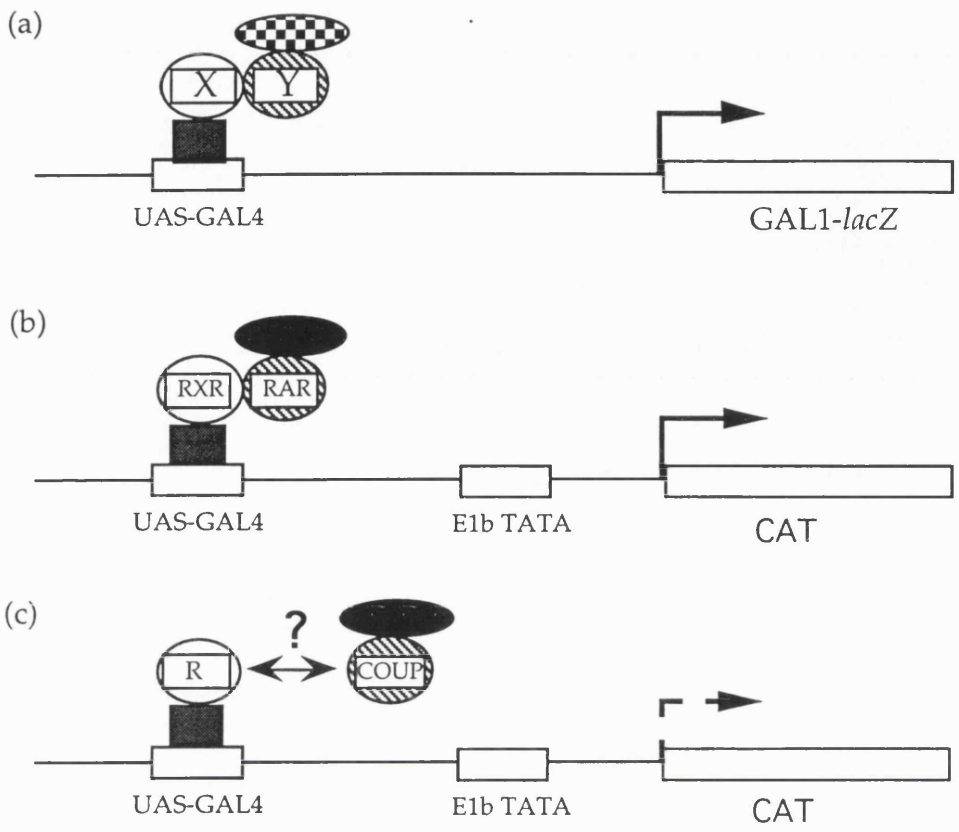
To investigate whether the COUP-TF II homodimers and heterodimers observed *in vitro* were also formed *in vivo* a two hybrid assay was used in transfected cells. The original two hybrid assay was devised by Fields and Song (Fields and Song 1989) to detect protein-protein interactions in the yeast *Saccharomyces cerevisiae*. The strategy of their system is outlined in Figure 4.7a and involves, as the name implies, two hybrid proteins, one containing the GAL4 DNA binding domain fused to protein X and the other containing the GAL4 activation domain fused to protein Y. Although neither chimeric protein can activate transcription alone, if protein X and protein Y interact the GAL4 activation domain is targeted to the GAL4 responsive reporter gene, *lac*Z. when the two proteins are coexpressed. The transcriptional activation of the *lac*Z reporter can be monitored by assaying for the production of β-galactosidase activity. The two hybrid strategy has since been applied to detect protein- protein interactions in mammalian cells with protein X fused to the GAL4 DNA binding domain, protein Y fused to the acidic activation domain of the herpes simplex virus transcriptional activator VP16 and a ~~GAL4 responsive~~ containing a GAL4 response element cloned upstream of the promoter reporter gene such as the chloramphenicol acetyltransferase (CAT) used to detect the interaction (Finkel *et al* 1993, Nagpal *et al* 1993, Tone *et al* 1994). To determine whether COUP-TF II could form homodimers and heterodimers *in vivo* the ability of COUP-TF II, RXRα and TRβ fused to GAL4 to recruit COUP-TF II tagged with the VP16 activation domain was examined. The assay was validated by demonstrating that the formation of heterodimers

between RXRα and RARα and between RXRα and TRβ could be detected confirming previous results (Nagpal *et al* 1993, Tone *et al* 1994). The assay is illustrated in Figure 4.7b and c. The chimeric receptor constructs used in the work described in this chapter and Chapter 6 are represented schematically in Figure 4.8.

Transient transfection assay

The results presented for this assay were obtained from transient transfection experiments using chicken embryo fibroblast cells (CEF) although similar results were obtained using HeLa cells. The DNA was transfected using the HBS-calcium phosphate coprecipitation method with a reporter gene plasmid pGE1bCAT (5 μg)(Lillie and Green 1989), containing one GAL4 site upstream of the adenovirus E1b TATA box linked to the CAT gene, an internal control plasmid pJ7lacZ (0.5 μg)(Morgenstern and Land 1990), the appropriate expression vectors (100 ng -1 μg) and pSG5 to a total of 10 μg/6 cm dish (see methods). Preliminary experiments were performed to determine the amount of input GAL4-RXR(DE) or GAL4-TR(DE) expression vector that gave a sub-maximal transcriptional response upon addition of their respective ligand. This was carried out to ensure that the amount of GAL4 fusion protein produced did not saturate the reporter gene plasmid. These results showed that the transcriptional activation obtained with 100 ng/dish of pGAL4-RXR(DE)/pGAL4-TR(DE) expression vector was within the linear range (data not shown). The fold induction was calculated by dividing the normalised CAT values obtained in the presence of the fusion proteins by that obtained with the reporter cotransfected with empty pSG5 expression vector ('reporter alone').

To judge if the levels of expression of the GAL4-fusion proteins were similar the expression plasmids (15 μg) and an internal control plasmid (pJ7lacZ, 1μg) were transiently transfected in COS-1 cells, the levels of expression in CEF cells being too low to allow detection. For each expression plasmid the transfected COS-1 cells were plated down on two 10 cm dishes and the cells from one plate harvested for determination of β-galactosidase activity and the second plate used to make whole cell extract. The β-galactosidase activities were used to correct for transfection efficiency and within a set of transfections were very similar. The protein concentration of each whole cell extract was determined and equal amounts of protein from each extract were analysed by gel shift analysis using an oligonucleotide probe that contained a GAL4 binding site (provided by P. Danielian,



GAL4 DNA binding domain (amino acids 1- 147)

GAL4 DNA binding site
UAS-GAL4

GAL4 activation domain

VP16 activation domain (amino acids 410- 490)

R Nuclear receptor (RXRα , TRβ or COUP-TF II)

Figure 4.7 Schematic representation of the two hybrid assay
(a) The Fields and Song two hybrid assay.
(b) Interaction of the fusion proteins GAL4-RXR and VP16RAR stimulates transactivation of the GAL4 responsive CAT reporter gene.
(c) Design of the two hybrid assay used to examine if COUP-TF II forms homodimers and/or heterodimers with RXRα or TRβ.

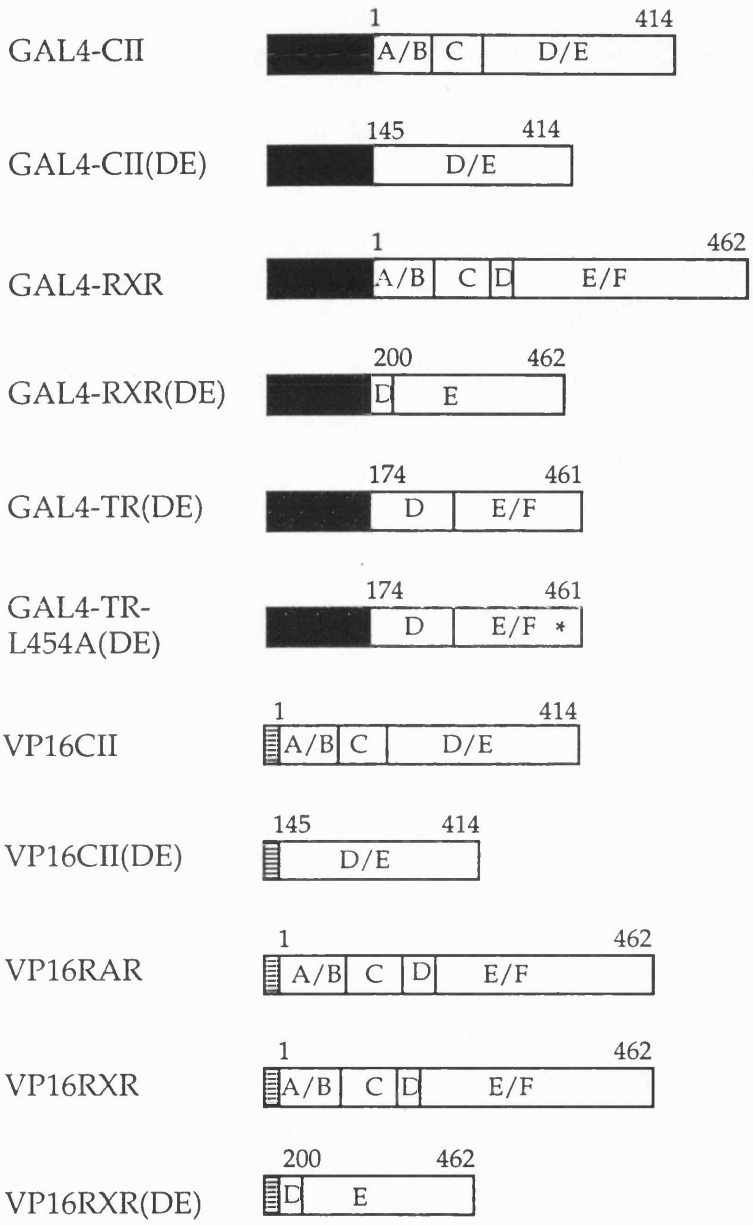


Figure 4.8 Schematic representation of the chimeric receptors used in the two hybrid assay.
The numbers indicate the amino acid position in the wild type protein, the shaded box represents the GAL4 DNA binding domain (amino acids 1-147) and the striped box represents the activation domain of VP16 (amino acids 410-490). The asterix (*) represents an amino acid substitution. CII; human COUP-TF II, RXR; human RXRα , TR; human TRβ, RAR; human RARα.

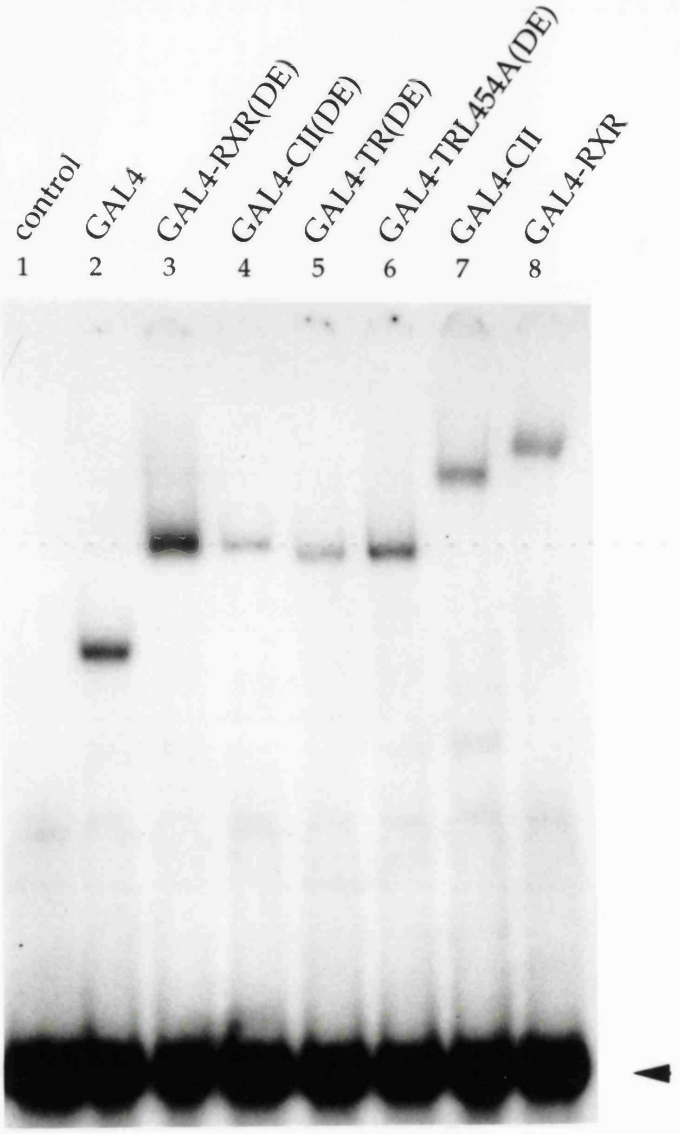


Figure 4.9 DNA binding activity of GAL4 chimeras.
Equal amounts of whole cell extracts (1 μg) from COS-1 cells transfected with expression vectors for the indicated GAL4 chimeric receptors (tracks 3-8), GAL4 (track 2) or control (no expression vector, track 1) were tested for DNA binding activity. The labelled DNA probe was an oligonucleotide containing a GAL4 binding site.
The arrowhead indicates the free probe.

Molecular Endocrinology Laboratory, ICRF). The amount of [^{32}P]-labelled oligonucleotide bound by each GAL4-fusion was quantified by phosphoimage analysis of the gel. The transfection was repeated three times and the mean value of the amount of [^{32}P]-labelled oligonucleotide bound for each of the GAL4-fusion proteins varied up to 4 fold. A typical gel shift analysis is shown in Figure 4.9. To assess the levels of expression of the VP16-fusion proteins the corresponding expression vectors were expressed in COS-1 cells and whole cell extracts prepared as described for the GAL4-fusion proteins. Equal amounts of protein from each extract were resolved by SDS-PAGE and subjected to western blotting using an antibody raised against the activation domain of VP16. Although the fusion proteins seemed to be expressed at similar levels the antibody also reacted with a number of non specific bands (data not shown). The amount of the VP16 fusion protein expression vectors to be used were therefore determined empirically and was routinely 1 μg/dish. This amount gave maximal transcriptional activation when cotransfected with the corresponding GAL4-fusion expression vector (100 ng). For example, 1 μg/dish of VP16RXR expression vector gave maximal activation when cotransfected with 100 ng/dish of GAL4-TR(DE) expression vector.

COUP-TF II homodimers can be detected *in vivo*

To investigate the formation of COUP-TF II homodimers *in vivo* expression vectors were constructed for the chimeric proteins GAL4-CII, comprising the DNA binding domain of GAL4 fused to full length COUP-TF II, and VP16CII, that consists of the acidic activation domain of VP16 fused to full length COUP-TF II (Figure 4.8 and appendices A6 and A11). The ability of GAL4-CII to recruit VP16CII and thereby stimulate transcription from the GAL4 responsive reporter was tested. The transcriptional activity of VP16CII or GAL4-CII alone was negligible when they were transiently transfected into CEF cells (Figure 4.10 tracks 1 and 3). This indicated that, under these conditions, GAL4-CII did not contain a constitutive activation domain and that VP16CII could not be recruited to the GAL4 responsive reporter non-specifically, through either its DNA binding domain or interaction with endogenous factors. However, expression of VP16CII did cause repression of the activity of the internal control reporter (pJ7lacZ) and therefore transfections that included VP16CII were normalised to the total amount of protein (see methods). Cotransfection of GAL4-CII and VP16CII resulted in an increase in transcription from the reporter indicating that the two fusion

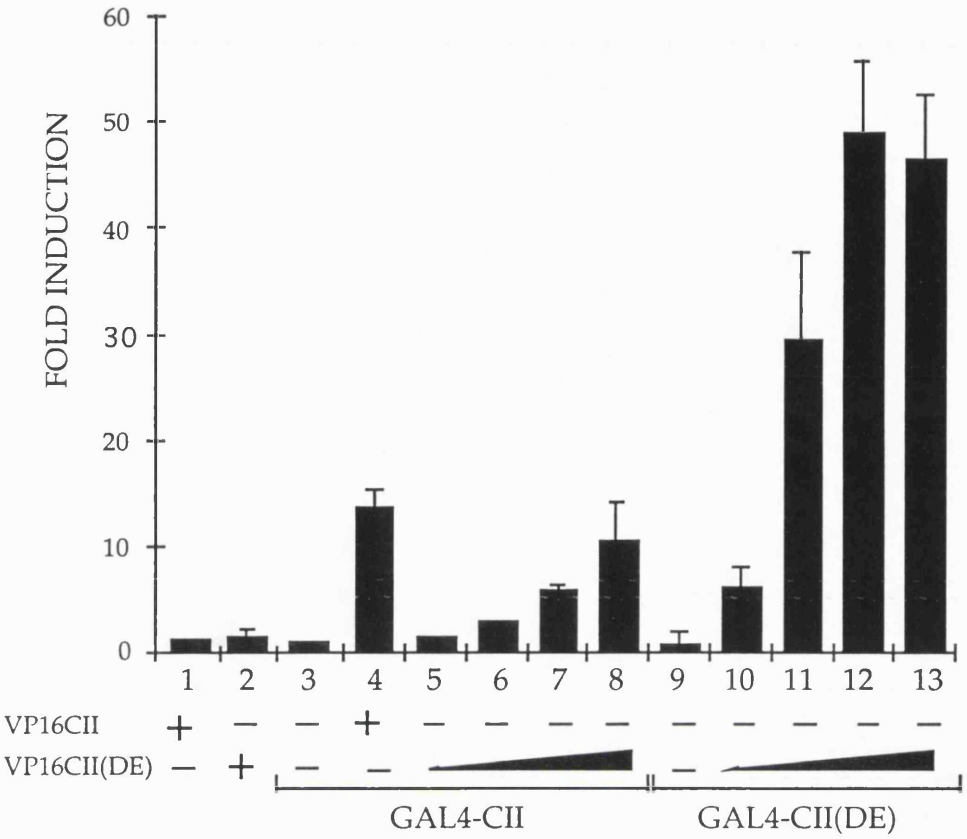


Figure 4.10 Formation of COUP-TF II homodimers in intact cells. CEF cells were transiently transfected with the GAL4 reporter pGE1BCAT (5 μg) and either 100 ng of GAL4-CII (tracks 3-8) or GAL4-CII(DE) (tracks 9-13) expression vectors. The effect of 1 μg of VP16CII expression vector was tested (track 4) and VP16CII(DE) was tested at increasing concentrations of expression vector, namely 1 ng (tracks 5 and 10), 10 ng (tracks 6 and 11), 100 ng (tracks 7 and 12) and 1000 ng (tracks 8 and 13). As a control, 1 μg VP16CII and VP16CII(DE) were transiently transfected alone (track 1 and 2 respectively). These results represent the mean values of at least three independent experiments each done in duplicate; the bars indicate standard deviations.

proteins could interact to recruit the VP16 acidic activation domain to the reporter (track 4).

Transfection of the expression vector, VP16CII(DE), that consists of the acidic activation domain of VP16 fused to the C-terminal region (DE) of COUP-TF II (Figure 4.8 and appendix A12) did not effect the transcriptional activity of the internal control vector (pJ7LacZ). Thus, transfections using this vector could be normalised for transfection efficiency using the β-galactosidase activity of the cell extract. Expression of VP16CII(DE) alone had a negligble effect on the transcriptional activity of the reporter (Figure 4.10 track 2). However, when both GAL4-CII and VP16CII(DE) were coexpressed there was a marked increase in transcription from the reporter that was dependent on the amount of input VP16CII(DE) expression vector, consistent with dimerisation of the chimeric proteins (Figure 4.10 tracks 5-8). Coexpression of either the GAL4 DNA binding domain and VP16CII(DE) or GAL4-CII and VP16 had no effect on transcription from the reporter (data not shown) suggesting that the recruitment of VP16CII(DE) was dependent on the presence of the COUP moiety in both fusion proteins. To confirm that this recruitment was mediated through the C-terminal region (DE) of COUP-TF II the chimeric protein GAL4-CII(DE) (Figure 4.8 and appendix A7) was tested and shown to be sufficient to recruit VP16CII(DE) and stimulate transcription (tracks 10-13). Thus the two hybrid data implies that COUP-TF II can form homodimers *in vivo* and that the (DE) region of this orphan receptor is sufficent to mediate the interaction. Coexpression of VP16CII(DE) with GAL4-CII(DE) stimulated transcription more than coexpression with GAL4-CII (compare tracks 6 and 11 and tracks 7 and 12). The increase, which was not due to differences in the amounts of the GAL4 fusion proteins as judged by gel shift analysis (Figure 4.9 tracks 4 and 7) suggests that GAL4-CII(DE) is able to recruit more VP16CII(DE). The reduced dimerisation by GAL4-CII may reflect an effect of the N-terminal and/or DNA binding domains on the conformation of the C-terminal dimerisation domain (DE).

RXRα/RARα but not RXRα/COUP-TF II heterodimers can be detected *in vivo*

To investigate the ability of COUP-TF II to form heterodimers with RXR the expression vector GAL4-RXR, comprising the GAL4 DNA binding domain fused to full length RXRα was constructed(Figure 4.8 and appendix A8). Transient expression of this chimeric receptor with reporter alone in CEF cells had no effect on reporter activity (Figure 4.11 track 2). This

confirmed that the treated serum used in these experiments lacked endogenous retinoids (see methods). Cotransfection of GAL4-RXR with increasing amounts of VP16CII(DE) (Figure 4.11 tracks 4-6) or VP16CII (data not shown) did not significantly increase the transcriptional activity of the reporter. This suggests that the RXR moiety in the GAL4 fusion could not interact with COUP-TF II to recruit the VP16 activation domain to the reporter.

In the previous section, cotransfection of VP16CII(DE) with a GAL4 chimera containing only the C terminal region of COUP-TF II, GAL4-CII(DE), had been shown to result in greater stimulation of the reporter than when full length COUP-TF II fused to GAL4, GAL4-CII, was used. The ability of the chimera GAL4-RXR(DE), containing only the C-terminal region of RXRα fused to the GAL4 DNA binding domain, was therefore tested (Figure 4.8 and appendix A9). As expected, expression of GAL4-RXR(DE) alone had a negligible effect on the transcriptional activity of the GAL4 responsive reporter (track 7). Coexpression of GAL4-RXR(DE) and VP16CII(DE) also had no significant effect on the reporter (Figure 4.11 tracks 8-11) indicating that GAL4-RXR(DE) was unable to recruit VP16CII(DE). To demonstrate that the dimerisation interface of RXR was functional within the chimera the expression vector VP16RAR, comprising full length RARα fused to the C-terminus of the acidic activation domain of VP16 (Figure 4.8 and appendix A13), was cotransfected with GAL4-RXR(DE). A 19 fold activation of the reporter was observed (Figure 4.11 track 12) confirming that RXR/RAR heterodimers can be detected using this assay, as previously reported (Nagpal *et al* 1993).

The transcriptional activity of GAL4-RXR(DE) obtained in the presence of 100 nM 9-*cis* retinoic acid was unaffected by cotransfection of VP16CII(DE) (Figure 4.11 tracks 13 and 14). It seems therefore, that there was no ligand dependent formation of heterodimers and moreover that VP16CII(DE) was not interacting with GAL4-RXR(DE) in such a manner as to sequester it off DNA. The failure to detect an interaction between GAL4-RXR(DE) and VP16CII(DE) indicated that in intact cells COUP-TF II and RXRα do not form heterodimers efficiently in solution.

Figure 4.11 RXRα /RARα but not RXRα/COUP-TF II heterodimers are detected in intact cells.

CEF cells were transiently transfected with the GAL4 reporter pGE1BCAT (5 μg) and 100 ng of GAL4-RXR expression vector in the absence (track 3) and presence of increasing amounts of VP16CII(DE), namely, 10 ng (track 4), 100 ng (track 5) and 1000 ng (track 6). GAL4-RXR(DE) expression vector (100 ng) was similarly tested by cotransfection with the GAL4 reporter pGE1BCAT (5 μg) in the absence (track 7) and presence of increasing amounts of VP16CII(DE), namely, 1 ng (track 8), 10 ng (track 9), 100 ng (track 10) and 1000 ng (tracks 11 and 14). As a positive control GAL4-RXR(DE) was cotransfected with
1 μg VP16RAR (track 12) and as negative controls, 1 μg VP16CII(DE) alone (track 1) or VP16RAR alone (track 2) were transiently transfected. The effect of 100 nM 9-*cis* retinoic acid (9-*cis* RA) on GAL4-RXR(DE) was tested in the absence and presence of 1 μg VP16CII(DE) (tracks 13 and 14). These results represent the mean values of at least three independent experiments each done in duplicate; the bars indicate standard deviations.

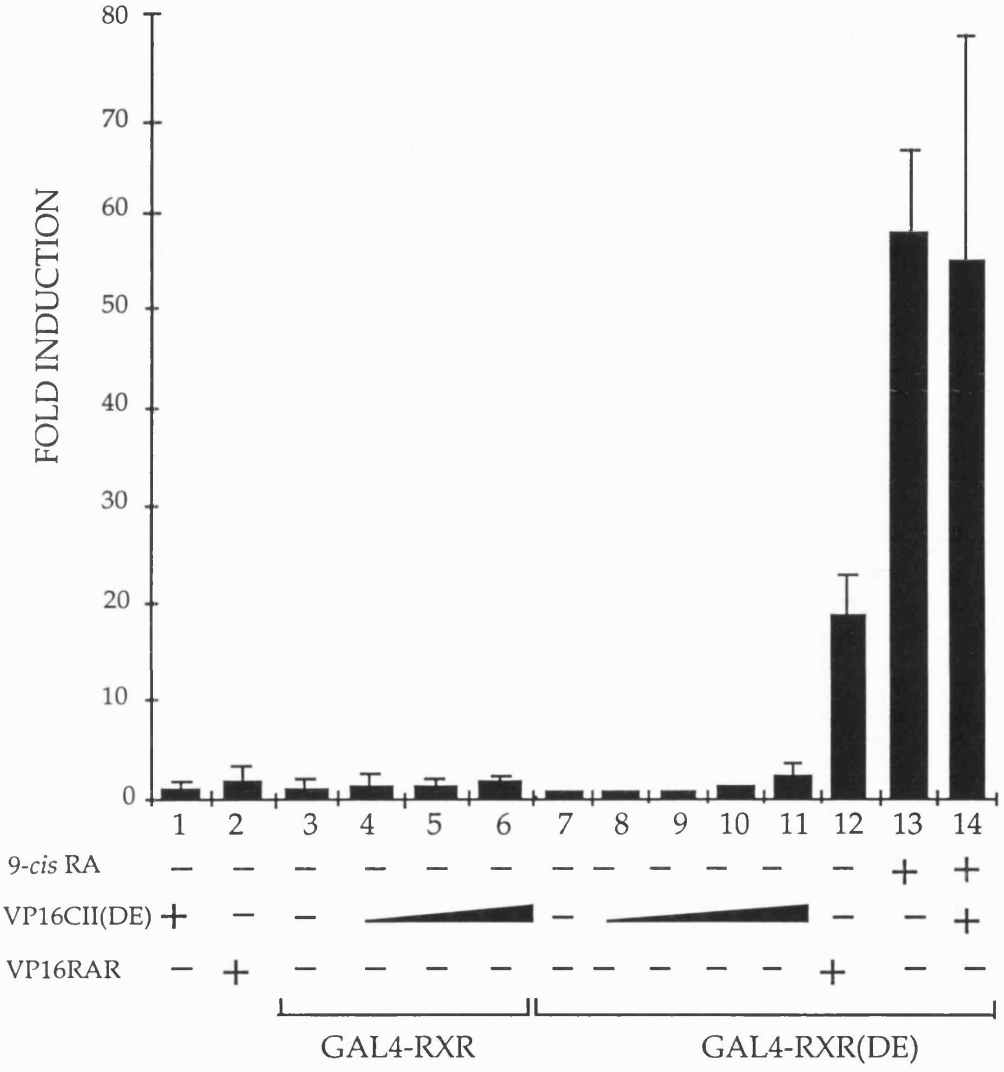


Figure 4.11 RXRα/ RARα but not RXRα/ COUP-TF II heterodimers are detected in intact cells.

RXRα/TRβ but not COUP-TF II/TRβ heterodimers are formed efficiently *in vivo*

Using the same approach, the ability of COUP-TF II to form heterodimers with TRβ *in vivo* was investigated. Expression of GAL4-TR(DE), comprising the GAL4 DNA binding domain fused to the C terminal region of TRβ (Figure 4.8)(Tone *et al* 1994), resulted in a reduction in activity of the reporter to 0.5 fold of the basal level (Figure 4.12a track 3). The decrease in reporter activity was more marked when a reporter gene containing 5 GAL4 binding sites was used (pG5E1bCAT) was used (see later). This negative effect on transcription by unliganded TR has been reported by others and postulated to be mediated by a silencing domain in the receptor (Baniahmad *et al* 1992a)(Baniahmad *et al* 1992b). Cotransfection of GAL4-TR(DE) and VP16CII(DE) had no effect on reporter activity (Figure 4.12a tracks 4-7) indicating that GAL4-TR(DE) failed to recruit VP16CII(DE). Cotransfection of GAL4-TR(DE) and VP16RXR, however, resulted in a 13 fold increase in transcription from the reporter (Figure 4.12a track 8). Hence GAL4-TR(DE) was able to recruit VP16RXR showing that TRβ could form heterodimers with RXRα in this assay, demonstrating that the TR dimerisation domain within the GAL4 fusion protein was functional.

In the presence of 200 nM T3, GAL4-TR(DE) stimulated transactivation 40 fold above the basal level (Figure 4.12b track 2) and this was further increased to 110 fold by cotransfection of VP16-CII(DE) (track 3). These data suggested that ligand dependent heterodimers were being formed between COUP-TF II and TRβ. To monitor the T3 dependent recruitment of VP16CII(DE) independently of the T3 induced TRβ transcriptional activity the TRβ mutant, L454A was used. This mutant retains its T3 binding activity and is able to recruit RXRα to a similar extent to that of the wild type receptor but is transcriptionally defective (Figure 4.12a track 11) (Tone *et al* 1994). It was predicted that if the increase in T3 dependent transactivation observed with cotransfection of VP16CII(DE) was due to ligand dependent recruitment of VP16CII(DE) by GAL4-TR(DE), a similar increase in transcription should be observed if GAL4-TRL454A(DE) was cotransfected with VP16CII(DE). As expected, the GAL4-TRL454A(DE) displayed only a weak transcriptional reponse to the addition of T3 stimulating transcription from approximately 0.3 to 1.4 fold induction. In contrast to the marked increase obtained with GAL4-TR(DE), cotransfection of VP16CII(DE) with GAL4-TRL454A in the presence of ligand enhanced the transcriptional

Figure 4.12 RXRα/TRβ but not COUP-TF II /TRβ-heterodimers are formed efficiently in intact cells.
(a) Transactivation in the absence of ligand. CEF cells were transiently transfected with the GAL4 reporter pGE1BCAT (5 μg) and either 100 ng of GAL4-TR(DE) (tracks 3-8) or 100 ng GAL4-TRL454A(DE) (tracks 9-11) expression vector in the absence (tracks 3 and 9) or presence of VP16CII(DE), 1 ng (track 4), 10 ng (track 5), 100 ng (track 6) and 1000 ng (track 7 and 10). As controls, 1000 ng of VP16CII(DE) (track 1) and VP16RXR (track 2), expression vectors were analysed and as positive controls GAL4-TR(DE) and GAL4-TRL454A(DE) were cotransfected with 1000 ng VP16RXR (tracks 8 and 11) expression vector.
(b) Transactivation in the presence of T3. CEF cells were transiently transfected with the reporter pGE1BCAT (5 μg) and 1000 ng of VP16CII(DE) expression vector (track 1), 100 ng of GAL4-TR(DE) expression vector (track 2) or 100 ng GAL4-L454A(DE) expression vector (track 4). 100 ng of GAL4-TR(DE) or GAL4-TRL454A(DE) were cotransfected with 1000 ng VP16CII(DE) (tracks 3 and 5, respectively). T3 was added at 200 nM to tracks 1-5.

These results represent the mean values of at least three independent experiments each done in duplicate; the bars indicate standard deviations.

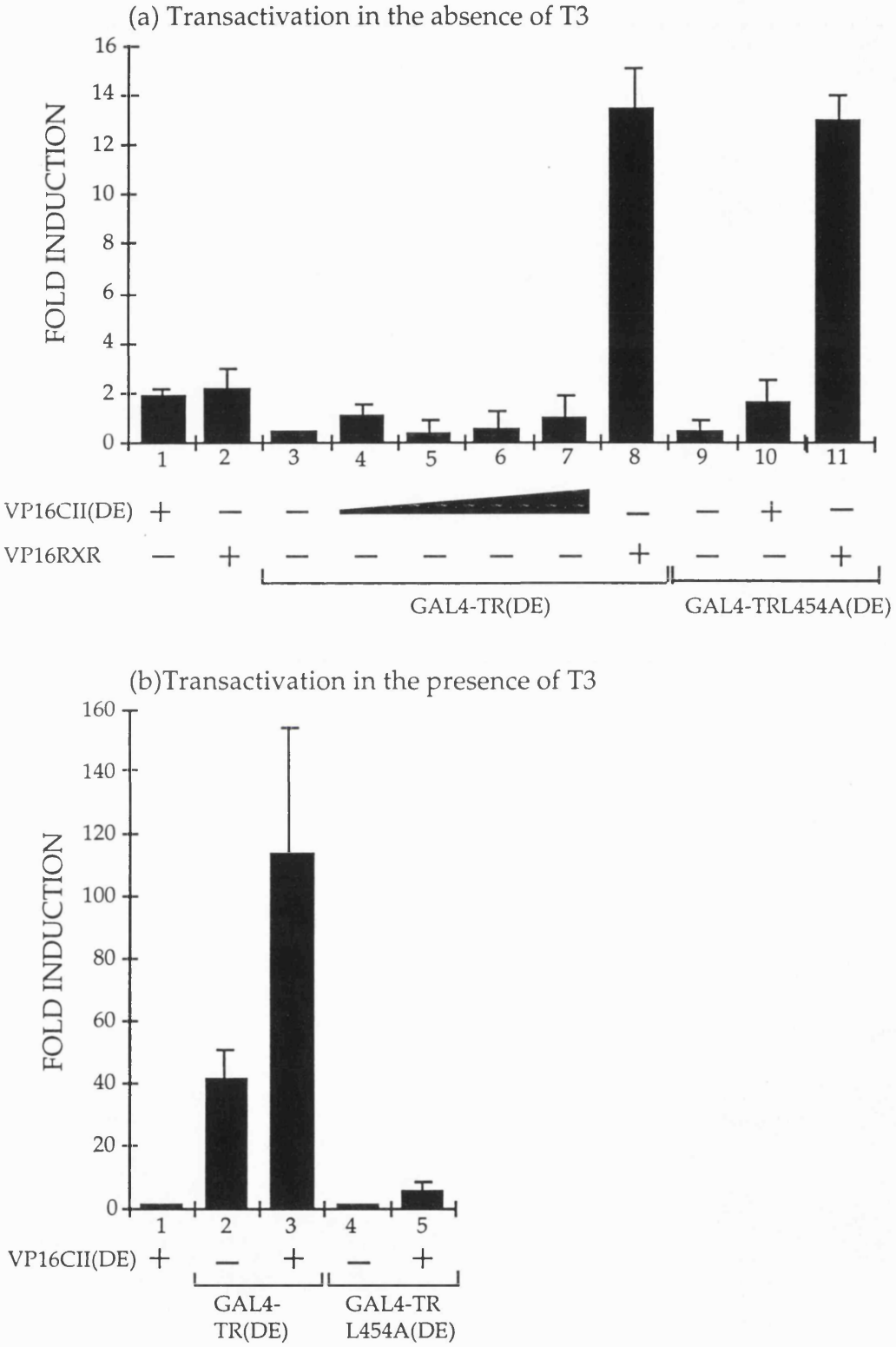


Figure 4.12 RXRα/TRβ but not COUP-TF II /TRβ-heterodimers are formed efficiently in intact cells.

activity of the reporter to only 5 fold above the basal level (Figure 4.12b tracks 4 and 5). This increase is presumably due to some T3 dependent recruitment of VP16CII(DE) but cannot explain the pronounced enhancement obtained with GAL4-TR(DE). This effect is presumably due to a synergistic effect on reporter transcription by the ligand activated TR and the VP16 activation domain.

Although the addition of T3 seemed to promote the recruitment of VP16CII(DE) by GAL4-TRL454A(DE) the activation observed was significantly less than that observed for the recruitment of VP16RXR(DE) in the presence of T3 (see Figure 6.2 track 7) or for the recruitment of VP16CII(DE) by GAL4-CII(DE) (Figure 4.10 track 13). It is likely therefore, that the formation of RXRα/TRβ heterodimers and COUP-TF II homodimers are favoured over COUP-TF II/TRβ heterodimers.

The deletion mutant CII(DE) does not inhibit transcriptional activation by GAL4-TR(DE)

The detection, albeit at low levels, of T3 dependent heterodimers between VP16CII(DE) and GAL-TR(DE) in the two hybrid assay raised the possibilty that COUP-TF II could form inactive heterodimers with liganded TRβ.in intact cells. To examine this potential the ability of COUP-TF II to inhibit T3 induced transcriptional activation by the GAL4-TR(DE) chimera was tested. Neither COUP-TF II nor the deletion mutant CIIDE, that contained the region of COUP-TF II present in the VP16CII(DE) fusion (appendix A15) repressed the T3 stimulation of reporter activity when coexpressed with GAL4-TR(DE) (Figure 4.13 track 7 and 5 respectively). Indeed, the T3 stimulation was enhanced (compare tracks 3, 5 and 7). The reason for this increase is unclear, but may be due to the titration of an inhibitory factor by COUP-TF II. Whatever the explanation, COUP-TF II and CII(DE) fail to repress the transcriptional activity of GAL4-TR(DE) suggesting that the T3 dependent interaction detected between the CII(DE) and TR(DE) domains in the two hybrid assay is insufficient to inhibit the ligand induced transcriptional activation of TRβ.

The deletion mutant CII(DE) does not inhibit 9-*cis* retinoic acid stimulated transactivation from the ApoAI-RARE

Mutants of RAR that have lost the ability to bind DNA but retain a functional C-terminal dimerisation domain have been demonstrated to act as dominant negative receptors (Durand *et al* 1994) and references therein).

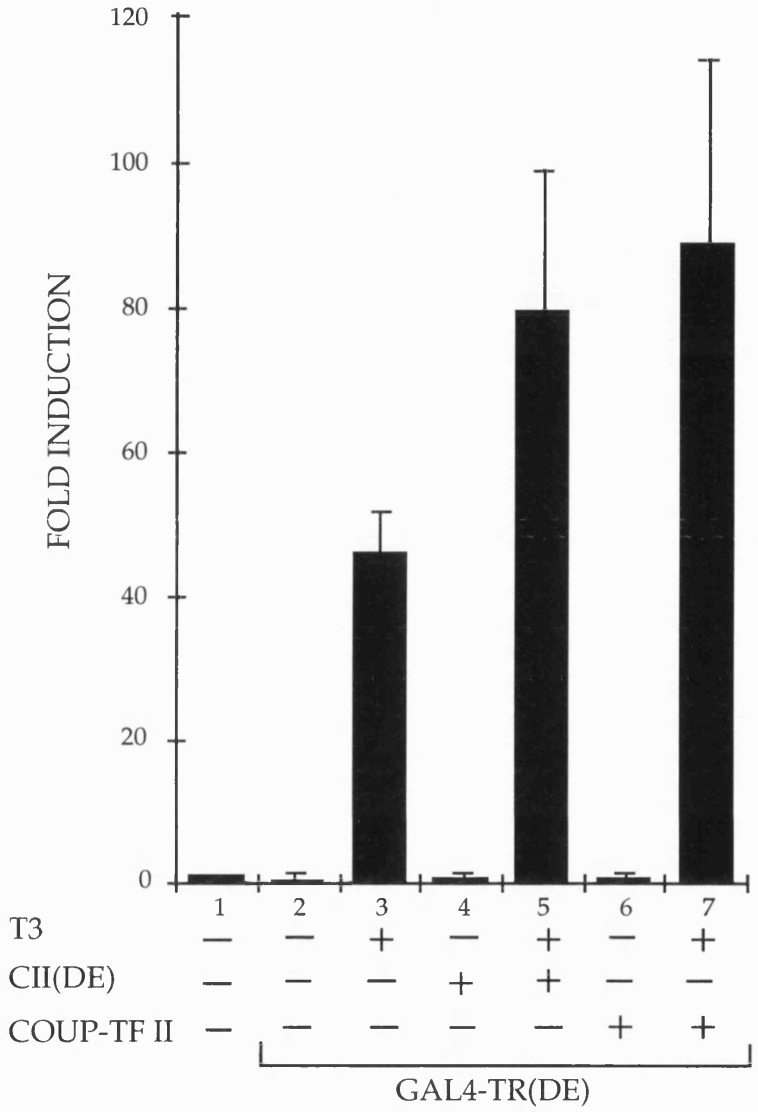


Figure 4.13 Cotransfection of COUP-TF II or CII(DE) enhances transactivation mediated by GAL4-TR(DE).
CEF cells were transiently transfected with the GAL4 reporter pGE1BCAT (5 μg) and 100 ng of GAL4-TR(DE) (tracks 2-7) in the absence (tracks 2 and 3) or presence of 1 μg of the expression vectors pSG5-CII(DE) (tracks 4 and 5) or pSG5-COUP-TF II (tracks 6 and 7). T3 was added at 200 nM to tracks 3, 5 and 7.
These results represent the mean values of three independent experiments each done in duplicate; the bars indicate standard deviations.

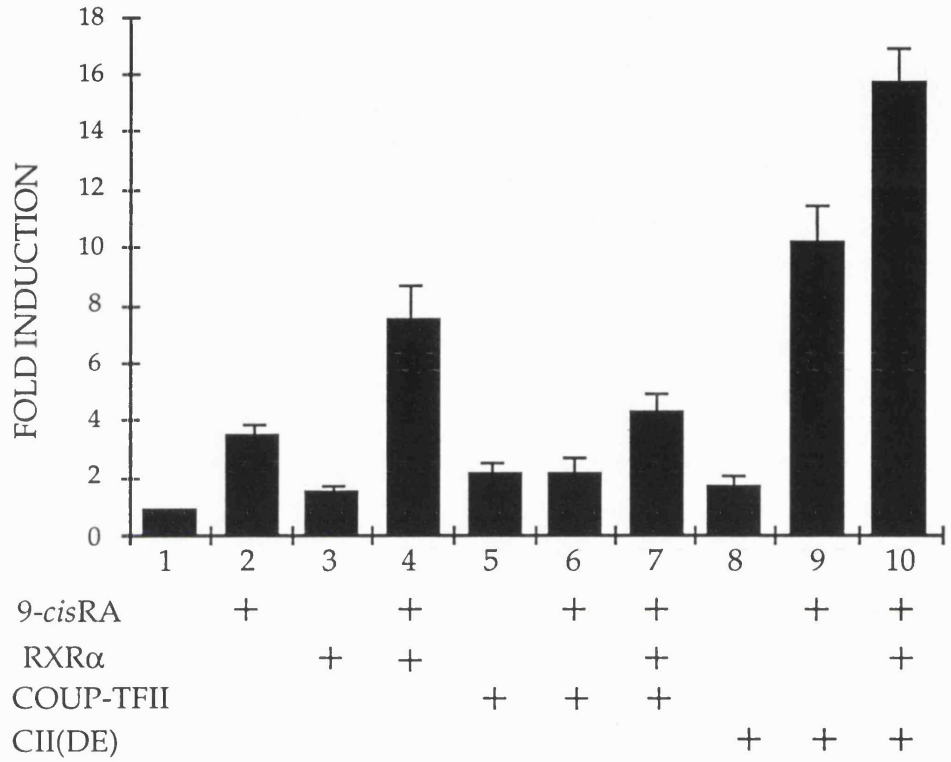


Figure 4.14 COUP-TF but not the mutant CII(DE) represses 9 *-cis* retinoic acid transactivation through the ApoAI-RARE.
CEF cells were transiently transfected with the reporter pGL2-ApoAI-RARE-TKLuc (5 μg) and expression vectors for RXRα (200 ng) COUP-TFII (2 μg) and CII(DE) (2 μg) as indicated. 9-*cis* retinoic acid (9-*cis* RA) was added at 50 nM as indicated. These results represent the mean values of at least three independent experiments each done in duplicate; the bars indicate standard deviations.

This inhibition of retinoic acid induced transcriptional activation is likely to be mediated by the sequestration of RXR into non DNA binding heterodimers, thereby preventing the formation of active RXR heterodimers with wild type RAR. The inability to detect COUP-TF II/RXR heterodimers in the two hybrid assay suggests that a non DNA binding form of COUP-TF II would be unable to act like the dominant negative RARs described above and sequester RXR off DNA. To test this hypothesis the deletion mutant CII(DE) was examined for its ability to repress 9-*cis* retinoic acid stimulated transcription. This mutant COUP-TF II retains the dimerisation interface but lacks the DNA binding domain. The effect of CII(DE) on transcription from the reporter plasmid pGL2-ApoAI-RARE-TKLuc (see appendix A17 for construction of reporters) was examined by transient transfection in CEF cells (Figure 4.14). Addition of 50 nM 9-*cis* retinoic acid stimulated transcription from the reporter approximately 3.5 fold (track 2). Cotransfection of the expression vector for RXRα (pSG5-RXRα) increased both the basal (to 1.6 fold, track 3) and ligand stimulated transcription (to 7.5 fold, track 4). As expected expression of wild type COUP-TF II repressed the 9-*cis* retinoic acid stimulation of the reporter (tracks 6 and 7). In contrast, the expression of the mutant CII(DE) not only failed to repress but actually enhanced the 9-*cis* retinoic acid stimulated transcription by both endogenous RARs and RXRs and the transiently transfected RXRα (tracks 9 and 10). The enhanced 9-*cis* retinoic acid stimulation in the presence of CII(DE) was unexpected and may be due to the formation of DNA binding deficient dimers between CII(DE) and endogenous COUP-TFs. These COUP-TF/CII(DE) dimers could sequester the COUP-TFs off the ApoAI-RARE and prevent them from competing with the endogenous retinoid reecptors or the coexpressed RXRα for binding to the reporter gene. The inability of CII(DE) to repress the 9-*cis* retinoic acid stimulated gene transcription showed that the dimerisation interface is insufficient to mediate repression and suggests that the DNA binding activity of COUP-TF II is required.

COUP-TF II does not recruit VP16RXR to an ApoAI-RARE or a DR+5 response element

The data from the two hybrid assay and experiments using the deletion mutant CII(DE) suggested that in intact cells COUP-TF II does not form heterodimers with RXRα or TRβ in solution. It is conceivable, however, that such heterodimers could be formed when the receptors are bound to DNA. This would contrast with the formation of RXR

heterodimers with RAR, TR, NGFI-B, NURR1 and LXR. These receptors can all form heterodimers with RXR in the absence of DNA, through the C-terminal interface, and can be detected using the two hybrid assay (Forman *et al* 1995, Nagpal *et al* 1993, Perlmann and Jansson 1995, Tone *et al* 1994, Willy *et al* 1995). To investigate the possibility that COUP-TF II heterodimers form on DNA *in vivo* the interaction between RXRα and COUP-TF II on DNA in intact cells was investigated. The ability of the two receptors to bind cooperatively was initially tested on the ApoAI-RARE. COUP-TF II/RXRα heterodimers have been reported to bind this site with an approximately ten fold greater affinity than either homodimer (COUP-TF II/RXRα K_d = 0.6 nM, COUP-TF II K_d = 5.2 nM and RXRα K_d = 8.8 nM) (Widom *et al* 1992) To investigate if the heterodimer also binds this site with greater affinity *in vivo*, the ability of coexpressed COUP-TF II to recruit VP16RXR to the reporter gene plasmid pGL2-ApoAI-RARE-TKLuc was determined. If the ApoAI-RARE is bound preferentially by COUP-TF II/RXRα heterodimers it would be expected that coexpression of COUP-TF II would increase the recruitment of VP16RXR to the reporter. Hence coexpression of COUP-TF II would result in increased stimulation of the reporter over that observed when VP16RXR is expressed alone. However, when a fixed amount of VP16RXR expression vector was cotransfected with an equal amount of COUP-TF II expression vector (pSG5-COUP-TF II) there was no increase in the transcriptional activity of the reporter above that seen with expression of VP16RXR alone (Figure 4.15a tracks 3 and 1 respectively). When ten fold more pSG5-COUP-TF II expresion vector was cotransfected the reporter activity was in fact decreased slightly (track 4). It seems therefore, that COUP-TF II does not recruit VP16RXR and the slight decrease presumably reflects partial displacement of VP16RXR by COUP-TF II homodimers. It has been reported that COUP-TF I can inhibit the VP16 activation domain when it is part of a GAL4-TR-VP16 fusion through dimerisation with the TR moiety (Casanova *et al* 1994) (discussed in Chapter 7). It is unlikely that COUP-TF II is acting in such a manner in this assay as the mutant CII(DE) (that retains the dimerisation domain but not the DNA binding domain) does not cause a decrease in reporter stimulation (data not shown).

To confirm that recruitment of VP16RXR by cotransfection of a partner receptor was possible in this assay the effect of cotransfection of RARα was assayed. RXRα/RARα heterodimers have also been shown to bind more efficiently to the ApoAI-RARE *in vitro* than either RXRα or RARα alone (Widom *et al* 1992). Unlike COUP-TF II, cotransfection of RARα

Figure 4.15 RARα but not COUP-TF II can recruit VP16RXR on DNA.
(a) VP16RXR recruitment on the ApoAI response element. CEF cells were transiently transfected with the reporter pGL2-ApoAI-RARE-TKLuc (5 μg) and expression vectors as indicated, VP16RXR (200 ng tracks 1, 3, 4, 6 and 7), COUP-TF II (200 ng track 3 and 2 μg tracks 2 and 4) and RARα (200 ng track 6 and 2μg tracks 5 and 7). These results represent the mean values of at least three independent experiments each done in duplicate; the bars indicate standard deviations.
(b) VP16RXR recruitment on a DR+5 response element. CEF cells were transiently transfected with the reporter pGL2-DR+5-TKLuc (5 μg) in the presence or absence of VP16RXR, COUP-TF II and RARα as described in (a). These results represent the mean values of at least three independent experiments each done in duplicate; the bars indicate standard deviations.

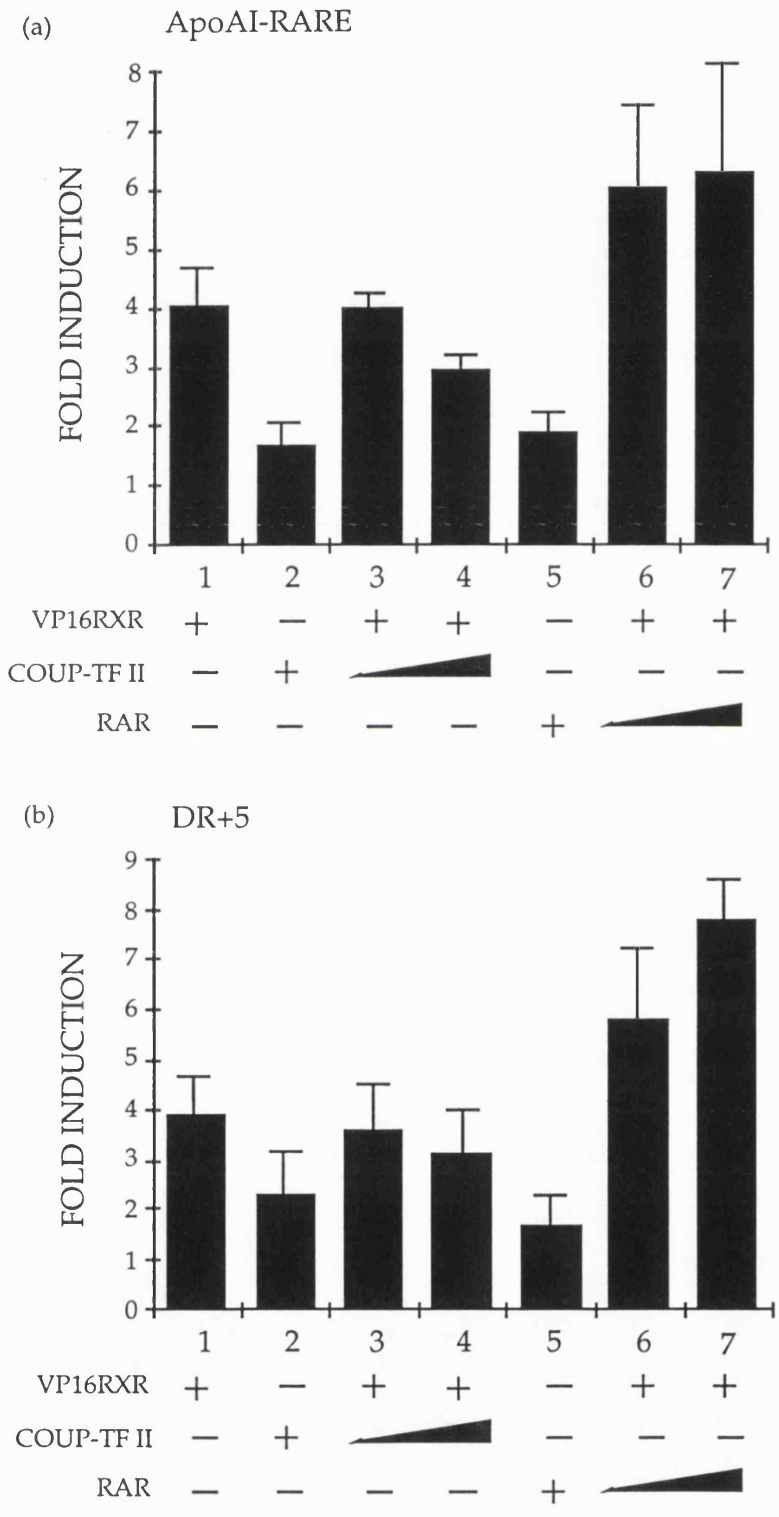


Figure 4.15 RARα but not COUP-TF II can recruit VP16RXR on DNA.

did result in an increase in reporter activity (Figure 4.15a tracks 6 and 7) implying that RARα can increase the amount of VP16RXR bound to the reporter.

The reporter gene plasmid pGL2-DR+5-TKLuc containing a DR+5 RARE, was also tested. This site binds RXR/RAR heterodimers preferentially over either homodimer *in vitro* (Figure 4.4 tracks 17, 19 and 21) (Yu *et al* 1991). COUP-TF I and II have been reported to repress retinoic acid stimulated transcription from the DR+5 element (Cooney *et al* 1993, Cooney *et al* 1992, Kadowaki *et al* 1992) and can bind *in vitro* to the response element as a homodimer (Cooney *et al* 1992, Kadowaki *et al* 1992) and as a heterodimer with RXRα (Figure 4.4 track 18). Cotransfection of COUP-TF II failed to recruit VP16RXR to the DR+5 element (Figure 4.15b tracks 3 and 4) although, as before, cotransfection of RARα did stimulate transcription. Similar results were also obtained with the reporter gene plasmid pGL2-DR+1-TKLuc containing a DR+1 element (data not shown). These data suggest that although COUP-TF II can form heterodimers with RXRα on the ApoAI-RARE and DR+5 response elements *in vitro,* homodimers are preferentially formed in intact cells. Such an interpretation favours a model for COUP-TF mediated repression that involves COUP-TF homodimers competing for binding to DNA rather than the formation of inactive COUP-TF heterodimers.

Silencing of transcription by COUP-TF II

The previous work in this chapter was designed to investigate the mechanism by which COUP-TF mediates passive repression of the transcriptional activity of other nuclear receptors. In the following section the ability of COUP-TF II to function as an active repressor of gene transcription will be described.

Repression of basal level transcription by GAL4-CII(DE)

It has been shown that an active transcriptional silencing domain located within the C-terminus of TRβ can be transferred to the heterologous DNA binding domain GAL4 and repress basal transcription from a GAL4 responsive reporter gene (Baniahmad *et al* 1992a). To examine whether COUP-TF II possessed a similar silencing function the effect of GAL4-CII(DE) on basal transcription was investigated. Initial experiments carried out while studying the ability of COUP-TF II to form homodimers and heterodimers in intact cells showed that expression of GAL4-CII(DE) (or GAL4-CII) did not

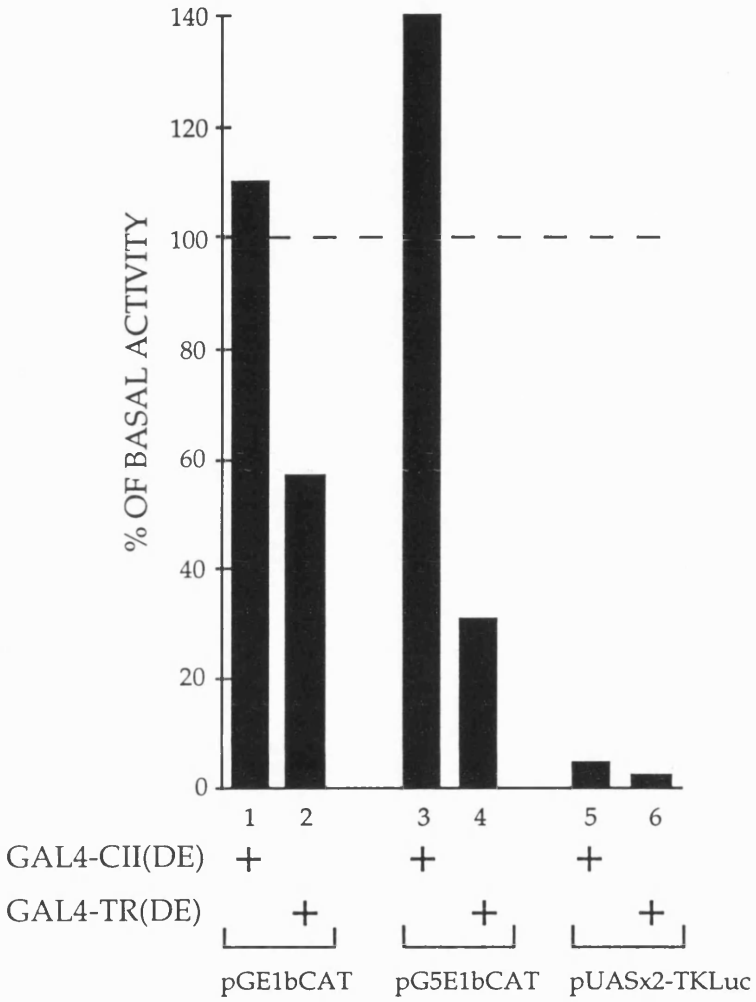


Figure 4.16 Comparison of the silencing activity of GAL4-CII(DE) and GAL4-TR(DE) on the reporters pGE1bCAT, pG5E1bCAT and pUASx2-TKLuc.

CEF cells were transiently transfected with 5 μg of the GAL4 reporter pGE1BCAT (tracks 1 and 2), pG5E1bCAT (tracks 3 and 4) or pUASx2-TKLuc (tracks 5 and 6) in the presence of 100 ng of GAL4-CII(DE) (tracks 1, 3 and 5) or 100 ng GAL4-TR(DE) (tracks 2, 4 and 6). The transcriptional modulation of the reporter is expressed as a percentage of the transcriptional activity of the reporter in the absence of cotransfected GAL4 expression vector. These results were derived from two independent experiments each done in duplicate. The relative levels of transcription varied less than 25%.

significantly alter the basal level of transcription from the pGE1bCAT reporter (Figure 4.16 track 1 and Figure 4.10). Expression of GAL4-TR(DE) under similar conditions repressed basal transcription of the reporter by approximately 55% (Figure 4.16 track 2 and Figure 4.12a). Two other reporters was also tested, the reporter gene plasmid, pG5E1bCAT, containing 5 multimerised GAL4 sites upstream of the adenovirus E1b TATA box linked to the CAT gene and the reporter gene plasmid, pUASx2-TKLuc, containing two GAL4 sites upstream of the herpes simplex virus thymidine kinase (TK) promoter linked to the luciferase gene. As shown in Figure 4.16, the ability of GAL4-CII(DE) and GAL-TR(DE) to repress basal transcription was tested by transient transfection in CEF cells. Expression of GAL4-TR(DE) resulted in repression of transcription from the pG5E1bCAT reporter to 30% of the basal level and to 2% of the basal level from the pUASx2-TKluc reporter (tracks 4 and 6 respectively). Expression of GAL4-CII(DE) also resulted in repression of basal level transcription from the pUASx2-TKluc reporter to approximately 5% of the basal level (track 5). In contrast, GAL4-CII(DE) stimulated transcription from the pG5E1bCAT reporter to approximately 140 % of the basal level (track 3).

These data suggest that the C-terminal domain of COUP-TF II contains a transcriptional silencing domain that acts in a promoter specific manner being active on the TK promoter based reporter (pUAS-TKluc) but not on the adenovirus E1b TATA box based reporters (pG5E1bCAT/pGE1b-CAT).

Effect of COUP-TF II on basal transcription

Next, the ability of the C-terminus of COUP-TF II to function as a transcriptional silencer in the context of the wild type protein was analysed. The ability of COUP-TF II to repress basal transcription was examined in a transient transfection assay. A variety of different reporter gene plasmids were tested that contained either one or two COUP-TF II binding sites upstream of the TK promoter linked to the luciferase gene (see appendix A17). Cotransfection of larger amounts (500 ng) of the COUP-TF II expression vector (pSG5-COUP-TF II) repressed expression of the β-galactosidase expression vector, pJ7lacZ. The cell extracts from this set of transfection experiments were therefore normalised for protein content rather than β-galactosidase activity. Cotransfection of 50 ng of the expression vector for

COUP-TF II in CEF cells[1] resulted in a modest, 1.7 fold, increase in the transcriptional activity of the parental reporter plasmid that lacks an inserted response element, pGL2-TKluc (Figure 4.17a track 2). This suggests that COUP-TF II can weakly stimulate transactivation from the TK promoter, perhaps by binding to a cryptic site in the vector or alternatively by sequestering a repressor that targets the promoter. In contrast, cotransfection of 500 ng of pSG5-COUP-TF II repressed the reporter activity to 0.6 fold of the basal level (track 3). This inhibtion may be due to the overexpressed COUP-TF II squelching a factor necessary for transcription. However, this interpretation is complicated by the observation that transcription from the plasmid pJ7lacZ, which contains the cytomegalovirus (CMV) promoter linked to the β-galactosidase gene, was simultaneously increased.

Cotransfection of the COUP-TF II expression vector with the pGL2-TKLuc reporter plasmid containing either a TREpal element, a DR+5 element or one or two copies of the ApoAI-RARE gave essentially the same result as with the parental reporter (Figure 4.17a). In contrast, transcriptional activation of the reporter was decreased upon cotransfection of 50 ng of the COUP-TF II expression vector when either a DR+1 element or two copies of a DR+5 element were upstream of the promoter (tracks 5 and 14 respectively). Similar results were obtained using the cell line JEG-3 (data not shown). It is possible that the transcriptional properties of COUP-TF II are influenced by the type of response element bound, however, the effects observed in these experiments are modest and any interpretation must be necessarily cautious.

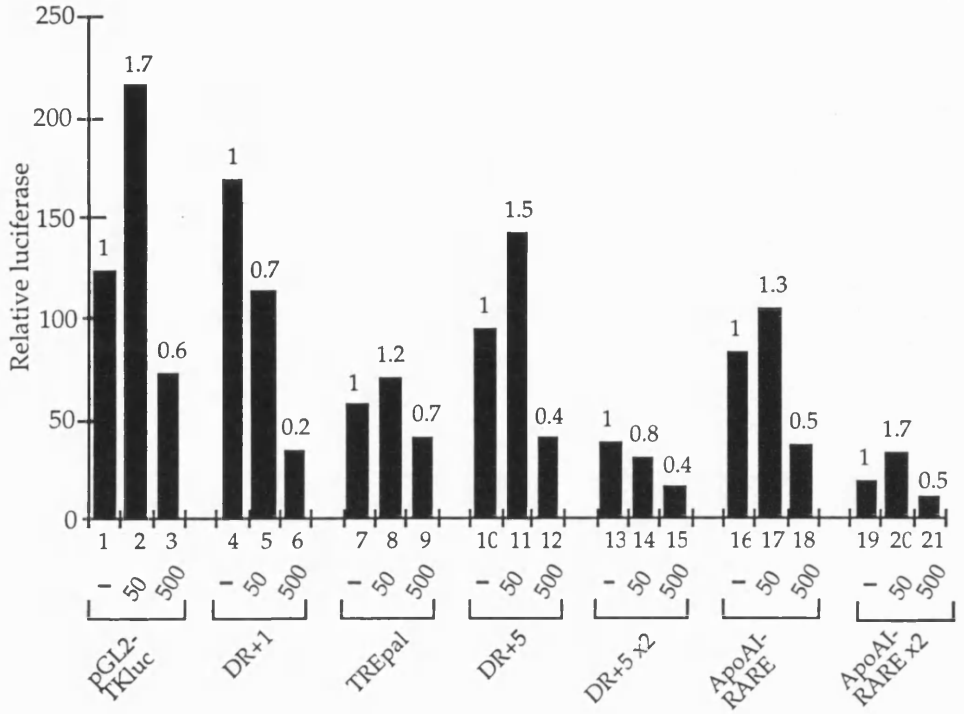
The transcriptional activity of COUP-TF II was also tested in Hela cells. Cotransfection of 50 ng of COUP-TF II expression vector resulted in stimulation of transcriptional activation from all the reporters tested, including the parental reporter plasmid, pGL2-TKLuc (Figure 4.17b). Cotransfection of 500 ng of COUP-TF II expression vector caused a further increase in the transcriptional activation. In addition, expression from the CMV promoter of the reporter plasmid pJ7lacZ was also increased. Thus, expression of COUP-TF II in HeLa cells appears to stimulate transcription from both the TK and CMV promoters in the absence of a defined binding site.

[1] In this series of transfections CEF cells were transfected with a total of 1.6 μg DNA in 24 well microtitre plates using the BBS calcium phosphate coprecipitation protocol, as for the HeLa cells (see methods).

Figure 4.17 Analysis of COUP-TF II transcriptional activity.
The ability of COUP-TF II to modulate transcription was tested in transient transfection experiments using (a) CEF cells and (b) HeLa cells. Reporter gene plasmids were used that contained either no insert (parental reporter gene plasmid, pGL2-TKluc) or characterised COUP-TF II binding sites as indicated. The CEF and Hela cells were transfected[2] with 1 μg of reporter plasmid either alone or with the COUP-TF II expression vector (pSG5-COUP-TF II), 50ng (tracks 2, 5, 8, 11, 14, 17 and 20) or 500ng (tracks 3, 6, 9, 12, 15, 18 and 21). In all transfection experiments the luciferase values were normalised using the protein content of the cell extract. For each reporter construct tested the fold inductions were calculated by dividing the corrected luciferase value observed in the presence of cotransfected COUP-TF II expression vector by that observed in the absence and are indicated above the bars. The results shown were derived from two independent experiments each carried out in duplicate. The levels of transcription stimulated for each point varied less than 20%.

[2] Note in these experiments CEF cells were transfected using the BBS calcium phosphate precipitation method rather than the HBS method with a total of 1.6 μg DNA in 24 well microtitre plates (see methods).

(a) CEF cells



(b) HeLa cells

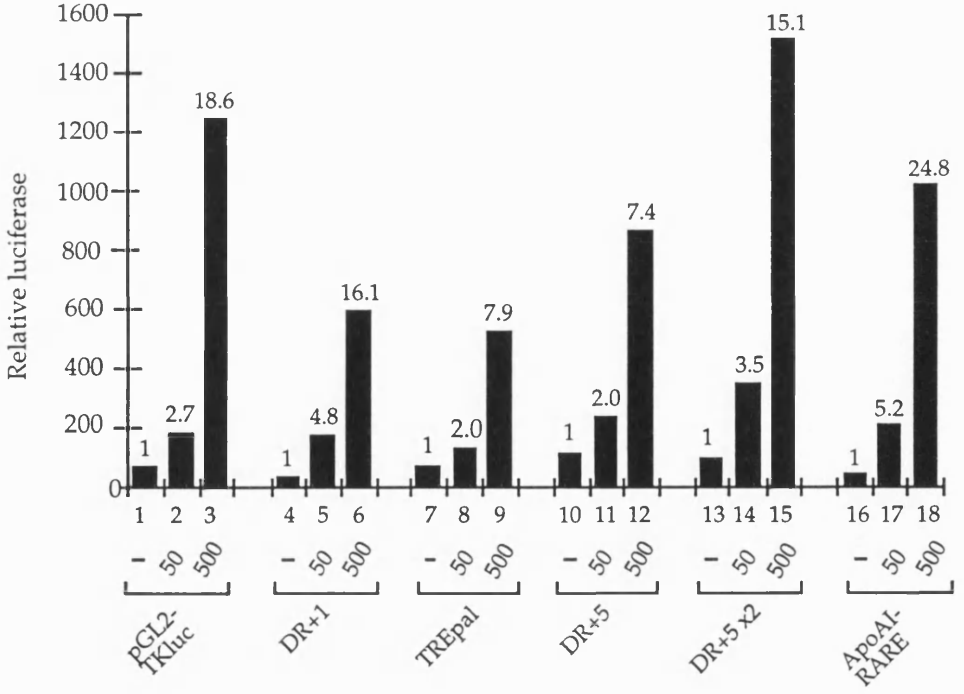


Figure 4.17 Analysis of COUP-TF II transcriptional activity.

From these transfection experiments it seems that COUP-TF II can modulate transcription from both the TK and CMV promoters. These effect can be observed in the absence of a defined COUP-TF II response element and are cell type specific. These responses may be due to the binding of COUP-TF II to crytic sites in the reporter gene plasmids or could occur off DNA, perhaps by squelching a cofactor involved in transcription.

Summary and conclusions

Using gel shift analysis COUP-TF II was shown to form both homodimers and heterodimers with RXRα and TRβ *in vitro*. The COUP-TF II/RXRα heterodimer was observed on all the response elements tested but formed most strongly on the DR+1 site. The COUP-TF II/TRβ heterodimer was detected on a TREpal but not on a DR+4 element. COUP-TF II homodimers bound with high affinity to all the sites tested (discussed in Chapter 5). These data illustrate that, potentially, COUP-TF II could function *in vivo* as a homodimer and/or as a heterodimer. To assess the formation of COUP-TF II homodimers and heterodimers *in vivo* the interaction of COUP-TF II, TRβ and RXRα were investigated using a two hybrid assay in CEF cells.

In the two hybrid assay COUP-TF II homodimers and RXRα/RARα heterodimers were detected but a stable interaction between COUP-TF II and RXRα was not observed, regardless of the addition of 9-*cis* retinoic acid. It is therefore unlikely that COUP-TF II sequesters RXRα off DNA. Similarly, under conditions that allowed the detection of RXRα/TRβ heterodimers COUP-TF II/TRβ heterodimers were not formed in the absence of T3. Although the addition of T3 promoted the formation of COUP-TF II/TRβ heterodimers it seems that COUP-TF II homodimers and RXRα/TRβ heterodimers are favoured as they generated higher levels of transcriptional activation in this assay.

The inability to detect the formation of COUP-TF II/RXRα heterodimers in the two hybrid assay conflicted with the formation of heterodimers in the gel shift assay. One possible explanation for this discrepancy is that the heterodimers are only formed when the receptors are bound to DNA. The importance of the DNA binding domain of COUP-TF II was also suggested by the observation that the mutant CII(DE), that lacks the DNA binding domain was unable to repress 9-*cis* retinoic acid mediated transactivation. However, in transiently transfected cells, RARα but not COUP-TF II was able to recruit VP16 tagged RXRα to an RARE containing

reporter. This suggests that although COUP-TF II can form heterodimers with RXRα *in vitro* homodimers are preferentially formed *in vivo* .

The C-terminus of COUP-TF II fused to the DNA binding domain of GAL4 was shown to repress basal level transcription from a reporter containing the TK promoter but not from a reporter containing the adenovirus E1b TATA box. It is unclear whether in the context of the wild type protein bound to a response element the C-terminal domain can actively repress transcription as transient expression of COUP-TF II in CEF and Hela cells modulated the transcriptional activity of both the TK and CMV promoters in the absence of defined binding sites.

CHAPTER 5

DNA BINDING PROPERTIES OF COUP-TF II

Introduction

COUP-TF I and II can bind to a diverse array of response elements such as the DR+1 type response element in the chicken ovalbumin promoter, DR+2 type response element in the apolipoprotein AI promoter, DR+6 type response element in the rat insulin promoter and the inverted repeat separated by 9 nucleotides in the HIV-1 LTR (Cooney *et al* 1991, Hwung *et al* 1988, Ladias *et al* 1992, Orchard *et al* 1992, Sagami *et al* 1986). Such degenerate binding site preference is unusual and suggests a remarkably flexible mode of DNA binding by the COUP-TF homodimer. This chapter describes the characterisation of the DNA binding activity of COUP-TF II and identification of the dimerisation interfaces formed on the different types of response element.

COUP-TF II homodimers can bind to response elements differing in the spacing and orientation of the half site sequence AGGTCA

A number of nuclear receptors recognise response elements that contain two (A/G)GGTCA motifs (see Chapter 1), the ability of COUP-TF II homodimers to bind to response elements with different arrangements of the half site sequence AGGTCA was assessed *in vitro* using competitive gel shift analysis. A series of oligonucleotides were synthesised (provided by I. Goldsmith, ICRF) that contained either direct repeats of the nucleotide sequence AGGTCA with different spacings or an inverted repeat with 0 spacing (Table 5.1). The DR+1 element was labelled with [^{32}P]-dCTP and used as the probe in a series of gel shift assays. The effect of coincubation of five, twenty and one hundred fold excess of unlabelled competitor oligonucleotide on the amount of DR+1 probe bound by *in vitro* synthesised COUP-TF II was tested and a representative gel shift is shown in Figure 5.1. A retarded complex was observed when *in vitro* synthesised COUP-TF II was incubated with the DR+1 probe alone (Figure 5.1 track 3) and this complex was not observed when unprogrammed reticulocyte lysate was used (track 2). The DR+1 and DR+2 competitor oligonucleotides were the most effective at diminishing the COUP-TF II-DNA complex (tracks 7-9 and 10-12) suggesting that COUP-TF II recognised these sites with the greatest affinity. The oligonucleotides containing DR+0, DR+3, DR+4 and DR+5 sites all competed for binding of COUP-TF II to a similar degree as did the oligonucleotide containing the TREpal element (tracks 4-6 and 13-24). These data imply that COUP-TF II homodimers can recognise response elements composed of two

Table 5.1 Sequences of the oligonucleotide pairs used in gel shift analysis. The half site sequences are highlighted in bold type. The DR+ series and the TREpal oligonucleotides contain two copies of the consensus half site sequence AGGTCA. The CDE2 oligonucleotide contains a single GGGTCA half site. The ApoAI-RARE oligonucleotide contains the sequence from position -214 to -192 of the human apolipoprotein AI gene (Ladias and Karathanasis 1991). The underlined sequence indicates a putative half site not required for COUP-TF binding. The ovalbumin-RARE oligonucleotide contains the sequence from position -91 to -66 of the human ovalbumin gene (Hwung*et al* 1988). The β-RARE oligonucleotide contains the sequence from position -58 to -32 of the human RARβ gene (de The*et al* 1990). The -210 21OH FRE oligonucleotide contains the sequence from position -215 to -197 of the mouse steroid 21-hydroxylase (*Cyp*-21) gene (Schimmer and Parker 1992). The ERE oligonucleotide contains the sequence from -338 to -313 of the *Xenopus laevis* vitellogenin A2 gene containing a consensus ERE (Klein-Hitpass*et al* 1986).

DR+0	5'-CTAGTCTATG**AGGTCAAGGTCA**AACTTC-3' 3'-AGATAC**TCCAGTTCCAGT**TTGAAGGATC-5'
DR+1	5'-CTAGTCTATG**AGGTCAAAGGTCA**AACTTC-3' 3'-AGATAC**TCCAGTTTCCAGT**TTGAAGGATC-5'
DR+2	5'-CTAGTCTATG**AGGTCA**AG**AGGTCA**AACTTC-3' 3'-AGATAC**TCCAGT**TC**TCCAGT**TTGAAGGATC-5'
DR+3	5'-CTAGTCTATG**AGGTCA**AGGA**GGTCA**AACTTC-3' 3'-AGATAC**TCCAGT**TCC**TCCAGT**TTGAAGGATC-5'
DR+4	5'-CTAGTCTATG**AGGTCA**CAGG**AGGTCA**AACTTC-3' 3'-AGATAC**TCCAGT**GTCC**TCCAGT**TTGAAGGATC-5'
DR+5	5'-CTAGTCTATG**AGGTCA**CCAGG**AGGTCA**AACTTC-3' 3'-AGATAC**TCCAGT**GGTCC**TCCAGT**TTGAAGGATC-5'
TREpal	5'-CTAGTCTATG**AGGTCATGACCT**AACTTC-3' 3'-AGATAC**TCCAGTACTGGA**TTGAAGGATC-5'
CDE2	5'-TCGACCCGCG**GGGTCA**GCCCGGCCCAGCTGCA-3' 3'-GGGCGC**CCCAGT**CGGGCCGGGTCG-5'
ApoAI-RARE	5-CTAGACTCAGGGCAG**GGGTCAAGGGTTCA**GTTCGAT-3' 3-TGAGTCCCGTC**CCCAGT**TC**CCAAGT**CAAGCTAGATC-5'
Ovalbumin-RARE	5'-CTAGTCTATG**GTGTCAAAGGTCA**AACTTC-3' 3'-AGATAC**CACAGTTTCCAGT**TTGAAGGATC-5'
β-RARE	5'-CTAGGTAG**GGTTCA**CCGAA**AGTTCA**CTCGC-3' 3'-CATC**CCAAGT**GGCTT**TCAAGT**GAGCGGATC-5'
-210 21OH FRE	5'-CTAGTCTATGACAGAG**AGGTCA**GGGTCTTC-3' 3'-AGATACTGTCTC**TCCAGT**CCCAGAAGGATC-5'
ERE	5'-CTAGAAAGTC**AGGTCA**CAG**TGACCT**GATCAAT-3' 3'-TTTCAG**TCCAGT**GTC**ACTGGA**CTAGTTAGATC-5'

Table 5.1 Sequences of the oligonucleotide pairs used in gel shift analysis.

Figure 5.1 Competition for COUP-TF II binding by oligonucleotides containing variations in the spacing and orientation of the AGGTCA half site.

The ability of a series of oligonucleotides to compete with a labelled DR+1 containing oligonucleotide for COUP-TF II binding was analysed by gel shift analysis. Equal amounts of *in vitro* synthesised COUP-TF II were preincubated in the presence (tracks 4-24) or absence (track 3) of unlabelled oligonucleotides containing variations in the orientation and spacing of the AGGTCA half site sequence as indicated prior to the addition of [^{32}P] labelled oligonucleotide containing a DR+1 site. The competitor oligonucleotides were added in 5 fold (tracks 4, 7, 10, 13, 16, 19 and 22), 20 fold (tracks 5, 8, 11, 14, 17, 20 and 23) and 100 fold (tracks 6, 9, 12, 15, 18, 21 and 24) excess. As a control unprogrammed lysate was also incubated with the labelled oligonucleotide (track 2). The position of the COUP-TF II homodimer is indicated by a solid arrowhead and the asterix indictaes a degradation product.

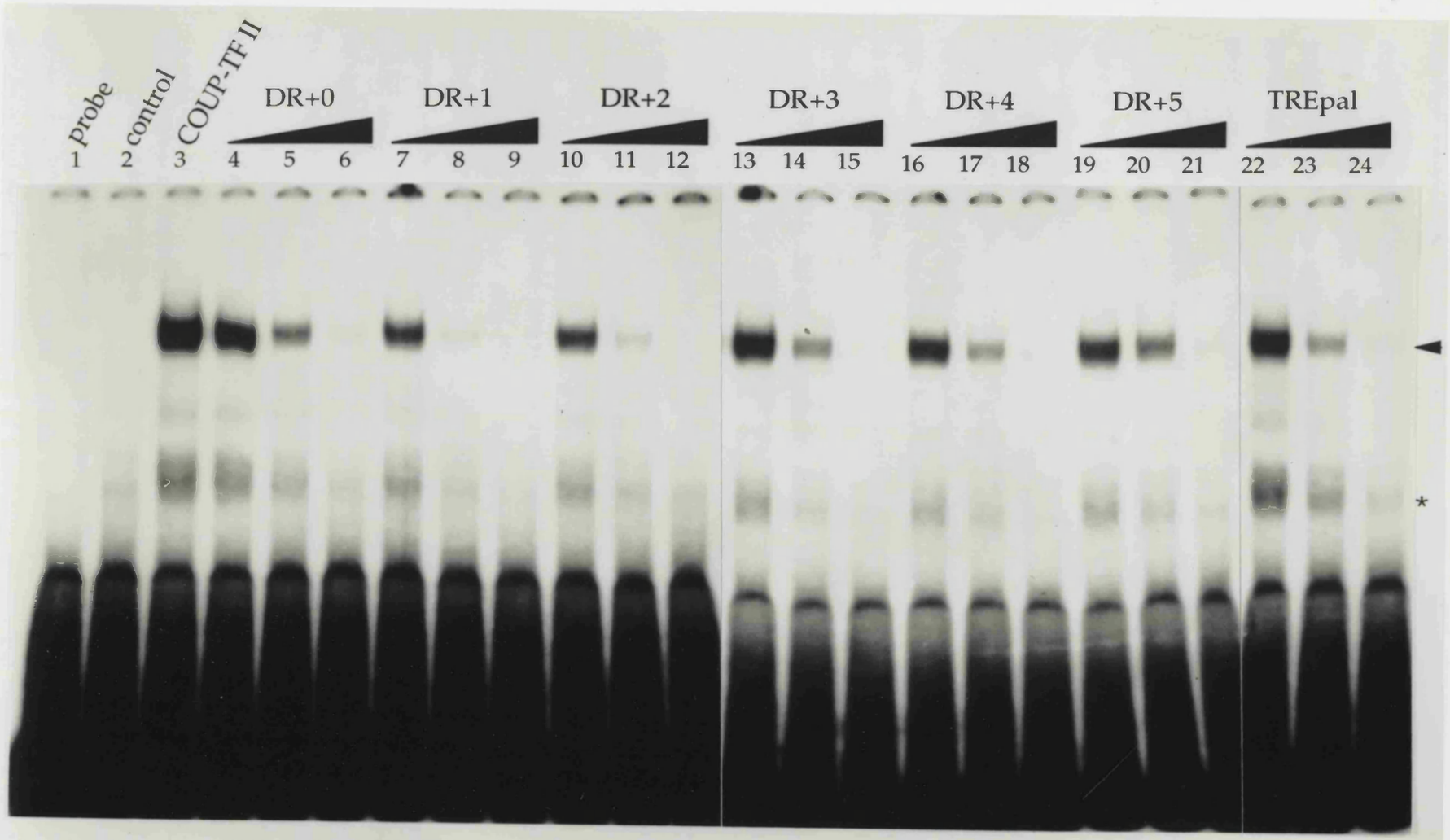


Figure 5.1 Competition for COUP-TF II binding by oligonucleotides containing variations in the spacing and orientation of the AGGTCA half site.

copies of the AGGTCA sequence arranged with different spacings or in different orientations. The ability of COUP-TF II homodimers to bind with similar efficiency to elements that differ in the spacing of the half sites contrasts with the selectivity exhibited by homodimers of other nuclear receptors (discussed in Chapters 1 and 7).

Oligonucleotides containing the ApoAI-RARE, a site identified in the apolipoprotein AI gene that is comprised of two half sites separated by two base pairs, and a consensus ERE (see Table 5.1) were also tested for their ability to compete for COUP-TF II binding. Addition of the ApoAI-RARE oligonucleotide significantly reduced the amount of retarded probe even at the lowest concentration tested (Figure 5.2 tracks 7-9) and was as efficient a competitor as the DR+2 oligonucleotide. This suggested that the imperfect response element, comprised of half sites that contain mismatches from the (A/G)GGTCA consensus sequence, was recognised by COUP-TF II with a similar affinity to the 'idealised' site. In contrast, the oligonucleotide containing an ERE, did not compete for COUP-TF II binding (Figure 5.2 tracks 4-6) although if this oligonucleotide was labelled and used as a probe in the gel shift assay COUP-TF II binding could be detected albeit it more weakly than with a DR+1 oligonucleotide probe (Figure 5.3 compare tracks 2 and 10). This suggests that although COUP-TF II can recognise the ERE element the interaction is less stable than with the DR+1 site. As mentioned the DR+3 oligonucleotide successfully competed for COUP-TF II binding (Figure 5.1 tracks 13-15) suggesting that with a three nucleotide spacing a direct repeat orientation of the half sites (DR+3) is bound with higher affinity than an inverted repeat (ERE). However, the ERE containing oligonucleotide contained different flanking nucleotides to the DR+ series (as did the ApoAI-RARE oligonucleotide) and it is possible that this contributed to the observed difference in affinity. It was noted that the DR+0 oligonucleotide competed to a similar extent as the TREpal oligonucleotide indicating that when there is no nucleotides between the half sites the orientation, direct or inverted, has no effect on the affinity of COUP-TF II recognition.

Examination of the binding of COUP-TF II homodimers to response elements that contain non consensus (A/G)GGTCA half sites

The natural response elements that COUP-TF I and II have been reported to bind do not contain repeats of the consensus (A/G)GGTCA sequence but rather are composed of imperfect repeats that contain a number of nucleotide mismatches from this sequence. The ability of the

oligonucleotide containing the ApoAI-RARE to compete as well as the oligonucleotides containing AGGTCA half sites suggested that COUP-TF II homodimers could bind this imperfect site with similar efficiency to an 'idealised' site. To extend this observation the binding of COUP-TF II to a number of other elements containing 'imperfect' half sites (Table 5.1) was judged by gel shift analysis. As shown in Figure 5.3, COUP-TF II homodimers bound as strongly to the ApoAI-RARE and the -210 element of the steroid 21 hydroxylase gene, response elements containing only one consensus half site, as to the DR+1 sequence. In contrast, COUP-TF II bound only weakly to the CDE2 element, which like the ApoAI-RARE contains a consensus GGGTCA half site sequence. This suggests that the sequence of the imperfect second half site influences the affinity with which the homodimer binds. This was supported by the observation that on another element that contained one copy of the half site sequence GGGTCA no binding of COUP-TF II could be detected (data not shown). This indicates that one half site is not sufficient to allow COUP-TF II binding and that sequence specific protein/DNA interactions are required at both half sites rather than one of the molecules of the dimer being tethered to the DNA through non specific contacts.

Analysis of the formation of COUP-TF II homodimers on the DR+1 and DR+5 response elements

The competitive gel shift experiments indicated that the binding site preference of COUP-TF II homodimers was more degenerate than that of other nuclear receptors. Methylation interference analysis of the binding of COUP-TF I or II to natural response elements had shown that the dimer makes specific contacts with both of the half site sequences (Carter *et al* 1994, Cooney *et al* 1991, Hwung *et al* 1988). Such data, and the inability of an oligonucleotide containing a single half site to compete for COUP-TF II binding, suggest that the dimer is unlikely to bind DNA with a fixed spacing between the individual molecules and one of the DNA binding domains forming non specific contacts on the DNA. The alternative possibility is that the dimerisation interface(s) is unusually flexible and can accommodate both alterations in the spacing and orientation of the two COUP-TF II molecules. To identify the dimerisation interface(s) formed on the different sites a series of C-terminal deletion mutants (generated by exonuclease III/mung bean nuclease digestion see Figure 3.1) were synthesised.

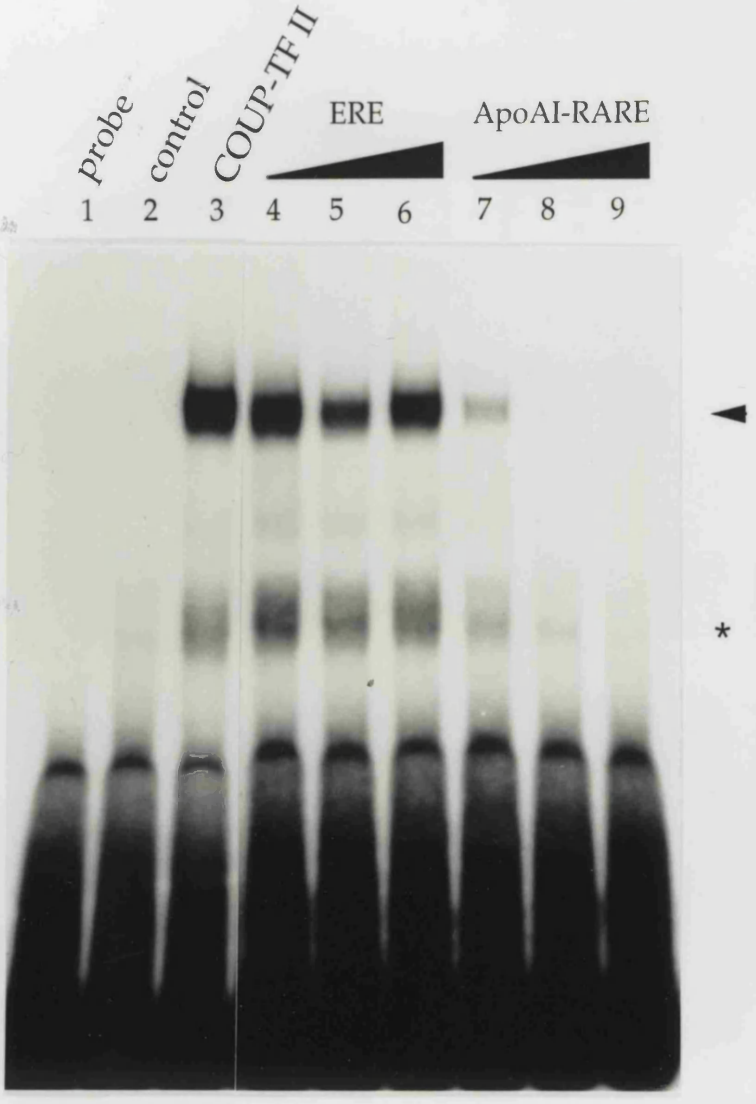


Figure 5.2 Competition for COUP-TF II binding by the natural response elements ApoAI-RARE and vitellogenin A2 ERE.
The ability of oligonucleotides containing an ERE or an ApoAI-RARE to compete with a DR+1 containing oligonucleotide for COUP-TF II binding was analysed by gel shift analysis. Equal amounts of *in vitro* synthesised COUP-TF II were preincubated in the presence (tracks 4-9) or absence (track 3) of competitor unlabelled oligonucleotides prior to the addition of labelled oligonucleotide containing a DR+1 site. The competitor oligonucleotides were added in 5 fold (tracks 4 and 7), 20 fold (tracks 5 and 8) and 100 fold (tracks 6 and 9) excess. As a control unprogrammed lysate was also incubated with the labelled oligonucleotide (track 2). The position of the COUP-TF II homodimer is indicated by a solid arrowhead and the asterix indicates a degradation product.

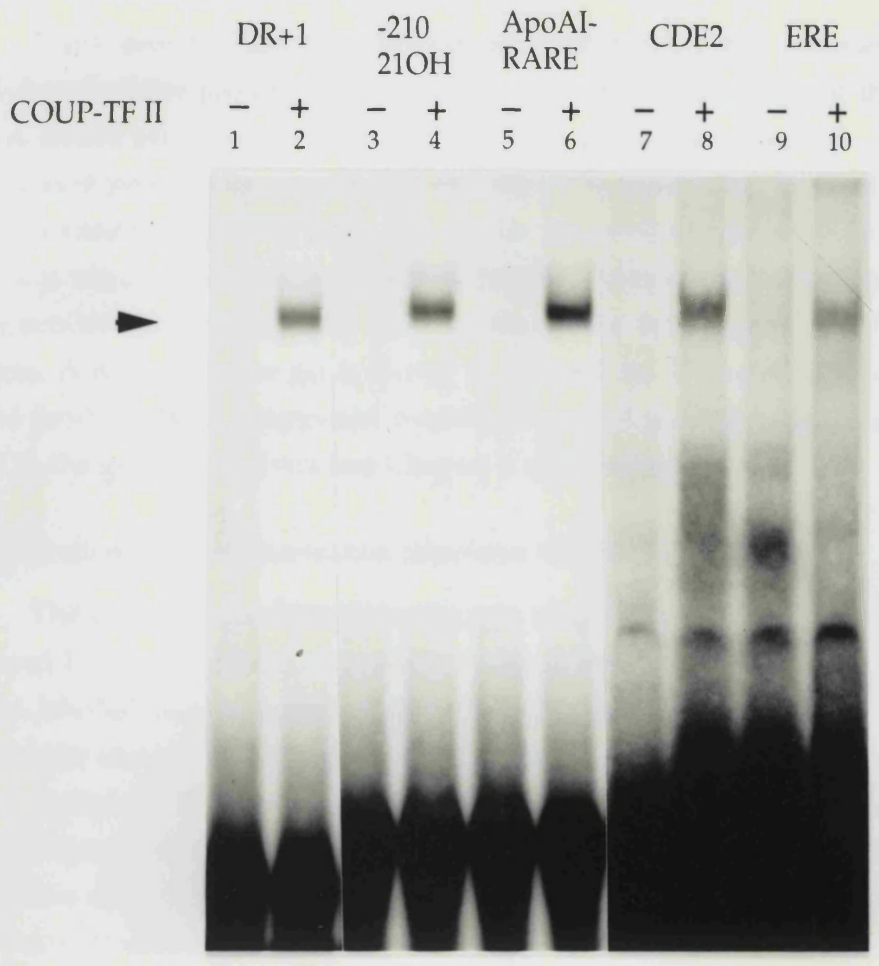


Figure 5.3 DNA binding activity of COUP-TF II homodimers to response elements that contain non consensus (A/G)GGTCA) half sites.
The DNA binding activity of COUP-TF II on the DR+1 and ERE response elements was compared with the binding to response elements that contained non-consensus (A/G)GGTCA half sites. Equal amounts of *in vitro* synthesised COUP-TF II or unprogrammed lysate as indicated were incubated with labelled DNA and analysed by gel shift analysis. The position of the COUP-TF II homodimer is indicated by a solid arrowhead. Tracks 7-9 have been exposed longer to detect the specific COUP-TF II complex.

Tracks 1-6 represent a 4 hour exposure and tracks 7-10 are from a 12 hour exposure (at room temperature).

The *in vitro* synthesis of COUP-TF II deletion mutants

The C-terminal deletion mutants were constructed in a pBluescript SKII- based vector (Stratagene) that allowed *in vitro* transcription of the cDNA clones using the bacteriophage T3 RNA polymerase. *In vitro* synthesised proteins were produced and the [^{35}S]-methionine labelled samples analysed by SDS-PAGE. The results indicated that the synthesised products were of the expected size and that the yields of the deletion mutants were similar although typically less than that of the full length COUP-TF II protein. A representative gel is shown in Figure 5.4a. The relative amounts of the products were determined quantitatively and the unlabelled proteins used in the gel shift analysis (see Chapter 4 and methods).

Identification of two dimerisation interfaces within COUP-TF II

The ability of the deletion mutants to bind DNA as a dimer was assessed by incubating equal amounts of the *in vitro* synthesised proteins with a labelled oligonucleotide that contained either a DR+1 or DR+5 site. The results showed that the deletion of 19 amino acids from the C-terminus had a negligible effect on the ability of this mutant (CII-395) to bind DNA as a dimer (Figure 5.4b compare tracks 2 and 3, tracks 8 and 9) whereas deletion of 108 amino acids (CII-306) prevented DNA binding on both the DR+1 and DR+5 sites (tracks 4 and 10). Further deletion to amino acid residue 190 restored DNA binding activity and the mutant CII-190 bound both the DR+1 and DR+5 elements with similar efficiency to wild type (compare tracks 2 and 5, 8 and 11). The inability of the mutant CII-306 to bind DNA may be due to masking of the DNA binding domain by the truncated C-terminus. Progressive deletion of the C-terminus of the ER revealed a similar pattern of DNA binding activity by the mutants with the loss and then the restoration of DNA binding activity (Fawell *et al* 1990a).

The behaviour of the deletion mutant CII-190 on the DR+1 and DR+5 elements was intriguing (Figure 5.4b compare tracks 5 and 11). This mutant bound as a dimer to the DR+1 element (some monomer binding was also detected, the amount varying between experiments) and exclusively as a monomer to the DR+5 element. This result implied that there was a dimerisation interface present in CII-190 that was sufficient for cooperative binding on a site that contained the AGGTCA repeats spaced by 1 but not 5 nucleotides. Further deletion to amino acid residue 138 (CII-138) abolished the ability of this mutant to bind to either probe (Figure 5.4b track 6 and 12).

This was expected as this mutant was truncated within the core DNA binding domain (see Chapter 1 and Figure 5.7).

To confirm that the mutant CII-190 could bind cooperatively to the DR+1 but not the DR+5 element increasing concentrations of CII-190 were incubated with the corresponding probes. Using the DR+1 probe and low concentrations of CII-190 two complexes were formed, corresponding to monomeric and dimeric forms of the mutant (Figure 5.5a). Increasing the concentration of CII-190 resulted in the preferential increase in the formation of the dimer relative to the monomer. In contrast, on the DR+5 probe only the monomeric complex was formed at low concentrations of CII-190 and the binding of dimers was only weakly observed with higher amounts of input protein (Figure 5.5b). Additional gel shift experiments showed that wild type COUP-TF II bound only as a dimer to either the DR+1 or DR+5 probe over a wide range of concentrations (Figure 5.5c). At higher concentrations of protein a higher mobility complex was observed that presumably corresponds to a COUP-TF II oligomer. These data indicate that COUP-TF II can bind cooperatively to both the DR+1 and DR+5 sites whereas CII-190 can only bind cooperatively to the DR+1 site.

Taken together these results suggest that the formation of COUP-TF II homodimers on the DR+5 element requires sequences located between amino acid residue 191 and 395 (figure 5.4 compare tracks 9 and 11). In contrast, this region is not essential for the binding of COUP-TF II homodimers to the DR+1 site (Figure 5.4 tracks 3 and 5 and Figure 5.5a). The ability of CII-190 to bind cooperatively to the DR+1 site but not the DR+5 site implied that in addition to the dimerisation interface identified between 190 and 395 (referred to as the C-terminal interface) there must be a second interface located N-terminal to residue 190. From the observation that the N-terminus of COUP-TF I was not required for dimer binding on a DR+1 response element (Cooney *et al* 1992) and by analogy to the steroid hormone receptors, the second dimerisation interface was predicted to be associated with the DNA binding domain. Computer modelling, using the coordinates of the ER DNA binding domain, showed that when the core DNA binding domains were arranged on a DR+1 element they were not in close enough proximity to make contact (data not shown). On the basis of homology it was predicted that the core DNA binding domain of COUP-TF II (amino acid residues 79-144) would also not be able to interact on the DR+1 site, implying that the second dimerisation region involved amino acid residues C-terminal to the core DNA binding domain.

To define more precisely the region of the DNA binding domain involved in the second interface, the ability of other C-terminal deletion mutants of COUP-TF II to form dimers on a DR+1 probe was analysed. Experiments showed that a mutant deleted to amino acid residue 170 was still capable of forming the second interface (data not shown). Therefore, a deletion mutant lacking a further 15 amino acids (CII-155) and another truncated at the end of the core DNA binding domain (CII-144) were constructed (appendix A16 and Figure 5.7). COS-1 cells were transiently transfected with the expression vectors for COUP-TF II, CII-155 and CII-144 (pSG5-COUP-TF II, pSG5-CII-155 and pSG5-CII-144) or the expression vector alone (pSG5) and these cells were harvested to make whole cell extracts. The expression of the wild type and mutant COUP-TF II proteins were assessed by western blotting and the proteins shown to be of the expected size and expressed at similar levels. This analysis was carried out for every batch of whole cell extracts prepared and a representative western blot is shown in Figure 5.6a. Equal amounts of the COS-1 extracts were then used in gel shift analysis. As shown in Figure 5.6b, the control extracts (COS-1 cells transfected with the expression vector pSG5) did not form any specific complex on the DR+1 probe. The mutant CII-155 retained the ability to dimerise on the DR+1 element and infact, surprisingly, bound more probe than COUP-TF II (compare tracks 2 and 3). This increase in binding might be explained if the C-terminal domain, corresponding to amino acid residues 156-414, in some way partially restricted the binding of COUP-TF II. Alternatively it is possible that a higher proportion of the mutant CII-155 protein was functional as compared to the wild type COUP-TF II. No specific complex was formed with the deletion mutant CII-144. When 10 fold more COS-1 expressed CII-144 was used, however, a faint complex was observed (Figure 5.6b track 5 and a longer exposure, track 6) and the mobility of this complex was consistent with the binding of monomer. These results suggest that the deletion of amino acid residues 145-155 disrupted the interface associated with the DNA binding domain.

The DNA binding activity of CII-155 and CII-144 was also examined on the DR+5 probe. The mutant CII-155 bound exclusively as a monomer on this site, indicating that no alternative dimerisation interface had been unmasked by the deletion of residues 156-190 (Figure 5.6b track 9). As with the DR+1 probe, binding of the mutant CII-144 was only observed when ten fold more protein was used and the weak complex detected corresponded to monomer binding (track 11 and longer exposure track 12). The observation

Figure 5.4 DNA binding activity of COUP-TF II C-terminal deletion mutants.

(a) Analysis of the relative levels of COUP-TF II and deletion mutants synthesised *in vitro*. Proteins were synthesised from the corresponding prokaryotic expression vector using a coupled *in vitro* transcription and translation system containing [^{35}S]-methionine. Aliquots (3 μl) of unprogrammed (control) or programmed lysates were analysed by SDS-PAGE and the resultant bands visualised by flurography and quantitated using phosphoimage analysis. The position of the molecular weight markers of the indicated size (kilodaltons) are marked.

(b)The COUP-TF II deletion mutants were tested in a gel shift assay. Equal amounts of *in vitro* translated mutant or wild type protein were incubated with a labelled oligonucleotide probe containing either a DR+1 (tracks 1-6) or DR+5 (tracks 7-12) response element. Unprogrammed lysate was also incubated with the probes (tracks 1 and 7).

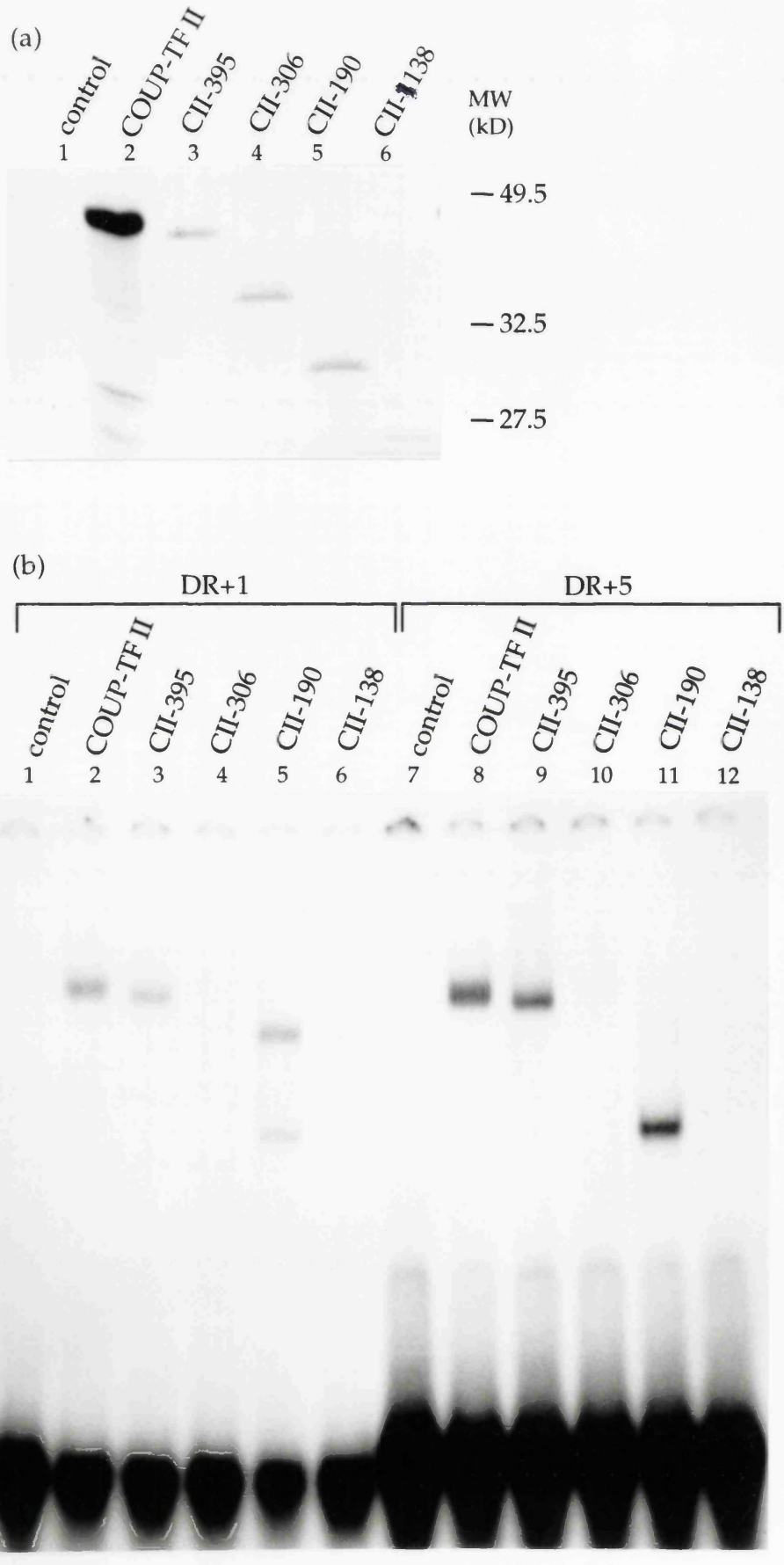


Figure 5.4 DNA binding activity of COUP-TF II C-terminal deletion mutants.

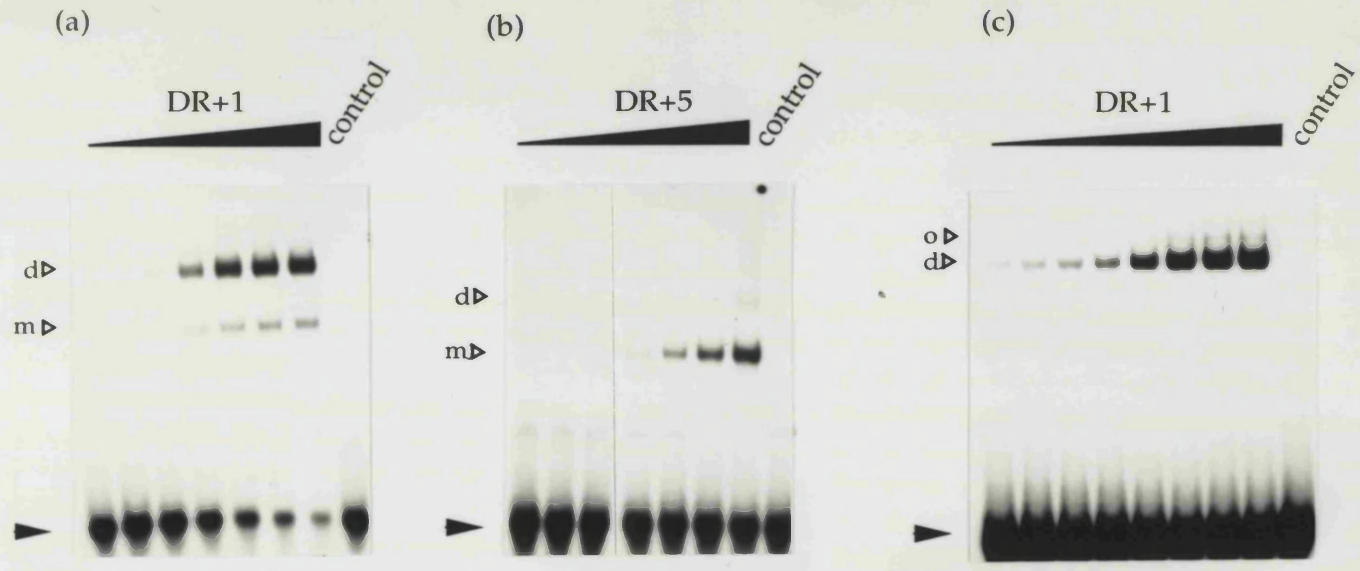


Figure 5.5 Cooperative DNA binding by COUP-TF II and the deletion mutant CII-190.
The ability of COUP-TF II and the deletion mutant CII-190 to bind cooperatively to DNA was assessed in a gel shift assay. Increasing amounts of *in vitro* synthesised CII-190 (a) and (b) or COUP-TF II (c) were incubated with a labelled oligonucleotide probe containing either a DR+1 or DR+5 response element as indicated. The total protein concentration in each reaction was maintained by the addition of unprogrammed reticulocyte lysate. Control indicates the incubation of unprogrammed lysate alone. The open triangle marked(d) indicates the dimeric complex, (m) the monomeric complex and (o) the oligomeric complex. The closed triangle indicates the free probe.

Figure 5.6 DNA binding activity of the deletion mutants CII-155 and CII-144.

(a) Western blot analysis of COUP-TF II, CII-155 and CII-144 expressed in COS-1 cells. Equal amounts of whole cell extracts (10 μg) prepared from COS-1 cells transiently transfected with pSG5-COUP-TF II, pSG5-CII-155, pSG5-CII-144 or the expression vector alone pSG5 were resolved by SDS-PAGE and subjected to western blotting. The antisera MP32 that recognises an epitope in the N-terminal region of COUP-TF II was used to detect the proteins. The positions of the molecular weight markers of the indicated size (kilodaltons) are marked.

(b) The DNA binding activity of the COUP-TF II C-terminal deletion mutants were compared in a gel shift assay using oligonucleotide probes that contained either a DR+1 (tracks 1-6) or DR+5 (tracks 7-12) response element. Equal amounts of whole cell extracts (0.5 μg) prepared from COS-1 cells transiently transfected with the expression vector alone pSG5 (control)(tracks 1 and 7), pSG5-COUP-TF II (tracks 2 and 8), pSG5-CII-155 (tracks 3 and 9) and pSG5-CII-144 (tracks 4 and 10 and 2 μg of extract track 5 and 11) were incubated with the oligonucleotide probe as indicated. Tracks 6 and 12 are longer exposures of tracks 5 and 11, under these conditions a non specific complex is also detected that is indictaed by an asterix.

Tracks 1-5 and 7-11 represent a 30 minute exposure and tracks 6 and 12 represent a 4 hour exposure (at room temperature).

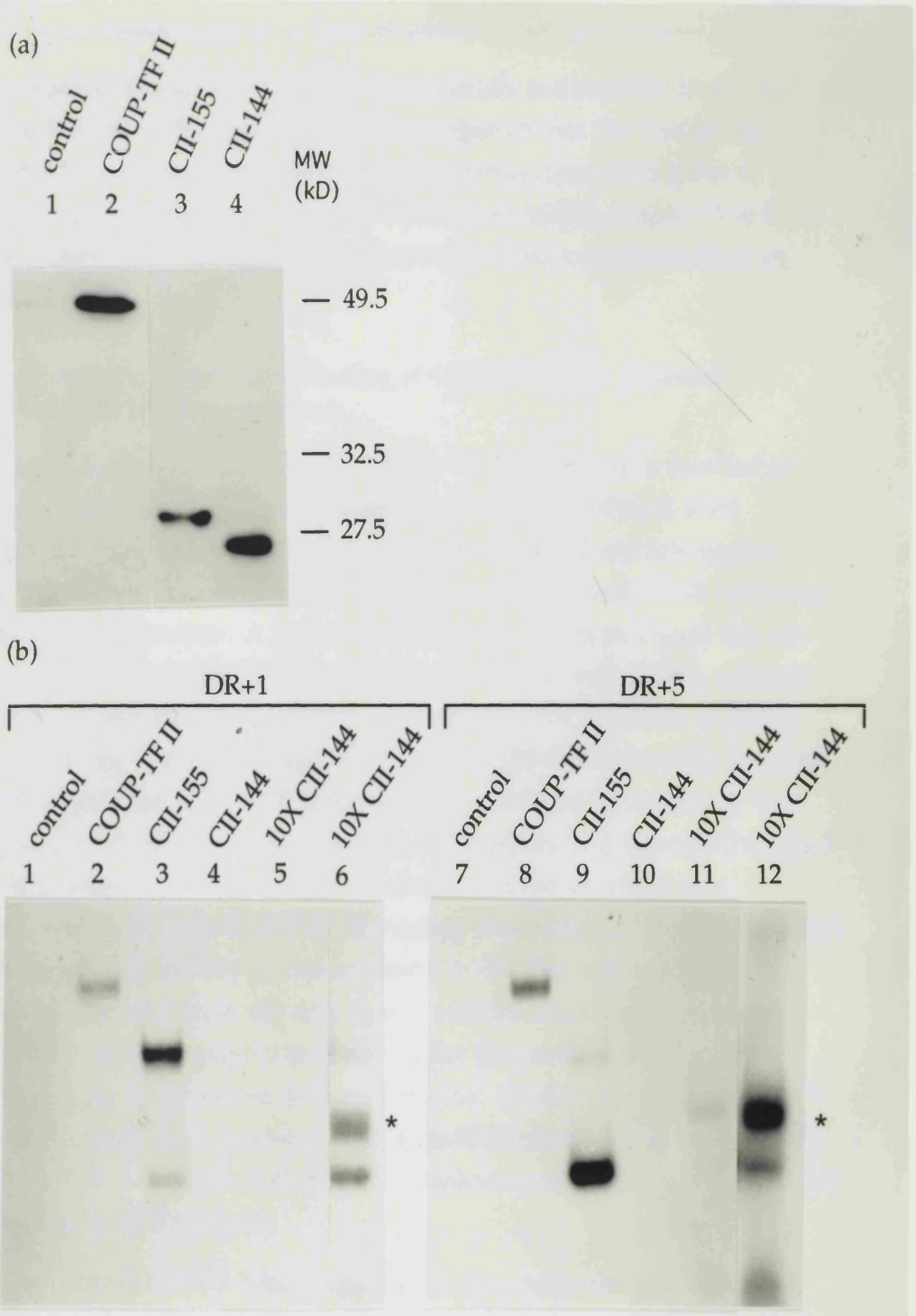


Figure 5.6 DNA binding activity of the deletion mutants CII-155 and CII-144.

that the deletion of amino acids 155-145 drastically reduced binding of the mutant to both the DR+1 and DR+5 sites suggested that the loss in high affinity DNA binding was a general property rather than specific for a particular response element and is discussed later in this chapter. The DNA binding properties of the deletion mutants discussed are summarised in Figure 5.7.

Examination of the cooperative binding of COUP-TF II on different AGGTCA response elements

The dimerisation interfaces of COUP-TF II formed on a number of alternative response elements composed of AGGTCA half sites were investigated by gel shift analysis. The batch of whole cell extracts used in these gel shift experiments was also tested on the DR+1 and DR+5 probes to allow a direct comparison. A representative gel is shown in Figure 5.8. On the DR+2 and TREpal elements the mutant CII-155 bound as both a dimer and monomer and the two complexes retarded similar amounts of probe (Figure 5.8 tracks 7 and 19 respectively). The CII-155 dimer retarded more of the DR+2 probe than COUP-TF II (compare tracks 6 and 7), similar to the binding of CII-155 with the DR+1 probe (see Figure 5.6 and Figure 5.8 tracks 2 and 3). Interestingly, on the TREpal element the opposite effect was observed, with wild type COUP-TF II binding more strongly than the mutant (compare tracks 18 and 19). As the mutant CII-155 only exhibited greater DNA binding activity than the wild type on certain sites this suggests that the difference may not simply reflect a higher propertion of the mutant protein being correctly folded. It is possible that the C-terminal region of COUP-TF II (amino acid residues 156-414) destabilises the binding of COUP-TF II to the DR+1 and DR+2 elements but enhances the affinity with which it can recognise the TREpal site.

The mutant CII-155 bound exclusively as a monomer on both the DR+4 and DR+5 containing oligonucleotides (Figure 5.8 tracks 11 and 15). It is particularly striking that the CII-155 monomer bound these probes with similar efficiency as the COUP-TF II dimer implying that cooperative binding is not required for a stable interaction between the DNA binding domain and the half site. Monomeric binding by the wild type COUP-TF II was not detected in these experiments (tracks 2, 6, 10, 14 and 18) nor on elements containing half site arrangements that the dimer bound poorly, such as the ERE containing oligonucleotide (Figure 5.3 track 10). This suggests that the C-terminal domain prevents the binding of monomer (discussed in Chapter 7).

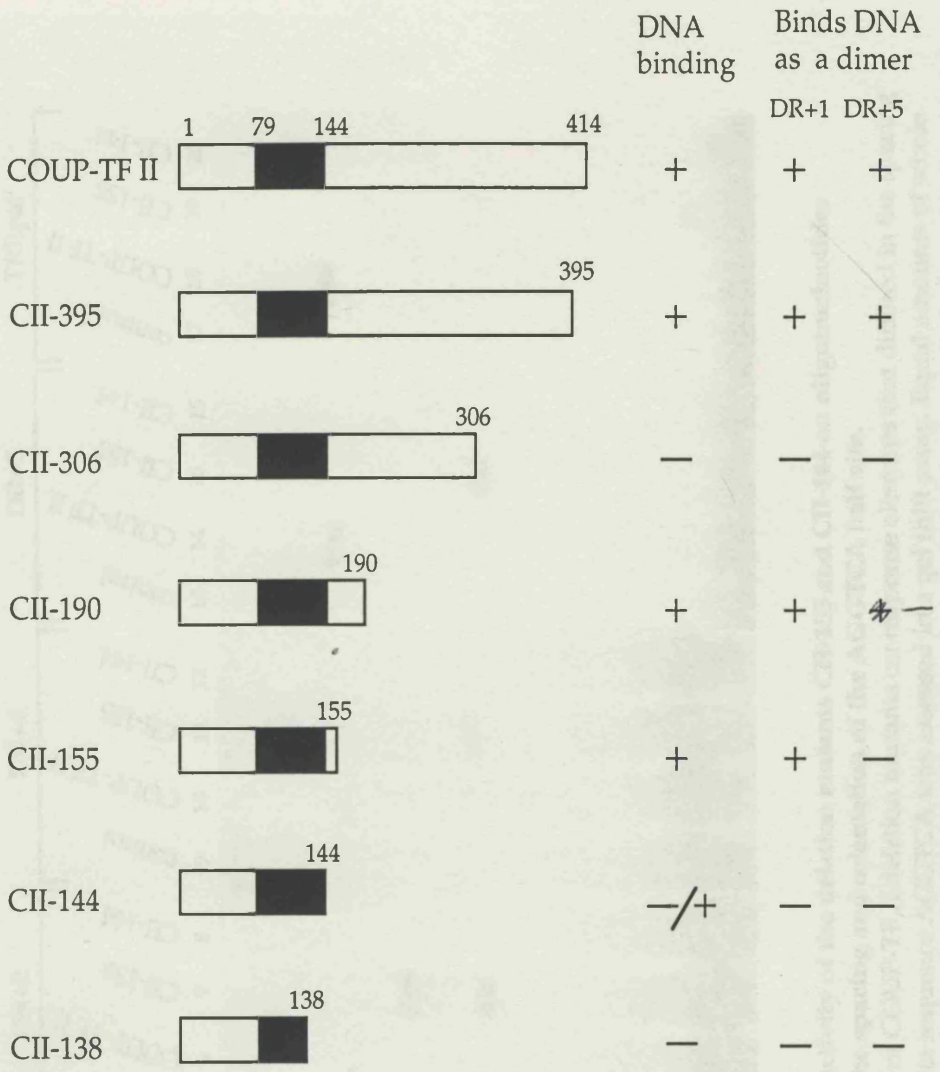


Figure 5.7 Summary of the DNA binding properties of the C-terminal COUP-TF II deletion mutants.

Schematic representation of the deletion mutants tested in gel shift analysis and their DNA binding properties. The black box indicates the DNA binding domain (amino acid residues 79-144). -/+ indicates that DNA binding was detected only at high concentrations of the mutant protein.

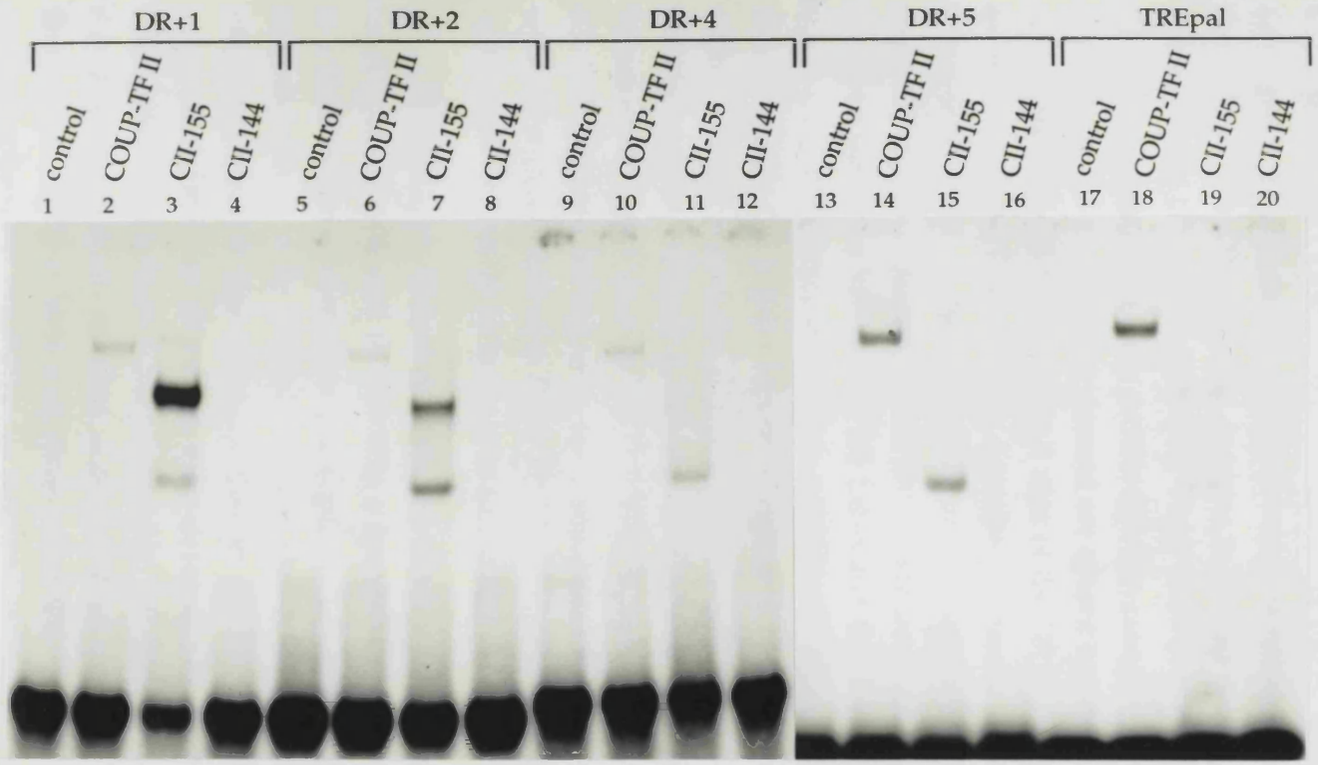


Figure 5.8 DNA binding activity of the deletion mutants CII-155 and CII-144 on oligonucleotides containing variations in the spacing and orientation of the AGGTCA half site.
The DNA binding activity of COUP-TF II deletion mutants on response elements that differed in the spacing or orientation of the half site sequence AGGTCA was assessed in a gel shift assay. Equal amounts of whole cell extracts (0.5 μg) prepared from COS-1 cells transiently transfected with pSG5- COUP-TF II , pSG5-CII-155, pSG5- CII-144 or the expression vector alone pSG5 (control) were incubated with a labelled oligonucleotide probe as indicated.

Taken together these data indicate that the mutant CII-155 forms dimers most efficiently on the DR+1 site and to a lesser extent on the DR+2 and TREpal sites and not at all on the DR+4 and DR+5 sites. The different spacings of the half sites in the DR+ series of oligonucleotides would result in the individual COUP-TF II molecules being placed different distances apart and with different stereoalignments. It is not surprising therefore that the ability of the DNA binding domains to interact is progressively lost as the spacing of the half sites is increased. From these results it is predicted that the DNA binding domains form protein/protein contacts most strongly when the half sites of the response element are spaced by one nucleotide. The arrangement of CII-155 on the half sites of the DR+2 response element does seem to allow the DNA binding domains to interact. This contact, however, is less stable than on the DR+1 element (perhaps involving different contacts) and both monomers and dimers are detected. In contrast, the arrangement of the half sites in the DR+4 and DR+5 elements does not allow the two CII-155 molecules to interact and hence the mutant binds exclusively as a monomer. On a direct repeat arrangement of half sites the two molecules bind asymmetrically, in a 'head to tail' orientation. It is predicted that the regions involved in forming the interface between the DNA binding domains will differ between the COUP-TF II molecule bound to the 5' of the site and the molecule bound to the 3' site. Hence it is likely that there are two separate regions within the DNA binding domain of CII-155 involved in making the protein/protein contacts (discussed in Chapter 7).

The TREpal element is composed of an inverted repeat and the DNA binding domains would be bound in a symmetrical arrangement on this site, in a 'head to head' orientation (see Figure 1.2). Surprisingly, CII-155 could bind as a dimer to the TREpal although, similar to the binding on the DR+2 site, the interaction was less stable than on the DR+1 element and similar amounts of dimer and monomer were detected. On the inverted repeat the DNA binding domains would, presumably, not be correctly aligned to form the same dimerisation interface as on the DR+1 element. This suggests that there is a third dimerisation interface within CII-155 that provides the interaction between symmetrically arranged molecules on the TREpal.

Examination of the cooperative binding of COUP-TF II on natural RAREs

The dimerisation interfaces formed by COUP-TF II on natural RAREs was also tested using gel shift analysis. These natural response elements all contained direct repeats, spaced by one nucleotide in the ovalbumin RARE,

two nucleotides in the ApoAI-RARE and five nucleotides in the β-RARE (Table 5.1). COUP-TF II bound as a homodimer to these RAREs (Figure 5.9). As expected, the mutant CII-155 bound exclusively as a monomer on the β-RARE (track 7) and also bound as a monomer to the ApoAI-RARE in contrast to the two complexes observed on the DR+2 (Figure 5.9 track 11 and Figure 5.8 track 7 respectively). On the ovalbumin RARE two complexes were observed indicating that both dimer and monomer complexes are formed (Figure 5.9 track 3). This contrasts with the DR+1 element, on which the mutant CII-155 bound exclusively as dimer (Figure 5.8 track 3). These data suggest that the protein/protein contacts between the DNA binding domains is influenced by the sequence of the response element as well as the spacing and orientation of the half sites.

The observation that the monomeric form of CII-155 binds more weakly to the ApoAI-RARE than the β-RARE indicates that the sequence of the half site and/or the flanking nucleotides dictates the strength of the interaction between the monomer and the DNA. The observation that full length COUP-TF II recognised these two elements with similar efficiency (Figure 5.9 tracks 10 and 6 respectively) suggests that the cooperative interaction between the C-terminal domains of the protein provides sufficient additional stability to the protein on the ApoAI-RARE to compensate for the weaker contacts of the DNA binding domain on this response element.

Identification of a region adjacent to the zinc finger motif of COUP-TF II that participates in DNA binding

The mutant CII-155 bound, as a monomer, to a number of different oligonucleotides such as the DR+2, DR+4 and DR+5 sites with similar efficiency as the wild type protein. Surprisingly, the monomer bound only weakly to the TREpal element (Figure 5.8 compare tracks 7, 11 and 15 with 19). As the TREpal element and the direct repeat elements contain the same half site sequences the difference in binding efficiency of the monomer is most likely due to the different flanking nucleotides.

Although the mutant CII-155 bound with efficiently to DNA, the mutant CII-144 that lacked a further eleven amino acid residues from the C-terminus bound very poorly to the oligonucleotide probes. When an amount of CII-144 protein equivalent to that used for CII-155 was incubated with any of the oligonucleotides tested no specific complexes were formed

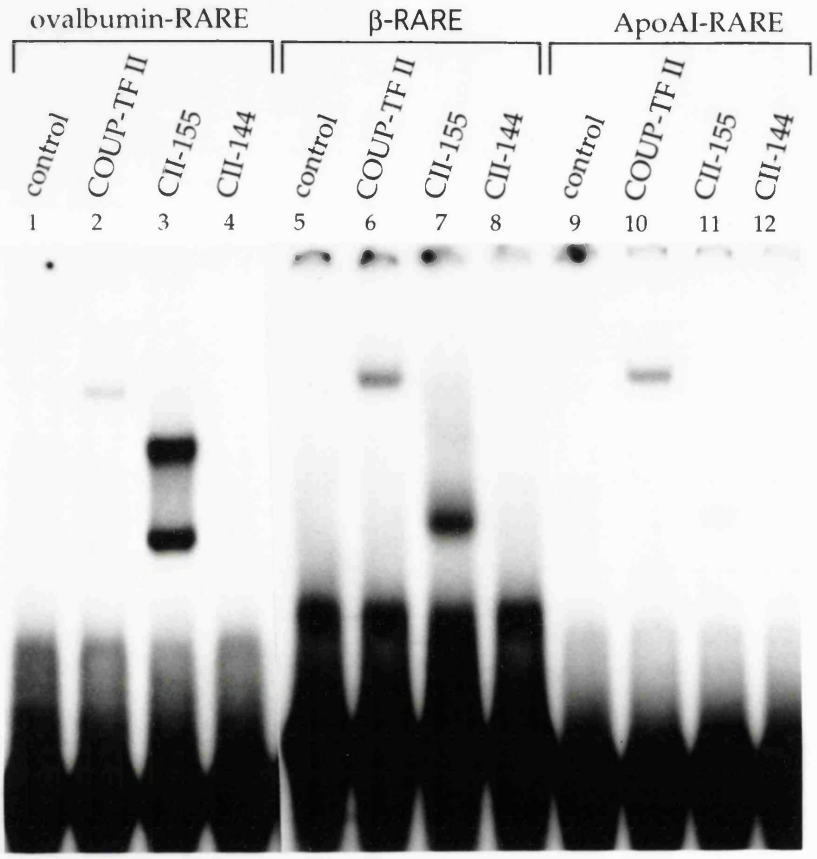


Figure 5.9 Analysis of the DNA binding activity of the deletion mutants CII-155 and CII-144 on natural RAREs.

The DNA binding activity of the COUP-TF II deletion mutants on response elements that differed in the spacing or orientation of the half site sequence AGGTCA was assessed in a gel shift assay. Equal amounts of whole cell extracts (0.5 μg) prepared from COS-1 cells transiently transfected with pSG5- COUP-TF II , pSG5-CII-155, pSG5- CII-144 or the expression vector alone pSG5 (control) were incubated with a labelled oligonucleotide probe as indicated.

(Figure 5.8 and Figure 5.9). Faint complexes were only observed when ten fold more extract was incubated with either the DR+1 and DR+5 probes (Figure 5.6 tracks 6 and 12). This suggests that sequences adjacent to the core DNA binding domain, between residues 144 and 155, are required for high affinity recognition of the half site sequence.

Summary and conclusions

COUP-TF II homodimers were shown to bind to oligonucleotides that contained direct repeats of the sequence AGGTCA separated by 0-5 nucleotides and also an inverted repeat arrangement with a 0 nucleotide spacing (TREpal). In addition to consensus response elements COUP-TF II was also shown to bind natural response elements that contain imperfect half site sequences .

Two regions in COUP-TF II were identified that were involved in the formation of homodimers, one in the C-terminus and one within the DNA binding domain. The region in the C-terminus was necessary for the formation of dimers on a DR+5 element whereas on a DR+1 element the interface within the DNA binding domain was sufficient. The interaction between the DNA binding domains required an eleven amino acid region (residues 145-155) adjacent to the core DNA binding domain that was also necessary for high affinity DNA binding. The deletion mutant CII-155, which lacked the C-terminal dimerisation domain, could bind as a dimer to both the DR+1 and TREpal elements. On the inverted repeat (TREpal) the DNA binding domains would be in a 'head to head' configuration in contrast to the 'head to tail' configuration on the direct repeat site (DR+1). It is likely therefore that an alternative dimerisation interface is formed between the DNA binding domains on the TREpal.

On the DR+ 2, DR+4 and DR+5 elements the DNA binding domains did not interact and CII-155 bound as a monomer, surprisingly, the mutant bound these sites with similar efficiency to the COUP-TF II homodimer. This suggests that an individual DNA binding domain makes sufficient protein/DNA contacts to form a stable complex in the absence of cooperative binding.

CHAPTER 6

THE ROLE OF LIGAND IN INFLUENCING THE FORMATION OF RXR/TR HETERODIMERS

CHAPTER 6

Introduction

Sequence analysis of TREs upstream of target genes has shown that the TR can bind to half site motifs arranged in a direct, inverted or everted repeat orientation. The TR can bind to DNA *in vitro* as a monomer, a homodimer or as a heterodimer (Kliewer *et al* 1992b, Lazar *et al* 1991, Leid *et al* 1992b, Marks *et al* 1992, Yu *et al* 1991). The formation of RXR/TR heterodimers modulates both the efficiency of DNA binding and the transcriptional activity of the TR (see Chapter 1). While investigating the dimerisation properties of COUP-TF II in a two hybrid assay, low levels of COUP-TF II/TR heterodimers were shown to be formed in a T3 dependent manner (see Chapter 4). This observation raised the possibility that ligand affected the formation of other heterodimers. This chapter describes the work carried out to assess the effect of T3 and 9-*cis* retinoic acid on the formation of RXRα/TRβ heterodimers both in intact cells and *in vitro*.

Effect of ligand on the formation of RXRα/TRβ heterodimers in intact cells

It was previously shown that the small amount of recruitment of VP16CII(DE) by GAL4-TR(DE) in the two hybrid assay required T3 (Figure 4.12b). As a positive control for these experiments, the ability of GAL4-TR(DE) to recruit VP16RXR was tested. RXRα/TRβ heterodimers were shown to be formed in the absence of ligand, resulting in a 14 fold increase in transcription (Figure 6.1 track 4 and Figure 4.12a). The effect of T3 on the recruitment of VP16RXR by GAL4-TR(DE) was also examined by transient transfection in CEF cells. In the presence of 200 nM T3, GAL4-TR(DE) stimulated transactivation of the reporter approximately 40 fold (Figure 6.1 track 3 and Figure 4.12b). When GAL4-TR(DE) and VP16RXR were coexpressed in the presence of T3 the increase in transcription was approximately 220 fold (track 5), significantly greater than the sum of the activation observed individually for GAL4-TR(DE) ligand activation (track 3) and the recruitment of VP16RXR in the absence of T3 (track 4). The larger increase in reporter transcription could be due to a T3 dependent increase in recruitment of VP16RXR. Alternatively, the increase could be the result of synergistic transactivation by the VP16 activation domain and the T3 dependent TR activation domain, AF-2. To assess the role of T3 in promoting recruitment of VP16RXR independently of inducing the TR AF2 activity the GAL4 fusion protein containing the C-terminal region of a transcriptionally defective TR mutant, TRL454A, was tested (see Chapter 4).

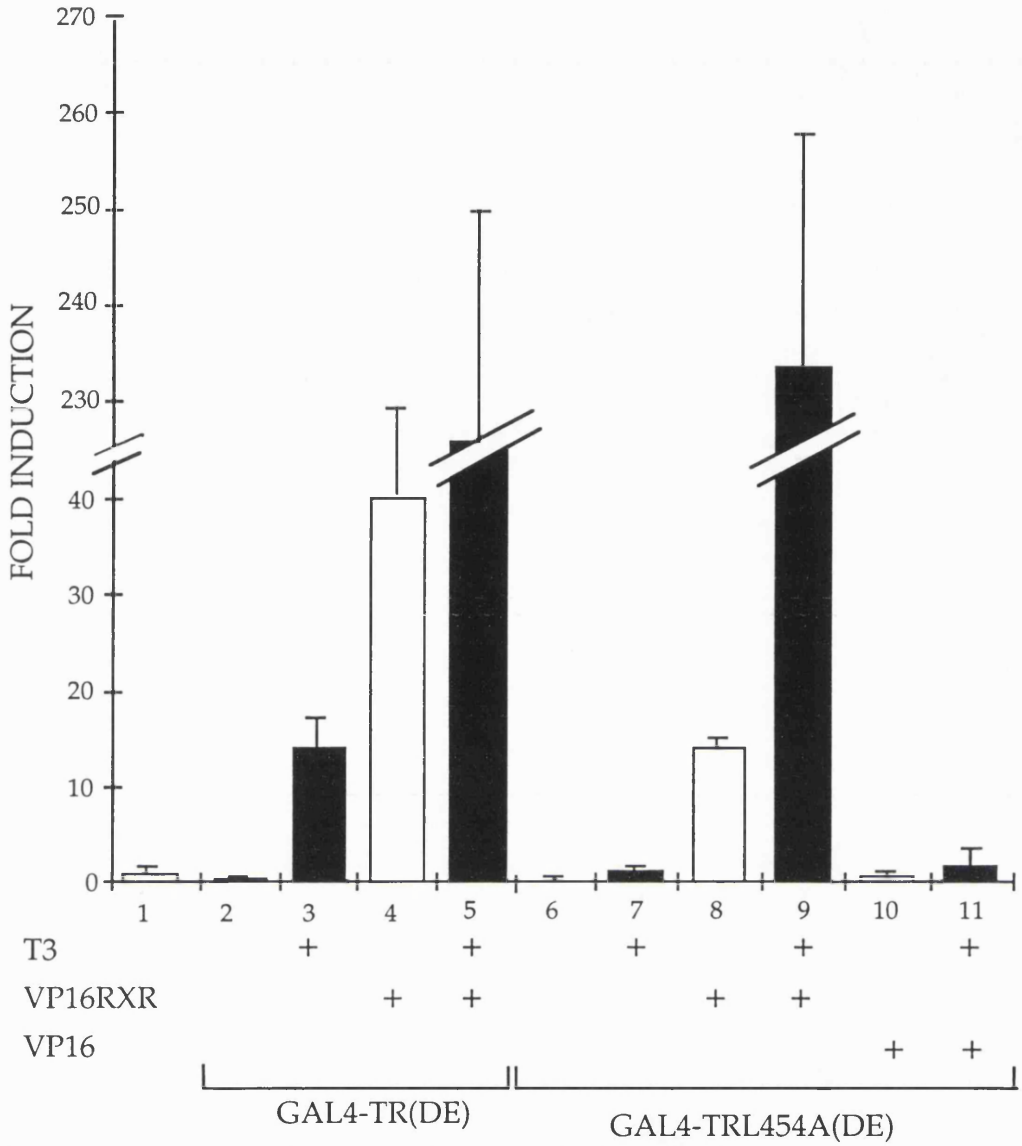


Figure 6.1 Effect of T3 on recruitment of VP16RXR by GAL4-TR(DE) and GAL4-TRL454A(DE).

CEF cells were transiently transfected with the reporter pGE1BCAT (5 µg) and either 100 ng GAL4-TR(DE) expression vector (tracks 2-5) or 100 ng GAL4- TRL454A(DE) expression vector (tracks 6-11) in the absence (tracks 2, 3, 6, 7, 10 and 11) and presence of 1000 ng of VP16RXR expression vector (tracks 4, 5, 8 and 9) or VP16 expression vector (tracks 10 and 11). As a control, 1000 ng of VP16RXR expression vector was analysed (track 1). T3 was added at 200 nM to tracks 3, 5, 7, 9 and 11 (solid bar). These results represent the mean values of three independent experiments each done in duplicate; the bars indicate standard deviations).

As expected, GAL4-TRL454A(DE) only weakly stimulated transcription in response to T3 (0.3 fold to 1.4 fold, Figure 6.1 tracks 6 and 7 and Figure 4.12). Coexpression of GAL4-TRL454A(DE) and VP16RXR resulted in a 14 fold induction of the reporter activity (track 8 and Figure 4.12). Addition of T3 resulted in a further increase to approximately 240 fold above the the basal level (Figure 6.1 track 9). Coexpression of the VP16 activation domain moiety with GAL4-TRL454A(DE) did not increase the transcriptional activity of the reporter above that seen with the GAL4 fusion protein alone, either in the absence or presence of T3 (compare tracks 6 and 10, 7 and 11). This suggested that the increase observed when cells cotransfected with VP16RXR and GAL4-TRL454A(DE) wre treated with T3 required the RXR moiety of the VP16RXR fusion, presumably due to the enhanced formation of RXRα/TRβ heterodimers.

To examine whether the recruitment of VP16RXR was through the C-terminal dimerisation interface of RXRα, the chimeric protein VP16RXR(DE) was tested (see appendix A14 for construction of the expression vector). Expression of GAL4-TRL454A(DE) alone resulted in a reduction in reporter activity to 0.3 fold (Figure 6.2 track 2), due to transcriptional silencing by the unliganded TR. Coexpression of VP16RXR(DE) relieved this repression but did not increase the transcriptional activity of the reporter above the basal level (track 6). This was in contrast to the 14 fold increase observed with VP16RXR (Figure 6.1 track 8). In the presence of T3, however, coexpression of VP16RXR(DE) stimulated the reporter activity approximately 330 fold (Figure 6.2 track 7), implying that recruitment of VP16RXR(DE) to the reporter required T3. These data suggest that the interaction observed between VP16RXR and GAL4-TRL454A(DE) in the absence of T3 involves other sequences of the RXR receptor in addition to the C-terminal (DE) domains. In the presence of T3, VP16RXR(DE) was recruited as efficiently as VP16RXR (compare Figure 6.2 track 7 with Figure 6.1 track 9). This suggests that the additional sequences required for the T3 independent interaction are not necessary once the TR has bound its ligand.

Having shown that T3 could promote the formation of RXRα/TRβ heterodimers in the two hybrid assay the effect of the ligand for RXR, 9-*cis* retinoic acid, was examined. When cells cotransfected with the expression vectors for GAL4-TRL454A(DE) and VP16RXR(DE) were treated with 100 nM 9-*cis* retinoic acid there was no significant increase in the level of reporter activity (track 8). Thus, the enhancement of the formation of RXR/TR

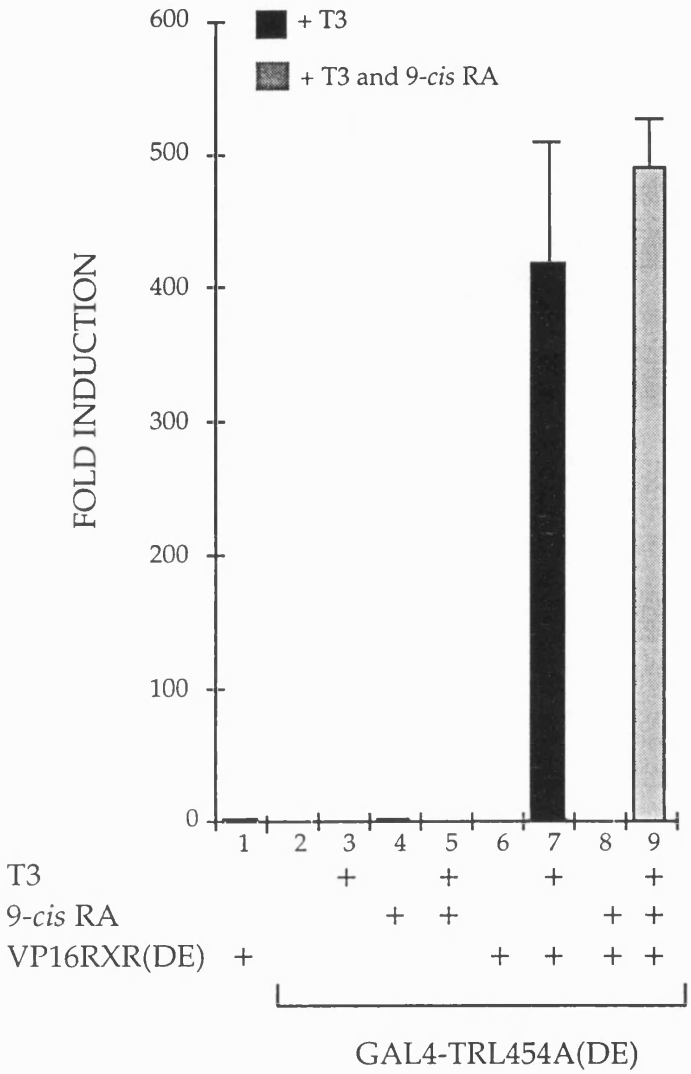


Figure 6.2 The effect of ligand on the recruitment of VP16RXR(DE) by GAL4-TRL454A(DE) .

CEF cells were transiently transfected with the reporter pGE1BCAT (5 µg) and 100 ng of the GAL4-TRL454A(DE) expression vector in the absence (tracks 2-5) and presence of 1000 ng of VP16RXR(DE) expression vector (tracks 6-9). As a control 1000ng ofVP16RXR(DE) was tested in the absence of GAL4-TRL454A(DE) (track 1). T3 was added at 200 nM to tracks 3, 5, 7 and 9 and 9-*cis* retinoic acid (9-*cis* RA) at 100 nM to tracks 4, 5, 8 and 9. These results represent the mean values of three independent experiments each done in duplicate; the bars indicate standard deviations).

heterodimers is only observed with the TR ligand. Addition of both T3 and 9-*cis* retinoic acid to the transfected cells activated the reporter to a similar level as T3 alone (track 9) indicating that 9-*cis* retinoic acid does not prevent the T3 dependent increase in recruitment of VP16RXR(DE) by GAL4-TRL454A(DE).

Analysis of the effect of ligand on the formation of RXRα/TRβ heterodimers *in vitro*

The initial approach used to examine whether ligand effected the formation of RXRα/TRβ heterodimers *in vitro* was to analyse its ability to alter the amount of heterodimer detected in a gel shift assay. The binding activity of RXRα/TRβ heterodimers on an oligonucleotide containing a DR+4 reponse element was monitored in the presence and absence of T3 and/or 9-*cis* retinoic acid (Figure 6.3). When incubated seperately neither RXRα or TRβ bound as a homodimers regardless of the addition of their respective ligands (tracks 2-5). When the two receptors were coincubated a heterodimeric complex was formed in the absence of ligand (track 6). The amount of this complex was unaffected by the addition of T3, although a slight increase in the mobility of this complex was observed (track 7) presumably due to a conformational change of the TR. In contrast, addition of 9-*cis* retinoic acid reduced the DNA binding activity of the heterodimer (track 8). Coincubation of T3 with 9-*cis* retinoic acid diminished the effect of 9-*cis* retinoic acid and the mobility of the complex was slightly increased (track 9), as observed with T3 treatment alone. Thus, in the gel shift assay T3 does not promote the formation of RXRα/TRβ heterodimers but appears to partially rescue the 9-*cis* retinoic acid induced decrease in DNA binding activity.

The effect of T3 on the formation of RXRα/TRβ heterodimers in solution was also examined *in vitro* using a 'pull down' assay. Coincubation at 37 ^{o}C with 200 nM T3 increased the amount of *in vitro* synthesised [^{35}S]-methionine labelled GAL4-TR(DE) that specifically interacted with bacterially expressed GST-RXR(DE) (K. Chatterjee, Addenbrokes Hospital, personal communication). It is possible that the discrepancy between the effect of T3 on the formation of RXRα/TRβ heterodimers in the pull down and gel shift assays reflects the different buffer or temperature conditions (room temperature for the gel shift assay and 37 ^{o}C for the pull down assay) used in the two assays. It should also be noted that in the 'pull down' assay (and the two hybrid assay) fusion proteins containing only the C-terminal domains of

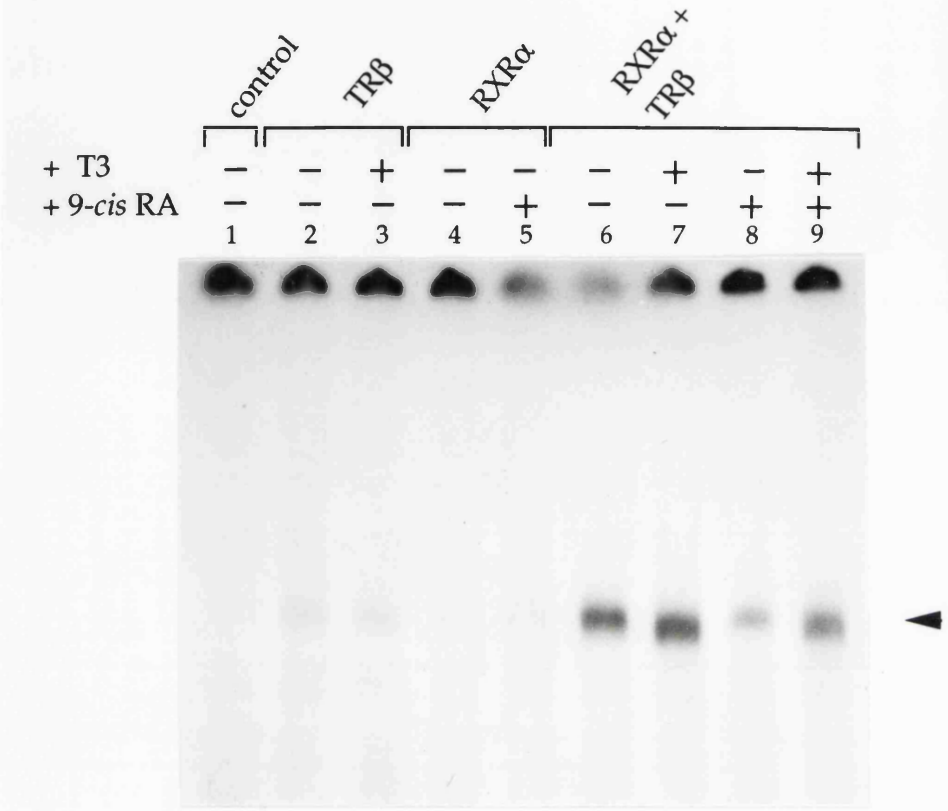


Figure 6.3 The effect of ligand on the formation of RXRα/TRβ heterodimers on the DR+4 response element. The effect of T3 and 9-*cis* retinoic acid on the DNA binding activity of RXRα/TRβ heterodimers was assessed by gel shift analysis using an oligonucleotide probe that contained a DR+4 response element. Equal amounts of *in vitro* synthesised RXRα and/or TRβ receptors were preincubated in the presence or absence of 9-*cis* retinoic acid (9-*cis* RA) (100 nM) and/or T3 (200 nM) prior to the addition of labelled DNA. The position of the heterodimer is indicated by a solid arrowhead. The free probe was run off the gel to allow better resolution of the complexes.

the receptors were used whereas in the gel shift assay wild type receptor proteins were used. Alternatively, the effect of T3 on the heterodimer may depend on whether the heterodimer is in solution (the two hybrid assay) or bound to DNA (the gel shift assay).

Summary and conclusions

The results from the two hybrid assay in intact cells suggest that T3 but not 9-*cis* retinoic acid promotes the formation of RXRα/TRβ heterodimers. When assayed *in vitro* the formation of RXR/TR heterodimers was increased by T3 when the receptors were incubated in solution but no increase was observed when the receptors were coincubated with DNA. It is unclear whether the stabilising effect of T3 on the heterodimer is dependent on the receptors being in solution or whether the difference is an artefact of the different assay conditions used. A more detailed analysis of the effect of T3 on heterodimer formation is required to clarify this point.

CHAPTER 7
DISCUSSION

CHAPTER 7

Introduction

The negative regulation of gene expression is an important regulatory process involved in generating the complex patterns of gene expression required for growth, differentiation and homeostasis. There are two main types of repression mediated by nuclear factors, so called passive and active repression (see Chapter 1). Members of the nuclear receptor family can inhibit transcription of certain genes in addition to their function as transcriptional activators. The inhibitory action includes passive repression not only of other nuclear receptors but also other transcription factors as well as active gene silencing. The phenomenon of interference between members of the same transcription factor family is a common regulatory process. The inhibitory protein Id forms DNA binding deficient heterodimers with other helix loop helix proteins such as MyoD (Benezra *et al* 1990). I-Pou negatively regulates POU homeodomain proteins by a similar mechanism (Treacy *et al* 1991). The transcription factor Mad inhibits the Myc/Max heterodimer by sequestering Max (Ayer *et al* 1993) and a truncated form of FosB, delta FosB, acts to inhibt Fos/Jun complexes by forming a transcriptionally defective Jun dimer (Nakabeppu and Nathans 1991).

Studies have shown that coexpression of hormone receptors can result in interference of the transcriptional activity of the individual receptors. Such behaviour may allow crosstalk between different ligand signalling pathways. The orphan receptors COUP-TF I and II have been shown to repress transactivation mediated by many different nuclear receptors on a variety of sites, contrasting with the more restricted inhibitory action of other family members. The ability of COUP-TF to act as a general inhibitor of nuclear receptor activity might place it in a central role in a number of signalling pathways. In such a model the intracellular concentration of COUP-TF would influence the activity of these pathways with alterations in the expression of this orphan receptor effecting the magnitude of the response.

Expression of COUP-TF

The mRNAs of COUP-TF I and II are widely expressed in adult tissues (Ladias and Karathanasis 1991, Miyajima *et al* 1988, cited in Schoorlemmer *et al* 1994) and protein has been detected in a number of cell types suggesting that at the protein level COUP-TF is also widely expressed. This is further supported by reports from a number of groups that implicate COUP-TF I

and/or II in repression of specific genes in various cell types (including (Bakke and Lund 1995, Galson *et al* 1995, Kimura *et al* 1993, Ladias *et al* 1992, Liu *et al* 1993, Miyata *et al* 1993, Schoorlemmer *et al* 1994, Wehrenberg *et al* 1994). The relative levels of protein differs between cell types and thus it is likely that the ability of COUP-TF to down regulate the activity of nuclear receptors displays cell specificity (figure 3.5) (Ladias *et al* 1992, Orchard *et al* 1992).

Many of the cell lines in which COUP-TF has been identified are cancer cell lines. The ability of this orphan receptor to inhibit the activity of nuclear receptors that have been implicated in the transformation of cells to a malignant phenotype makes it tempting to speculate that COUP-TF may also be involved in this process. The group of Kieback et al (Kieback *et al* 1993b) have shown using immunocytochemistry that COUP-TF expression in human ovarian cancer cell lines is inversely correlated with the expression of the ER. As this group have also demonstrated that ER level has a direct prognostic correlation in advanced human ovarian cancer (Kieback *et al* 1993a) they speculate that COUP-TF may be involved in either the metabolic or dedifferentiation processes of these cancers. However, as only four cell lines were analysed in this report the inverse correlation between COUP-TF and ER expression will need to be confirmed in a more comprehensive study. COUP-TF was also detected in a panel of breast and endometrial tumours with higher levels of expression in the endometrial tumours than the breast tumour samples (Figure 3.6). It would be of interest to discern whether the different levels of COUP-TF correlate with differences in the magnitude of the cellular response to oestrogen and/or retinoids.

In Chapter 3 the expression of COUP-TF in the breast cancer cell lines MCF-7 and ZR-75-1 was shown to be down regulated by oestrogen and upregulated by retinoic acid (9-*cis* or all-*trans*) (Figure 3.7). The action of COUP-TF is therefore modulated by two of the signalling molecules it acts to repress. The inhibitory effect of COUP-TF on the activity of the ER would presumably be restricted by the oestrogen mediated decrease in its expression whereas in the case of retinoic acid treatment COUP-TF expression would be stimulated and hence its capacity to function as a repressor increased. Both COUP-TF I and II mRNAs have been shown to be induced by retinoic acid in mouse P19 embryonal carcinoma cells, the the kinetics of this up-regulation shows a delayed response, only being observed after 24-48 hours in contrast to the immediate induction of other retinoic acid inducible genes such as the RARβ gene (Jonk *et al* 1994). As well as COUP-TF and RARβ, retinoic acid

also regulates the expresion of RXR genes in P19 EC cells, such that RXRα is induced, RXRγ is down regulated and RXRβ is unaffected (Jonk *et al* 1994). It is also interesting to note that although COUP-TF can bind the RARE in the promoter of the RARβ gene it has been reported to repress expression of the gene relatively inefficiently (Kliewer *et al* 1992a, Tran *et al* 1992). Thus, retinoic acid treatment results in a complex pattern of altered RAR, RXR and COUP-TF gene expression .

The expression of COUP-TF may also be regulated by other signalling molecules. This prediction is supported by the observation that grafting of an ectopic notochord results in induction of the chicken COUP-TF II gene (Lutz *et al* 1994). Differential expression of COUP-TF, both temporally and spatially, would provide one mechanism for the diversification of the cellular response to hormones such as retinoic acid.

Transcriptional repression by nuclear receptors

The different mechansims by which COUP-TF may repress nuclear receptor mediated transactivation are now discussed with reference to the inhibitory action of other nuclear receptors.

Formation of inactive heterodimers

The observation that COUP-TF I and II were able to form heterodimers with RXRα on a DR+1 element suggested that COUP-TF might repress the transcriptional activity of RXR by sequestering it into an inactive heterodimer (Figure 4.3) (Kliewer *et al* 1992a)(Cooney *et al* 1993). COUP-TF I and II have also been shown to form heterodimers with RXRα on the ApoAI-RARE element (Widom *et al* 1992) and DR+1, DR+2, DR+4 and DR+5 elements (Figure 4.4) while heterodimers with TRα, TRβ and RARβ were detected on a TREpal element (Figure 4.5) (Berrodin *et al* 1992). It is conceivable that the formation of transcriptionally inactive COUP-TF heterodimers may therefore represent a general mechanism by which COUP-TF modulates the cellular responses to retinoids and T3. Receptors that bind to DNA as heterodimers with RXR could be inhibited through the formation of an inactive COUP-TF heterodimer either with the specific receptor or with RXR (Figure 4.1a and b). When the concentration of RXR within a cell is limiting it is predicted that receptors whose activity depends on the formation of heterodimers with this protein will compete for RXR. As a result of the lack of available RXR the transcriptional activity of these receptors would be reduced, particularly those receptors that compete

inefficiently for RXR. This idea is supported by the observation that addition of RXR specific ligands which promote the formation of RXR homodimers reduce the transcriptional activity of the TR, possibly due to the lack of available RXR with which to bind DNA (Lehmann *et al* 1993). Sequestration of RXR by COUP-TF would therefore have wider consequences on gene expression than titration of one of the receptors that dimerises with RXR.

COUP-TF II/RXRα heterodimers were not detected using a two hybrid assay in intact cells (Figure 4.11) and although some T3 dependent heterodimers with TRβ were formed it was at significantly lower levels than the formation of RXRα/TRβ heterodimers (compare Figures 4.12 and 6.2). These experiments suggest that COUP-TF II is unlikely to sequester either RXRα or TRβ off DNA. The inability of COUP-TF II to interact with RXR in solution *in vivo* is supported by the failure of the C-terminal domain of COUP-TF II to inhibit the transcriptional activity of RXRα (Figure 4.14). The corresponding regions of RAR and TR, that can interact with GAL4-RXR(DE) in the two hybrid assay, act as a dominant negative inhibitor of RXR presumably through the formation of DNA binding deficient heterodimers (Barettino *et al* 1993, Durand *et al* 1994, Forman *et al* 1995). As isoforms of the TR, RAR and ER exist that do not bind DNA these may act as naturally ocuring dominant negative receptors (Bigler *et al* 1992, Wang and Miksicek 1991, Zelent *et al* 1991).

The inability of COUP-TF II to form heterodimers with TR or RXR in solution when assayed in intact cells conflicts with a report from Muscat et al (Muscat *et al* 1995) in which increasing amounts of bacterially expressed COUP-TF II tagged with glutathione-S-transferase (GST-COUP-TF II) blocked the binding of TR and RXR/TR heterodimers to DR+4 type TREs. There are no other examples of COUP-TF sequestering TR or RXR off DNA *in vitro*, rather there is evidence to the contrary (Figure 4. 5) (Cooney *et al* 1993, Tran *et al* 1992). As bacterially expressed GST alone was not tested in the study it is possible that the inhibition of DNA binding was a non specific effect of the GST moiety or due to a contaminant in the bacterial extract.

Although COUP-TF II preferentially forms homodimers in the two hybrid assay, in which neither of the receptors being tested is directly bound to DNA, it is possible that the formation of heterodimers are favoured when the receptors are bound to hormone response elements (HREs). This would explain the discrepancy between the inability to detect COUP-TF II heterodimers in the two hybrid assay and the detection in the gel shift assay

of heterodimers with RXRα and TRβ (Figure 4.3, 4.4 and 4.5b). Recently the group of Kurokawa et al (Kurokawa *et al* 1994) have demonstrated two novel mechanisms by which the activity of receptors within a heterodimer can be inhibited suggesting two possible models for the inactivation of a receptor if it bound DNA as a COUP-TF heterodimer. Firstly they reported that, although an RXRα homodimer can bind ligand and activate transcription from a DR+1, within an RXRα/RARα heterodimer RXRα is prevented from binding ligand and is therefore transcriptionally silent. This modification of the ligand binding properties of RXRα occurs when the heterodimer is bound to DNA (on a DR+1 or DR+5 response element) but is not observed when RXRα and RARα are coincubated in solution. Similarly, displacement of ligand from RXR has since been reported for the RXRα/TRα heterodimer on a DR+4 element (Forman *et al* 1995). It seems likely that when the RXRα heterodimer binds DNA the conformation of the ligand binding domain of RXRα is altered such that it can no longer bind ligand with high affinity.

Work from three groups has suggested that the activity of RXRα can also be suppressed by RAR and TR when neither of the receptors are directly bound to DNA. Coexpression of RARα, TRα and TRβ has been demonstrated to inhibit the transcriptional activity of RXR when it is tethered to DNA via the heterologous GAL4 DNA binding domain (Forman *et al* 1995, Perlmann and Jansson 1995, Qi *et al* 1995). As the reporter plasmids used lack any defined HRE any interactions between the coexpressed receptors are presumably mediated through the ligand binding domains of the receptors in solution. Thus, inhibition of the transcriptional activity of the RXR chimera does not depend on the binding of the heterodimer to an HRE. It is feasible that *in vivo* the formation of heterodimers with RAR and TR in solution prevents the RXR chimera binding ligand although *in vitro*, when RXR/RAR heterodimers are formed off DNA, RXR retains the ability to bind ligand (Forman *et al* 1995, Kurokawa *et al* 1994). It is also possible that the interaction between the ligand binding domains blocks the ability of the RXR chimera to be transcriptionally active at a different step. The partner receptor may prevent an interaction between RXR and a protein required for transactivation such as a cofactor or basal transcription factor, either by inducing an inappropriate conformation or by steric interference.

Samuels and coworkers (Casanova *et al* 1994) have proposed an alternative model in which the unliganded C-terminal domain of TRα

silences the activity of RXR through the recruitment of an inhibitory factor. This factor is then proposed to dissociate from the heterodimer upon addition of T3. The activation domain of VP16 was shown to be inhibited when it was fused with a GAL4-TRα chimera (GAL4-TR-VP16) and this could be relieved by coexpression with unliganded TR presumably by titration of the inhibitory factor. Although it was suggested that COUP-TF was a candidate for the inhibitory factor no interaction between TRβ and COUP-TF II has been observed in intact cells in the absence of ligand and in fact some ligand dependent formation has been detected (Figure 4.12). Furthermore, there are a number of pieces of evidence that suggest the addition of T3 actually promotes inactivation of RXR within a RXR/TR heterodimer. Firstly, the addition of T3 to a heterodimer on DNA enhanced the inhibition of ligand binding by RXR (Forman *et al* 1995). Secondly, when TR and RXR or GAL4-RXR(DE) are coexpressed the addition of 9-*cis* retinoic acid or the RXR specific ligand LG69 with T3 does not increase the reporter activity over that seen with T3 alone, infact a decrease is observed (Forman *et al* 1995). The simplest interpretation of these data is that in the presence of T3 and 9-*cis* retinoic acid the heterodimer selectively binds T3 and that the TR but not RXR is able to stimulate transcription. By analogy to the inhibition of RXR, it is conceivable that the formation of a heterodimer with COUP-TF could inactivate a hormone receptor by inducing a conformational change that blocked either ligand binding or a subsequent step in transactivation.

The second mechanism by which a receptor in a heterodimer can be repressed is illustrated by the ability of RARα in a RXRα/RARα heterodimer to activate transcription from a DR+5 element but not a DR+1 element (Kurokawa *et al* 1994). When an RXRα/RARα heterodimer binds to a DR+5 element, RARα binds to the downstream half site and upon ligand binding is able to stimulate transcription. However, on a DR+1 element RARα binds to the upstream half site and fails to transactivate demonstrating that the polarity of binding can modulate the transcriptional activity of RAR. By analogy, the transcriptional activity of a receptor could be inhibited if it bound DNA as a heterodimer with COUP-TF and was placed on the 'silent' half site of a response element.

Although other RXR heterodimers only bind with high affinity to specific classes of response element there is no evidence that binding to DNA is required for the formation of the dimer. Rather it is thought that the heterodimers are formed in solution (Kliewer *et al* 1992b, Leid *et al* 1992b,

Marks *et al* 1992) and bind selectively to those response elements on which the DNA binding domains interact cooperatively (Kurokawa *et al* 1993, Mader *et al* 1993b, Perlmann *et al* 1993, Zechel *et al* 1994a). In support of this prediction, the formation of RXR heterodimers with RAR, TR and the orphan receptors NGFI-B, NURR1 and LXR have all been detected in a two hybrid assay (Forman *et al* 1995, Nagpal *et al* 1993, Perlmann and Jansson 1995, Tone *et al* 1994, Willy *et al* 1995). If COUP-TF II does form DNA dependent heterodimers *in vivo* this would imply that COUP-TF II undergoes a conformational change upon binding DNA that converts its dimerisation interface(s) from being homodimer specific to being competent for both homodimer and heterodimer formation.

It is difficult to discriminate between the binding of COUP-TF homodimers and heterodimers to DNA *in vivo*. In Chapter 4 an indirect assay was used to monitor the formation of COUP-TF II/RXRα heterodimers on DNA in transfected cells. The ApoAI-RARE is bound by COUP-TF II/RXRα heterodimers *in vitro* with ten fold greater affinity than either homodimer (Widom *et al* 1992). If this site is also bound preferentially by the heterodimer *in vivo* then more RXRα should be bound in the presence of COUP-TF II than when RXRα is expressed alone. The amount of transfected RXRα bound to a reporter gene plasmid containing an ApoAI-RARE was monitored by tagging the receptor with the activation domain of VP16. The ability of cotransfected COUP-TF II to increase the amount of VP16RXR bound to the reporter was then tested and from these experiments it appears that COUP-TF II is unable to recruit VP16RXR to the ApoAI-RARE (Figure 4.15a). In contrast RARα, which can form heterodimers with RXR in the two hybrid assay, is able to recruit VP16RXR to this site. Similar results were obtained using a DR+5 response element (Figure 4.15b) or a DR+1 response element (data not shown). Thus, while it remains feasible that COUP-TF II might form heterodimers with certain receptors, it is extremely doubtful whether the formation of inactive heterodimers is a general mechanism by which it mediates repression. This is further supported by the failure to detect COUP-TF heterodimers *in vitro* on sites through which it can mediate repression *in vivo*, such as the SF-1 response elements in the steroid 21-hydroxylase gene (-210 21OH element) (Figue 4.6) and the steroid 17α-hydroxylase genes (Bakke and Lund 1995), the DR+7-RARE in the arrestin gene (Lu *et al* 1994) and the peroxisome proliferator responsive element in the hydratase dehydrogenase gene (Miyata *et al* 1993).

Competition for DNA binding

An alternative mechanism for COUP-TF mediated repression involves COUP-TF homodimers competing with the transcriptionally active receptor for DNA binding (Figure 4.1c). In such a model COUP-TF homodimers would have to recognise a variety of response elements and in support of this prediction COUP-TF homodimers have been shown to bind with similar affinity to oligonucleotides containing direct repeats of the (A/G)GGTCA motif separated by 0-5 nucleotides and to an inverted repeat with 0 nucleotide spacing (TREpal) (Figure 5.1) (Cooney *et al* 1992, Kadowaki *et al* 1992). These sites are consensus response elements for a group of nuclear receptors that includes the TR, RAR, RXR, VDR and a number of orphan receptors (see Chapter 1). In addition to the consensus sequences COUP-TF II homodimers could recognise with similar affinity natural response elements that contain imperfect half site sequences (Figure 5.3). The ability of COUP-TF to mediate repression in transient transfection assays through a diverse set of response elements may reflect the promiscuous DNA binding activity of the COUP-TF homodimer. Due to the overlap in sequence recognition between various nuclear receptors competition for DNA binding sites is a common model for many of the examples of repression within this family.

TRα and RXRβ can block transcriptional activation by the ER from a reporter containing the vitellogenin A2 ERE (Glass *et al* 1988, Holloway *et al* 1990, Segars *et al* 1993). Both TRα and RXRβ bind to the ERE with high affinity *in vitro* and it has been proposed that these receptors inhibit ER activity by displacing it from the ERE by direct competition for DNA binding (Glass *et al* 1988). Addition of T3 appears to either be required for (Glass *et al* 1988, Segars *et al* 1993), or to strongly enhance (Holloway *et al* 1990), the repression of TR mediated repression. The role of T3 in this process is unclear as *in vitro* studies have demonstrated that ligand is not required for DNA binding by the TR. One possibility is that *in vivo* the unliganded TR only weakly recognises the ERE and the ligand is required to stabilise the interaction or, alternatively, the binding of ligand may be required for a subsequent step involved in the repression. Inhibition mediated by RXRβ is independent of its ligand however, as with TRα, a functional DNA binding domain is not sufficient for repression and both the N and C-terminal domains of RXRβ are also required (Segars *et al* 1993). These domains may make specific protein/protein contacts that stabilise the binding of RXRβ to the ERE or alternatively they might recruit a negative cofactor. Intriguingly,

although *in vitro* both TRα or RXRβ can bind the ERE with high affinity neither is able to stimulate transcription from the ERE containing reporter in transfected cells suggesting that when bound to this site both the TR and RXR adopt a transcriptionally inactive conformation.

In addition to the negative effect on oestrogen action the TR can also inhibit the effects of retinoic acid. Coexpression of TRα or TRβ in the absence of T3 can block all-*trans* retinoic acid stimulation by RARβ from a TREpal response element and upon addition of T3, TR mediated transactivation of the reporter is observed (Graupner *et al* 1989). RAR and TR bind the TREpal element preferentially as heterodimers with RXR (Kurokawa *et al* 1993, Leid *et al* 1992b, Marks *et al* 1992, Yu *et al* 1991) and it is likely that the inactive RXR/TR heterodimer displaces the active RXR/RAR heterodimer by direct competition for DNA binding (Au *et al* 1993). Unliganded TRα can also suppress the retinoic acid induction of the RARβ promoter through a DR+5 type RARE (β-RARE). However, although the TRα can recognise this site as an RXRα/TRα heterodimer it binds with considerably lower affinity than the RXRα/RARβ heterodimer (Bugge *et al* 1992). Furthermore, a TRα deletion mutant that lacks the DNA binding domain retains the ability to suppress the retinoic acid induction (Barettino *et al* 1993). While these data do not rule out that direct competition for DNA binding between RXR/TR and RXR/RAR can occur they illustrate that DNA binding by the TR is not required for repression. The observations that *in vitro* TRα could compete with RARβ for RXRα and that coexpression of RXRα in transfected cells could alleviate the inhibitory effects of the unliganded TRα suggests an alternative mechanism whereby the TR inhibts RAR function by sequestering the common partner RXR.

As well as inhibitory effects between different hormone receptors antagonsim also occurs between different isoforms of the same receptor. The TRα splice variant TRα-2, which differs from TRα-1 in the C-terminal region, fails to bind hormone and is consequently unresponsive to T3. Cotransfection of TR-α2 results in inhibition of T3 stimulation of gene expression (Koenig *et al* 1989, Lazar *et al* 1989a). Work by Lazar (Katz *et al* 1992) and coworkers has shown that this isoform does not form heterodimers with either other forms of the TR or with other receptor types. Two pieces of evidence suggest that TRα-2 inhibits the response to T3 by direct competition for DNA binding. Firstly, the DNA binding domain of TRα-2 is required for the dominant negative activity of this receptor and

secondly, a mutant TRα-2 that binds with higher affinity to a TRE is a more effective inhibitor of T3 activation (Katz and Lazar 1993).

Squelching

Squelching has been proposed to explain the observed inhibitory behaviour displayed by steroid hormone receptors. Coexpression of the ER in the presence of oestrogen has been shown to inhibit the activity of the PR and conversely in the presence of its specific ligand, the PR could similarly repress the oestrogen stimulation from a reporter gene containing the vitellogenin A2 ERE (Meyer *et al* 1989). DNA binding of the receptor was not required and the amino terminus or the ligand binding domain alone could mediate the effect. Progestins could also block the ability of oestrogen to stimulate pS2 transcription in MCF7 cells when the receptors were not overexpressed indicating that this type of inhibition could be observed at physiological concentrations of receptor and effect endogenous gene expression. As no physical interaction between the ER and PR was detected it is unlikely that the inhibition is due to the formation of inactive heterodimers. A model is favoured in which the receptor acts by squelching a common cofactor(s) necessary for ligand activated transcription. The ability of endogenous levels of PR to inhibt the activity of the ER suggests that this cofactor(s) must be present in limiting concentrations in MCF7 cells and possibly other cell types.

In addition to splice variants of TRα it has been proposed that N-terminally truncated TRα isoforms are generated by alternative translational initiation (Bigler *et al* 1992). These smaller proteins contain the C-terminal region but lack the DNA binding domain and although no detectable heterodimers are formed with the wild type receptor they can inhibit its activity. These truncated mutants may cause repression by squelching a cofactor necessary for the transcriptional activation.

The deletion mutant CII(DE), that contains only the C-terminal region of COUP-TF II, fails to inhibit 9-*cis* retinoic acid stimulated transcription suggesting that COUP-TF II is unlikely to sequester a cofactor necessary for RXR mediated transactivation. Furthermore, as wild type COUP-TF II is unable to inhibit the T3 stimulation of GAL4-TR(DE) (Figure 4.13) or the 9-*cis* retinoic acid stimulation of GAL4-RXR(DE) (data not shown) it is unlikely that a region outside the C-terminus can mediate squelching. Taken together these data show that the dimerisation interface of COUP-TF II is insufficient

to mediate repression and suggest that COUP-TF II needs to be bound to DNA to inhibit the hormone induction of transcription.

In summary, the model favoured for the general mechanism by which COUP-TF II represses nuclear receptor mediated transcription involves COUP-TF II homodimers competing for DNA binding sites. This type of repression, displacement of the active factor from DNA, is probably the most common mechanism used by nuclear receptors to down regulate the activity of other family members. A variation on this model is illustrated by the inhibtion of the oestrogen stimulation of the mouse lactoferrin gene by COUP-TF. The repression of the activity of the ER on this gene appears to be mediated by COUP-TF binding to a site that overlaps the ERE (Liu *et al* 1993).

While there are less reported examples of the formation of inactive heterodimers the recent discovery that the ligand binding activity of RXR can be inhibited by particular heterodimer partners (RAR and TR) suggests that this mechansim may play a significant role in restricting the activity of RXR and perhaps other hormone receptors. This type of repression provides a more subtle control of gene transcription because although the response to one receptor is inhibited, the expression remains sensitive to the partner receptor. Similarly, the polarity with which a heterodimer binds to DNA may turn out to be a commonly used mechanism for inactivating one of the receptors in a heterodimer. For instance, it is already known that on direct repeat response elements RARα seems only to be transcriptionally active when bound to the 3' site and it is feasible that other receptors might only be active on the 3' or indeed the 5' half site while others might be active on either. Lastly, although steroid hormone receptors may cause mutual inhibition through squelching of a necessary cofactor the general physiological significance of this mechanism is less clear.

Repression of other transcription factors

Nuclear receptors not only inhibit the transcriptional activity of other nuclear receptors but also that of a number of other transcription factors. The potential mechanisms involved are similar and include competiting for DNA binding to overlapping response elements, the formation of inactive complexes and squelching of a common cofactor.

The interference of AP-1 activity is probably the best studied example of a transcription factor inhibited by nuclear receptors and can involve either

DNA binding dependent or independent mechanisms. The GR, RAR, RXR and TR have been shown to reduce the activity of AP-1 complexes, the mechanism involved in the inhibition is thought to be dictated by the type of response element tested. The so-called 'simple response elements' such as that in the collagenase promoter contain only an AP-1 binding site and DNA binding by the nuclear receptor is not required for repression. Various models have been proposed for the mechanism of this type of repression. These include squelching by the receptor of a cofactor necessary for AP-1 mediated activation. Alternatively, the nuclear receptor may bind to the AP-1 complex and inactivate it, perhaps by physically blocking the interaction with the transcription machinery. (Jonat *et al* 1990, Salbert *et al* 1993, Yang *et al* 1990). The 'composite response elements' such as the AP-1 site in the proliferin promoter contain both an AP-1 site and a binding site for the negatively acting nuclear receptor. The binding of the GR to the proliferin promoter has been proposed to block the transcriptional activity of the AP-1 complex bound at an adjacent site (Diamond *et al* 1990, see Miner and Yamamoto 1991). However, it has not yet been demonstrated that the GR mediated repression *in vivo* requires binding of the receptor to the GRE (Diamond *et al* 1990, Jonat *et al* 1990, Yang *et al* 1990, see Akerblom and Mellon 1991, Saatcioglu *et al* 1994 for reviews of nuclear receptor mediated repression of AP-1 activity).

Other transcription factors have also been reported to be repressed by nuclear receptors including, CREB (CRE-binding protein) (Akerblom *et al* 1988), the lymphocyte specific factor Oct-2A (Wieland *et al* 1991) and the pituitary specific factor-1 (pit 1) (Adler *et al* 1988).

Repression of basal transcription- transcriptional silencing

The C-terminus of TR has been demonstrated to contain an active transcriptional silencing domain (see Chapter 1) (Baniahmad *et al* 1992a, Baniahmad *et al* 1992b). The ability of COUP-TF to act analagously and repress basal transcription has also been investigated. Chimeric forms of the COUP-TF proteins, containing the GAL4 DNA binding domain, have been used for these studies. As mammalian cells do not contain a GAL4 homologue inhibition of the reporter activity through the GAL4 site is indicative of active rather than passive repression (see Chapter 1). The C-terminal domains of both COUP-TF I and II when fused to the GAL4 DNA binding domain could silence transcription from the TK promoter (Figure 4.16)(Cooney *et al* 1993). The GAL4 chimera of COUP-TF II did not, however,

silence transcription from a GAL4 responsive reporter containing a minimal promoter comprised of the adenovirus E1b TATA box (Figure 4.16) suggesting that the silencing activity of COUP-TF is promoter specific. In contrast, the C-terminal domain of the TR fused to the GAL4 DNA binding domain is able to actively repress transcription from both reporters, although the silencing activity is most marked on the reporter that contains the TK promoter (Figure 4.16).

Whether the full length COUP-TF protein can act as an active silencer of transcription is not clear. In Chapter 4 an attempt was made to investigate the behaviour of cotransfected COUP-TF II on basal transcription on a number of different types of response element upstream of the TK promoter (Figure 4.17). Unfortunately, the transcriptional properties of COUP-TF II on these various elements were difficult to assess because the activity of the parental reporter gene plasmid, that lacks a defined COUP-TF binding site, was also altered by COUP-TF II. It is unlikely that COUP-TF II is binding to a cryptic site in the TK promoter because other reports have shown that COUP-TF II has no effect on the TK promoter albeit in different cell types to that used in this study (Ge *et al* 1994, Kadowaki *et al* 1995). In addition, COUP-TF II stimulated expression from the internal control vector which contained the CMV promoter. Thus overexpression of COUP-TF II appears to alter transcription in a relatively non specific manner and independently of defined binding sites, possibly through the sequestering of transcription cofactors.

There is some circumstantial evidence consistent with, although not proof of, a silencing activity within COUP-TF. The first is the observation by Kadowaki et al (Kadowaki *et al* 1995) that activation mediated by COUP-TF I from a site downstream of the mouse mammary tumour virus (MMTV) promoter (transcriptional activation by COUP-TF is discussed later) could be inhibited by COUP-TF I bound to a site upstream of this promoter. Secondly, Ge and coworkers (Ge *et al* 1994) have demonstrated that the isolated DNA binding domain of COUP-TF II, which binds with comparable affinity to wild type protein, is insufficient to repress expression of the apolipoprotein AI gene. This indicates that other regions of the protein are required to mediate the inhibitory effect. However, COUP-TF II does not reduce the transcription levels below that of the promoter lacking the COUP-TF binding site suggesting that the additional regions of COUP-TF II are involved in passive rather than active repression of transcription. Taken together the data suggest that in general COUP-TF does not interfere with basal transcription

although it remains possible that on certain sites it can act to silence transcription. The silencing activity of the C-terminal domains of COUP-TF I and II, observed when they are fused to the GAL4 DNA binding domain may be masked in the full length protein due to an induced conformational change or by steric hindrance.

COUP-TF as a transcriptional activator

O'Malley and coworkers made the original observation that COUP-TF could function as a transcriptional activator. Using an *in vitro* transcription assay they showed that activation from the chicken ovalbumin promoter required both COUP-TF, which recognised a specific site in the promoter, and TFIIB. COUP-TF I and II can physically interact with TFIIB *in vitro* (Ing *et al* 1992, Malik and Karathanasis 1995) although the region(s) of TFIIB that are contacted have not been determined. As for the other members of the nuclear receptor family, the functional significance of this interaction is not clear. More recently, COUP-TF II has also been reported to stimulate transcription *in vitro* (Malik and Karathanasis 1995). In this study both the wild type COUP-TF II and GAL4 chimeric proteins, containing either the C-terminus of COUP-TF II or the N-terminus and DNA binding domain, were shown to be capable of stimulating transcription. Thus, COUP-TF II contains two independent domains that are capable of stimulating transcription *in vitro*. Tran et al (Tran *et al* 1992) have shown that the COUP-TF binding site in the ovalbumin promoter, used by O'Malley and coworkers in the *in vitro* assay, can function as an RARE in transiently transfected cells. They also demonstrated that coexpression of COUP-TF I repressed the retinoic acid stimulation from this element and similarly, COUP-TF II repressed the retinoic acid stimulation from the ApoAI-RARE in intact cells and activated through this site *in vitro* (Malik and Karathanasis 1995, Widom *et al* 1992). These results need not be contradictory if the transcriptional activity of COUP-TF is less potent than that of the RXR/RAR heterodimer. In such a case, transcriptional activation by COUP-TF *in vivo* would be masked by the simultaneous inhibition of the activity of ligand activated RAR and RXR.

There are several reports that support a positive role for COUP-TF in transcriptional regulation *in vivo*. Overexpression of COUP-TF II in HeLa cells resulted in non specific activation (Figure 4.17b) which is intriguing as HeLa nuclear extracts were used in the *in vitro* transcription assays. In addition to this non specific activation, there are an increasing number of reports that suggest that COUP-TF can stimulate gene expression through

specific response elements in transfected cells (Kliewer *et al* 1992a, Lu *et al* 1994, Miyata *et al* 1993, Rottman and Gordon 1993). It is possible that *in vivo* COUP-TF functions as either a transcriptional activator or repressor depending on certain factors such as the particular response element and cell type.

The transcriptional activity of COUP-TF appears to be modulated by a variety of factors including cell type, phosphorylation and response element sequence and position. Although no ligand has been identified for COUP-TF it remains a further possibility that, analagous to the hormone receptors, its activity could be stimulated by the binding of an exogenous ligand. An example of the cell specificty of COUP-TF transcriptional activity is illustrated by the ability of COUP-TF II to stimulate transcription from an upstream element in the rat intestinal fatty acid binding protein gene in Caco-2 cells (human colon adenocarcinoma) but not in CV1 cells (african green monkey kidney cells) (Rottman and Gordon 1993). The lack of activity in CV1 cells may reflect either the presence of a competing transcription factor or the absence of a putative endogenous ligand (or other type of modification, discussed later) or a cofactor required for activation. It should be noted, however, that COUP-TF can mediate transcriptional activation in this cell line, albeit weakly, when a different reporter construct is tested (Lu *et al* 1994).

While searching for an exogenous ligand for COUP-TF, O'Malley and coworkers identified a ligand independent mechanism for COUP-TF activation. They discovered that treatment of CV1 cells with the neurotransmitter dopamine stimulated the transcriptional activity of a COUP-TF I chimera in which the DNA binding domain had been swapped for that of the PR (Power *et al* 1991a). This activation required the C-terminal domain of COUP-TF I suggesting that the effect of dopamine was not through some unrelated transcription factor nor through the DNA binding domain of the PR. Dopamine does not alter the expression levels of the COUP-TF I chimera nor does it bind to COUP-TF and its effect can be mimicked by a protein kinase A stimulator or a protein phosphatase inhibitor most likely being mediated by phosphorylation (Power *et al* 1991a). The transcriptional activity of COUP-TF may therefore be controlled, at least partially, by the activity of a kinase(s) within the cell. Ligand independent activation is not restricted to COUP-TF but has also been shown to occur with the PR and ER (Power *et al* 1991b). Intriguingly *seven-up*, the *Drosophila* homologue of COUP-TF requires ras activation for its function

suggesting that this protein may also be activated by phosphorylation (Begemann *et al* 1995).

COUP-TF only stimulates transcription from a subset of its binding sites. As COUP-TF assumes alternative conformations on the different arrangements of the half site motifs (Cooney *et al* 1992) it is conceivable that COUP-TF only binds to certain sites in a transcriptionally competent form. This is similar to the behaviour of the hormone receptors which do not activate all the sites they recognise. The TR, for example, binds but does not activate from an ERE, however, if the C-terminal domain is replaced by that of the ER then the chimera is transcriptionally active (Hirst *et al* 1992). The GRE in the promoter of the proopiomelanocortin (POMC) gene which contains three half sites is an example of a 'null' site, unusally three molecules of the GR bind with high affinity to this site but are transcriptionally inactive (Drouin *et al* 1993). More recently, the position of the response element has been proposed to dictate whether COUP-TF can act as a positive or negative factor. As mentioned previously, a COUP-TF response element has been identified downstream of the transcriptional start site of the MMTV promoter through which COUP-TF I can stimulate transcription (Kadowaki *et al* 1995). Intriguingly, if a COUP-TF binding site such as DR+1, TRE or ERE is placed upstream of the MMTV promoter the positive effect of COUP-TF I from the downstream site is blocked. The authors suggest that the downstream position of the site is important for the positive effect of COUP-TF I as it fails to transctivate when the site is upstream of the TK promoter (cited in Kadowaki *et al* 1995).

The region(s) of COUP-TF involved in mediating transactivation are not known but by analogy to the hormone receptors it could be through an activation domain in the N-terminus, AF-1, or in the C-terminus, AF-2. GAL4 chimeras containing either the C-terminus of COUP-TF II or the N terminus and DNA binding domain could stimulate transcription *in vitro*. The isolated N-terminal domains of COUP-TF I and II have not been tested *in vivo* for transcriptional activity. As these domains contain the highest degree of divergence between the two proteins it is tempting to speculate that they might exhibit different transcriptional properties to correlate with their differential tissue and developmental expression.

The AF-2 function of hormone receptors is characterised by a conserved motif present in the C-terminal part of the E domain of the hormone receptors that is required for ligand dependent activation

(Danielian *et al* 1992) (see Chapter 1). This motif is predicted to form an amphipathic α-helical structure and contains hydrophobic residues flanking a highly conserved glutamic acid residue and a less well conserved second acidic amino acid following these residues. In the corresponding sequence of COUP-TF II there are two notable differences, the highly conserved central acidic amino acid glutamate is replaced by aspartate and the second less well conserved acidic amino acid is substituted for a leucine. This region of COUP-TF II has been tested for transactivating properties (Durand *et al* 1994).When the AF-2 sequence of RARα was replaced by that of COUP-TF II, ligand activation by the mutant RAR was severly compromised, however, when the two conserved acidic residues of the motif were reintroduced into the mutant then ligand stimulation was regained. In contrast when the two conserved acidic residues were introduced into wild type COUP-TF II no transcriptional activation was observed (Durand *et al* 1994). The failure of the COUP-TF II mutant to activate transcription could be due to the requirement for a ligand or some other posttranslational modification such as phosphorylation to unmask the AF-2 domain. The observation that the chimeric RAR containing the AF-2 motif of COUP-TF II is inactive, however, suggests that this orphan receptor may not in fact contain a transcriptionally competent AF-2 domain.

DNA binding properties of COUP-TF homodimers

COUP-TF homodimers can bind to various response elements that are composed of half sites arranged in different orientations and with different spacings. The effect of the different arrangements of the half sites on the affinity with which COUP-TF homodimers bind has been assessed using consensus half site sequences. COUP-TF II homodimers were shown to bind with similar affinity to direct repeats of the half site sequence AGGTCA separated by 0-5 nucleotides and to an inverted repeat with no nucleotides separating the half sites (Figure 5.1). Moreover, COUP-TF I and COUP-TF II bound the sequence GGGTCAnAGGTCA spaced by up to 6 nucleotides with greater than 60% relative binding affinity as compared to a 1 nucleotide spacing. If the spacing was increased to 11 nucleotides COUP-TF still bound, although the affinity was decreased to 10%. In addition, the half sites arranged as an inverted repeat with no spacing were recognised with 40% relative binding affinity (Cooney *et al* 1992). Thus, although COUP-TF binds most strongly to a DR+1 arrangement of half sites it can also bind to many other arrangements of the half sites. Such a degenerate binding site

preference is unusual and suggests a flexible mode of DNA binding by the COUP-TF homodimer. This contrasts with the behaviour of steroid hormone receptor homodimers, which bind selectively to inverted repeats with a three nucleotide spacing, and RXR homodimers that bind preferentially to direct repeats with a one nucleotide spacing. Those receptors that bind DNA as heterodimers with RXR also show selectivity, at least partially, for a particular spacing between the half sites with, for example, an RXR/VDR heterodimer preferentially binding a direct repeat with a three nucleotide spacing and an RXR/TR heterodimer to a four nucleotide spacing (Naar *et al* 1991, Umesono *et al* 1991). On the other hand, RXR/RAR heterodimers do display a more degenerate preference, binding with high affinity to elements with a one or five nucleotide spacing as well as to a two nucleotide spacing with lower affinity (Mader *et al* 1993b) (see Chapter 1).

The selectivity for the spacing between the half sites is dictated by the cooperative interaction of the DNA binding domains (see Chapter 1). The formation of this dimerisation interface requires the DNA binding domains to be correctly orientated relative to one another on the DNA and this only occurs on certain conformations of the half site motifs. These additional protein/protein contacts presumably confer increased stability to the receptor/DNA complex. The DNA binding domain of COUP-TF II, like that of RXR, can bind cooperatively to a DR+1 but not to a DR+5 element. Unlike RXR, however, full length COUP-TF II can bind the DR+5 with high affinity as a homodimer implying that COUP-TF II does not require protein/protein contacts between the DNA binding domains to bind cooperatively to direct repeats (figure 5.4) (Lee *et al* 1993).

The deletion mutant CII-155, that lacked the C-terminal dimerisation domain, bound to the DR+5 element as a monomer with similar affinity to the wild type COUP-TF II homodimer (Figure 5.6). This is in marked contrast to the diminshed binding detected for C-terminal deletions of RXR, ER and GR (Lee *et al* 1993, Mader *et al* 1993a). It suggests that the DNA binding domain of COUP-TF II binds to the half site strongly enough to obviate the need for additional stability provided by cooperative binding. The TR DNA binding domain can also bind to DNA as a stable monomer due to the extensive number of DNA contacts formed (Rastinejad *et al* 1995). The increased number of contacts made by the TR DNA binding doamin compared with the DNA binding domains of RXR, ER and GR are provided by its A-helix which makes contact both with the half site motif and nucleotides upstream (see Chapter 1). This explains why nucleotides 5' of the

half site can also influence the affinity with which the TR binds. It is possible that the DNA binding domain of COUP-TF II binds stably as a monomer because, like the TR, it forms a sufficient number of DNA contacts to stabilise the interaction. It is unlikely, however, that the equivalent A box region of COUP-TF II is involved in mediating these contacts as this region can be deleted without affecting high affinity DNA binding (Figure 5.6, mutant CII-155 lacks the A-box).

In the same experiments that monomer binding by the deletion mutant CII-155 was observed full length COUP-TF II bound exclusively as a homodimer (figure 5.6). The reason for the absence of monomer binding by the wild type COUP-TF II could be due to the formation of stable homodimers in solution with little dissociation of monomers available for DNA binding. Alternatively, the C-terminus may mask the DNA binding domain of the monomer. In contrast to COUP-TF, full length TR has been shown to bind DNA as both monomer and homodimer as well as a heterodimer (Katz and Koenig 1994b, Lazar *et al* 1991, Schrader *et al* 1994a).

In addition to preventing the binding of monomers the C-terminus of COUP-TF II seems to restrict the DNA binding activity of the homodimer. On the DR+1 and DR+2 elements the deletion mutant CII-155, lacking the C-terminus, bound more strongly as a homodimer than the wild type. This suggests that the C-terminus restricts the binding of COUP-TF II homodimers to these sites and may explain why the homodimer only shows a modest preference for the DR+1 element despite this being the only spacing on which there is a cooperative interaction between the DNA binding domains. It seems likely that the increased stability afforded by the additional protein/protein contacts is tempered by the unfavourable conformation of the half sites for the dimerisation interface in the C-terminus. In the absence of the C-terminus the protein/protein contacts formed when the DNA binding domains bind cooperatively does result in enhanced DNA binding of COUP-TF II, demonstarted by the mutant CII-155 which has a marked preference for the DR+1 element compared with sites on which the DNA binding domains do not interact (Figure 5.8). On the TREpal element, however, the deletion mutant binds weakly and on this site the C-terminus acts to promote DNA binding by forming stable homodimers (Figure 5.8).

Amino acids C-terminal to the core DNA binding domain (residues 145-155) are required for high affinity binding to DNA by COUP-TF II. As mentioned previously the A box of COUP-TF II is C-terminal to residue 155

and does not seem to be required. However, the region 146-153 is highly homologous to the T box of RXR (Figure 7.1). This region is required for high affinity binding of the homodimer to a DR+1 site and is predicted to make contacts with the nucleotides 5' of the half sites (see Chapter 1). Furthermore, in both RXRα and COUP-TF II, this region is also involved in cooperative binding of the homodimer to the DR+1 element. As the individual DNA binding domains bind to direct repeats in an asymmetric arrangement the cooperative interaction must involve two regions of the protein, one region in the receptor bound to the 5' site and a different region in the receptor bound to the 3' site. Computer modelling predicts that the T-helix of the 3' RXR monomer would make specific contacts with second zinc finger of the monomer on the 5' half site (Lee *et al* 1993). The DNA binding domain of COUP-TF II may also contain such a T-helix, which on the DR+1 site would be predicted to make contacts with the second zinc finger of the 5' COUP-TF II monomer. On other direct repeats the T-helix would be predicted to contact the DNA 5' of the half sites but be unable to contact the 5' monomer and hence cooperative DNA binding would not be observed. One exception may be the DR+2 element on which a protein interface is formed between the DNA binding domains although not as stably as on the DR+1 site (Figure 5.8). As the COUP-TF II DNA binding domain but not that of RXR can bind stably as a monomer implies that the T-box of COUP-TF II makes a greater number of DNA contacts than for RXR providing sufficient free energy for stable complex formation.

On an inverted repeat such as the TREpal the monomers will be arranged in a symmetrical manner, 'head to head' as opposed to the asymmetric 'head to tail' arrangement on direct repeats (Figure 1.2). The C-terminal domain of COUP-TF can interact on both these conformations suggesting that there is a flexible 'linker' between the DNA binding domains and the C-terminus that allows rotation of the two domains approximately 180^{o}, alternatively, different dimerisation interfaces may be formed on the two types of site. Studies by Glass and colleagues (Kurokawa *et al* 1993) on the dimerisation properties of the TR suggest that within the C-terminus a common dimerisation interface is used both on direct and inverted repeats.

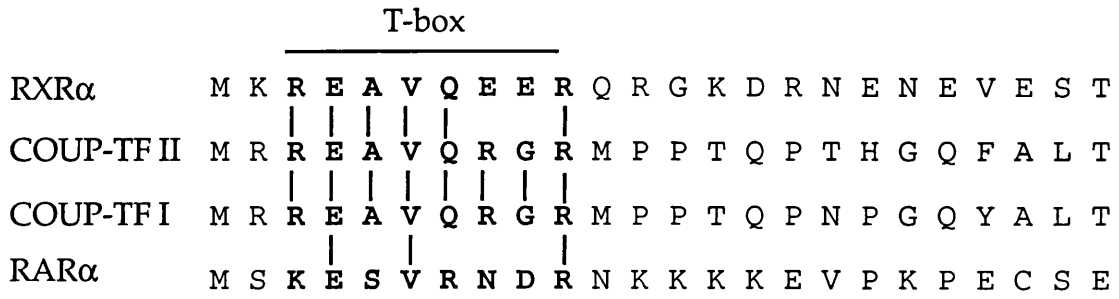


Figure 7.1 Sequence alignment of the T box region of members of the nuclear receptor family.

The sequences shown are human; RXRα (residues 200-223, Mangelsdorf *et al* 1990), COUP-TF II (residues 144-67, Ladias and Karathanasis 1991), COUP-TF I (residues 151-174, Miyajima *et al* 1988) and RARα (residues 153–176, Giguére *et al* 1987). The T-box region is highlighted.

Furthermore, their data suggests that in the RXR/TR heterodimer, bound to a DR+4 element, the C-terminus of the TR is rotated approximately 180° relative to the DNA binding domain as compared with RXR. By analogy, the C-terminal domain of COUP-TF may also exhibt such rotational flexibility to allow it to dimerise on both direct and inverted repeats. Intriguingly, the DNA binding domain of COUP-TF II can bind cooperatively on the TREpal element although this is less efficient than on the DR+1 site (Figure 5.8). This implies that both a symmetric and an asymetric dimerisation interface can be formed between the DNA binding domains. The DNA binding domain of the TR can also bind cooperatively to the TREpal and this seems to involve the D box (Hirst *et al* 1992). This region, within the second zinc finger motif, was originally shown to be required for dimeric binding of the steroid hormone receptor DNA binding domains to inverted repeats with a 3 base pair spacing (discussed in Chapter 1).

The role of ligand in dimer formation

The ligand binding domain and major dimerisation of nuclear receptors colocalise to the C-terminus (see Chapter 1). Ligand binding by a receptor can effect its dimerisation properties, vitamin D has been demonstrated to decrease homodimer binding of VDR to DNA and to enhance the formation of RXR/VDR heterodimers (Cheskis and Freedman 1994)(MacDonald *et al* 1993). T3 similarly destabilises TR homodimer binding but has no effect on RXR/TR heterodimers (Andersson *et al* 1992, Yen *et al* 1992b) while the RXR ligand, 9-*cis* retinoic acid, destabilises both RXR/VDR (Cheskis and Freedman 1994) and RXR/TR heterodimers (Lehmann *et al* 1993). This effect of 9-*cis* retinoic acid may be explained by the observation that it promotes the formation of RXR homdimers (Lehmann *et al* 1993, Zhang *et al* 1992b). The stability of PR and ER homodimers *in vivo* and under certain conditins *in vitro* also appears to be stabilised by their respective hormones (Metzger *et al* 1995, Ylikomi *et al* 1992). A more marked effect of ligand on the formation of dimers on DNA has been observed with some mutant nuclear receptors with the identification of mutant TR and RAR receptors which form homodimers in a ligand independent manner but require their respective ligand to form heterodimers with RXR (Au *et al* 1993). Mutant forms of the ER have been characterised that require oestrogen for homodimer binding under conditions that allow ligand independent DNA binding of the wild type ER (Danielian *et al* 1993). In contrast, a mutant RXR has been described that

forms homodimers in the absence but not the presence of, specific ligand, the complete opposite of the behaviour of wild type receptor (Zhang *et al* 1994). These data suggest that binding of ligand causes a structural change of the receptor which results in an altered dimer interface. Conversely, it seems that dimerisation can alter the ligand binding activity of receptors with the striking observation that the formation of a DNA bound heterodimer with RAR or TR inhibits high affinity ligand binding by RXR (Forman *et al* 1995, Kurokawa *et al* 1994).

There is evidence that ligand can cause structural changes in some receptors. Hormone is known to alter the migration of receptor/DNA complexes, such as the ER and TR, suggesting that they induce a conformational change (Andersson *et al* 1992, Lees *et al* 1989, Toney *et al* 1993, Yen *et al* 1992b). Furthermore, the ligand binding domains of the PR, RAR, and RXR show increased resistance to protease digestion when bound by ligand (Allan *et al* 1992, Keidel *et al* 1994, Leid 1994, Leng *et al* 1995). Interestingly, when progestin antagonists are bound to the PR a different sized fragment is protected from protease digestion suggesting that an alternative conformation of the receptor is induced (Allan *et al* 1992). The resolving of the crystal structure of the C-terminal domain of RXR revealed that the ligand binding site is located away from the dimerisation interface (Bourguet *et al* 1995). This motif is likely to have been conserved within the nuclear receptor family and implies that any ligand induced conformational changes at the dimerisation interface would be an indirect effect mediated through a number of amino acid residues.

In a two hybrid assay T3 but not 9-cis retinoic acid was shown to promote the recruitment of VP16 tagged RXR by a GAL4-TR chimera (Figure 6.1 and 6.2). The ligand 9-*cis* retinoic acid has been reported to stabilise the formation of RXR homodimers *in vitro* and *in vivo* (Lehmann *et al* 1993, Zhang *et al* 1992b) and it has been suggested that this ligand can inhibt the transcriptional activity of the TR by reducing the amount of available RXR with which to form heterodimers (Lehmann *et al* 1993). In the two hybrid assay, coincubation of 9-*cis* retinoic acid with T3 had no effect on the ability of the TR ligand to promote RXR/TR heterodimers indicating that the stabilising effect of T3 on the RXR/TR heterodimer is dominant over the negative effect of 9-*cis* retinoic acid.

The formation of the RXR/TR heterodimer *in vitro* was also enhanced by the addition of T3 to the receptors in solution (K. Chatterjee,

Addenbrooke's Hospital, personal communication) whereas in a gel shift assay the DNA binding activity of the heterodimer was not enhanced by the ligand. It is possible that under different *in vitro* conditions, such as altered temperature, the binding of RXR/TR heterodimers could be affected by T3. Ligand enhanced DNA binding by the ER *in vitro* has recently been shown to be temperature dependent with addition of oestrogen to receptor pre-incubated at 37°C but not 4°C enhancing DNA binding (Metzger *et al* 1995). Alternatively, the heterodimer may be more stable when bound to DNA than in solution and consequently less affected by the binding of T3. The role of ligand on dimerisation of nuclear receptors has been poorly defined *in vitro*primarily because of the difficulty of reproducing physiological conditions. Although a more thorough *in vitro* analysis of the effect of T3 on TR dimerisation is clearly warranted it is *in vivo* studies that will provide the most significant evidence. As the two hybrid system is performed in intact cells the conditions of this assay mimics to a certain extent the conditions *in vivo* although it does utilise overexpressed chimeric proteins. To further characterise the role of T3 in TR heterodimer formation additional studies will be required *in vivo* that can monitor the effect of T3 on the dimerisation properties of the TR both in solution and on DNA.

APPENDIX

KEY TO APPENDIX


ColEI origin of replication


F1 filamentous phage origin of replication


β-lactamase coding sequence (Amp^r)


SV40 origin of replication and early promoter


Intron II of rabbit β-globin gene


SV40 large T polyadenylation signal


T3 or T7 RNA polymerase promoter


Human COUP-TF II coding sequence


Human COUP-TF I coding sequence


Human RXRα coding sequence


Human RARα coding sequence


5' untranslated region


GAL4 coding sequence


VP16 coding sequence


LacZ coding sequence


Thymidine kinase promoter

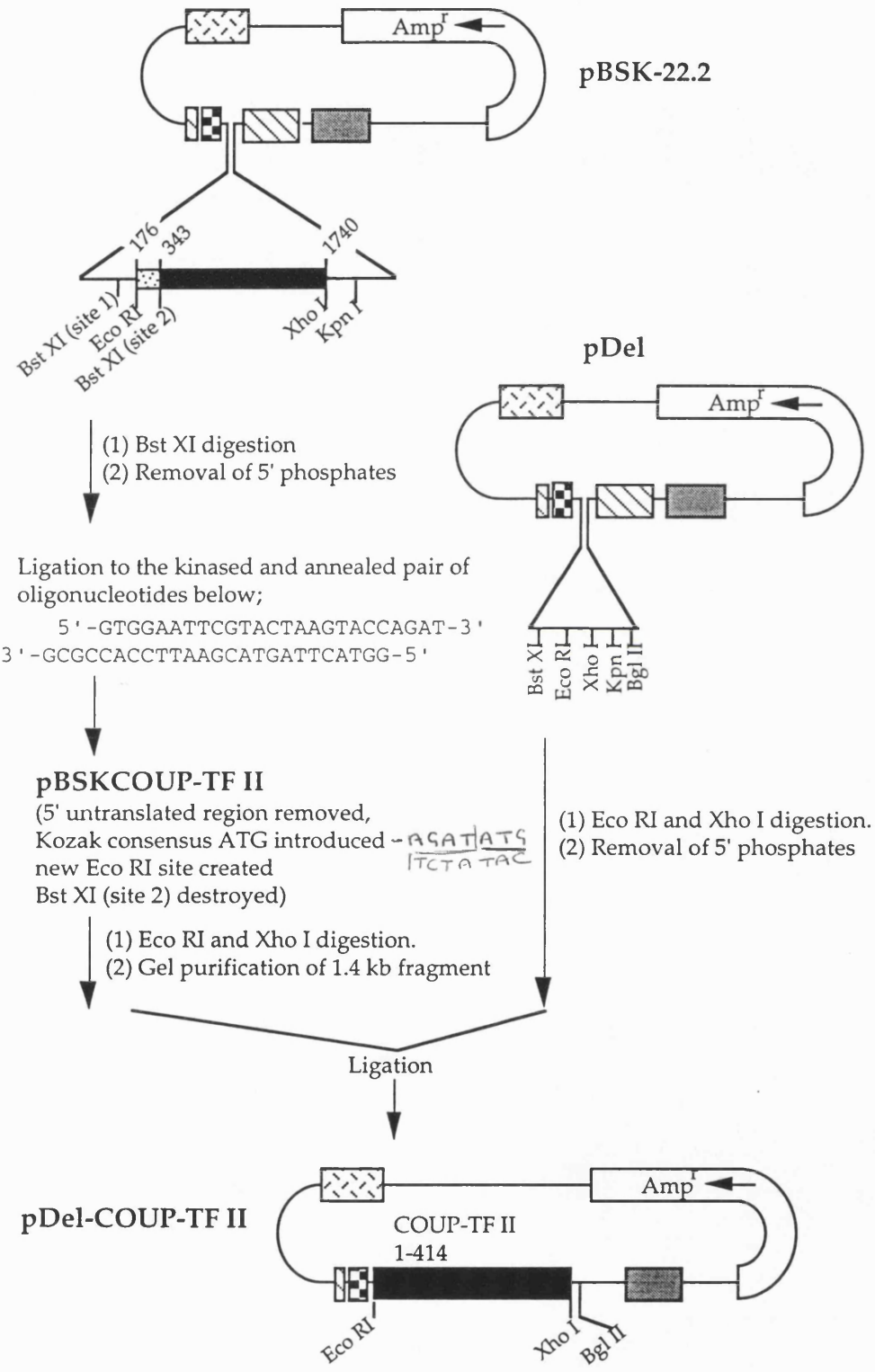

Luciferase or CAT reporter genes

A1 Construction of pBSKCOUP-TF II and pDel-COUP-TF II.

To remove the 168 bp of 5' untranslated region, pBSK-22.2 (3 μg) was digested with Bst XI and treated with calf intestinal alkaline phosphatase (CIP) to remove the 5' phosphates. The kinased and annealed pair of oligonucleotides shown were subcloned into the vector to create pBSKCOUP-TF II. Recombinants were identified by the loss of an approximately 210 bp Bst XI fragment.

pBSKCOUP-TF II (3 μg) was digested with Eco RI and Xho I and the products separated on a 1% agarose gel. The 1.4 kb fragment was isolated using NA 45 paper and subcloned into pDel (see appendix A2) that had been digested with Eco RI and Xho I and treated with CIP. Recombinants were identified by the presence of a 1.4 kb Eco RI/Bgl II fragment and the sequence at the 5' junction verified by sequencing.

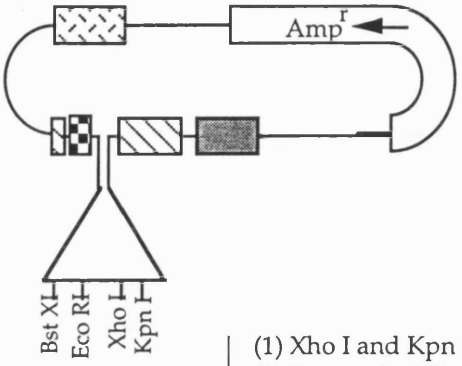
A1 Construction of pBSKCOUP-TF II and pDel-COUP-TF II



A2 Construction of pDel.

pBSKII- (2 μg) was digested with Xho I and Kpn I and treated with CIP. The kinased and annealed oligonucleotides shown were subcloned into this vector. The original Kpn I site was replaced and a Bgl II site generated. Recombinants were identified by the introduction of the Bgl II site. Stop codons in all three frames (highlighted in bold) were introduced between the Kpn I and Bgl II sites.

A2 Construction of pDel



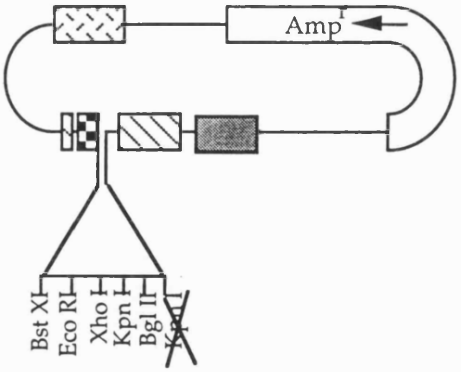
pBSKII-

(1) Xho I and Kpn I digestion.
(2) Removal of 5' phosphates.

Ligation to the kinased and annealed pair of oligonucleotides below;

```
5'-TCGAGCATCGTGGTACCTAACTGAGTAAGTACTAGCTACGAGATCTCGTAC-3'
    3'-CGTAGCACCATGGATTGACTCATTCATGATCGATGCTCTAGAG-5'
```

pDel

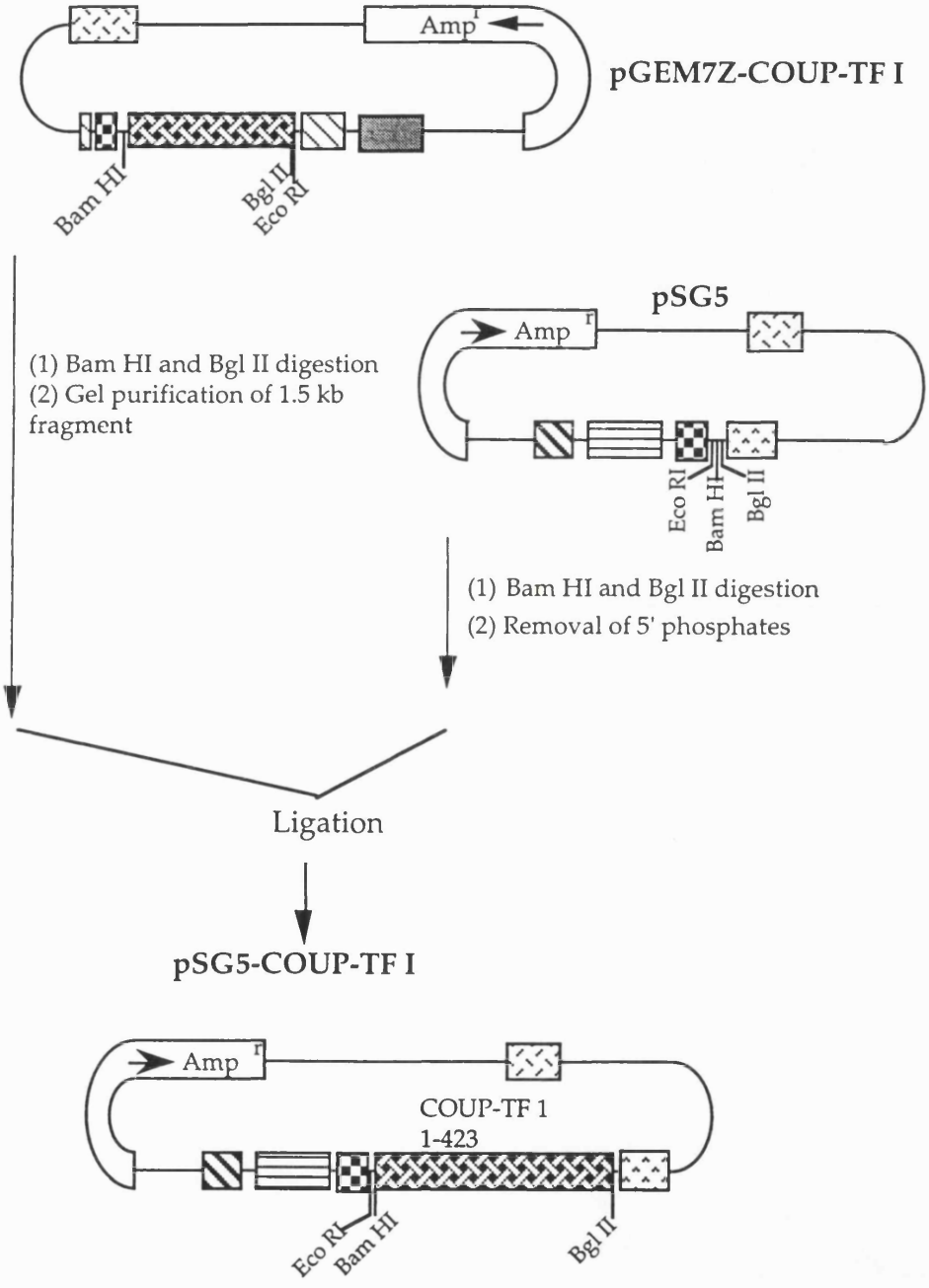


Contains new Kpn I and Bgl II sites with stop codons in all three frames between those two sites.

A3 Construction of pSG5-COUP-TF I

pGEM7Z-COUP-TF I (3 μg) was digested with Bam HI and Bgl II and the products separated on a 1% agarose gel. The 1.5 kb fragment was isolated using NA 45 paper and subcloned into pSG5 that had been digested with Bam HI and Bgl II and treated with CIP. Recombinant clones were identified by Bam HI/Eco RI digestion.

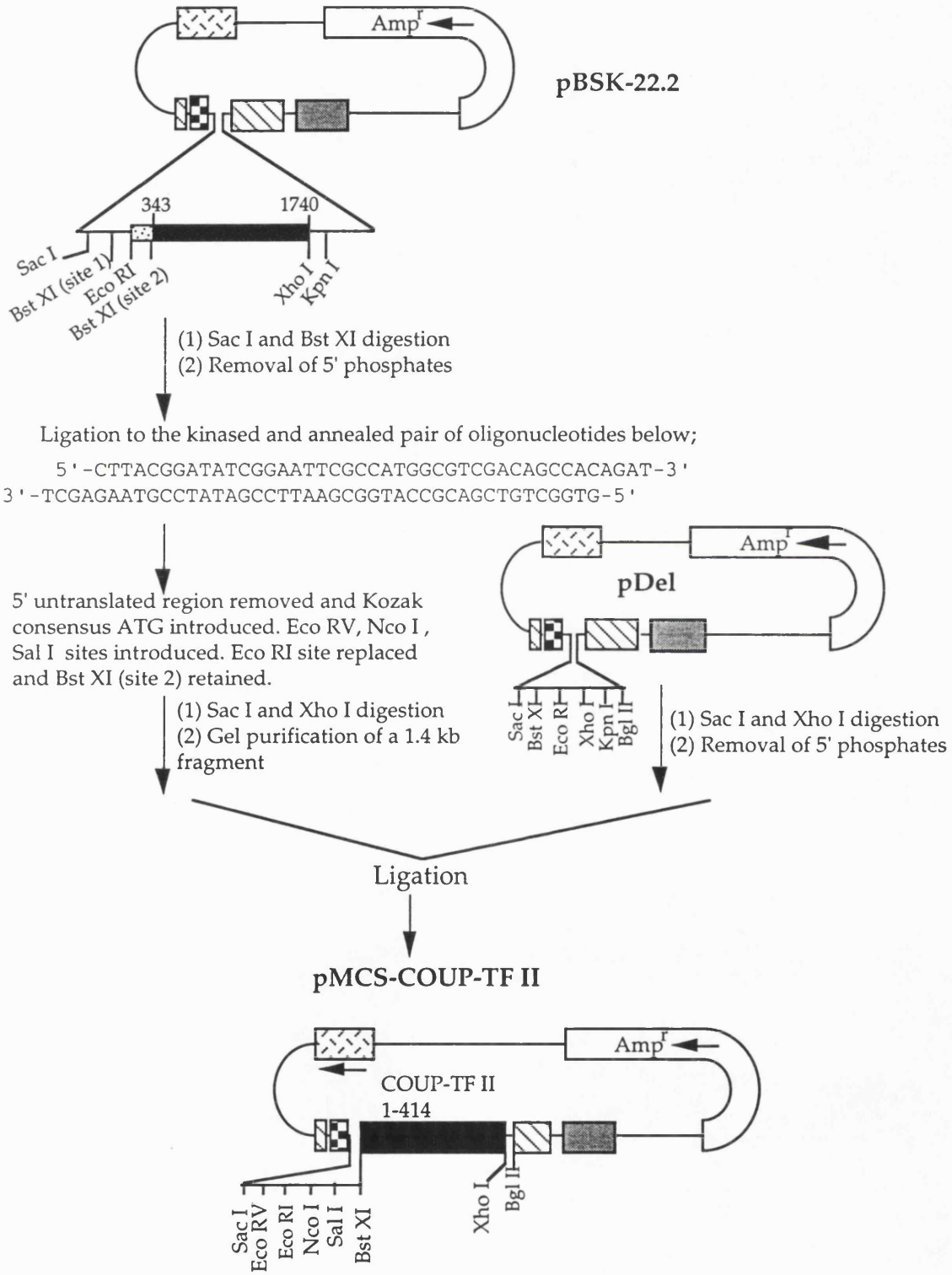
A3 Construction of pSG5-COUP-TF I



A4 Construction of pMCS-COUP-TF II.

pMCS-COUP-TF II was generated to place suitable restriction enzyme sites upstream of the COUP-TF II coding sequence which could be used for cloning COUP-TF II, in the correct reading frame, into GAL4 and VP16 expression vectors. pBSK-22.2 (2 μg) was digested with Sac I and Bst XI and treated with CIP. The kinased and annealed pair of oligonucleotides shown were subcloned into this vector. These oligonucleotides were designed to remove the 5' untranslated region of COUP-TF II. The original Eco RI site was replaced andthe Bst XI (site 1) was lost. Eco RV, Nco I and Sal I sites were introduced 5' of the COUP-TF II coding sequence. Recombinant clones were identified by Eco RI/Xho I digestion. This vector was then digested with Sac I and Xho I and the products separated on a 1% agarose gel. The 1.4 kb fragment was isolated using NA 45 paper and subcloned into pDel (see appendix A2) that had been digested with Sac I and Xho I and treated with CIP. The recombinant clones were identified by the presence of a 1.4 kb Eco RI/Bgl II fragment and the sequence at the 5' junction verified by sequencing.

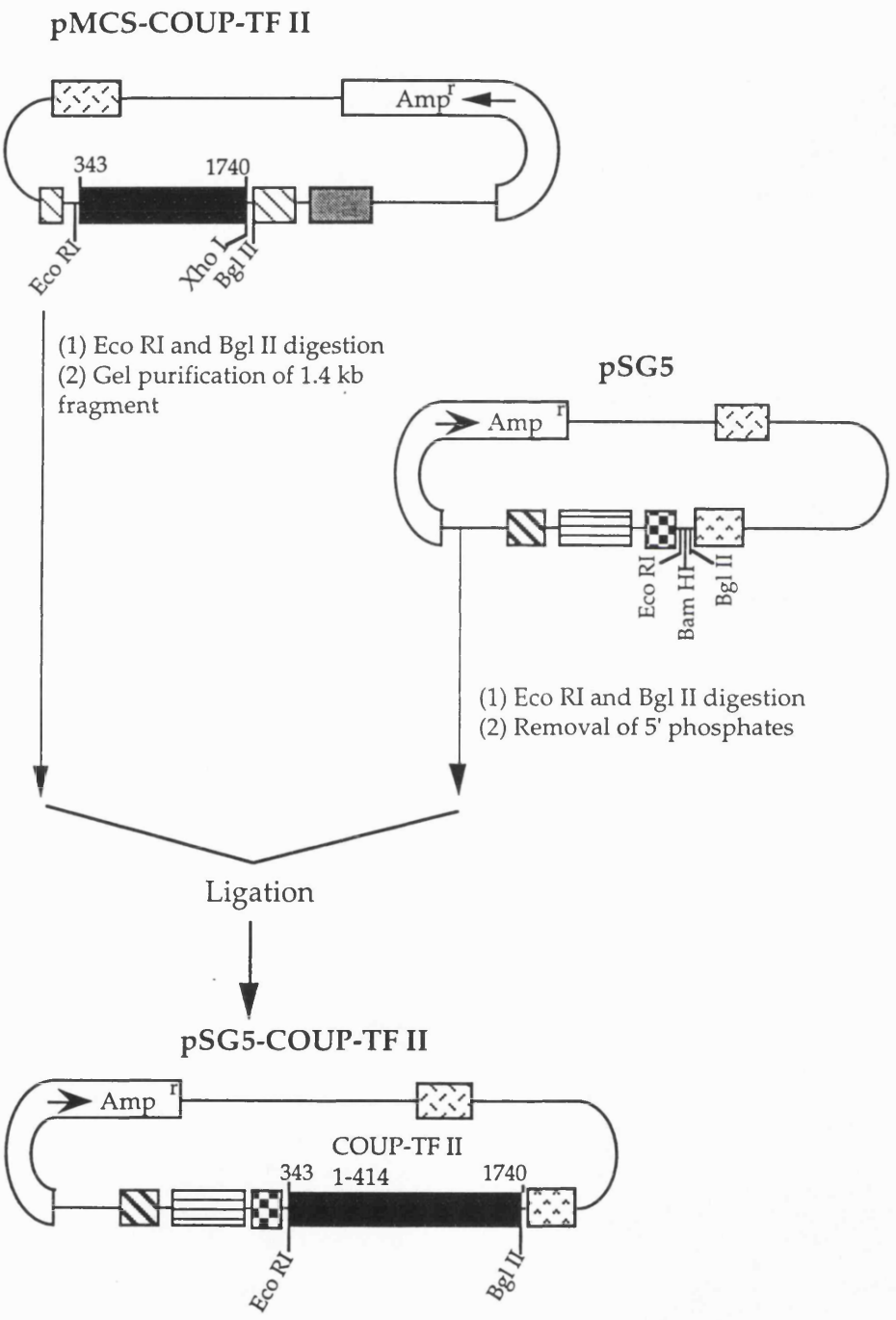
A4 Construction of pMCS-COUP-TF II



A5 Construction of pSG5-COUP-TF II.

pMCS-COUP-TF II (3 μg) (see appendix A4) was digested with Eco RI and Bgl II and the products separated on a 1% agarose gel. The 1.4 kb fragment was isolated using NA 45 paper and subcloned into pSG5 that had been digested with Eco RI and Bgl II and treated with CIP. Recombinant clones were identified by Eco RI/Bgl II digestion.

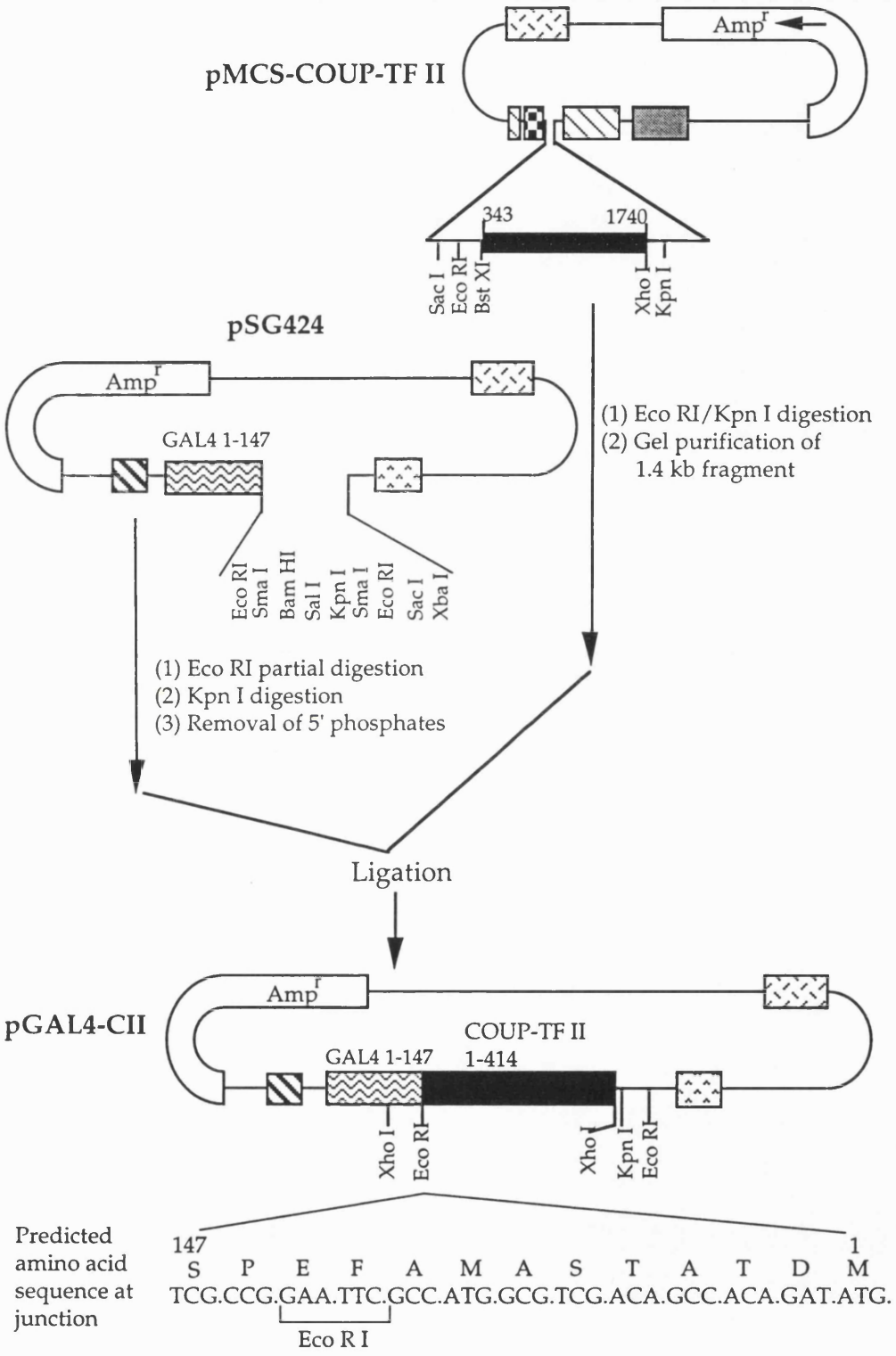
A5 Construction of pSG5-COUP-TF II



A6 Construction of pGAL4-CII.

GAL4-CII contains amino acid residues 1-414 of COUP-TF II fused to the C-terminus of the GAL4 DNA binding domain (resiues 1-147). To generate the expression vector pGAL4-CII the vector pMCS-COUP-TF II (5 μg) (see appendix A4) was digested with Eco RI and Kpn I and the products separated on a 1% agarose gel. The 1.4 kb fragment was isolated using NA 45 paper. The vector pSG424 (20 μg) was digested with Eco RI at room temperature and aliquots removed at 0, 2, 5, 10, 15 and 20 minutes, transferred to eppendorfs and extracted with a 50:50 mix of phenol/chloroform. The DNA was ethanol precipitated and one third of each of the aliquots was analysed by gel electrophoresis. The 5 minute sample was chosen because this sample contained a high proportion of plasmid that had been cut once. This DNA was then digested with Kpn I, treated with CIP and the 1.4 kb fragment subcloned. Recombinant clones were identified by the presence of a 1.5 kb Xho I fragment. The sequence at the junction of the GAL4 and COUP-TF II coding sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 147 represents the C-terminal GAL4 amino acid and the residue marked 1 is the start of the COUP-TF II coding sequence.

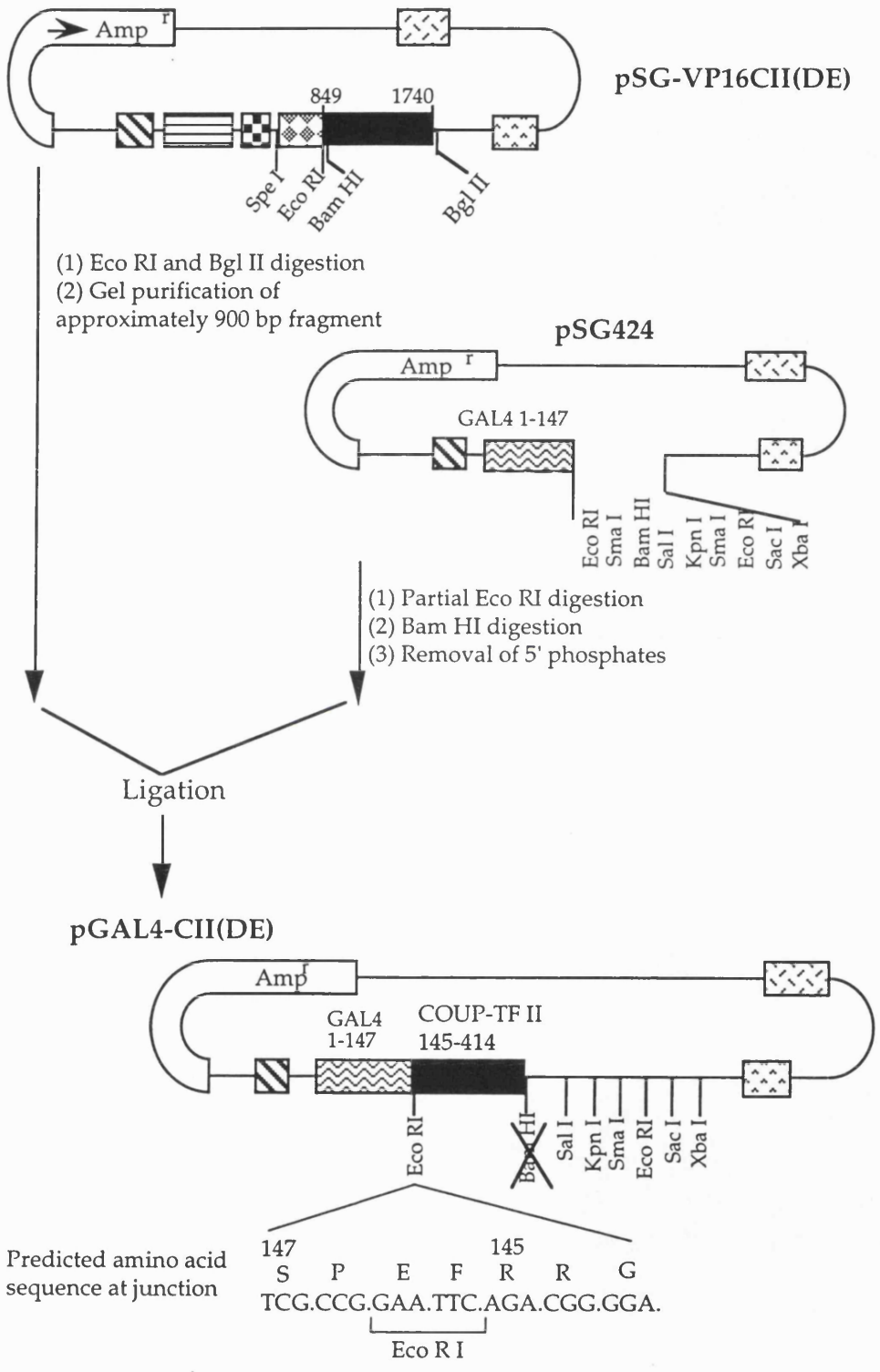
A6 Construction of pGAL4-CII



A7 Construction of pGAL4-CII(DE).

GAL4-CII(DE) contains amino acid residues 145-414 of COUP-TF II fused to the C-terminus of the GAl4 DNA binding domain. To generate the expression vector pGAL4-CII(DE), pSG-VP16CII(DE) (3 μg) (see appendix A11) was digested with Eco RI and Bgl II and the products separated on a 1% agarose gel. The 900 bp fragment was isolated using NA 45 paper and subcloned into pSG424 that had been partially digested with Eco RI (see appendix A7) and then digested with Bam HI and treated with CIP. Recombinant clones were identified by the loss of the Bam HI site. The sequence at the junction of the GAL4 and COUP-TF II coding sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 147 represents the C-terminal GAL4 amino acid and the residue marked 145 is the start of the COUP-TF II coding sequence.

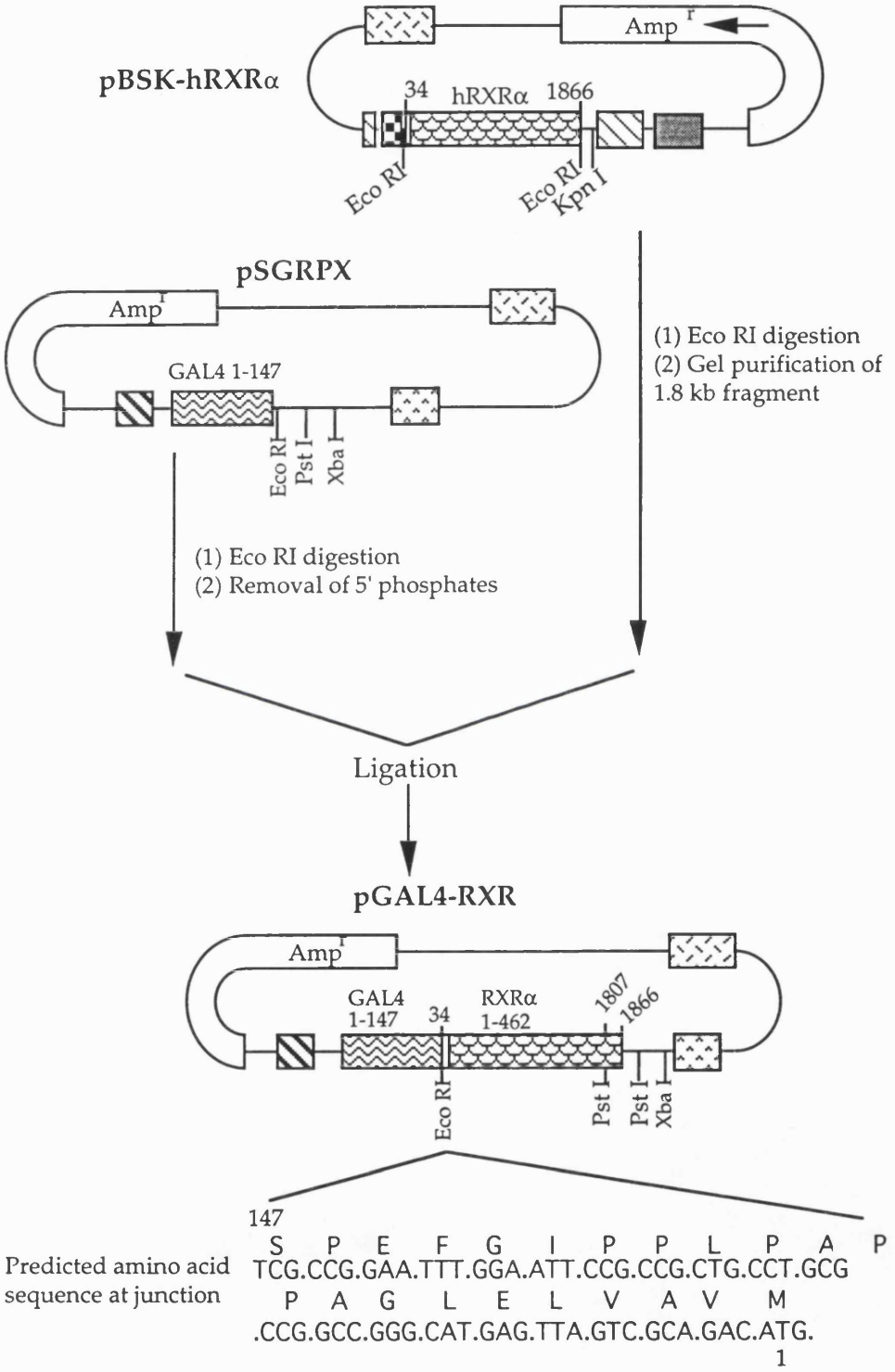
A7 Construction of pGAL4-CII(DE)



A8 Construction of pGAL4-RXR.

GAL4-RXR contains amino acids 1-462 of hRXRα fused to the C-terminus of the GAL4 DNA binding domain. To generate the expression vector pGAL4-pBSK-hRXRα was digested with Eco RI and the products separated on a 0.8% agarose gel. The 1.8 kb fragment was isolated using NA 45 paper and subcloned into pSGRPX (Danielian 1993) that had been digested with Eco RI and treated with CIP. Recombinant clones were identified by Pst I digestion. The sequence at the junction of the GAL4 and RXRα coding sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 147 represents the C-terminal GAL4 amino acid and the residue marked 1 is the start of the RXRα coding sequence.

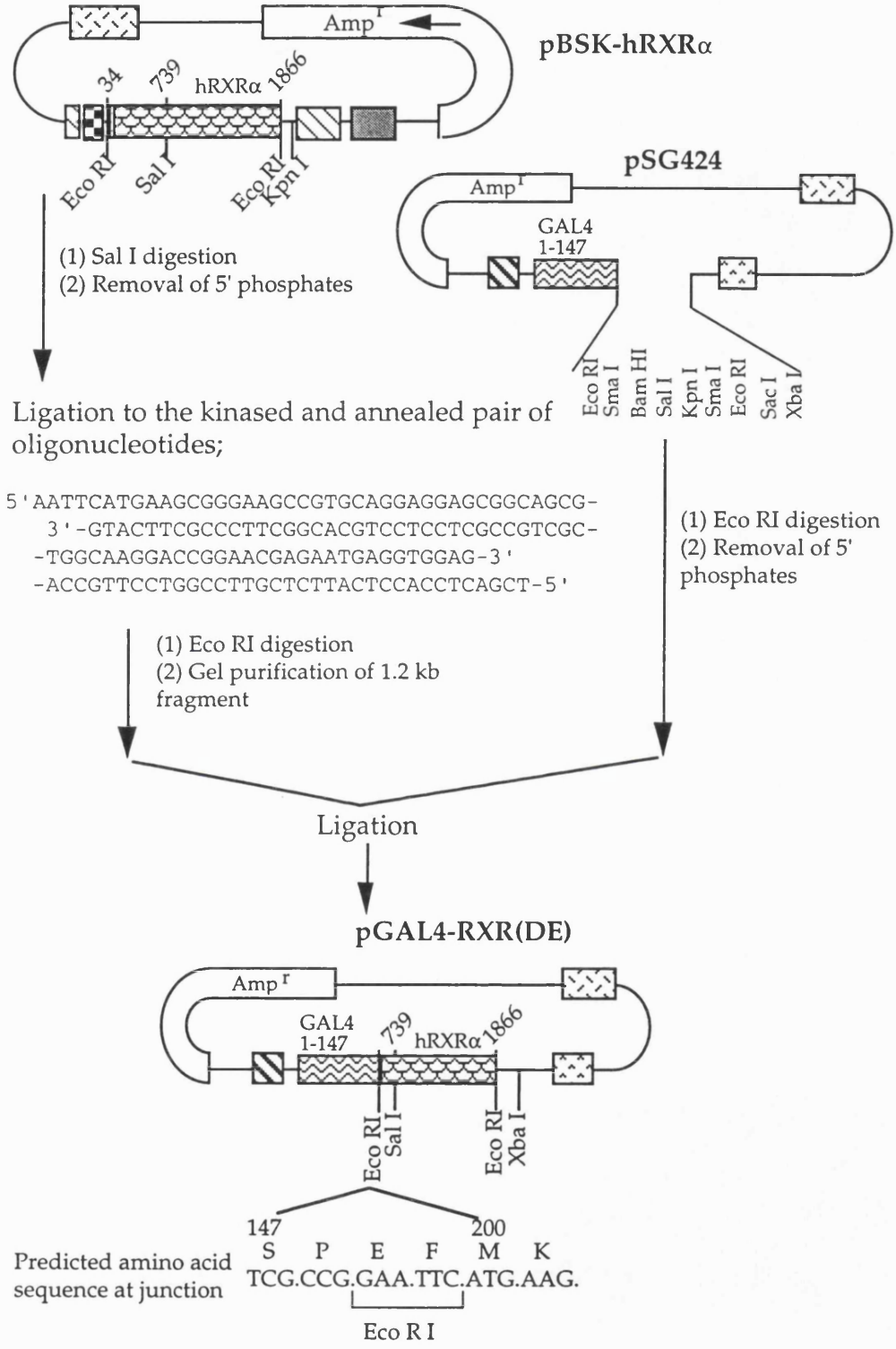
A8 Construction of pGAL4-RXR



A9 Construction of pGAl4-RXR(DE).

GAL4-RXR(DE) contains amino acids 200-462 of hRXRα fused to the C-terminus of the GAL4 DNA binding domain. To generate pGAL4-RXR(DE) the vector, pBSK-hRXRα (5 μg) was digested with Sal I and treated with CIP. The pair of kinased and annealed oligonucleotides shown were ligated to the vector. This was then digested with Eco RI and the products separated on a 1% agarose gel. The 1.2 kb fragment was isolated using NA 45 paper and subcloned into pSG424 that had been digested with Eco RI and treated with CIP. Recombinant clones were identified by Sal I/Xba I digestion. The sequence at the junction of the GAL4 and RXRα coding sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 147 represents the C-terminal GAL4 amino acid and residue marked 200 is the start of the RXRα coding sequence.

A9 Construction of pGAL4-RXR(DE)



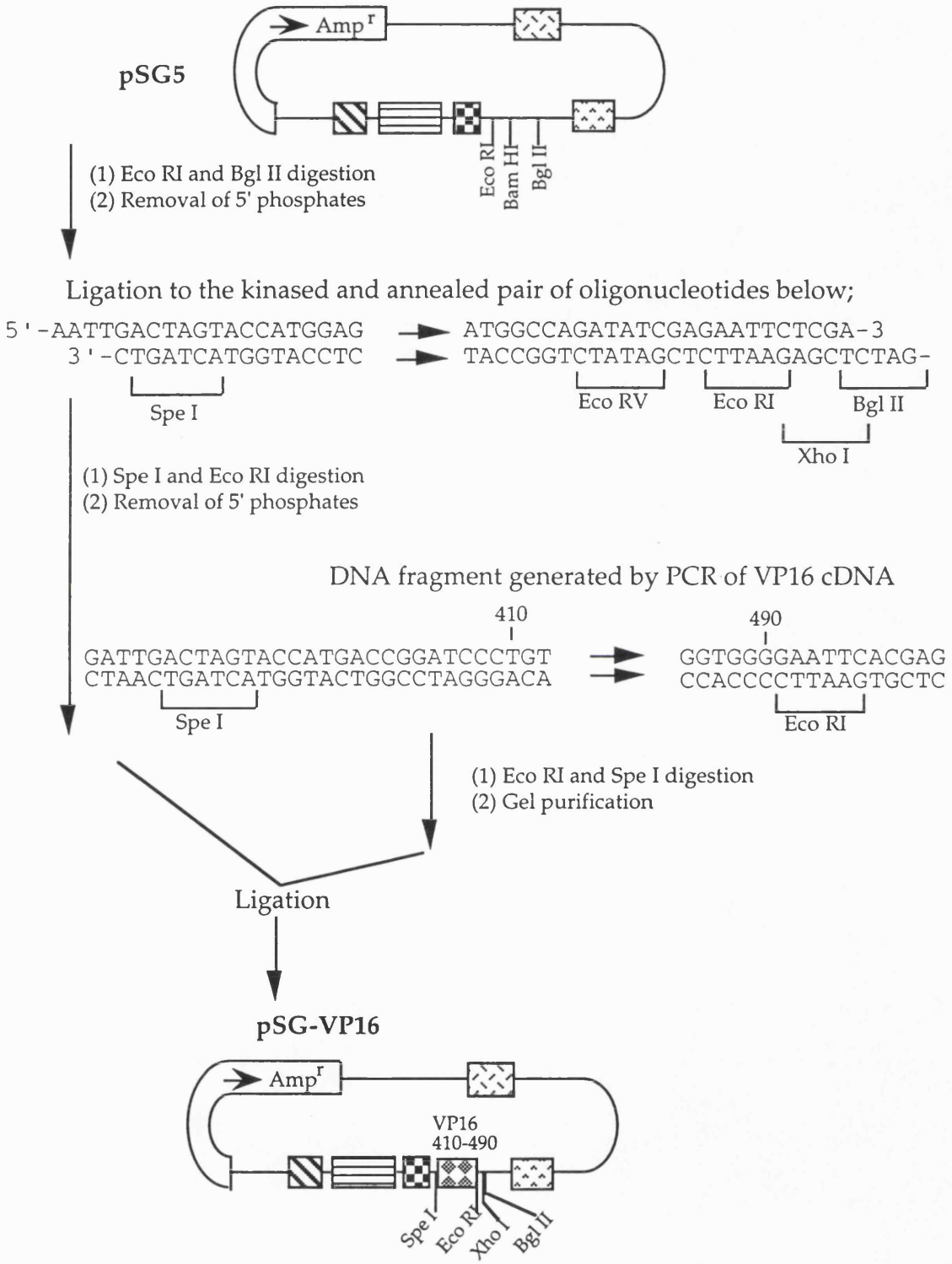
A10 Construction of pSG-VP16.

To generate pSG-VP16 it was necessary to introduce unique restriction enzyme sites into the expression vector pSG5. pSG5 (2 μg) was digested with Eco RI and Bgl II and treated with CIP. The kinased and annealed oligonucleotides shown below were subcloned into this vector;

5'AATTGACTAGTACCATGGAGCAGAAGCTGATCTCCGAGGAGGACCTGAAC
ATGGCCAGATATCGAGAATTCTCGA-3'
3'CTGATCATGGTACCTCGTCTTCGACTAGAGGCTCCTCCTGGACTTGTACCGG
TCTATAGCTCTTAAGAGCTCTAG-5'

The original Eco RI site was replaced and Spe I, Eco RV and Xho I sites introduced. Recombinants were identified by the introduction of the Spe I site. The acidic activation domain of VP16 (amino acids 410- 490) was amplified from pSDO6 (Dalton and Treisman 1992) by PCR using the primers 5'-GATTGACTAGTACCATGACCGGATCCCTGTCG-3' and 5'-CTCGTGAATTCCCCACCGTACTCGTCAATT-3'. The PCR product was digested with Spe I and Eco RI and the products separated on a 2% agarose gel. The 260 bp fragment was isolated using NA 45 paper and subcloned into the modified pSG5 vector that had been digested with Spe I and Eco RI and treated with CIP. Recombinant clones were identified by the presence of an approximately 260 bp Spe I/Eco RI fragment and the VP16 sequence was verified by DNA sequencing.

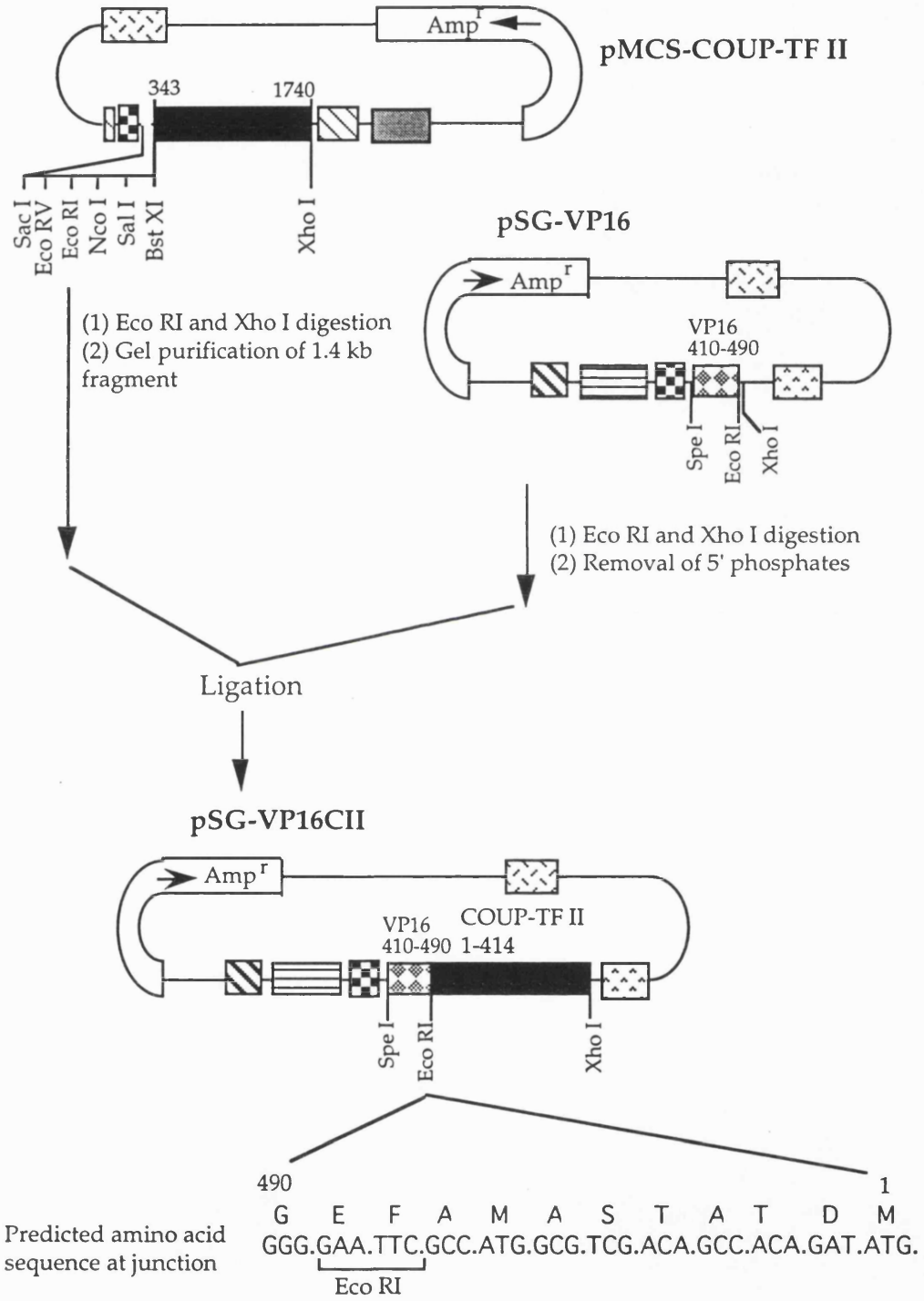
A10 Construction of pSG-VP16



A11 Construction of pSG-VP16CII.

VP16CII contains amino acids 1-414 of COUP-TF II fused to the C-terminus of the VP16 activation domain (residues 410-490). pMCS-COUP-TF II (3 μg) (see appendix A4) was digested with Eco RI and Xho I and the products separated on a 1% agarose gel. The 1.4 kb fragment was isolated using NA 45 paper and subcloned into pSG-VP16 (see appendix A10) that had been digested with Eco RI and Xho I and treated with CIP. Recombinant clones were identified by Spe I/Xho I digestion. The sequence at the junction of the VP16 and COUP-TF II coding sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 490 represents the C-terminal VP16 amino acid and the residue marked 1 is the start of the COUP-TF II coding sequence.

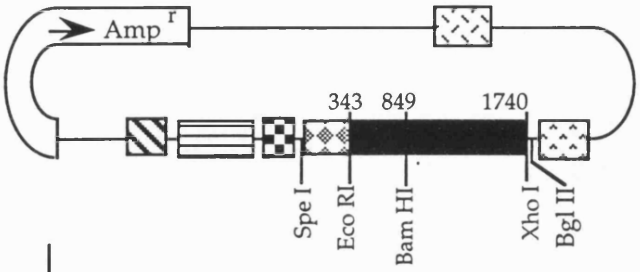
A11 Construction of pSG-VP16CII



A12 Construction of pSG-VP16CII(DE).

VP16CII(DE) contains amino acids 145-414 of COUP-TF II fused to the C-terminus of the VP16 activation domain. pSG-VP16CII (2 μg) was digested with Eco RI and Bam HI and treated with CIP. The kinased and annealed oligonucleotides shown were subcloned into this vector. Recombinants were identified by Eco RI/Bgl II digestion. The sequence at the junction of the VP16 and COUP-TF II coding sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 490 represents the C-terminal VP16 amino acid and the residue marked 145 is the start of the COUP-TF II coding sequence.

A12 Construction of pSG-VP16CII(DE)



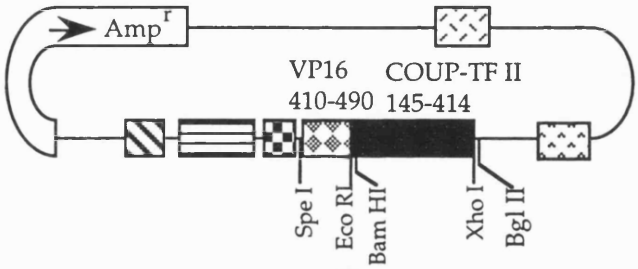
pSG-VP16CII

(1) Eco RI and Bam HI digestion
(2) Removal of 5' phosphates

Ligation to the kinased and annealed pair of oligonucleotides below;

5'-AATTCAGACGGGAAGCGGTGCAGAGGGGCAGGATGCCGCCGACCCAGCCGACCC
ACGGGCAGTTCGCGCTGACCAACGGG-3'
3'-GTCTGCCCTTCGCCACGTCTCCCCGTCCTACGGCGGCTGGGTCGGCTGGGTGCC
CGTCAAGCGCGACTGGTTGCCCCTAG-5'

pSG-VP16CII(DE)



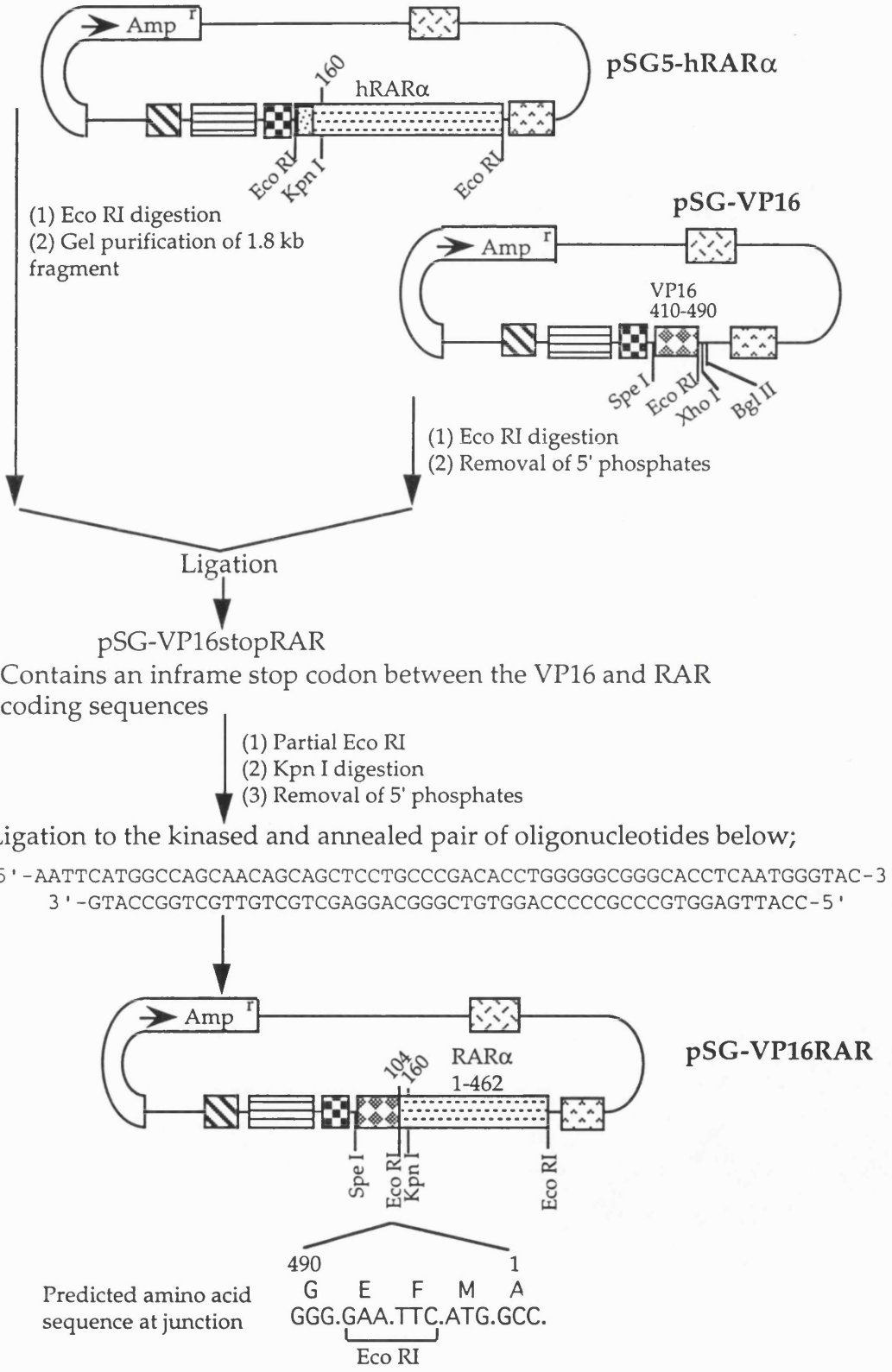
Predicted amino acid sequence at junction

490 145
G E F R R G
GGG.GAA.TTC.AGA.CGG.G(
Eco RI

A13 Construction of pSG-VP16RAR.

VP16RAR contains amino acids 1-462 of hRARα fused to the C-terminus of the VP16 activation domain. pSG5-hRARα (3 μg) was digested with Eco RI and the products separated on a 1% agarose gel. The 1.4 kb fragment was isolated using NA 45 paper and subcloned into pSG-VP16 (see appendix A10) that had been digested with Eco RI and treated with CIP. Recombinant clones were identified by Spe I/Kpn I digestion. The 5' untranslated sequence of hRARα was then removed. The vector (20 μg) was digested with Eco RI at room temperature and aliquots removed at 0, 2, 5, 10, 15 and 20 minutes, transferred to eppendorfs and extracted with a 50:50 mix of phenol/chloroform. The DNA was ethanol precipitated and one third of each of the aliquots was analysed by gel electrophoresis. The 2 and 5 minute samples were chosen because these samples contained a high proportion of plasmid that had been cut once. This DNA was then digested with Kpn I and treated with CIP. The pair of kinased and annealed oligonucleotides shown were subcloned into this vector. Recombinant clones were identified by the presence of an approximately 260 bp Spe I/Kpn I fragment. The sequence at the junction of the VP16 and hRARα sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 490 represents the C-terminal VP16 amino acid and the residue marked 1 is the start of the hRARα coding sequence.

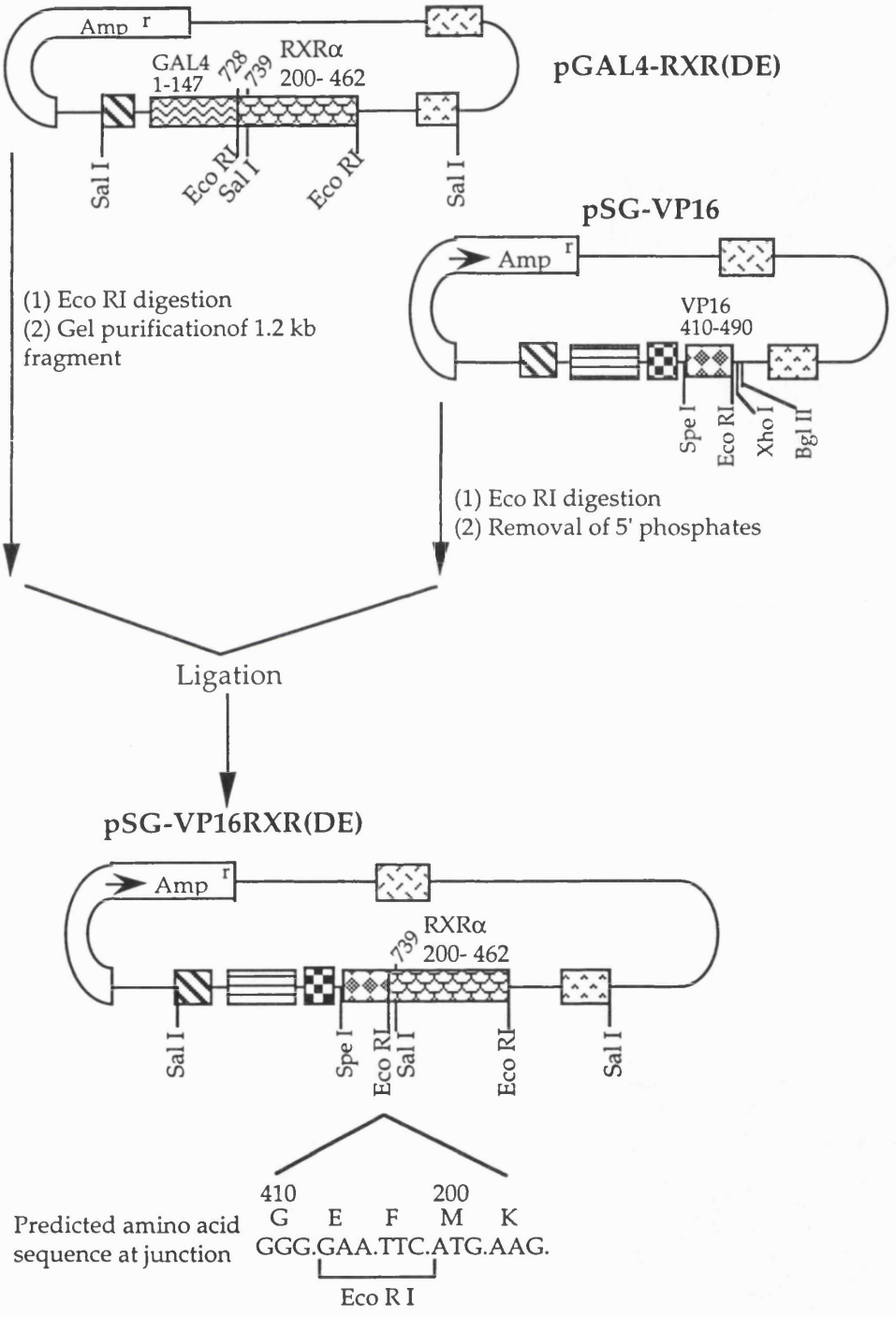
A13 Construction of pSG-VP16RAR



A14 Construction of pSG-VP16RXR(DE).

VP16-RXR(DE) contains amino acids 200-462 of hRXRα fused to the C-terminus of the VP16 activation domain. pGAL4-RXR(DE) (3 μg) was digested with Eco RI and the products separated on a 1% agarose gel. The 1.2 kb fragment was isolated using NA 45 paper and subcloned into pSG-VP16 (see appendix A10) that had been digested with Eco RI and treated with CIP. Recombinant clones were identified by Sal I digestion. The sequence at the junction of the VP16 and RXRα coding sequences was verified by DNA sequencing. The predicted amino acid sequence at the junction is indicated, the residue marked 490 represents the C-terminal VP16 amino acid and residue marked 200 is the start of the RXRα coding sequence.

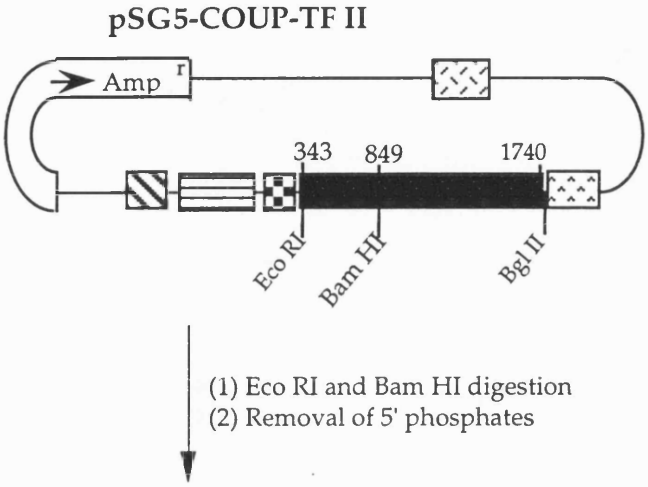
A14 Construction of pSG-VP16RXR(DE)



A15 Construction of pSG5-CII(DE).

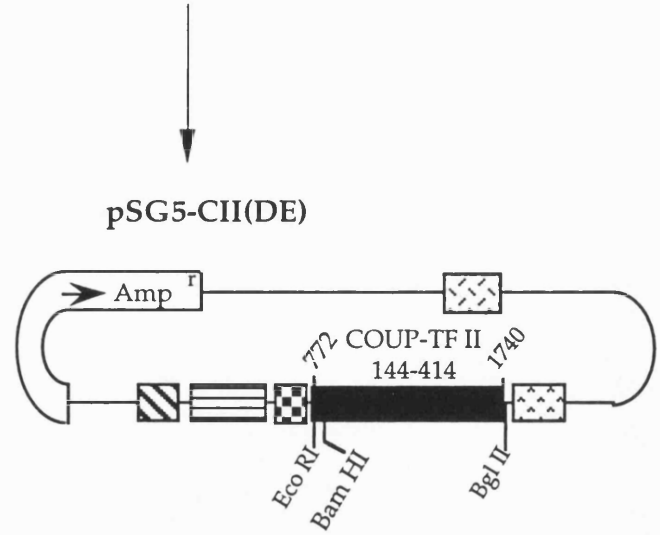
The deletion mutant CII(DE) was constructed by deleting the nucleotide sequence corresponding to amino acids 1-143 of COUP-TF II. pSG5-COUP-TF II (3 μg) was digested with Eco RI and Bam HI and treated with CIP. The pair of kinased and annealed oligonucleotides shown were subcloned into this vector. Recombinants were identified by the presence of a 1 kb Eco RI/Bgl II fragment. The sequence of the oligonucleotide cassette was verified by DNA sequencing.

A15 Construction of pSG5-CII(DE)



Ligation to the kinased and annealed pair of oligonucleotides below;

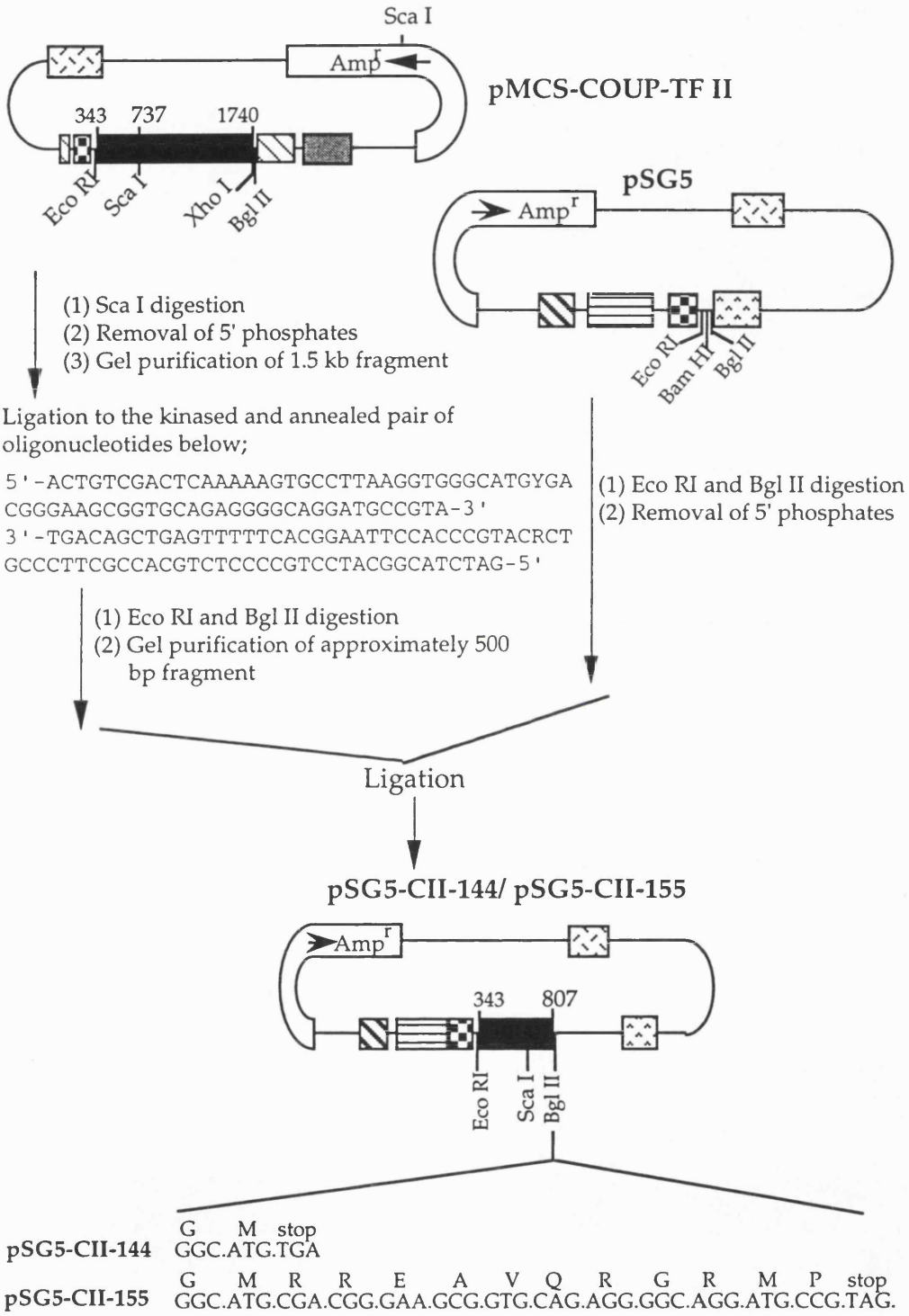
5'-AATTCATGAGACGGGAAGCGGTGCAGAGGGGCAGGATGCCGCCGACCCAGCCGA
CCCACGGGCAGTTCGCGCTGACCAACGGG-3'
3'-GTACTCTGCCCTTCGCCACGTCTCCCCGTCCTACGGCGGCTGGGTCGGCTGGGT
GCCCGTCAAGCGCGACTGGTTGCCCCTAG-5'



A16 Construction of pSG5-CII-144 and pSG5-CII-155.

pMCS-COUP-TF II (8 μg) (see appendix A4) was digested with Sca I, treated with CIP and the products separated on a 0.8% agarose gel. The 1.5 kb frgament was isolated using NA 45 paper and the pair of kinased and annealed oligonucleotides shown were ligated. The ligated products were then digested with Eco RI and Bgl II and the products separated on a 2% agarose gel. The 0.5 kb fragment was isolated using NA 45 paper and subcloned into the expression vector pSG5 that had been digested with Eco RI and Bgl II and treated with CIP. Recombinant clones were identified by the presence of the o.5 kb Eco RI/Bgl II fragment and pSG5-CII-144 and pSG5-CII-155 clones distinguished by DNA sequencing. The nucleotide and predicted amino acid sequence at the 3' end of the cloned fragment is indicated. Y indicates T or C and R indicates A or G.

A16 Construction of pSG5-CII-144 and pSG5-CII-155

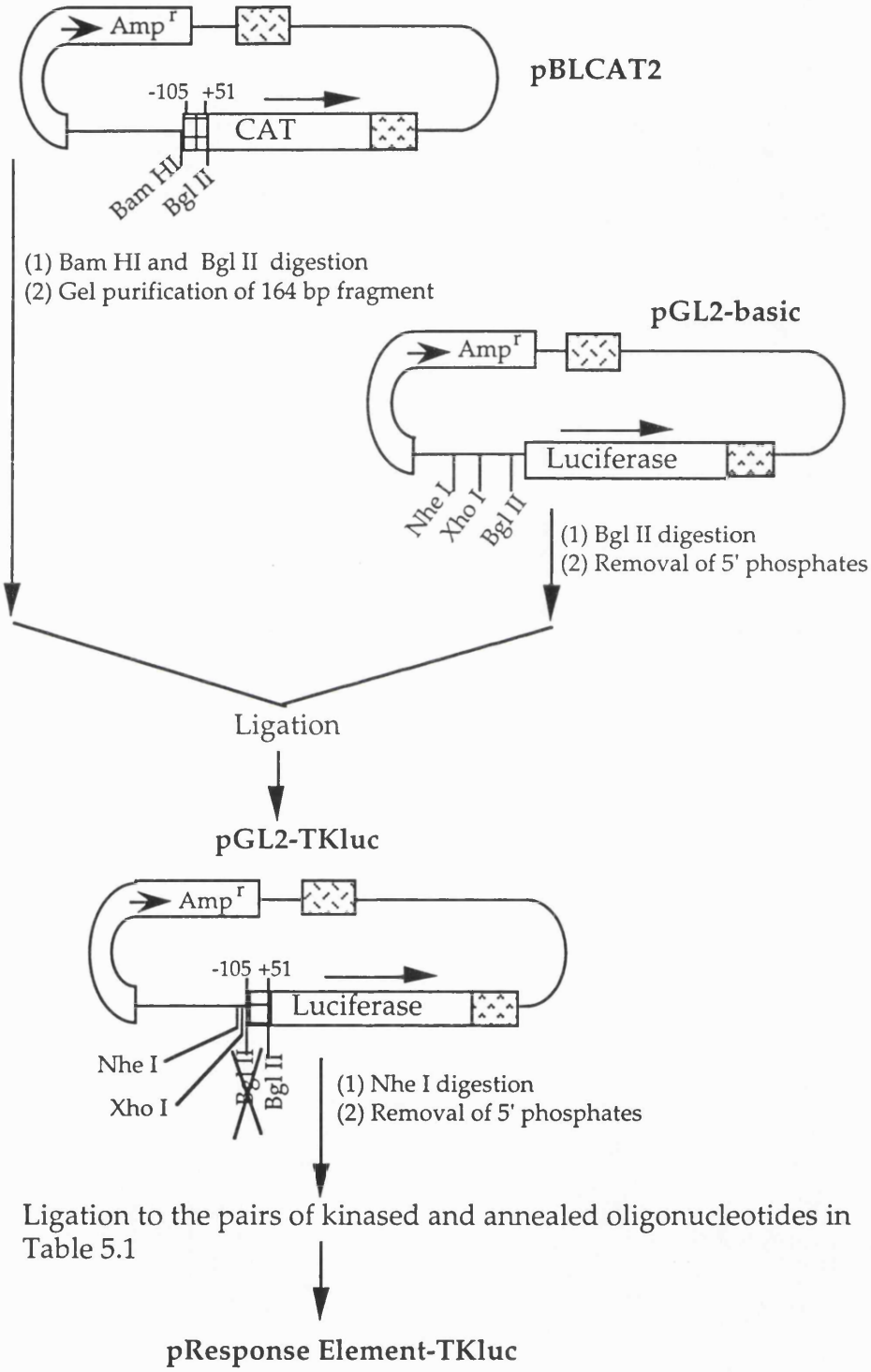


A17 Construction of pGL2-TKLuc reporter gene plasmids.

The herpes simplex virus thymidine kinase (TK) promoter (-105 to +51) was isolated from the vector pBLCAT2 (Luckow and Schutz 1987) and cloned upstream of the luciferase reporter gene in pGL2-basic. pBLCAT2 (6 μg) was digested with Bam HI and Bgl II and the products separated on a 2% gel. The 164 bp fragment was isolated using NA 45 paper and subloned into pGL2 that had been digested with Bgl II and treated with CIP. Recombinant clones were identified by the presence of a 168 bp Bgl II/Xho I fragment.

Pairs of kinased and annealed oligonucleotides (shown in Table 5.1) were subcloned into pGL2-TKLuc that had been digested with Nhe I and treated with CIP. Recombinant clones were identified by the loss of the Nhe I site and the response element sequence verified by DNA sequencing.

A17 Construction of pGL2-TKLuc reporter gene plasmids.



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