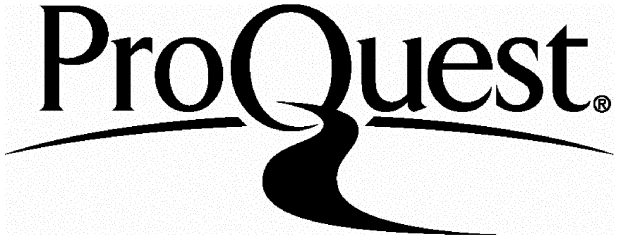
Transcriptional Activation by the Mouse Oestrogen Receptor

Paul Shemavon Danielian

Thesis presented in partial fulfilment of the degree of
Doctor of Philosophy at the University of London
June 1993

Molecular Endocrinology Laboratory
Imperial Cancer Research Fund
Lincoln's Inn Fields
London

Biochemistry Department
University College London
Gower Street
London



Dedicated to my parents

All the experiments included in this thesis were performed by myself unless indicated otherwise in the text.

Abstract

The oestrogen receptor is a ligand inducible transcription factor that belongs to a family of nuclear receptor proteins. Previous studies have shown that in transient transfection experiments two regions of the oestrogen receptor protein are capable of stimulating transcription. One, located N-terminal to the DNA binding domain called transcriptional activation function 1, TAF-1, can stimulate transcription constitutively whilst TAF-2, located in the C-terminal hormone binding domain requires oestrogen binding for its activity. Analysis of mutant mouse oestrogen receptors has identified a region of the hormone binding domain important for TAF-2. The amino acid sequence in this region is conserved in other members of the nuclear receptor family. Point mutation of conserved hydrophobic residues practically abolished hormone dependent transcriptional stimulation by TAF-2 without affecting oestradiol or DNA binding. The ability of TAF-2 and TAF-1 to cooperate in stimulating transcription in the full-length receptor was also abolished by mutation of the conserved hydrophobic residues but not conserved acidic residues. Mutagenesis of the corresponding residues in the glucocorticoid receptor showed similar results indicating that these residues may be important for ligand inducible transcription by other members of the nuclear receptor family.

A yeast genetic screen was established to identify target(s) for TAF-2 that were important for its ability to stimulate transcription. No candidate targets were identified but analysis of the TAF-2 defective mutants suggested that the mechanism whereby TAF-2 stimulates transcription in mammalian cells is not conserved in yeast.

Finally the effects of mutations in the steroid binding domain upon transcriptional activation by the oestrogen receptor have been examined. One finding of these studies was that some mutations, that abolished oestradiol binding and transcriptional activation, did not affect transcriptional activation induced by another ligand indicating that while the binding sites for different ligands overlap they are distinct.

Acknowledgements.

Firstly I wish to thank Malcolm Parker for his encouragement and advice during the last four years. I would also like to thank past and present members of the Molecular Endocrinology, Viral Carcinogenesis and Transcription laboratories for their material and spiritual assistance and their company. In particular I would like to thank Roger White, Jackie Lees, Steve Fawell, Sophie Dauvois, Sue Hoare and Anna Florence for their help, ideas and patience. I also thank Ian Goldsmith for synthesising oligonucleotides and many others for providing invaluable reagents. I also thank my parents and friends for their support and understanding. Finally I also wish to express my gratitude to the Imperial Cancer Research Fund and to those who give time and money towards its progress.

Contents

Chapter 2: Materials and Methods.

Materials.

Methods.

Abbreviations.

ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CEF	chicken embryo fibroblast
cpm	counts per minute
CTD	carboxyl-terminal domain
C-terminal	carboxyl-terminal
CYC 1	iso-1-cytochrome C gene
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	diethylaminoethyl
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	oestrogen receptor
ERE	oestrogen response element
FCS	foetal calf serum
GRE	glucocorticoid response element
GTP	guanosine 5'-triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
hsp	heat shock protein
kb	kilobase
Kd	dissociation constant
lacZ	β–galactosidase gene
MMTV	mouse mammary tumour virus
MOPS	morpholinopropanesulphonic acid
MOR	mouse oestrogen receptor
mRNA	messenger RNA

NP40	nonidet P40
N-terminal	amino-terminal
OD_x	optical density at a wavelength of x nm
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PBSA	phosphate buffered saline A
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PRE	progesterone response element
RAR	retinoic acid receptor
RNA	ribonucleic acid
RNase	ribonuclease
RXR	retinoid X receptor
S	Svedberg units
SDS	sodium dodecyl sulphate
SRE	serum response element
SRF	serum response factor
SV40	simian virus 40
T_3	3, 5, 3'-triiodo-L-thyronine
TAF	transcriptional activation function
TBP	TATA binding protein
TEMED	*N'N'N'N'*-tetramethylethylenediamine
tk	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	octyl phenoxy polyethoxyethanol
Tween 20	polyoxyethylene sorbitan monolaurate
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

Chapter 1

Introduction

The survival of most multicellular organisms relies on the ability of cells to respond to intercellular signalling molecules. In many cases these signalling molecules bind to receptors that cause changes in the expression of appropriate genes. Receptors for intercellular signalling molecules located at the cell surface induce changes in gene expression indirectly through other molecules that "transmit" the signal to the nucleus. However, some signalling molecules, that are able to diffuse across cell membranes, bind to intracellular receptors that function as transcription factors, regulating gene expression directly. This thesis describes a study of one intracellular receptor, that for the steroid hormone oestradiol.

The oestrogen receptor belongs to a large family of nuclear hormone receptors that contains receptors for steroid hormones, vitamin D, retinoids and thyroid hormones that play important roles in growth, development and homeostasis. The receptors for the gonadal steroid hormones, oestrogens, progestins and androgens are essential for the development and function of mammalian reproductive systems (King and Mainwaring, 1974; Cunha *et al.*, 1991). The adrenal steroid hormones, glucocorticoids, regulate carbohydrate and protein metabolism and the mineralocorticoids control water and electrolyte metabolism whilst vitamin D is important for bone formation (Hughes and O' Malley, 1991) and the absorption of calcium ions and inorganic phosphate from the intestine. The insect steroid hormone ecdysone also binds a member of the nuclear hormone receptor family and initiates changes in development during moulting and metamorphosis (Andres and Thummel, 1992). The affects of retinoids and thyroid hormones, that are important for the growth and development of mammals are also mediated by members of the nuclear receptor family (reviewed in Ragsdale and Brockes, 1991; Chaterjee and Tata, 1992; Maden and Holder, 1992).

Intracellular steroid hormone receptors.

Although steroid hormones are capable of entering most cells physiological responses are restricted to certain tissues. By following the fate of injected ^{3}H hexoestrol and ^{3}H oestradiol in mammals Glascock and Hoekstra, (1959) and Jensen and Jacobson, (1962) found that the labelled steroid was concentrated in responsive tissues. A weak oestrogen, oestrone, was retained less efficiently by the responsive tissues (Jensen and Jacobson, 1962) and the uptake of ^{3}H oestradiol could be blocked by antioestrogens (cited in Emmens *et al.*, 1962). These results provided the first evidence for a tissue specific receptor. This discovery was followed by the biochemical analysis of receptors, that could be monitored using labelled ligands. Cell fractionation studies indicated that the receptor hormone complex was, initially, located in the cytoplasm and had a sedimentation of 9 S, this form of the receptor then disappeared with the concomitant increase of a nuclear 5 S form (Gorski *et al.*, 1968;

Jensen *et al.*, 1968). These observations gave rise to the two-step model which proposed that receptor in the cytoplasm (the 9 S form) bound oestradiol and then migrated to the nucleus where it was detected, transformed to a 5 S form. Similar observations were also made of the progesterone, androgen and glucocorticoid receptors and further studies suggested that the 4-5 S form of the receptor bound to DNA or chromatin (reviewed in King and Mainwaring, 1974).

Biochemical analysis indicated that the 8-9 S forms of the steroid receptors contained a common component (Joab *et al.*, 1984; Schuh *et al.*, 1985) that was subsequently identified as the heat shock protein hsp90 (for examples see Catelli *et al.*, 1985; Snachez *et al.*, 1985; Denis *et al.*, 1987; Redeuilh *et al.*, 1987; Sanchez *et al.*, 1987). Ligand binding and/or thermally induced transformation of the receptor to the 5 S form resulted in the dissociation of hsp90 (Sanchez *et al.*, 1985; Denis *et al.*, 1987; Redeuilh *et al.*, 1987; Sanchez *et al.*, 1987; Denis *et al.*, 1988; Howard and Distelhorst, 1988; DeMarzo *et al.*, 1991). This transformation process also appeared to involve receptor dimerisation (Miller *et al.*, 1985b; DeMarzo *et al.*, 1991). Pulse-chase labelling experiments using ^{35}S methionine (Howard and Distelhorst, 1988) showed that labelled hsp90 could be co-immunoprecipitated with the glucocorticoid receptor from extracts of untreated cells but not from cells treated with dexamethasone, a synthetic glucocorticoid. Rexin *et al.*, (1988, 1992) showed that the glucocorticoid receptor could be cross-linked to hsp90 when the cross-linking was performed using intact cells. These two reports suggest that the glucocorticoid receptor associated with hsp90 *in vivo* rather than, non-specifically, during the extraction procedure.

A major role of hsp90 appears to be to maintain the receptor in an inactive state in the absence of ligand. On the basis of other studies hsp90 may also be important for folding of the receptor protein and/or transport across membranes (reviewed in Smith and Toft, 1993). In the case of the glucocorticoid receptor the binding of hsp90 appears to be important for high affinity ligand binding (Bresnick *et al.*, 1989; Nemoto *et al.*, 1990). All these possibilities are supported by studies of yeast containing exogenous glucocorticoid and oestrogen receptors where lowering the level of hsp90 reduces the ability of the receptor to stimulate transcription (Picard *et al.*, 1990a). The analysis of deletion mutants of the glucocorticoid and oestrogen receptors have shown that main binding site of hsp90 is the hormone binding domain (Pratt *et al.*, 1988; Chambraud *et al.*, 1990; Housley *et al.*, 1990; Howard *et al.*, 1990; Schlatter *et al.*, 1992). Steroid receptors may also associate with a number of other proteins including hsp70, that may also be involved in protein folding and/or transport across membranes, p59 (also called hsp56) that is a member of the immunophilin family, p54, p50 and p23 (for a review see Smith and Toft, 1993). The function of these proteins is, as yet, unclear.

Although cell fractionation experiments suggested that steroid receptors were located in the cytoplasm and migrated to the nucleus upon the binding of ligand immunocytochemical studies have indicated that the oestrogen (King and Greene, 1984; Picard *et al.*, 1990b) and progesterone receptors are predominantly nuclear in the absence or presence of ligand (for examples see Perrot-Applanat, 1985; Guiochon-Mantel *et al.*, 1989). In contrast, the glucocorticoid receptor is predominantly cytoplasmic and translocates to the nucleus in the presence of ligand (Govindan, 1980; Papamichail *et al.*, 1980; Picard and Yamamoto, 1987; Wikstrom *et al.*, 1987). In the cases of the oestrogen and progesterone receptor the cell fractionation results may reflect "tight nuclear binding" in the presence of ligand.

Nuclear localisation signals have been identified in the glucocorticoid (Picard and Yamamoto, 1987), progesterone (Guiochon-Mantel *et al.*, 1989) and oestrogen receptors (Picard *et al.*, 1990b; Ylikomi *et al.*, 1992). One, that is conserved in many members of the nuclear receptor family, located near the C-terminus of the DNA binding domain is composed of groups of basic amino acids (reviewed in Dingwall and Laskey, 1991) whilst a second may exist in the hormone binding domain and require the binding of hormone for its activity. Recent studies indicate that the progesterone, glucocorticoid and oestrogen receptors cycle between the nucleus and the cytoplasm (Guiochon-Mantel *et al.*, 1991; Madan and DeFranco, 1993; S. Dauvois, ICRF, personal communication). The differences between the localisation of receptors may be related to the ability of the hormone binding domain to repress (in the absence of ligand) the function of the nuclear localisation signal near the DNA binding domain (see Ylikomi *et al.*, 1992) thus altering the efficiency of receptor import into the nucleus.

Steroid hormone regulated genes.

Initial biochemical analysis of steroid hormone induced proteins indicated that one of the first changes induced by the hormone was an increase in the amount of RNA and that the induction could be prevented by inhibitors of transcription such as actinomycin D (for examples see Walters *et al.*, 1985). These data along with evidence that indicated that the transformed receptor was able to bind DNA suggested that steroid receptors may be able to stimulate transcription of specific genes. Following the examples of prokaryotic and viral transcription factors the possibility that high affinity DNA binding sites for receptors were located near responsive genes was examined.

Using partially purified glucocorticoid receptor Payvar *et al.*, (1981) showed, by filter binding assay, that the receptor bound specifically to a cloned DNA fragment of the mouse mammary tumour virus (MMTV) that was known to be hormone responsive in cells. Subsequent nuclear run-off assays indicated that dexamethasone

caused an increase in the amount of mRNA from several MMTV genes by increasing the efficiency of initiation of transcription (Ringold *et al.*, 1977; Ucker and Yamamoto, 1984). The initial report of Payvar *et al.*, (1981) was followed by the analysis of mainly glucocorticoid and oestrogen responsive genes that will be reviewed in the following sections.

Glucocorticoid responsive genes.

As indicated above some of the initial analyses were performed using cloned MMTV DNA that contained genes whose transcription was increased by glucocortcoids *in vivo* (Ringold *et al.*, 1977; Young *et al.*, 1977; Grove *et al.*, 1980). High affinity binding sites for the glucocorticoid receptor were found in the long terminal repeat (LTR) sequences upstream of the promoter as well as positions far from the promoter (Geisse *et al.*, 1982; Govindan *et al.*, 1982; Pfahl *et al.*, 1982; Payvar *et al.*, 1983). Scheidereit *et al.*, (1983) mapped some of these binding sites in the LTR to between positions -202 and -50 relative to the transcription initiation site. The ability of these sequences to confer hormone induced transcription upon other genes was confirmed in transfection experiments (Buetti and Diggelmann, 1981; Lee *et al.*, 1981; Ponta *et al.*, 1985) and deletion analysis identified a DNA fragment of a few hundred base pairs 5' of the MMTV promoter that conferred glucocorticoid induction of gene transcription upon the Herpes simplex virus thymidine kinase gene promoter (Chandler *et al.*, 1983; Hynes *et al.*, 1983; Majors and Varmus, 1983). This sequence conferred hormone responsive transcriptional activation when joined to the promoter in either orientation or when placed over 1 kb upstream (Chandler *et al.*, 1983) thus functioning as a classical enhancer (Muller *et al.*, 1988).

The positions of the glucocorticoid receptor binding sites in responsive genes were determined by DNase I footprinting and methylation interference in the mouse mammary tumour virus DNA (Payvar *et al.*, 1983; Scheideriet *et al.*, 1983; Scheideriet and Beato, 1984), sequences upstream of the human metallothionein-II_A gene (Karin *et al.*, 1984), the lysozyme (Renkawitz *et al.*, 1984) and uteroglobin genes (Cato *et al.*, 1984) and in the first intron of the growth hormone gene (Moore *et al.*, 1985). Based on these studies the binding site for the glucocorticoid receptor was suggested to be 15 base pairs that contained an imperfect inverted repeat of TGTTCT with a three nucleotide gap between the repeats (Scheidereit *et al.*, 1986). Subsequent studies showed that a 15 bp glucocorticoid response element, GRE, was sufficient to confer glucocorticoid induction upon a heterologous promoter (Klock *et al.*, 1987; Strahle *et al.*, 1987). The importance of these sequences for hormone induced transcription of the MMTV genes was verified by deletion and linker scanning mutagenesis although the replacement of individual binding sites with unrelated sequence did not abolish induction suggesting that several regions of the

MMTV LTR between positions -201 and -58 were important (Buetti and Kuhnel, 1986; Kuhnel *et al.*, 1986; Chalepakis *et al.*, 1988).

This region of the MMTV LTR contains four potential binding sites for the glucocorticoid receptor one distal to the promoter, centred at position -177 and three proximal, centred at positions -120, -100 and -85. The distal sequence is an imperfect inverted repeat of the TGTTCT sequence whilst the proximal sites lack an inverted repeat in the correct position also the number of receptors that bind to the proximal sites is unclear (Chalepakis *et al.*, 1988). The deletion and linker scanning mutagenesis described above also showed that mutation of the binding site of another transcription factor, nuclear factor I (CTF/NFI), located at position -70 reduced the level of induction approximately 10-fold. The importance of the CTF/NFI binding site was confirmed by mutagenesis and transient transfection experiments using cells with low CTF/NFI levels (Cato *et al.*, 1988a; Bruggemeier *et al.*, 1990). Two binding sites for another transcription factor Oct-1 have also been found in the MMTV promoter between positions -57 and -37 (note that these were not present in the sequences tested by some groups) and the mutation of the distal site reduces the level of glucocorticoid induction 4-to 5-fold whilst mutation of the proximal site has little affect (Bruggemeier *et al.*, 1991). Mutation of the CTF/NFI and Oct-1 binding sites virtually abolishes induction in response to glucocorticoids (Bruggemeier *et al.*, 1991).

Glucocorticoid response elements have also been found near other genes whose transcription is increased by glucocorticoids. In the tryptophan oxygenase gene response elements were found 450 and 1, 200 base pairs upstream of the site of initiation of transcription (Danesch *et al.*, 1987). The DNA sequence near position -450 also contains a binding site for the CACCC box binding factor that is able to cooperate with the glucocorticoid receptor to stimulate transcription (Danesch *et al.*, 1987; Schule *et al.*, 1988b). In the case of the tyrosine aminotransferase (TAT) gene two GREs were found approximately 2.5 kb upstream of the cap site (Becker *et al.*, 1986; Jantzen *et al.*, 1987). Jantzen *et al.*, (1987) demonstrated the proximal element was not sufficient to confer glucocorticoid induction alone but in combination with the distal element enhances the level of induction in response to dexamethasone suggesting that glucocorticoid receptors cooperated to stimulate transcription. In contrast to the proximal element a 22 base pair oligonucleotide containing the distal GRE was sufficient to confer glucocorticoid inducibility upon a heterologous promoter. This distal GRE was located near a CCAAT box that was subsequently shown to be important for glucocorticoid induction of transcription when the response element was placed a few hundred base pairs upstream of the transcription start site (Strahle *et al.*, 1988). In addition a CACCC box is located near the proximal GRE in the TAT gene and additional elements are important for tissue specific and temporal

expression (reviewed in Nitsch *et al.*, 1991).

Further studies indicated that a GRE and a TATA box were sufficient to obtain hormone induced stimulation of gene transcription only if the GRE was close to the TATA box (Bradshaw *et al.*, 1988; Ham *et al.*, 1988; Strahle *et al.*, 1988). The progesterone (Thomson *et al.*, 1990; Meyer *et al.*, 1992) and oestrogen receptors (Tora *et al.*, 1989b) can also stimulate transcription from promoters containing the receptor binding site and a TATA box only. Hormone dependent transcriptional activation when binding sites were located far from the TATA box could be obtained using two or more GREs or a GRE in combination with a binding site for another transcription factor (Schule *et al.*, 1988a, b; Strahle *et al.*, 1988). These results suggested that the glucocortcoid receptor could cooperate with other transcription factors to stimulate transcription.

Surprisingly the same DNA sequences identified as GREs could also mediate an increase in gene transcription in response to progestins, androgens and mineralocorticoids, for example those in the MMTV LTR were shown to be responsible for induction by all three of these steroids (for examples see Cato *et al.*, 1986; Darbre *et al.*, 1986; Arriza *et al.*, 1987; Chalepakis *et al.*, 1988; Ham *et al.*, 1988) whilst a GRE alone was shown to be sufficient to confer progesterone and glucocorticoid and androgen induction of transcription upon a heterologous promoter (Strahle *et al.*, 1987; Ham *et al.*, 1988). Although the DNA sequence of the binding sites for these receptors are similar DNase I footprinting and methylation protection studies have shown differences between the binding of the progesterone and glucocorticoid receptors to the MMTV LTR (von der Ahe *et al.*, 1986; Chalepakis *et al.*, 1988). Chalepakis *et al.*, (1988) and Cato *et al.*, (1988a) also identified point mutations in the MMTV LTR that did not significantly alter the affinity of the receptors for the binding sites but affected the induction in response to progestins, glucocorticoids and possibly androgens differently.

Oestrogen responsive genes.

In oviparous vertebrates the synthesis of yolk proteins such as vitellogenin and ovalbumin is induced by oestradiol (for a review see Whali, 1988). Following the cloning of the vitellogenin genes A1, A2, B1 and B2 from *Xenopus laevis* and the chicken vitellogenin II and apo-very low density lipoprotein (VLDL) II genes, Walker *et al.*, (1984) derived a putative sequence for an oestrogen response element (ERE) by searching for similar sequences in the 5' flanking regions of these genes.
The authenticity of the sequence suggested by Walker *et al.*, (1984) was verified by subsequent functional analysis of the *Xenopus* vitellogenin A2 gene that identified 35 base pairs containing the sequence 5'-GGTCACAGTGACC-3' that conferred oestrogen induction upon a heterologous promoter (Klein-Hitpass *et al.*, 1986).

Functional analysis of the 5' flanking region of the *Xenopus* vitellogenin B1 gene identified a 33 base pair sequence that was able to confer oestrogen inducible transcription when placed in either orientation upstream of a heterologous promoter or when placed 1.7 kb downstream of the reporter gene (Seiler *et al.*, 1986; Martinez *et al.*, 1987; Martinez and Wahli, 1989). This sequence contained two 13 bp imperfect inverted repeats both of which were required for oestrogen induced stimulation of transcription. The ERE described by Klein-Hitpass *et al.*, (1986) contained a palindrome of the sequence TGACC similar to that of a GRE, in fact changing two base pairs in the palindrome of the ERE 5'-AGGTCACAGTGACCT-3' to 5'-AGAACACAGTGTTCT-3' (Figure 1.1, see later) is sufficient to convert the ERE into a GRE (Klock *et al.*, 1987; Martinez *et al.*, 1987). Further mutational analysis of the ERE from the vitellogenin A2 gene identified base pairs important for receptor binding *in vitro* and oestrogen receptor induced transcription *in vivo* based on these results the sequence GGTCANNNTGACC was proposed as a consensus ERE where N indicates any nucleotide (Klein-Hitpass *et al.*, 1988).

Oestrogen response elements have also been found in sequences flanking other oestrogen responsive genes such as the chicken ovalbumin gene (Tora *et al.*, 1988a) and the human pS2 gene (Berry *et al.*, 1989). These EREs, however, are not perfect inverted repeats. In the case of the ovalbumin gene two regions 5' of the start of transcription have been identified as being important for oestrogen regulation. One is located close to the transcription start site and contains two GGTCA motifs (Tora *et al.*, 1988a) whilst the second is located 3.3 kb from the transcription start site contains four half-palindromes (TGACC sequences) in a region of 675 bp (Kato *et al.*, 1992). Combining these two elements results in a highly oestrogen inducible promoter (~80-fold induction). In combination with the distal element that functions as an ERE the proximal element can be replaced by the binding site for other transcription factors suggesting that the oestrogen receptor can cooperate with other transcription factors to stimulate transcription (Kato *et al.*, 1992). The proximal ERE in the pS2 gene can confer oestrogen induction upon a heterologous promoter although the level of induction is 3-4 times lower than that conferred by a consensus ERE (Berry *et al.*, 1989). This may be a reflection of the fact that the affinity of the oestrogen receptor for this ERE, as judged *in vitro*, is approximately 5 times lower than that for a consensus ERE (Kumar and Chambon, 1988; Berry *et al.*, 1989).

Retinoic acid, thyroid hormone and vitamin D response elements.

The analysis of natural response elements from retinoic acid and thyroid hormone responsive genes as well as artificial DNA binding sites resulted in Umesono *et al.*, (1991) proposing that the vitamin D, thyroid hormone and retinoic acid receptors could function by binding direct repeats of the sequence AGGTCA

DNA binding site sequences	Receptors
AGAACAnnnTGTTCT	GR, PR, AR, MR.
AGGTCAnnnTGACCT	ER.
AGGTCAnAGGTCA	RXR, COUP-TF/RXR RAR/RXR, RXR/PPAR.
AGGTCAnnAGGTCA	RAR/RXR.
AGGTCAnnnAGGTCA	VDR/RXR.
AGGTCAnnnnAGGTCA	TR/RXR.
AGGTCAnnnnnAGGTCA	RAR/RXR.
AAAAGGTCA	NGFI-B.

Figure 1.1. DNA binding sites for nuclear receptors.
Idealised DNA binding sites for the following nuclear receptors are shown: glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), mineralocorticoid receptor (MR), oestrogen receptor (ER), retinoid X receptor (RXR), chicken ovalbumin upstream transcription factor (COUP-TF), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), vitamin D receptor (VDR), thyroid hormone receptor (TR), nerve growth factor induced clone B (NGFI-B). The inverted or direct repeats are indicated by the arrows, a n indicates any nucleotide and a receptor heterodimer with RXR is represented as receptor/RXR.

spaced by 3, 4 or 5 nucleotides respectively (note that EREs are also composed of similar sequence arranged as an inverted repeat with a three nucleotide gap, see Figure 1.1). Further studies, however, have shown that this is not always the case and is further complicated by the fact that vitamin D, thyroid hormone and retinoic acid receptors can bind DNA as heterodimers with the retinoid X receptor, RXR (for examples see Yu *et al.*, 1991; Bugge *et al.*, 1992; Kliewer *et al.*, 1992b; Leid *et al.*, 1992b; Marks *et al.*, 1992; Rosen *et al.*, 1992; Zhang *et al.*, 1992). For example the studies of Carlberg *et al.*, (1993) showed that maximal transcriptional activation by the vitamin D receptor from a response element (from the osteopontin gene) occurred in the presence of RXR, suggesting that the presence of heterodimers, whilst maximal transcriptional activation from a different response element (found in the osteocalcin gene) occurred in the presence of the vitamin D receptor only suggesting that this element was bound as a homodimer. The retinoid X receptors bind a naturally occurring isomer of all-*trans* retinoic acid, 9-*cis* retinoic acid (Levin *et al.*, 1992; Heyman *et al.*, 1992) whilst the retinoic acid receptor binds both these molecules.

Response elements found in retinoic acid regulated genes are often composed of direct repeats of PuGGTCA as well as PuGTTCA sequences separated by 1, 2, 4 or 5 nucleotides, Pu indicates a purine nucleotide (for examples see Leid *et al.*, 1992a; Mader *et al.*, 1993a). In transfection experiments the highest levels of transcriptional activation are often observed from direct repeats separated by 1, 2 or 5 nucleotides (Durand *et al.*, 1992; Mader *et al.*, 1993a). In these cases it is possible that the retinoic acid receptor (RAR) is bound to DNA as a heterodimer with RXR. RXR alone appears to preferentially act through to direct repeats spaced by only 1 or 2 nucleotides (Manglesdorf *et al.*, 1991; Mader *et al.*, 1993a). In the case of the thyroid hormone receptor natural response elements are often composed of direct imperfect repeats of AGGTCA or AGGTAA sequences spaced by 4 nucleotides but other arrangements do exist (Umesono *et al.*, 1991; Kim *et al.*, 1992; Williams *et al.*, 1992). For example in the rat growth hormone gene a response element 5' to the gene is composed of a direct repeat (spaced by 4 nucleotides) as well as an inverted repeat with no nucleotide gap (described in Kim *et al.*, 1992) whilst a response element in the third intron is composed of two direct repeats with no nucleotide gap (Sap *et al.*, 1990).

Functional analysis of orphan receptors (members of the nuclear receptor family for which no ligand has been identified) has indicated that some also bind repeats of sequences similar to AGGTCA. For example COUP-TF (chicken ovalbumin upstream promoter transcription factor) appears to be able to bind several GGTCA based response elements (Cooney *et al.*, 1992; Kadowaki *et al.*, 1992). COUP-TF and a related orphan receptor ARP-1 can repress transcriptional activation by retinoic acid/retinoid X, thyroid hormone and vitamin D receptors (Cooney *et al.*,

1992; Kliewer *et al.*, 1992a; Tran *et al.*, 1992) and the orphan receptor HNF-4 (Ladias *et al.*, 1992; Mietus-Snyder *et al.*, 1992). COUP-TF and ARP-1 can also heterodimerise with RXR and therefore may repress transcription by direct competition for the DNA binding site or possibly by competing for RXR (Berrodin *et al.*, 1992; Kliewer *et al.*, 1992a; Widom *et al.*, 1992). The orphan peroxisome proliferator-activated receptors appear to bind imperfect direct repeats of AGGTCA sequences spaced by one nucleotide (Dryer *et al.*, 1992; Kliewer *et al.*, 1992c; Tugwood *et al.*, 1992) possibly as heterodimers with RXR (Kliewer *et al.*, 1992c; Gearing *et al.*, 1993; Keller *et al.*, 1993). In regulated genes response elements composed of direct repeats can be found in either DNA strand, whether this influences the response is as yet unclear.

Interestingly some orphan receptors bind to slightly different response elements. For example NGFI-B and Rev-ErbAα bind elements composed of a single GGTCA sequence preceded by a short A/T rich sequence (Wilson *et al.*, 1991; Harding and Lazar, 1993) whilst FTZ-F1 and ELP/SF-1 bind PyCAAGGPyC based sequences and Ad4BP binds this and PuPuAGGTCA sequences, Pu and Py indicate purine and pyrimidine nucleotides respectively (described in Lala *et al.*, 1992; Tsukiyama *et al.*, 1992; Honda *et al.*, 1993). Some idealised DNA binding sites for some nuclear receptors are shown in figure 1.1.

The nuclear receptor family.

A comparison of the predicted protein sequences of the glucocorticoid, oestrogen and thyroid hormone receptors identified several regions of similar sequence suggesting that these proteins may be members of a superfamily of ligand inducible transcription factors. On the basis of this sequence homology other cDNA clones were isolated that encoded similar proteins. These included retinoic acid receptors and a number of orphan receptors for which, as yet, a ligand has not been identified (for reviews see Evans, 1988; Green and Chambon, 1988; Beato, 1989; Ham and Parker, 1989; Oro *et al.*, 1992). To date over 30 members of the nuclear receptor family have been cloned that may have evolved from a single common ancestor (Amero *et al.*, 1992; Laudet *et al.*, 1992).

Analysis of genomic clones for members of the nuclear receptor family, including the human androgen, glucocorticoid, oestrogen and retinoic acid receptors (types γ and β) reveals a similar exon/intron structure also suggesting that they may be derived from a common ancestor (see van der Leede *et al.*, 1992). For example in all these cases each zinc finger in the DNA binding domain is encoded by an exon and the hormone binding domain is encoded by five exons. However, at least one member of the nuclear receptor family, COUP-TF, has a different gene structure, being encoded by only three exons (Ritchie *et al.*, 1990).

All members of the nuclear receptor family, on the basis of sequence homology, have a similar DNA binding domain composed of two zinc finger motifs and in most cases contain a C-terminal ligand binding domain. The high sequence homology in the DNA binding domain is reflected in the similarity of the sequences bound by these receptors (described above). Functional analysis of the members of the nuclear receptor family has also indicated similarities in their mechanisms of action, these studies are described elsewhere in this chapter and chapter 6. In contrast, however, the region N-terminal to the DNA binding domain is, in general, poorly conserved in sequence or size between members of the nuclear receptor family. For example in the vitamin D receptor this region is composed of only 24 amino acids in comparison to 603 amino acids in the mineralocortcoid receptor (Evans, 1988). This region may therefore be important for receptor, tissue and/or gene specific regulation of transcription by nuclear receptors.

Functional analysis of oestrogen and glucocorticoid receptors.

The first members of the nuclear receptor family for which cDNA clones were isolated included the human glucocorticoid (Hollenberg *et al.*, 1985) and oestrogen receptors (Green *et al.*, 1986). The aim of this section is to review the subsequent analyses of mainly these receptors to provide a general idea of nuclear receptor structure and function.

A cDNA encoding the human glucocorticoid receptor was isolated in 1985 using specific antiserum to screen an expression library made from a lymphoid cell line (Hollenberg *et al.*, 1985). The protein encoded by this cDNA, called hGRα, was synthesised *in vitro* and shown to bind glucocorticoids with high affinity and specificity. Hollenberg *et al.*, (1985) also isolated a second clone (hGRβ) that encoded a protein that had a different C-terminus from hGRα. This protein failed to bind glucocorticoids suggesting that the hormone binding domain was located in the C-terminus of the molecule. Analysis of the predicted protein sequence indicated that the C-terminal half of the glucocorticoid receptor was related to the product of the *v-erbA* gene found in avian erythroblastosis virus (see Krust *et al.*, 1986).

Based on studies of transcription factor IIIA (TFIIIA), isolated from *Xenopus laevis*, a region highly conserved between the glucocorticoid receptor and v-erbA proteins, rich in the amino acids cysteine, lysine and arginine, was proposed to be a DNA binding domain. Functional analysis of TFIIIA, that contained nine repeats of a cysteine and histidine rich region, indicated that these repeats bound zinc ions and that the binding of zinc was important for DNA binding (Miller *et al.*, 1985a). In each repeat in TFIIIA, two cysteine and two histidine residues were proposed to bind a zinc ion forming a DNA-binding "finger" (Miller *et al.*, 1985a; Berg *et al.*, 1988).

The human oestrogen receptor cDNA, cloned by Green *et al.*, (1986) also encoded a protein that was related to the product of the *v-erbA* gene. Comparison of the protein sequences of the human and chicken oestrogen receptors, v-erbA and the glucocorticoid receptor revealed that the cysteine, lysine and arginine rich region was highly conserved between all these proteins and that a slightly less well conserved region, suggested to be a ligand binding domain, was located C-terminal to the putative DNA binding domain (see Krust *et al.*, 1986). In the former region 9 cysteine residues were conserved in all of these proteins and these were proposed to form two zinc fingers.

On the basis of the homology between the chicken and human oestrogen receptors the protein sequences were divided into six regions, A to F (Krust *et al.*, 1986). The clones for the cellular homologues of v-erbA were also isolated and found to encode receptors for thyroid hormones (Sap *et al.*, 1986; Weinberger *et al.*, 1986). Following the successful expression of functional receptors from these cDNA clones in heterologous cells and *in vitro* mutant cDNA clones were generated to assess the role of different regions of the receptor proteins.

DNA binding.

The highly conserved cysteine rich region, described as region C by Krust *et al.*, (1986), was first shown to contain the protein sequences necessary for target gene recognition by "domain swapping" experiments (Green and Chambon, 1987). In these experiments region C of the oestrogen receptor was replaced with that of the glucocorticoid receptor. This chimeric receptor, in the presence of oestradiol, stimulated the transcription from a glucocorticoid but not oestrogen responsive reporter plasmid. The converse replacement was subsequently shown to confer dexamethasone inducible transcription from an oestrogen responsive element upon a chimeric glucocorticoid receptor (Kumar *et al.*, 1987). Green and Chambon, (1987) also showed that the replacement of two highly conserved cysteines with other amino acids in region C of the oestrogen receptor abolished the stimulation of gene transcription but not oestradiol binding.

By analysing the ability of rat glucocorticoid receptor mutants to co-immunoprecipitate a fragment of the MMTV-LTR DNA containing GREs Rusconi and Yamamoto, (1987) showed that approximately 140 amino acids encompassing the cysteine rich region was sufficient to bind specifically to MMTV LTR DNA with a similar efficiency to that of the wild-type receptor. One mutation that replaced four amino acids, including one of the highly conserved cysteines, abolished DNA binding verifying the importance of the cysteine rich region. Similarly a 157 amino acid fragment of the oestrogen receptor was capable of binding to an ERE in a band shift assay (Kumar and Chambon, 1988).

Structural analysis of a fragment of the rat glucocorticoid receptor containing the DNA binding domain using visible light spectroscopy and extended X-ray absorption fine structure spectroscopy suggested that this polypeptide coordinated two zinc ions each of which was bound by four cysteine residues (Freedman *et al.*, 1988). The binding of zinc was also shown to be required for the folding of this polypeptide into a DNA binding form. The replacement of 8 of the 9 conserved cysteine residues with serine and/or alanine abolished receptor function whilst replacement of some of the critical cysteines residues with histidine residues, that are able to coordinate zinc ions, did not abolish function (Severne *et al.*, 1988).

Mutational analysis of region C in the oestrogen receptor indicated that both putative zinc fingers were required for the ability of the receptor to stimulate transcription in transfected cells and that up to 6 amino acids could be inserted between the zinc fingers without reducing the ability of the receptor to stimulate transcription more than 3-fold (Green *et al.*, 1988). This report also showed, using "swapping" experiments, that the N-terminal zinc fingers of the oestrogen and glucocorticoid receptors were sufficient to confer transcriptional activation from oestrogen and glucocorticoid responsive elements respectively.

The subsequent point mutagenesis of the N-terminal zinc finger identified amino acids important for discriminating between an ERE and a GRE (Danielsen *et al.*, 1989; Mader *et al.*, 1989; Umesono and Evans, 1989). The replacement of the two amino acids between the third and forth cysteine residues of the N-terminal zinc finger of glucocorticoid receptor DNA binding domain with the corresponding amino acids of the oestrogen receptor resulted in a mutant that stimulated transcription poorly from a glucocorticoid responsive gene but efficiently from a oestrogen responsive gene (Danielsen *et al.*, 1989; Umesono and Evans, 1989). In the case of the oestrogen receptor the corresponding amino acids and one located between the zinc fingers (these amino acids constitute the P box) were identified as being important for discriminating between oestrogen and glucocorticoid response elements (Mader *et al.*, 1989). Discrimination between these binding sites in the glucocorticoid receptor appears to rely upon both positive contacts with specific base pairs and inhibitory (negative) contacts with base pairs composing EREs (Zilliacus *et al.*, 1992). Interestingly in thyroid hormone and retinoic acid receptors two amino acids in the P box are the same as those in the oestrogen receptor and these receptors bind similar response elements based on repeated AGGTCA sequences (described above) whilst Ad4BP, ELP and FTZ-F1 that can bind different DNA sequences have different amino acids in the P box (Tsukiyama *et al.*, 1992; Ueda *et al.*, 1992; Honda *et al.*, 1993).

The replacement of sequences in the C-terminal zinc finger of the glucocorticoid receptor with the corresponding amino acids in the thyroid hormone

receptor suggested that residues between the first and second cysteine residues (called the D box) are important for determining the recognition of response elements having none (some thyroid response elements) or three nucleotides (as in EREs and GREs) between the palindromic sequences (Umesono and Evans, 1989). In the oestrogen receptor these amino acids have been shown to be important for dimerisation of the DNA binding domain and their replacement with the corresponding residues in the thyroid hormone or retinoic acid receptor abolishes dimerisation, of the DNA binding domain alone, on an ERE (Hirst *et al.*, 1992; Mader *et al.*, 1993b). These residues may therefore also be involved in discriminating between EREs and other binding sites. Mader *et al.*, (1993b) also showed that the 30 amino acids C-terminal to the zinc fingers in the oestrogen receptor were important for DNA binding especially to imperfect palindromes. Amino acids in this region had also been shown to be important for DNA binding by NGFI-B and H-2RIIBP (retinoid X receptor type β) (Wilson *et al.*, 1992) and FTZ-F1 (Ueda *et al.*, 1992).

The structure of the DNA binding domain of the glucocorticoid receptor bound to DNA has been determined by X-ray crystallographic analysis (Luisi *et al.*, 1991) and two-dimensional nuclear magnetic resonance spectroscopy has been used to assess the structures of the DNA binding domains of the oestrogen (Schwabe *et al.*, 1990) and glucocorticoid receptors (Hard *et al.*, 1990). These studies indicate that the DNA binding domain contains two amphipathic α-helices that contain the C-terminal pair of zinc binding cysteines of each "finger". The N-terminal helix, that contains the amino acids identified as being important for discriminating between GREs and EREs, lies in the major groove of the DNA helix containing the response element. The X-ray crystallographic analysis shows that each DNA binding domain binds to half of the palindrome and that residues in the D box are part of a dimerisation interface.

Hormone binding.

As indicated above analysis of a mutant glucocorticoid receptor, hGRβ, indicated that sequences important for hormone binding were located in the C-terminus of the molecule (Hollenberg *et al.*, 1985). By analysing the consequences of deletions or insertions throughout the oestrogen and glucocorticoid receptors it was shown that a region of ~250 amino acids (region E in Krust *et al.*, 1986), located C-terminal to the DNA binding domain was sufficient for high affinity hormone binding (Giguére *et al.*, 1986; Kumar *et al.*, 1986; Godowski *et al.*, 1987; Hollenberg *et al.*, 1987; Rusconi and Yamamoto, 1987). These results agreed with studies that showed that 27 to 28 kilodalton proteolytic fragments of the oestrogen receptor (Katzenellenbogen *et al.*, 1987) and the glucocorticoid receptor (Eisen *et al.*, 1985) retained the ability to bind hormone. By using radiolabelled ligands that can be

covalently linked to cysteine residues such as ketonoestrol aziridine (an agonist) and tamoxifen aziridine (an antagonist) Harlow *et al.*, (1989) showed that the cysteine residue 530 of the human oestrogen receptor could be labelled suggesting that it was close to the ligand in the hormone binding site. This amino acid, however, does not play a direct role in ligand binding since its replacement with an alanine or serine does not alter the affinity of the receptor for oestradiol significantly (Fawell *et al.*, 1990a; Reese and Katzenellenbogen, 1991b).

The C-terminal boundary of the ligand binding domain of the mouse oestrogen receptor has been located by deletion analysis to be between 61 and 77 amino acids from the C-terminus (Lees *et al.*, 1989; Fawell *et al.*, 1990a). Subsequent mutagenesis of this region identified amino acids important for oestradiol binding (Fawell *et al.*, 1990a). In the case of the glucocorticoid receptor, however, deletion of only 5 amino acids from the C-terminus reduces the affinity for dexamethasone approximately 25-fold whilst binding is essentially abolished by deletion of 29 amino acids (Rusconi and Yamamoto, 1987). Analysis of mutant glucocorticoid receptors has identified amino acids throughout the hormone binding domain that are important for ligand binding. (Danielsen *et al.*, 1986; Byravan *et al.*, 1991; Garabedian and Yamamoto, 1992).

Dimerisation.

Early biochemical studies suggesting that the oestrogen receptor existed as a homodimer (Notides *et al.*, 1981; Miller *et al.*, 1985b) were verified by subsequent experiments that also demonstrated that steroid hormone receptors bound response elements as homodimers (Kumar and Chambon, 1988; Tsai *et al.*, 1988). The functional analysis of mutant receptors has indicated that the DNA binding domain contains a weak dimerisation domain (Kumar and Chambon, 1988; Tsai *et al.*, 1988; Dahlman-Wright *et al.*, 1991) whilst the major dimerisation domain, at least in the oestrogen receptor, is located in the hormone binding domain (Kumar and Chambon, 1988; Guiochon-Mantel *et al.*, 1989; Fawell *et al.*, 1990a). Fawell *et al.*, (1990a) identified a region within the hormone binding domain of the mouse oestrogen receptor that was essential for receptor DNA binding *in vitro*. This region contains a sequence of amino acids conserved in other members of the nuclear receptor family and the mutagenesis of some of these conserved amino acids prevented receptor dimerisation and DNA binding *in vitro* without abolishing ligand binding (Fawell *et al.*, 1990a). Subsequent studies showed that a 22 amino acid peptide corresponding to this region was sufficient to confer DNA binding upon a DNA binding defective mutant receptor suggesting that this region of the oestrogen receptor was part of the dimer interface (Lees *et al.*, 1990). The hormone binding domain is also important for dimerisation of other members of the nuclear receptor family such as the thyroid

hormone, retinoic acid and retinoid X receptors (for examples see Forman *et al.*, 1989; Glass *et al.*, 1989; Selmi and Samuels, 1991; Spanjaard *et al.*, 1991; Leid *et al.*, 1992b; Marks *et al.*, 1992) that, in contrast to the steroid hormone receptors, can bind to response elements as heterodimers.

The association of a dimerisation function with the hormone binding domain is supported by some studies using the antioestrogen, ICI 164, 384 that show it prevents receptor dimerisation and DNA binding *in vitro* (Fawell *et al.*, 1990b; Arbuckle *et al.*, 1992). *In vivo* and in cultured cells this antioestrogen appears to promote degradation of receptor protein but whether this is a consequence of preventing receptor dimerisation or another property of this ligand is unclear (Gibson *et al.*, 1991; Dauvois *et al.*, 1992; Reese and Katzenellenbogen, 1992b).

The dimerisation function located in the hormone binding domain may also be important in determining the transcriptional response once a receptor has bound DNA. In the presence of hormone nuclear receptors in some cases, rather than stimulate gene transcription, can repress or not stimulate transcription significantly. For example the thyroid hormone receptor fails to stimulate transcription when bound to an oestrogen response element and the oestrogen receptor does not stimulate transcription when bound to a thyroid hormone response element (Holloway *et al.*, 1990). This study also showed that a chimeric receptor containing the N-terminus and DNA binding domain of the thyroid hormone receptor and the hormone binding domain of the oestrogen receptor only stimulated transcription when bound to the oestrogen response element. Similarly the reciprocal chimera only stimulated transcription from the thyroid hormone response element. These results suggest that dimerisation of the hormone binding domain directs upon which elements the receptor is transcriptionally active.

Transcriptional activation.

Identification of transcriptional activation domains.

The regions of the glucocorticoid and oestrogen receptor proteins important for transcriptional activation have been identified by analysing the ability of mutant receptors to stimulate transcription in transient transfection experiments. The initial studies of the human oestrogen receptor indicated that removal of the region N-terminal to the DNA binding domain had little effect on the ability of the receptor to stimulate transcription in a hormone dependent manner when using the promoter from the *Xenopus* vitellogenin A2 gene (vit-tk-CAT) whilst mutants lacking the hormone binding domain (in the C-terminus) stimulated transcription, constitutively, to only 5% the level of the wild-type receptor in the presence of oestradiol (Kumar *et al.*, 1987; Bouquel *et al.*, 1989). In contrast the deletion of the region N-or C-terminal to the DNA binding domain reduced the level of transcriptional activation

significantly when the reporter plasmid containing promoter sequences from the pS2 gene (pS2-CAT) was used (Kumar *et al.*, 1987). These results suggested that the region of the protein that overlapped the hormone binding domain, in the C-terminus of the receptor, was essential for efficient hormone dependent transcriptional activation and that the requirement for the region N-terminal to the DNA binding domain for maximal transcriptional activation was promoter specific.

Subsequent studies showed that the region N-terminal to the DNA binding domain of the oestrogen receptor that stimulated transcription constitutively, at a low level, in HeLa cells but stimulated transcription efficiently in chicken embryo fibroblast (CEF) cells (Tora *et al.*, 1989b; Berry *et al.*, 1990) and GC2 cells (Waterman *et al.*, 1988) suggesting that it may contain a cell specific activation domain. The N-terminal activation domain is called transcriptional activation function 1 (TAF-1) whilst the hormone dependent transcriptional activation domain located in the C-terminus was named transcriptional activation function 2 (TAF-2) (Tora *et al.*, 1989b; Berry *et al.*, 1990). In HeLa cells, on some promoters, TAF-1 and TAF-2 in the oestrogen receptor appear to synergise (or cooperate) to stimulate transcription in the full-length receptor (Tora *et al.*, 1989b). Analysis of the protein sequence of the oestrogen receptor suggested that these activation domains are not composed of a high proportion of acidic amino acids or glutamine residues as found in some transcription factors. TAF-1, however, is slightly proline rich whilst TAF-2 does not fall into any of these categories. Additionally based on their ability to cooperate with or inhibit (by squelching, see later) transcriptional activation by themselves and transcription factors that contained acidic activation domains TAF-1 and TAF-2 were suggested to be functionally distinct from each other and from acidic activation domains (Tora *et al.*, 1989b; Tasset *et al.*, 1990; Oehler and Angel, 1992).

Further studies showed that the ability of deletion mutants containing TAF-2 to stimulate transcription varied depending upon the promoter used suggesting that the level of transcriptional activation may be affected by the presence of endogenous transcription factors bound to the promoter (Tora *et al.*, 1989b, Berry *et al.*, 1990). The hormone binding domains of the oestrogen and glucocorticoid receptors were shown to contain a ligand dependent transcriptional activation domain that functioned when attached to the DNA binding domain of the yeast transcription factor GAL4 (Webster *et al.*, 1988b). Transcriptional activation by these chimeric proteins was observed in the presence of oestradiol and dexamethasone but not in the presence of the antioestrogen tamoxifen or the antiglucocorticoid RU486. In the presence of these antagonists the GAL4 oestrogen or glucocorticoid receptor chimeras were able to inhibit transcriptional activation by full-length GAL4 suggesting that antagonists promoted DNA binding by the chimeric proteins. This suggested that these compounds functioned by preventing transcriptional activation by these domains

(Webster *et al.*, 1988b). In an attempt to locate the region of the hormone binding domain of the oestrogen receptor important for TAF-2 each of the exons that encoded the hormone binding domain were tested for their ability to stimulate transcription (Webster *et al.*, 1989). As none of the exons stimulated transcription significantly the authors suggested that elements throughout the hormone binding domain formed TAF-2 upon the binding of oestradiol.

Analysis of the mouse oestrogen receptor also showed that the region N-terminal to the DNA binding domain stimulated transcription constitutively to a low level and that the region C-terminal to the DNA binding domain contained a ligand dependent transcriptional activation domain (Lees *et al.*, 1989). The examination of C-terminal deletion mutants of the mouse receptor showed that TAF-2 activity could be reduced without significantly affecting high affinity oestradiol and DNA binding (Lees *et al.*, 1989). In these studies as well as those described in Kumar *et al.*, (1987) and Webster *et al.*, (1989) the DNA binding domain failed to stimulate transcription significantly.

The analysis of the human glucocorticoid receptor mutants identified two activation domains one N-terminal to the DNA binding domain (called $\tau 1$) and one located in the N-terminal region of the hormone binding domain (called $\tau 2$) (Giguére *et al.*, 1986; Hollenberg and Evans, 1988). Both of these activation domains, in contrast to those in the oestrogen receptor, were reported to be acidic activation domains. Further studies of the human, mouse and rat receptors suggested that the activation domain located N-terminal to the DNA binding domain may be responsible for the majority of the transcriptional activation stimulated by the wild-type receptor since deletion of the hormone binding domain could result in a constitutively active mutant that stimulated transcription to nearly wild-type levels (Danielsen *et al.*, 1987; Godowski *et al.*, 1987; Hollenberg *et al.*, 1987). The study of Godowski *et al.*, (1988) using chimeric proteins containing the DNA binding domain of the bacterial repressor lexA and the N-or C-terminal domains of the glucocorticoid receptor also indicated that an activation domain in the N-terminal region was responsible for the majority of the transcriptional activation by the receptor.

Two reports suggested that the DNA binding domain contains an activation domain, Miesfeld *et al.*, (1987) tested this directly by examining a mutant that contained the DNA binding domain only whilst Hollenberg *et al.*, (1987) concluded this from the fact that deletion of either the N-or C-terminal domains did not completely abolish transcriptional activation. The possibility that the DNA binding domain is involved in transcriptional activation is supported by reports of mutations in the DNA binding domain that do not significantly affect the ability of the receptor to bind DNA *in vitro* but reduced the ability of the full-length receptor (Hollenberg and Evans, 1988) or mutant containing only the N-terminal transcriptional activation

domain to stimulate transcription in mammalian cells (Schena *et al.*, 1989; Zandi *et al.*, 1993). A hormone dependent transcriptional activation domain in the glucocorticoid receptor has been identified by joining the region C-terminal to the DNA binding domain to heterologous DNA binding domains (Hollenberg and Evans, 1988; Webster *et al.*, 1988b).

In summary the oestrogen and glucocorticoid receptors contain at least two transcriptional activation domains, one located N-terminal to the DNA binding domain that can function without hormone and a second located in the hormone binding domain that requires the binding of hormone for its activity. The contribution of these domains to transcriptional activation by the wild-type receptor appears to be receptor, promoter and cell specific (Bouquel *et al.*, 1989 and see above). This is also true for other nuclear receptors that have been examined and will be discussed in chapter 6. For example, the naturally occurring A form of the progesterone receptor can stimulate transcription from the MMTV promoter and the promoter from the chicken ovalbumin gene whilst the B form (that has a slightly longer N-terminus than the A form) only stimulates transcription efficiently from the MMTV promoter (Tora *et al.*, 1988b). The region N-terminal to the DNA binding domains of the retinoic and retinoid X receptor proteins are also important for promoter specific transcriptional activation (Nagpal *et al.*, 1992).

Synergistic transcriptional activation by steroid hormone receptors.

Analysis of steroid hormone responsive genes has indicated that, in many cases, the hormone induced gene transcription requires several transcription factor binding sites. In these cases transcription factors synergise (or cooperate) to stimulate gene transcription. Synergy is said to occur when the level of transcription observed in the presence of two (or more) transcription factors is greater than the sum of the levels of transcription stimulated by the factors when tested individually (Ptashne, 1988). Cooperation can occur at two levels, firstly transcription factors may bind to DNA in a cooperative manner and secondly the transcription factors may cooperate to stimulate gene transcription.

Oestradiol induced transcription of the vitellogenin B1 gene (Martinez *et al.*, 1987; Martinez and Wahli, 1989) requires the presence of two imperfect EREs. The mutation of one response element virtually abolishes hormone dependent transcriptional activation suggesting that receptors cooperate to stimulate gene transcription. Glucocorticoid induced transcription from the MMTV promoter also appears to involve cooperation between receptors binding to the distal binding site and proximal binding sites (Chalepakis *et al.*, 1988). Additionally the glucocorticoid and oestrogen receptors can also cooperate with one another to stimulate transcription from the chicken vitellogenin II gene (Ankerbauer *et al.*, 1988; Cato *et al.*, 1988b).

In the case of the vitellogenin B1 gene studies have shown that the binding of oestrogen receptors to the imperfect EREs in the promoter is cooperative and this may account for the level of synergy observed in transcriptional activation (Martinez and Wahli, 1989).

Steroid receptors also cooperate with other transcription factors in stimulating gene transcription. As described previously the glucocorticoid receptor appears to cooperate with CACCC and possibly CCAAT box binding factors to stimulate transcription from the tryptophan oxygenase and tyrosine aminotransferase genes respectively (Danesch *et al.*, 1987; Schule *et al.*, 1988b; Strahle *et al.*, 1988). Further analysis has indicated that cooperation between the CACCC box binding factor and the glucocorticoid receptor was optimal when the binding sites were aligned on the same side of the DNA helix raising the possibility that the cooperation may depend upon protein-protein interactions between these factors (Schule *et al.*, 1988b). This study also showed that both of these transcription factors bound DNA efficiently in the absence of the other suggesting that the cooperation was not at the level of DNA binding. The glucocorticoid receptor can also cooperate with the transcription factor Oct-I to stimulate transcription from the MMTV promoter, this cooperation appears to be at the level of DNA binding, (Bruggemeier *et al.*, 1991).

The ability of steroid receptors to cooperate with themselves and other transcription factors in stimulating transcription has also been examined using artificial DNA constructs (for examples see Kakidani and Ptashne, 1988; Schule *et al.*, 1988a; Strahle *et al.*, 1988). Schule *et al.*, (1988a) reported that the glucocorticoid receptor and the progesterone receptor cooperated with transcription factors that bound to Sp1, NFI or octamer sites and CCAAT or CACCC-boxes. In contrast to the results of Martinez and Wahli, (1989) described above Ponglikitmongkol *et al.*, (1990) did not observe cooperative DNA binding of oestrogen receptors and suggested that cooperation between oestrogen receptors bound to pairs of perfect or imperfect EREs occurs at the level of transcriptional activation. This report also showed that maximal cooperation occurred when the EREs were aligned on the same face of the DNA helix suggesting a requirement for protein-protein interactions either between the receptor dimers or between the receptors and factors bound at the promoter. Synergy between the oestrogen receptor and a chimeric protein containing the DNA binding domain of GAL4 joined to transcriptional activation domain of CTF/NFI also appears to occur at the level of transcriptional activation (Martinez *et al.*, 1991). The analysis of deletion mutants of steroid hormone receptors have suggested that in some cases the cooperation may depend upon different activation domains depending upon the cooperating transcription factor (Cato and Ponta, 1989; Tasset *et al.*, 1990).

Gene regulation by specific steroid hormones.

The receptors for glucocorticoids, androgens, progestins and mineralocorticoids, as described previously, can stimulate transcription by binding to identical response elements, whilst other nuclear receptors appear to bind similar response elements. These observations raise the question of how a single ligand regulates the transcription of only a subset of genes that contain these response elements as enhancers? This may be accomplished by controlling the secretion and/or metabolism of hormones or by the regulation of the types of functional receptors present in cells. Additionally differences between response elements and/or surrounding DNA sequences could result in an element being bound preferentially by a particular receptor. Many of the studies, described above, showing that several receptors can stimulate transcription from the same response element used transient transfection experiments where the DNA is probably not, or incorrectly, organised into chromatin (Bresnick *et al.*, 1990; Archer *et al.*, 1992, see later). These experiments may be misleading since Hager and Archer, (1991) have reported that transcription from the MMTV promoter in stable minichromosomes was refractory to stimulation by progesterone but responsive to glucocorticoids whilst the transiently transfected template was responsive to both these hormones. This would provide a mechanism whereby specific genes may be regulated by specific hormones although how this occurs is not clear. However, this mechanism may not occur universally since El-Ashry *et al.*, (1989) reported that stably transfected MMTV is responsive to progestins and Strahle *et al.*, (1989) demonstrated that the progesterone receptor, introduced into a cell line that lacked these receptors, could stimulate transcription of endogenous genes that were normally glucocorticoid regulated.

Another mechanism whereby gene transcription is regulated specifically by a particular receptor involves receptor specific interactions with other cellular factors that may or may not bind to the responsive gene. For example part of the enhancer of the *Slp* gene, that encodes the sex-limited protein, contains a response element that is bound by the glucocorticoid and androgen receptors but in transient transfection experiments is only responsive to androgens (Adler *et al.*, 1992). The response element isolated from this fragment could mediate transcriptional activation by both receptors and further analysis suggested that an additional DNA binding factor was required to obtain androgen receptor specific transcriptional activation. These studies also showed that replacement of the region N-terminal to the DNA binding domain of the androgen receptor with that of the glucocorticoid receptor resulted in a chimeric receptor that failed to stimulate transcription from the *Slp* gene suggesting that the N-terminus of the androgen receptor was important for transcriptional activation from the *Slp* promoter.

In transfection experiments transcriptional activation by different steroid receptors can be altered by the presence of components of the AP-1 transcription factor (reviewed in Miner and Yamamoto, 1991; Schule and Evans, 1991). AP-1 was originally identified as a complex that bound to phorbol ester-response elements and can be composed of heterodimers between members of the Fos and Jun transcription factor families (reviewed in Angel and Karin, 1991). This raises the possibility that the presence of differing amounts of Fos and Jun family members in cells could modulate steroid hormone responses to allow transcriptional regulation of a gene by only one type of receptor. For example Jun appears to inhibit transcriptional activation by the glucocorticoid receptor but stimulate transcription by the androgen receptor (Shemshedini *et al.*, 1991). These effects were also cell and promoter specific implicating additional regulatory mechanisms (Shemshedini *et al.*, 1991).

In the case of the proliferin gene a response element (plfG) has been identified that when attached to a heterologous promoter can mediate repression or stimulation of transcription in response to glucocorticoids depending upon the cell type (Diamond *et al.*, 1990). This response element can be bound by AP-1 and the glucocorticoid receptor and further studies indicated that altering the ratio of Fos to Jun switches the transcriptional response from repression to stimulation (Diamond *et al.*, 1990). These and several other studies have proposed that this event relies on a direct interaction between members of the nuclear receptor family and members of the Fos and Jun families (reviewed in Miner and Yamamoto, 1991; Schule and Evans, 1991). In contrast to the glucocorticoid receptor the mineralocorticoid receptor is unable to repress proliferin gene (pflG) transcription under similar conditions (Pearce and Yamamoto, 1993). Although the report did not show that these two receptors bound this element with similar affinity the functional analysis of chimeric proteins showed that the region N-terminal to the DNA binding domain of the glucocorticoid receptor was sufficient to confer regulation upon the mineralocorticoid receptor DNA and hormone binding domains suggesting that it did bind to the plfG element. The authors suggested that the N-terminal region of the glucocorticoid receptor was important for interaction with a component of AP-1 that caused the alterations in gene transcription. Members of the Jun and Fos families also differ in their ability to regulate transcription by glucocrticoid receptors (Lucibello *et al.*, 1990; Miner and Yamamoto, 1992).

In summary ligand specific changes in gene expression may occur for one or more of the following reasons. The responsive cell does not contain competing receptors either due to the lack of those receptors or due to the absence of their ligands. Competing receptors are unable to bind to chromatin templates or due to differences in the response element and/or surrounding DNA sequences do not compete efficiently for the response elements. Only one particular receptor is able to

regulate gene transcription appropriately due to interactions with cell or promoter/enhancer specific transcription factors or the activity of receptors is altered by specific posttranslational modifications. Additionally, as described previously, for some members of the nuclear receptor family heterodimerisation is important for obtaining specific gene expression and this may be another level at which regulation could occur. It is also conceivable that a single response element at different times will bind different receptors that may repress or induce gene expression providing further regulatory mechanisms.

Eukaryotic transcription factors.

Gene expression is, in part, controlled by proteins that bind to specific DNA sequences near a gene and directly regulate its transcription (reviewed in Johnson and McKnight, 1989). These proteins, transcription factors, are often composed of two, separable, domains one of which is required for sequence specific DNA binding and another that is able to stimulate transcription (Frankel and Kim, 1991). Transcription factors are proposed to increase gene transcription by protein-protein interactions between the transcriptional activation domain of the transcription factor and proteins that contact or are part of the RNA polymerase pre-initiation complex (Ptashne and Gann, 1990; Roeder, 1991; Pugh and Tjian, 1992).

The functional analysis of mutant transcription factors in transfection and *in vitro* transcription experiments has identified a large number of polypeptides that are able to stimulate gene transcription. Some of these activation domains contain a high proportion of acidic amino acids, glutamine residues or proline residues (reviewed in Mitchell and Tjian, 1989). For example the transcriptional activation domain of CTF/NFI is proline rich (Mermod *et al.*, 1989). Some activation domains do not fit in any of these categories for example that of the adenovirus E1a protein (Martin *et al.*, 1990) or TAF-2 of the oestrogen receptor (Tora *et al.*, 1989b; Webster *et al.*, 1989).

The initial functional analysis of acidic activation domains from the yeast transcription factor GAL4 suggested that transcriptional activation depended upon the presence of acidic amino acids *per se* (Gill and Ptashne, 1987; Gill *et al.*, 1990). This was supported by the finding that from a pool of random peptides many that stimulated transcription, in yeast, contained acidic amino acids and a net negative charge (Ma and Ptashne 1987; Ruden *et al.*, 1991). These results indicated that the structure of the activation domain may not be critical and that it may contact target(s) by means of multiple non-specific hydrogen bonds (reviewed in Sigler, 1988).

However, an amphipathic α–helix containing acidic amino acids but not a peptide of identical amino acid composition that was not predicted to form an α-helix stimulated transcription in yeast suggesting that the structure of the domain may also be important (Giniger and Ptashne, 1987). Recent functional analysis has altered the

view of these domains further by proposing that the structure of the activation domain (GAL4 was used in this study) rather than the presence of acidic amino acids is important for transcriptional activation (Leuther *et al.*, 1993). This proposal is also supported by the fact that polypeptides composed entirely of acidic, glutamine or proline amino acids fail to stimulate transcription significantly in transfection experiments whilst similar sized polypeptides representing amino acid sequences found in activation domains of transcription factors that contain a high proportion of these amino acids stimulate transcription (Seipel *et al.*, 1992). Transcriptional activation by a portion of the acidic activation domain of the Herpes simplex virus protein VP16 can be essentially abolished by the replacement of non-acidic amino acids also suggesting that the structure of the activation domain is important (Cress and Triezenberg, 1990; Regier *et al.*, 1993). The authors suggested that a pattern of bulky hydrophobic amino acids, conserved in other transcription factors, was important for transcriptional activation by VP16. Mutational analysis of activation domains has identified amino acids important for transcriptional activation but it is impossible to draw any conclusions about activation domains in general and this may reflect the diversity of target(s) contacted. To describe mechanisms whereby eukaryotic transcription factors simulate gene transcription the following section reviews, briefly, the formation of the pre-initiation complex and initiation of transcription by RNA polymerase II which is responsible for the transcription of most mRNA encoding genes.

Transcription by RNA polymerase II.

The biochemical fractionation of nuclear cell extract has identified seven activities named TFIIA to IIJ (using the nomenclature described in Zawel and Reinberg, 1992; no IIC, IIG or II I exist) that are required for accurate basal (uninduced) transcription by RNA polymerase II (reviewed in Sawadogo and Sentenac, 1990; Zawel and Reinberg, 1992). These activities are often called basal transcription factors. Some of these activities have been cloned and others purified to constituent polypeptide(s) (Zawel and Reinberg, 1992). One of the initial steps in the formation of a pre-initiation complex is the binding of TFIID to the TATA DNA element that is located in the promoter approximately 30 base pairs upstream of the start site of transcription (Nakajima *et al.*, 1988; Van Dyke *et al.*, 1988), for a review of the promoter sequences required for accurate transcription see Breathnach and Chambon, (1981). The binding of TFIID to DNA may be assisted by a second fraction TFIIA (Buratowski *et al.*, 1989; Maldonado *et al.*, 1990). The protein in the TFIID fraction that binds the TATA element, the TATA binding protein (TBP), has been cloned from several species (for references see Pugh and Tjian, 1992). The DNA bound complex containing TFIID and IIA is bound by TFIIB this complex then

recruits RNA polymerase II in the presence of TFIIF (Buratowski *et al.*, 1989; Buratowski *et al.*, 1991; Flores *et al.*, 1991). The remaining activities TFIIE, IIH and IIJ associate sequentially to form a competent pre-initiation complex (Flores *et al.*, 1992).

TFIIF is composed of two RNA polymerase associated proteins (RAPs), RAP30 and RAP74 of which RAP30 appears to decrease the affinity of RNA polymerase II for non-specific DNA (Killeen and Greenblatt, 1992). Other reports have suggested that an ATP dependent DNA-helicase activity is associated with TFIIF and/or TFIIE that may cause the unwinding of DNA helix in the promoter forming an open complex ready for transcription (Sopta *et al.* 1989; Buratowski *et al.*, 1991). At or shortly after the initiation of transcription the hydrolysis of ATP (or GTP) is required to allow phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II by TFIIH (Lu *et al.*, 1992). *In vivo* RNA polymerase II is found in two forms one of which is phosphorylated to a greater extent than the other, this phosphorylation increase appears to be due to the phosphorylation of the C-terminal domain of the largest subunit (for a review see Corden, 1993). This domain, in mammals, contains 52 repeats of the heptapeptide YSPTSPS (one letter amino acid code). The lesser phosphorylated form is preferentially recruited into the pre-initiation complex (Lu *et al.*, 1991) whilst the highly phosphorylated form is associated with the transcribing enzyme (Corden, 1993). The possibility that this phosphorylation is required for the initiation of transcription has been supported by *in vitro* studies that show that antibodies that bind to the unphosphorylated form of the CTD prevent initiation but not elongation (Laybourn and Dahmus, 1989; Moyle *et al.*, 1989; Thompson *et al.*, 1989 and see Corden, 1993). Additionally deletion of the CTD from yeast RNA polymerase is lethal (see Corden, 1993) and mutations in the yeast transcription factor GAL4 that reduce its ability to stimulate transcription can be partially compensated for by increasing the number of repeats in the CTD suggesting a role for the CTD in the stimulation of gene transcription (Allison and Ingles, 1989).

Recently the CTD has been reported to associate with the TATA binding protein, TBP (Usheva *et al.*, 1992). This study showed that the unphosphorylated form of RNA polymerase II was active in this respect whilst the phosphorylated form failed to bind TBP. The authors suggested that elongation and transcription by the RNA polymerase complex may occur after phosphorylation of the CTD and its disengagement from TBP. To increase the transcription of a gene any of the steps described above could be affected, in the following section evidence indicating which activities and/or steps may be affected by transcription factors will be reviewed.

Transcriptional activation.

Early studies of the mammalian transcription factors USF (Sawadogo and Roeder, 1985), ATF (Horikoshi *et al.*, 1988b) and the pseudorabies immediate early protein (Abmayr *et al.*, 1988; Workman *et al.*, 1988) suggested that they may cause an increase in gene transcription by promoting DNA binding by TFIID. Additionally the yeast transcription factor GAL4 was able to alter the footprint of TFIID (as did ATF) suggesting it may interact directly with TFIID (Horikoshi *et al.*, 1988a). The possibility that direct interactions were important for transcriptional activation was supported by other studies that showed that the overexpression of a transcription factor could inhibit transcriptional activation (Gill and Ptashne, 1988; Sadowski *et al.*, 1988; Meyer *et al.*, 1989) possibly by binding limiting target proteins preventing their use by transcription factors bound near the promoter. This phenomenon was called squelching (Ptashne, 1988). Additionally experiments showing synergy were proposed to also support the possibility that transcription factors stimulated transcription by contacting either directly or indirectly components of the pre-initiation complex (Carey *et al.*, 1990; Lin *et al.*, 1990). Synergy may occur when the simultaneous binding of a target (that is required for transcriptional activation) by several transcription factors results in a dramatically more stable interaction than that achieved by a single transcription factor or possibly when different activation domains act at different independent steps required to obtain the initiation of transcription (for a review see Herschlag and Johnson, 1993).

Following the cloning of the component in the TFIID fraction that bound to the TATA element, the TATA binding protein (TBP), Stringer *et al.*, (1990) showed that the activation domain of the Herpes simplex virus protein VP16 could bind to the mammalian TFIID fraction and bound yeast TBP directly suggesting that this protein was the target for VP16. Further studies indicated that VP16 mutants bound TBP with an efficiency that correlated, roughly, with their ability to stimulate transcription in cells (Ingles *et al.*, 1991). In contrast using resin immobilised DNA templates that could be separated from nuclear extract by centrifugation Lin and Green, (1991) showed that two acidic activation domains including that of VP16 stimulated transcription by recruiting the general transcription factor TFIIB to the promoter template. Recombinant TFIIB was also shown to interact directly with the activation domain of VP16 and again mutations that reduced transcriptional activation in cells reduced the efficiency of the interaction *in vitro* (Lin *et al.*, 1991). In addition to VP16 several other transcription factors interact with TFIID *in vitro* (for a list see Hagemeier *et al.*, 1993).

The possibility that TBP or TFIIB is a target for transcription factors has been questioned by *in vitro* transcription experiments in which the replacement of the TFIID fraction with TBP resulted in pre-initiation complexes only capable of basal

level (uninduced) transcription that were refractory to stimulation by the mammalian transcription factors such as Sp1 and USF as well as VP16 (White *et al.*, 1991 and see Pugh and Tjian, 1992). These data suggested that additional activities apart from the putative targets of VP16 (TBP and TFIIB) and the other proteins necessary for basal transcription were required for transcriptional activation. This possibility was supported by transcription interference (squelching) experiments which showed that partially purified fractions that were not required for basal transcription were able to prevent squelching by VP16 possibly because these fractions contained target(s) for VP16 (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Flanagan *et al.*, 1991; Berger *et al.*, 1992). By using a genetic screen in yeast Berger *et al.*, (1992) isolated a putative target, ADA2, that was required for transcriptional activation by VP16 and GCN4 (a yeast transcription factor) but not some other yeast transcription factors suggesting that target(s) may be factor specific.

Subsequent biochemical analysis showed that human and *Drosophila* TFIID is a large macromolecular complex composed of at least seven polypeptides, called TBP associated factors, that are tightly bound to TBP (Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; see Weinzierl *et al.*, 1993 for further references). These studies demonstrated that the addition of these purified factors to recombinant TBP and the basal transcription factors restored the ability of transcription factors to stimulate transcription *in vitro*. Recent studies have shown that two recombinant factors are sufficient to partially restore Sp1 mediated transcriptional activation to a pre-initiation complex containing TBP (Weinzierl *et al.*, 1993). One of these factors (a 110 kilodalton protein) is bound by Sp1 and may therefore be a target of Sp1 and the second factor (a 250 kilodalton protein) is able to bind TBP directly and recruits the first factor to TBP (see Weinzierl *et al.*, 1993). Chromatographically separable complexes of TBP that contain common and unique TBP associated factors appear to mediate transcriptional activation *in vitro* by some transcription factors and not others suggesting that transcription factors may function by interacting with specific TBP associated factors (Brou *et al.*, 1993).

The possibility that different transcription factors stimulate gene transcription by contacting different factors is also supported by transcriptional interference (squelching) assays. For example the adenovirus transcription factor E1a is unable to inhibit transcription by an artificial acidic activation domain suggesting that E1a binds factors that are not required for the stimulation of transcription by the acidic activation domain (Martin *et al.*, 1990). This appears not to be due to the acidic activation domain binding the factors with a higher affinity than E1a since E1a when bound to the promoter stimulated transcription much more efficiently than the acidic domain. Similarly the acidic activation domain of VP16 does not interfere with transcriptional activation by TAF-2 of the oestrogen receptor whilst it can interfere

with transcriptional activation by the N-terminal domain of the glucocorticoid receptor that contains an acidic activation domain (Tasset *et al.*, 1990).

How these interactions increase the amount of gene transcription is as yet unclear but *in vitro* studies have suggested that transcription factors are required early in pre-initiation complex formation and alter the number of productive initiation complexes rather than the rate of formation. This is in agreement with the described studies that indicate that transcription factors act at the level of TFIID and/or IIB, possibly at "decision points" in the pathway of initiation complex formation to prevent the assembly of non-productive complexes (reviewed in Herschlag and Johnson, 1993).

Chromatin and transcriptional activation

Gene expression *in vivo* is often correlated with the appearance of DNase I hypersensitive sites suggesting a link between alterations in chromatin structure and the conversion of a gene from a non transcribed to a transcribed state (reviewed in Grunstein, 1990). For some time the link between chromatin and transcription was only a correlation but recent experiments indicate that chromatin plays an active role in the regulation of gene expression *in vivo* (for reviews see Fedor, 1992; Felsenfeld, 1992; Svaren and Hörz, 1993).

In vitro analysis of the role of chromatin in transcription.

In vitro experiments indicate that the binding of nuleosomes, components of chromatin, to template DNA can repress the initiation of transcription and that this repression may be counteracted by transcription factors (for examples see Workman and Roeder, 1987; Workman *et al.*, 1988; Workman *et al.*, 1990; Laybourn and Kadanoga, 1991). In the absence of nucleosomes transcription occurs at a high basal level whilst the addition of histones, components of nucleosomes, represses transcription efficiently. Workman *et al.*, (1991) showed that the addition of a GAL4-VP16 chimera but not the DNA binding domain of GAL4 alone, during nucleosome assembly, preserved transcription from the template. These and other data suggested that the presence of an activation domain was necessary to prevent complete repression of transcription indicating that, in part, a transcription factor *in vivo* may act by promoting basal transcription factor binding to the promoter in place of competing nucleosomes. Studies using purified components sufficient for basal transcription suggested that additional fractions were required for transcriptional activation in the presence of nucleosomes or histone H1(see Fedor, 1992). A partially purified fraction that restored transcriptional activation to this system was found to contain RNA and was inactivated by treatment with RNase A (Croston *et al.*, 1992). The authors suggested that this activity may function as a histone acceptor and that

transcription factor function may require an association of altered or displaced histone H1 with this acceptor that would relax the chromatin structure assisting the binding of basal transcription factors to the promoter and the initiation of transcription.

Genetic analysis of the relationship between chromatin and transcription in yeast.

The possibility that chromatin is required for gene repression has been supported by the observation that deletion of one copy of the histone H4 gene results in the expression of several yeast genes under non-inducing conditions (Han *et al.*, 1988; Han and Grunstein, 1988; Durrin *et al.*, 1992). Removal of the N-terminal sequence from histone H4, but not other histones, or mutation of the acetylation sites in this region of histone H4 reduces the transcriptional activation of several yeast genes (Johnson *et al.*, 1990; Durrin *et al.*, 1991). Acetylated histones are usually associated with expressed genes (reviewed in Turner, 1991). These results suggest that the transcription of a previously repressed gene may require the acetylation of histone H4 which alters the chromatin structure, possibly allowing basal and/or classical transcription factor access to the DNA.

Additional genetic analysis in yeast has identified several genes important for transcriptional activation that link this process to chromatin structure. The mutation of members of two sets of genes named *SWI* and *SNF* reduces the extent of induced transcription of a number of yeast genes and these mutations can be partially suppressed by mutations in two other genes *SIN1* and *SIN2* (reviewed in Winston and Carlson, 1992; Svaren and Hörz, 1993). The genes *SIN1* and *SIN2* encode for a high mobility group (HMG 1) like protein, a component of chromatin (Kruger and Herskowitz, 1991) and histone H3 (cited only) respectively. Additionally mutations in one set of genes that encode histone H2A and histone H2B can suppress mutations in *SNF2/SWI2* (*SNF2* and *SWI2* are identical) and *SNF5* (Hirschhorn *et al.*, 1992). The SWI and SNF proteins have not been found to bind DNA but SNF2/SWI2 and SNF5 can stimulate transcription when attached to a heterologous DNA binding domain in the presence of other SWI/SNF proteins (reviewed in Winston and Carlson, 1992). Since in the absence of the SWI and SNF proteins transcription of suppressed genes could be restored by mutations that lead to a disorganised chromatin structure these and other results suggested that the SWI/SNF proteins acted with transcription factors to suppress the repression of transcription by chromatin (see reviews for references).

Steroid hormone induced alterations in chromatin structure.

The analysis of steroid hormone responsive genes has shown that the induction of some DNase I hypersensitive sites is strictly dependent upon the presence of hormone. These alterations in chromatin structure have been examined in detail in the mouse mammary tumour virus promoter (MMTV) and the tyrosine

aminotransferase (TAT) gene. In the case of the MMTV promoter the template *in vivo* is associated with six specifically positioned nucleosomes of which nucleosome B is altered upon addition of glucocorticoids (reviewed in Hager and Archer; 1991 and see Bresnick *et al.*, 1992b). Nucleosome B includes the binding sites for the glucocorticoid receptor and the transcription factor CTF/NFI. In the absence of glucocorticoids the binding of neither of these factors is detected whilst in the presence of hormone CTF/NFI but not the glucocorticoid receptor can be detected bound to DNA (Cordingley *et al.*, 1987; Archer *et al.*, 1992). *In vitro* analysis has shown that in contrast to the glucocorticoid receptor CTF/NFI is unable to bind to reconstituted nucleosomes (Pina *et al.*, 1990; Archer *et al.*, 1991). Further studies have indicated that CTF/NFI and the glucocorticoid receptor are unable to bind simultaneously to naked DNA (Bruggemeier *et al.*, 1990; Pina *et al.*, 1990) suggesting that the glucocortcoid receptor may function transiently, altering chromatin structure to allow the binding of CTF/NFI. This, however, is probably not the sole function of the receptor since comparison of stably replicating chromatin templates and transiently transfected templates indicates that although CTF/NFI is constitutively bound to transiently transfected DNA glucocorticoids are still required for efficient stimulation of transcription also on the transient and stable templates the binding of basal transcription factors to the TATA element is only detected in the presence of dexamethasone (Cordingley *et al.*, 1987; Archer *et al.*, 1992). Transcriptional activation from a chromatin template *in vivo* can be inhibited by sodium butyrate, that induces the hyperacetylation of histones, but this treatment does not prevent glucocorticoid induction of transcription from a transiently transfected template also suggesting that the glucocorticoid receptor is able to stimulate gene expression directly (Bresnick *et al.*, 1990). One alteration in the chromatin structure of the MMTV promoter that has been shown to occur upon induction with glucocorticoids is the displacement of histone H1 (Bresnick *et al.*, 1992a). *In vitro* transcription in the presence of nucleosomes is inhibited dramatically upon the addition of histone H1 suggesting that it has a role in gene repression (Laybourn and Kadanoga, 1991).

In the case of the tyrosine aminotransferase gene (TAT) treatment with glucocorticoids also results in the alteration of chromatin structure near the glucocorticoid receptor binding sites and the binding of the glucocorticoid receptor is not detected due to the binding of another transcription factor, in this case HNF 5, to overlapping sites (Rigaud *et al.*, 1991). Reik *et al.*, (1991) showed that hypersensitive sites disappear rapidly upon hormone withdrawal or upon the addition of the antagonist RU486 suggesting that "acitve" glucocorticoid receptor is required to maintain the alterations in chromatin structure that may allow the binding of HNF 5. The binding of several putative transcription factors to the chicken apo-very low

density lipopoprotein II gene *in vivo* is also only observed after oestradiol treatment and one transcription factor appears to interfere with the binding of the oestrogen receptor to an ERE (Wijnholds *et al.*, 1991). In the chicken vitellogenin II gene *in vivo* oestradiol is required to induce footprints over the EREs as well as the binding of CTF/NFI to a distant binding site in this case, however, it is not clear if the receptor is excluded from its DNA binding site by another transcription factor (Bakker *et al.*, 1988; Philipsen *et al.*, 1988). Studies of the oestrogen receptor in yeast have been inconclusive suggesting that a functional activation domain of the oestrogen receptor was not required to induce alterations in chromatin structure (Pham *et al.*, 1991a; Gilbert *et al.*, 1992) or that the extent of these alterations was related to the strength of the activation domain (Pham *et al.*, 1991b). In summary steroid hormone receptors may therefore stimulate transcription by, firstly by altering chromatin structure to allow DNA binding by other, previously excluded, transcription factors (basal and/or classical transcription factors) and secondly by stimulating transcription directly.

Chapter 2

Materials and Methods

MATERIALS.

Chemicals.

All general chemicals and solvents were of analytical grade and obtained from either FSA Laboratory Supplies, Loughborough, England or Sigma Chemicals Ltd., Poole, England. Additional reagents are listed:

Absolute alcohol	Hayman Ltd, Witham, U.K.
Acetyl Co-enzyme A	Sigma, Poole, U.K.
Acrylamide, Protein Gel and Sequencing Gel mixes	Boehringer Mannheim, Lewes, U.K.
Agarose	FMC Bioproducts, Rockland, U.S.A.
Amino acid mixture (without methionine).	Promega, Southampton, U.K.
Ammonium persulphate	BDH.
Ampicillin	Beechams Research Laboratories, Brentford, England.
Amplify	Amersham plc, Amersham, U.K
Bacto-Agar	Difco Laboratories, Michigan, U.S.A.
Bromophenol blue	BDH, Poole, U.K.
Bovine serum albumin (fraction V).	Sigma.
Casamino Acids	Difco Laboratories.
Chloramphenicol	Sigma.
DEAE-dextran	Pharmacia P-L Biochemicals, Milton Keynes, U.K.
Diethyl pyrocarbonate	Sigma.
Dexamethasone (cell culture grade)	Sigma.
Dextran T-70	Pharmacia.
Diethylstilbestrol	Sigma.
DTT	BioRad, Hemel Hempstead, UK.
D (+) Galactose	Sigma.
Glycogen	Boehringer Mannheim, Lewes, U.K.
Human placental ribonuclease inhibitor (HPRI)	Amersham.
Luciferin (sodium salt)	Sigma.
Nonidet P-40	BDH.
Nucleotide triphosphates	Pharmacia.
17β-Oestradiol	Steraloids Inc., Wilton, New Hampshire, U.S.A.
ONPG	Sigma.

Picofluor scintillant	Packard Instruments, Groningen, Netherlands.
Poly (dI-dC)·(dI-dC)	Pharmacia
Polyethylene glycol #4000	BDH.
Poly-L-lysine	Sigma.
Protease inhibitors	Sigma.
RNA cap structure analogue	New England Biolabs Inc. Ma., U.S.A.
SDS	Serva Feinbiochemica GMBH & Co. (Supplied by Cambridge Bioscience, U.K.).
TEMED	BioRad.
Tween-20	BioRad.
L-Tyrosine	BDH.
Urea (enzyme grade)	GIBCO BRL Uxbridge UK.
X-gal	Calbiochem Novabiochem, Nottingham, U.K.
Xylene cyanol	BDH.
Yeast Nitrogen base (w/o amino acids)	Difco Laboratories.

Radiochemicals.

Amersham International PLC (Amersham,U.K.) supplied radiochemicals with the following specific activities:

[1-^{14}C] acetyl-coenzyme A	50-60 mCi/mmol.
[^{35}S] dATPαS	400 Ci/mmol
[α-^{32}P] dCTP	~3000 Ci/mmol.
[1,2,4,6,7,-^{3}H] dexamethasone	70-110 Ci/mmol.
Z-4-hydroxy [N-methyl-^{3}H] tamoxifen	71 Ci/mmol.
16α-[^{125}I] iodoestradiol	~2000 Ci/mmol.
L-[^{35}S] methionine	>1000 Ci/mmol.
[6,7,-^{3}H] oestradiol	40-60 Ci/mmol.

Enzymes.

Calf intestinal alkaline phosphatase (CIP), DNase 1 (RNase-free), Klenow enzyme (labelling grade) were supplied by Boehringer Mannheim. T4 polynucleotide kinase was from Pharmacia. RNase A and lysozyme were obtained from Sigma. Vent DNA polymerase and restriction enzymes were routinely purchased from Biolabs (Bishops Stortford, U.K.) apart from Sst I which was supplied by Gibco BRL. T4 DNA ligase was obtained from Gibco BRL and SP6 polymerase was supplied by Stratagene (Cambridge, U.K.).

Plasmids.

Many plasmids used in this thesis were kindly provided by the people listed below.

pERE MLTCAT, A. Thomson, Molecular Endocrinology Laboratory, ICRF.

pG5E1BCAT, N. Jones, Gene Regulation Laboratory, ICRF..

SRE TKCAT, C. Hill, Transcription Laboratory, ICRF.

pSPMORK, MMTV-CAT and pGEM3 MORK, R. White, Molecular Endocrinology Laboratory, ICRF.

pJ3Ω, pJ3MOR, 1-339, 91-599, 121-599 and pERE BLCAT, J. Lees, Molecular Endocrinology Laboratory, ICRF.

pJ3 luciferase, J. Ham, Molecular Endocrinology Laboratory, ICRF.

pSV2Wrec, M. Danielsen, Georgetown University Medical School, Washington, DC, U.S.A.

pSG424, K. Flint, Gene Regulation Laboratory, ICRF.

pSDO8, S. Dalton, Transcription Laboratory, ICRF.

Miscellaneous.

Beckman quick seal tubes (polyallomer)	Beckman, Palo Alto, U.S.A.
Film: Polaroid type 57	Polaroid.
RX	Fuji.
XAR	Kodak.
Filtration units	Nalge Company, Rochester, U.S.A.
FITC conjugated antibodies	Sigma.
Gene Pulser cuvettes (0.4 cm)	Biorad
Glass beads (425-600 μm)	Sigma.
Horseradish Peroxidase conjugated antibodies	Dako Ltd, High Wycombe, U.K.
Hybond-N nylon membranes	Amersham plc, Amersham,U.K.
Luminometer cuvettes	Labsystems Group, U.K.
NA-45 DEAE membrane	Schleicher & Schuell (Supplied by Andermann & Co. Ltd., Kingston-upon-Thames, UK.
Nitrocellulose	Schleicher & Schuell (as above).
Nonfat dried milk	Boots PLC, Nottingham, UK.
Oligonucleotides	Synthesised by I. Goldsmith, ICRF.
Rabbit reticulocyte lysate	Promega, Southampton, England.
Sequenase sequencing kit	U.S. Biochemical Corporation, Cleveland, USA.

Buffers.

All solutions were prepared using water that was quartz distilled and deionised (ddH_2O) and were stored at room temperature unless stated otherwise.

Band shift buffer (ER)(2x)	100 mM KCl, 40 mM HEPES pH 7.4, 2 mM DTT, 20% glycerol (stored at 4°C).
Band shift buffer (SRF)(2x)	100 mM KCl, 20 mM HEPES pH 7.9, 1 mM EDTA, 0.2 mM EGTA, 3 mM spermidine, 1 mg/ml ovalbumin (stored at 4°C).
CAP buffer (5x)	2.5 mM ATP, UTP and CTP, 250 μM GTP and 2.5 mM $m^7G(5')ppp(5')G$ (stored at -20°C).
CIP buffer (10x)	0.5 M Tris-HCl, 1 mM EDTA pH 8.5 (stored at 4°C).
DCC suspension	0.025% (w/v) dextran, 0.25% (w/v) charcoal suspended in TE, pH7.4 (stored at 4°C).
T4 DNA ligase buffer (5x)	0.25 M Tris-HCL (pH7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG # 8000 (stored at -20°C).
DNA loading buffer (5x)	0.25% bromophenol blue, 0.25% xylene cyanol, 25% (v/v) glycerol.
HBS (2x)	40 mM HEPES, 275 mM NaCl, pH 7.1.(stored at 4°C).
Kinase buffer (10x)	0.5 M Tris-HCl, pH 7.4, 100 mM $MgCl_2$, 1 mM EDTA, pH 8.(stored at -20°C).
Luciferase Lysis Buffer	0.65% NP40, 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl.
Luciferase Reaction Buffer	25 mM glcylglycine pH 7.8, 5 mM ATP pH 8.0, 15 mM $MgSO_4$ (stored at -20°C).
NTE (1x)	0.1 M NaCl in TE.
PBSA	140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2.
Phenol/chloroform	Redistilled phenol, equilibrated with 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, heated to 45°C for 10 minutes allowed to cool and stored at -20°C.
Protein loading buffer (4x)	4% SDS, 0.25 M Tris-HCl, pH 6.7, 20% glycerol, 0.05% bromophenol blue, 2% β-mercaptoethanol.
Repair buffer (10x)	0.5 M Tris-HCl, pH 7.4, 70 mM $MgCl_2$, 10 mM DTT (stored at -20°C).

Restriction enzyme buffers (stored at -20°C)	
Low salt (10x)	0.1M Tris-HCl, 0.1 M $MgCl_2$, 10 mM DTT, pH 7.5.
Medium salt (10x)	As low salt but 0.5 M NaCl
High salt (10x)	As low salt but 1 M NaCl .
Very high salt (10x)	As low salt but 1.5 M NaCl .
RIPA buffer (2x)	50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS (strored at 4°C).
Sequencing loading buffer	80% (v/v) deionized formamide, 10 mM NaOH, 1 mM EDTA, pH 8.0, 0.1% (v/v) xylene cyanol, 0.1% (v/v) bromophenol blue.
SDS-PAGE buffer	25 mM Tris base, 190 mM glycine, 0.1% (w/v) SDS.
STET buffer	8% sucrose (w/v), 0.5% Triton X-100 (v/v), 0.0 5M EDTA, 0.01 M Tris-HCL pH 8.0.
T buffer (5x)	200 mM Tris-HCl, pH 7.4, 30 mM $MgCl_2$, 10 mM spermidine (stored at -20°C).
TBE (10x)	0.89 M Tris base, 0.89 M boric acid, 25 mM EDTA.
TE (1x)	10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
TEL buffer	0.1 M Lithium acetate in TE pH 8.0.
Tfb 1	30 mM Potassium acetate, 100 mM RbCl, 10 mM$CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol. Add 0.2 M acetic acid to pH 5.8, filter sterilise, store at 4°C.
Tfb 2	10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl and 15% glycerol. pH to 6.5 with KOH, filter and store at 4°C.
Transfer buffer	192 mM glycine, 25 mM Tris base, 20% methanol (v/v), made just proir to use.
Whole Cell Extract Buffer	0.4 M KCl, 20 mM HEPES pH 7.4, 1 mM DTT, 20% glycerol, 0.5 mg/ml bacitracin, 40 µg/ml PMSF, 5 µg/ml pepstatin A, 5 µg/ml leupeptin (stored without protease inhibitors at 4°C).
X-gal solution	20 mg/ml X-gal in dimethylformamide.
Yeast lysis buffer. (for Western blotting)	50 mM HEPES pH 7.9, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.5 mg/ml bacitracin, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 10 µg/ml aprotinin (stored without protease inhibitors at 4°C).
Yeast lysis buffer (for β-gal assays)	0.1 M Tris-HCL pH 7.5, 0.05% (v/v) Triton X-100.

Z buffer	60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ pH 7.0.

Bacterial Media and Plates.

All the organic components listed below were obtained from Difco.

L-plates	1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.1% glucose, 1.5% (w/v) bactoagar.
L-broth	As for L-agar without agar.
Ψ plates	2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) $MgSO_4$, 1.4% (w/v) bactoagar. pH to 7.6 with KOH.
Ψ broth	Ψa plates without agar, filter sterilised prior to autoclaving.

Yeast Media and Plates.

YP	2.2% (w/v) Bacto-Agar, 2.2% (w/v) Bacto-peptone, 1.1% (w/v) Yeast extract, 0.0055% (w/v) Adenine Sulphate 2% glucose.
Glucose	20% solution ICRF media supplies.
Tryptophan minus agar.	2.2% (w/v) Bacto-Agar, 1.1% (w/v) Casamino acids, 0.8% (w/v) Nitrogen base, 0.014% (w/v) adenine, 0.011% (w/v) tyrosine, 0.011% (w/v) uracil, 0.01% (w/v) leucine, 2% carbon source (glucose or galactose).
Tryptophan minus media.	As above without agar.
Uracil minus agar/media.	As above without uracil and 0.011% (w/v)tryptophan.

Tryptophan,uracil minus media/agar.As above without uracil and tryptophan.

Cell culture media.

Chick serum	Sigma.
Dulbecco's modified Eagle's medium (DMEM)	ICRF media supplies.
Foetal calf serum (FCS)	Gibco, Gibco Ltd., Paisley, Scotland.
PBSA	ICRF media supplies.
Trypsin (Stored at -20°C)	ICRF media supplies [0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.01% (w/v) disodium hydrogen orthophosphate, 0.01% (w/v) dextrose, 0.3% (w/v) Tris-HCl, pH 7.7, 0.25% (w/v) trypsin, 0.01%(w/v) streptomycin, 100 U/ml penicillin, phenol red].
Versene	ICRF media supplies [0.02% (w/v) EDTA in PBSA, phenol red].

METHODS.

Bacterial Transformation.

Storage of bacteria.

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

Preparation of competent bacteria. (Unpublished method of M. Scott and V. Simanis, derived from that of Hanahan, 1983).

Bacteria were streaked out on a Ψ plates and incubated at 37°C until colonies reached approximately 2 mm in diameter. Single colonies were then inoculated into 5 mls of Ψ broth (in glassware prewashed with Ψ broth) and incubated with vigorous shaking at 37°C until the OD_{550} reached 0.3 (approximately 3 hours). This was then subcultured (1/20) into 100 ml of prewarmed Ψ broth and incubated until the OD_{550} was 0.48 (approximately 2 hours). The bacteria were then chilled on ice and pelleted by spinning for 10 minutes at 3000 rpm at 4°C. The pellet was resuspended in 40 ml of ice cold Tfb 1 (2/5 vol) and incubated on ice for 5 minutes. After respinning, the pellet was resuspended in 4 ml of ice cold Tfb 2 (1/25 of original vol) and the cell suspension snap frozen in 0.3 ml aliquots on cardice. Competent bacteria were stored at -70°C.

Transformation. (Unpublished method of M. Scott and V. Simanis).

Competent cells were thawed on ice and 25 μl of cells was added to each 10 μl prechilled DNA sample or ligation products (less than 1 ng DNA/μl cells). After 30 minutes on ice the cells were heat shocked for 5 minutes at 37°C and incubated at 37°C for 40 minutes after addition of 4 volumes of L-broth. Cells were then spread on L-plates containing 50-100 μg/ml ampicillin that were inverted and incubated overnight at 37°C. Competent DH5α cells typically gave between 10^6 and 10^8 bacterial colonies per μg of supercoiled DNA.

Preparation of plasmid DNA.

Plasmid DNA was prepared using both small and large scale methods to allow either rapid screening of bacterial colonies after transformation, or preparation of larger amounts of supercoiled DNA.

Small scale plasmid preparation (mini-prep).

Mini-preparations frequently yield between 2 and 10 μg of DNA. The "boiling" miniprep method was routinely used. L-broth (5 ml), 80 μg/ml ampicillin, was inoculated with a single bacterial colony and incubated overnight at 37°C with

vigorous shaking. Of this culture 1 ml was spun for 10 seconds in a microfuge and the cell pellet resuspended in 0.35 ml of STET buffer. After addition of 25 µl of 10 mg/ml lysozyme (in TE) and mixing the tube was placed in boiling water for 40 seconds and then spun for 10 minutes at room temperature in a microfuge. The pellet was removed and the plasmid DNA precipitated by addition of 40 µl of 3 M Sodium acetate pH 7.0 and 420 µl of isopropanol, vortexing and incubation on cardice for 10 minutes. The DNA was pelleted by centrifugation for 10 minutes at room temperature and after drying resuspended in distilled water.

Large scale plasmid preparation. (Unpublished method of D. Ish-Horowitz ICRF).

A 5 ml overnight culture was inoculated into 400 ml of L broth containing 80 µg/ml ampicillin and grown at 37°C until the OD_{550} was ~1 (approximately 6 hours). The culture was then grown overnight at 37°C (maximum of 16 hours) after addition of 2 ml of 34 mg/ml chloramphenicol (in ethanol). With high copy number plasmids chloramphenicol amplification was not required and a colony or approximately 50 µl of overnight culture was inoculated directly into 400 ml of ampicillin containing L-broth and incubated overnight at 37°C. The bacteria were harvested in 500 ml Sorvall bottles by centrifugation at 6, 000 g for 10 minutes. The bacterial pellet was resuspended in 20 ml of TE containing 0.05 M glucose and incubated for 10 minutes at room temperature after addition of 100 mg of lysozyme. 40 ml of freshly prepared 0.2 M NaOH, 1% SDS was then added and the suspension incubated for 5 minutes on ice after which 25 ml of 5M potassium acetate, pH 4.8 was added and the mixture kept on ice for 15 minutes with occasional mixing. The debris was removed by centrifugation at 7, 000 g for 10 minutes at 4°C and filtration through medical gauze. The plasmid DNA was then precipitated by the addition of 52 ml of propan-2-ol and harvested by respinning at 7, 000 g for 10 minutes at 4°C.

The dried pellet was resuspended in 8.5 ml TE to which 0.7 ml 0.5 M EDTA, pH8 and 240 µl of 1 M Tris base had been added. To this mixture 10.5 g of caesium chloride and 100 µl of 5 mg/ml ethidium bromide were added and the volume was adjusted to fill a Beckman 5/8 x 3" polyallomer Quick-seal centrifuge tube and balanced for spinning. The tubes were spun at 64, 000 rpm for 20-25 hours in a Beckman L3-50 ultracentrifuge using a 70 Ti rotor. The plasmid was visualised using a low wavelength ultra violet lamp and the supercoiled plasmid harvested with a 19 gauge needle and syringe. The DNA was added to 5 ml of distilled water and then mixed with an equal volume of isobutanol. The solvent phase, containing ethidium bromide, was discarded and the extraction performed twice more to remove the ethidium bromide. The DNA was then precipitated by addition of 2.5 volumes of ethanol and centrifugation at 10, 000 g for 10 minutes. The pellet was washed with ice-cold 70% ethanol until all traces of salt were removed, air dried and dissolved in 0.25-1 ml of distilled water. The DNA concentration and purity was determined by

measurement of the OD_{260} and OD_{280} as described in Sambrook *et al.* (1989). The DNA was run on an agarose gel and generally found to be 80-90% supercoiled plasmid.

DNA manipulation and subcloning.

Restriction endonuclease digestion.

Restriction enzyme digests were performed in low, medium, high or very high salt buffers according to the suppliers instructions apart from enzymes which were supplied with a buffer different to these. DNA was digested, at the temperature suggested by the supplier, with a 3-5 fold excess of enzyme. The volume of enzyme added did not exceed 1/10th of the final reaction volume. For analysis of digestion products by gel electrophoresis the digestion was stopped by the addition of DNA loading buffer to 25% (v/v). Alternatively digestions were stopped by extraction with an equal volume of phenol/chloroform and the aqueous phase transferred to a fresh Eppendorf tube after spinning for 2 minutes. This solution was adjusted to contain 0.1 M NaCl and 2.5 volumes of absolute ethanol added to precipitate the DNA and the solution incubated on cardice for 10 minutes. The tube was then spun for 10 minutes in an Eppendorf centrifuge. The pellet was washed with 180 µl of 70% ethanol (that had been stored at -20°C), respun for 5 minutes and the pellet dried and resuspended in distilled water.

Agarose gel electrophoresis. (McDonell *et al.*, 1977).

Agarose [0.8 - 2.5% (w/v)] was dissolved in 1x TBE by boiling in a microwave oven. The solution was allowed to cool and ethidium bromide added to 1 µg/ml before the gel was poured. DNA loading buffer was added to the DNA sample to 20% of the final volume and the samples loaded onto the gel submerged in 1x TBE. The gel was run at 7.5 V/cm until the DNA fragments were well separated. These were visualised by illumination with a long wave ultraviolet light source and photographed using a Polaroid camera. The size of fragments was determined by comparing their mobility to that of restriction fragments of known size (markers were typically Lambda phage DNA digested with Hind III and/or pAT153 digested with Hinf I).

Purification of restriction fragments.

Restriction fragments were purified from agarose gels using NA-45 DEAE membrane (Dretzen *et al.*, 1981). The membrane was presoaked in TE and inserted into a cut in the gel immediately in front of the fragment to be purified. A further piece of membrane was placed behind the band to prevent contamination by other fragments. The voltage was then reapplied to the gel for 5-10 minutes allowing the DNA to collect on the paper. The paper was then transferred to an Eppendorf tube containing 0.2 ml 1 M NaCl and the DNA eluted by heating at 70°C for 30 minutes.

The tube was then vortexed vigorously, the paper removed and the DNA recovered by ethanol precipitation, washed in 70% ethanol, dried and then resuspended in distilled water at the required concentration.

Preparation of vectors.

Routinely 1 to 2 µg of plasmid DNA was digested with the appropriate restriction endonuclease(s) and then extracted with phenol/chloroform, ethanol precipitated and washed with 70% ethanol. The DNA was resuspended in 35 µl of water and then incubated at 37°C for 45 minutes after the addition of 4 µl of 10x CIP buffer and 1 µl of calf intestinal alkaline phosphatase (24 units). This enzyme removes 5' terminal phosphates so that self ligation of the vector is prevented. The DNA was then re-extracted with phenol/chloroform, ethanol precipitated and 70% ethanol washed. Vectors were routinely resuspended at a final concentration of 5-10 ng/ml.

Oligonucleotide kinasing and annealing.

A number of experiments used oligonucleotides to introduce mutations in DNA sequence. The oligonucleotides were synthesised by I. Goldsmith (ICRF) with hydroxyl groups at both the 3' and 5' termini. In order to ensure efficient ligation the 5' ends were kinased prior to annealing. 10 ng of each oligonucleotide was mixed in 10 µl that was 1x kinase buffer, 1 mM dATP, 5 mM DTT and contained 1 µl of T4 polynucleotide kinase (approximately 5 units) and incubated at 37°C for 60 minutes. The oligonucleotides were then annealed after the addition of 10 µl of 10x NTE buffer and 80 µl of distilled water by heating the mixture at 80°C for 3 minutes and allowing it to cool to room temperature.

Ligations.

Ligations were usually carried out with 10 ng of vector and an equimolar and one to four ratio of vector to insert. The insert was generally a DNA fragment isolated from an agarose gel or an annealed pair of oligonucleotides. Ligations in a final volume of 10 µl that contained 2 µl of 5x ligase buffer and 1 µl of T4 DNA ligase (1 unit) were incubated for 2-4 hours at room temperature or overnight at 14°C.

Polymerase chain reactions.

Polymerase chain reactions were performed using Vent DNA polymerase in 50 µl reactions that contained: Biolabs vent buffer (1x), 0.2 mM nucleotides, 0.1 µM of each primer, 1x 10^6-10^7 molecules of plasmid DNA and 2 units of Vent polymerase. Mineral oil was then added to cover this mixture. The mixture was then subjected to 30 cycles of the following: 2 minutes at 94°C, 1 minute at 60°C and 3 minutes at 72°C. The last cycle was followed by 4 minutes at 72°C. The products were recovered and a portion analysed by gel electrophoresis, the remainder was treated with phenol/chloroform ethanol precipitated, washed dried and resuspended in distilled water.

DNA sequencing. (Sanger *et al.*, 1977).

Preparation of DNA and sequencing reactions.

2-4 µg of miniprep or caesium chloride banded DNA was routinely used for sequencing. The DNA was denatured by addition of NaOH (final concentration 0.2 M) and incubation at 70°C for 10 minutes. The single strands were then precipitated on cardice for 10 minutes by the addition of 8 µl of 5 M ammonium acetate, pH 5.4 and 120 µl of ethanol. The DNA was recovered by spinning for 10 minutes at room temperature, washed in cold 70% ethanol, dried and resuspended in 7 µl of distilled water. To this 2 µl of 5x Sequenase buffer and 2.5 ng of oligonucleotide primer (1 µl) were added. Double stranded sequencing was carried out exactly according to the Sequenase (USB)protocol.

Electrophoresis of sequencing reactions.

The reactions were boiled for 5 minutes to separate the freshly synthesised strands from the template and placed on ice just prior to loading on a 6% denaturing polyacrylamide gel. The gel solution contained:

42 g electrophoretic grade urea

15 ml acrylamide stock (38% acrylamide, 2% bis-acrylamide)

10 ml 10x TBE

This was made up to 100 ml with distilled water and polymerisation initiated by the addition of 80 µl of TEMED and 400 µl of 10% ammonium persulphate. This mixture was then poured between glass plates (20 cm x 30 cm) separated by 0.25 mm spacers and the blunt side of the sharks tooth comb inserted. Once the gel was set the comb was removed the well rinsed to remove unpolymerised acrylamide and the vacuum greased teeth of the comb inserted to form the sample wells. The gel was pre-run for 15 minutes at 45 mA in 1x TBE before the samples were loaded and after loading the gel run for the required time. The gel was fixed for 15 minutes in a fix solution, 10% methanol, 10% acetic acid, transferred to Whatmann 3MM paper and dried under vacuum at 80°C for 30 minutes. The labelled bands were visualised by autoradiography using Kodak XAR film.

***In vitro* protein analysis.**

Complementary RNA synthesis. (Modified from Melton *et al.*, 1984).

Complementary RNA (cRNA) was prepared to allow *in vitro* synthesis of receptor protein in a rabbit reticulocyte lysate system. For cRNA synthesis receptor cDNA clones in pSP64 which contains the bacteriophage SP6 RNA polymerase promoter were linearised using a unique restriction site 3' of the coding sequence. The linear DNA template was then phenol/chloroform extracted, ethanol precipitated and washed with 70% ethanol before being resupended in diethyl pyrocarbonate (DEPC)

treated distilled water to give a concentration of 1 mg/ml. Templates were transcribed using SP6 polymerase in the following reaction mix:

5 µg template DNA
20 µl 5x T buffer
20 µl 5x CAP buffer
1 µl 1 M DTT
43-48 µl DEPC treated water (to a total of 100 µl)
4 µl human placental ribonuclease inhibitor (HPRI)
2 units SP6 polymerase

This mix was incubated for 1 hour at 37°C. To digest the DNA template 5 µl DNase, RNase free (23 units/ml) was added along with 3 µl of HPRI and the incubation continued for 15 minutes at 37°C. The RNA was then extracted with phenol/chloroform and precipitated by the addition of 100 µl 5 M ammonium acetate, pH 5.4 and 400 µl ethanol, in the presence of 1 µl molecular biology grade glycogen. The mixture was chilled on cardice for 20 minutes prior to centrifugation for 20 minutes. The RNA pellet was washed with 70% ethanol dried in a speed vac for 5 minutes and resuspended in 25 µl DEPC treated water.

In vitro translation.

cRNA was translated *in vitro* both in the presence and absence of [^{35}S]methionine using a rabbit reticulocyte lysate system. A typical reaction contained 20 µl of rabbit reticulocyte lysate (with $ZnCl_2$ added to 0.1 mM), 3 ml cRNA (~500 ng) and 1 µl of a 1 mM 20 amino acid mix (without methionine), this was made up to 30 µl with DEPC treated water. Of this reaction mix 9 µl was transferred to a second Eppendorf tube containing 1 µl [^{35}S]methionine. To the remaining 21 µl of cold 1 mM methionine (1 µl) was added. These reactions were incubated in parallel for 1 hour at 30°C and stored at -70°C after the addition of glycerol to 15%. Translations labelled with [^{35}S]methionine were used to assess both the size, purity and yield of receptor proteins. Unlabelled translations were used for ligand binding and band shift assays.

SDS polyacrylamide gel electrophoresis. (Laemmli, 1970).

Proteins were analysed on discontinuous polyacrylamide gels using the Atto corporation AE-6220 dual slab chamber. The gel plates (14 cm x 16 cm) were separated by 0.75 mm spacers and sealed with silicon gaskets. Gels were prepared from two solutions forming the resolving and stacking gels respectively. The resolving gel routinely contained 10% acrylamide (30% acrylamide, 0.8% bis-acrylamide stock), 375 mM Tris-HCl, pH 8.8 and 1% SDS. To the mixture ammonium persulphate to 0.1% (w/v) and TEMED 0.1% (v/v) were added. The solution was poured between the plates to within 3 cm of the top and overlaid with water saturated isobutanol. Once the gel had set (~30 minutes) the isobutanol was

removed and the gel rinsed with distilled water. The gel was then rinsed once with the stacking gel solution [4% acrylamide, 125 mM Tris-HCl, pH 6.8, 1% SDS, 0.1% (w/v) ammonium persulphate and 0.1% (v/v) TEMED] before the stacking gel was poured and the comb inserted. The stacking gel was degassed for 15 minutes before the addition of SDS and polymerising agents. The gel was run within 30 minutes of the stacking gel setting. Samples in protein loading buffer were heated for 5 minutes at 95°C and 10-50 µg of total protein was routinely loaded per 6 mm well. In all cases molecular weight markers were run on the gel. The gel was run in 1x SDS-PAGE buffer at 250 V. Protein gels were fixed for 30 minutes in 10% glacial acetic acid, 30% methanol and then incubated in Amplify for 15 minutes before drying. Bands were then visualised by fluorography using Kodak XAR film. For western blotting the gels were not fixed but processed as described below.

Western blotting.

Proteins which had been resolved by SDS-PAGE were transferred to nitrocellulose using a wet blotting method described in Sambrook *et al.*, (1989). The gel was placed in a "sandwich" of a fibrous pad three pieces of Whatman 3MM paper the gel a piece of nitrocellulose, paper and pad. To remove air bubbles the "sandwich" was constructed submerged in transfer buffer and each layer above the gel rolled with a glass pipette. This "sandwich" was enclosed in a basket and placed in transfer buffer in a blotting tank (BioRad) with the nitrocellulose nearest the anode and the transfer performed at 140 mA overnight at 4°C.

The "sandwich" was dismantled and the nitrocellulose membrane washed with distilled water, incubated for a minute with a solution of 2% tricarboxylic acid, 0.2% Ponceau S and washed with water to remove any excess stain. Ponceau S stains transferred proteins allowing the efficiency of transfer and the position of the molecular weight markers to be evaluated (Sambrook *et al.*, 1989). The stain was removed by washing with PBSA and the membrane incubated with blocking solution, 4% (w/v) non-fat milk, 0.2% (v/v) Tween 20 in PBSA, for two hours at room temperature. The membrane was then placed in a bag with 2-4 mls of blocking solution containing an appropriate dilution of monoclonal antibody, immune or pre-immune sera and incubated for two hours at room temperature with occasional agitation. This solution was removed and the membrane washed four times (5 minutes each wash) with 100 ml of PBSA 0.2% (v/v) Tween 20. The membrane was then incubated with 2-4 mls of blocking solution containing an appropriate dilution of a horseradish peroxidase conjugated antibody against the primary antibody for 90 minutes. The membrane was then washed as described above with three additional washes with PBSA alone and processed for enhanced chemiluminescence (ECL) as described in the protocol, Amersham International plc, RX film was used.

In each case the antibodies were titrated to ensure that the amount used was not limiting.

Ligand binding. (Modified from Coffer *et al.*, 1980).

Reticulocyte lysate or COS-1 whole cell extract samples containing receptor proteins (typically 1-2 μg of protein) were incubated in duplicate for two hours at room temperature or overnight at 4°C in 50 μl containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8, 1 mM DTT, 1 mg/ml BSA and [6,7,-^{3}H] oestradiol or 16α-[^{125}I] iodoestradiol at the required concentration. Non-specific binding was determined by the inclusion of a 500 fold excess of diethylstilbestrol in parallel reactions. Free steroid was removed by a 10 minute incubation with 50 μl DCC suspension followed by 10 minute centrifugation. Of the supernatant 80 μl was counted directly [^{125}I] or added to 5 mls of scintillation fluid [^{3}H] and the samples counted, the total ligand added was also counted allowing the affinity of the receptor for oestradiol to be calculated by Scatchard analysis (Scatchard, 1949). Specific binding was determined by subtracting the counts obtained in the presence of cold competitor from those in the absence. For dexamethasone or 4-hydroxytamoxifen assays unlabelled (cold) ligand was used as a competitor. The dissociation constant (Kd) is the concentration of ligand at which the binding of ligand by the receptor is half maximal.

Band shift assay.

1-5 μl of *in vitro* translated receptor or 2-4 μg of whole cell extract was pre-incubated for 15 min at room temperature in 18 μl containing 1 μg poly (dI-dC)·(dI-dC), 0.1 mg BSA, 13.3 mM HEPES, pH 7.4, 67 mM KCl, and 13.3% (v/v) glycerol with or without the addition of pre-or immune sera, ligand or additional DNA. Radiolabelled oligonucleotide probe, 2 μl (1 ng) (see below) was then added and the samples incubated for a further 30 minutes at room temperature. Samples were then applied directly to pre-run (30 minutes at 120V) 6% polyacryamide (30% acrylamide, 0.8% bis -acrylamide stock), 0.5x TBE non-denaturing gels (apparatus as SDS-PAGE) and electrophoresed in 0.5x TBE at 250 V for 50 minutes. Gels were fixed for 15 minutes in 10% acetic acid, 30% methanol, dried and subjected to autoradiography (using RX film).

Probes were prepared by annealing oligonucleotides to form the binding site and these labelled by filling in the 5' overhanging ends in the presence of [α-^{32}P] dCTP. Routinely 200 ng of annealed oligonucleotide was incubated for 30 minutes at room temperature in 1x Repair buffer, 0.2 mM dATP, dGTP and dTTP and a two molar excess of [α-^{32}P] dCTP and four units of Klenow enzyme. The probe was then extracted twice with phenol/ chloroform, once with chloroform, an equal volume of 5 M ammonium acetate and 1 μl of glycogen added, mixed and ethanol precipitated. The pellet was washed with 70% cold ethanol, dried and resuspended in 20 μl of

distilled water. The ERE oligonucleotide pair contains the same sequence as that between positions -338 to -313 of the *Xenopus laevis* vitellogenin A2 gene (Klein-Hitpass *et al.*, 1986) containing a consensus ERE, shown below. The sequence of the ERE is underlined.

5'-CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3'
3'-TTTCAGTCCAGTGTCACTGGACTAGTTAGATC-5'

For band shifts of proteins containing a SRF DNA binding domain the procedure was similar apart from the use of a different buffer (see buffers) and a 4% acrylamide gel was used that was run in 1x TBE (pre-run at 100 V for 2 hours). The probe (provided by S. Nurrish, Transcription Laboratory, ICRF) was generated by PCR with [α-^{32}P] dCTP in the nucleotide pool and corresponds to nucleotides -387 to -250 of the c-*fos* promoter that contains an SRE.

Determination of protein concentration.

The concentration of protein of cell extracts was determined using a dye binding assay (Bradford, 1976). Dye concentrate was obtained from BioRad and used as directed in the protocol. Routinely duplica 2 and 5 µl aliquots of each extract (for the blank extract buffer alone was used) were added to 0.8 ml of water in a polystyrene cuvette and 0.2 ml of dye concentrate added. After mixing the cuvettes were left for 20 minutes at room temperature and then the OD595 measured. The OD595 of a series of dilutions of BSA standard were determined with each assay and a standard curve constructed, from this the protein concentration of the extracts was determined. The concentration of an extract was determined as the average of the determination for the 2 and 5 µl samples.

Yeast transformation and library screening.

The *Saccharomyces cerevisiae* strain used in this thesis (S62L, Dalton and Treisman, 1992) contains the serum response element (SRE) /*lacZ* reporter integrated at the URA 3 locus and was maintained on YP plates. S62L* is identical apart from containing changes in the SRE which prevent SRF binding. S62L was derived from the S50 strain (*HML*α, *MAT*α, *HMR*a, his-11,15, trp1-1, ade2-1, leu2-3, 112, URA3+ho, can1-100). Stocks of all yeast strains were stored in 10% glycerol at -70^{o}C and at 4^{o}C on agar plates.

Yeast transformation.

Transformations were by the lithium acetate method (Ito *et al.*, 1983) and are described below. A colony was added to 20 mls of YP broth or selective media e.g. trp^{-} media for yeast transformed with pSRF-ER, and incubated overnight at 30^{o}C with vigorous shaking. This was then subcultured 1 in 10 into 50 mls and incubated

until the OD_{660} was between 0.65 and 0.8. These cells were pelleted by centrifugation at 1500 rpm resuspended in 30 mls of water respun, resuspended in 15 ml of TEL, respun and resuspended at ~1x 10^9 cells/ml in TEL. An OD_{660} of 0.68 is ~1x 10^7 cells/ml.

The cells were then incubated in a 30°C water bath for 30 minutes with gentle agitation. For 0.1 ml of cells 1-5 μg of plasmid DNA or library DNA was added in 10 μl of water (when transforming with the plasmid cDNA library 15 μg of sheared salmon sperm DNA was added as carrier to increase transformation efficiency) and the cells incubated for a further 30 minutes. After the addition of 0.7 ml of 40% polyethylene glycol # 4000 in TEL and mixing, the cells were incubated for 50 minutes then heat shocked at 42°C for 5 minutes. To pellet the cells the tubes were spun at 6, 500 rpm for 10 seconds in a microfuge and the supernatant removed. The cells then washed twice in selective media and plated on selective plates. This technique normally gave 2-4x 10^4 transformants/μg of DNA. The plasmids containing SRF-ER or VP16-cDNA chimeras contain yeast centromere (CEN) and autonomously replicating sequences (ARS) resulting in mitotically stable, replicating plasmids maintained at 1 to 2 copies per yeast cell, described in Dalton and Treisman, (1992).

Library screening.

The strain S62L transformed with pSRF-ER (tryptophan, TRP, selectable marker) was transformed, as described above, with the VP16 tagged cDNA library (uracil, URA, selectable marker) and plated on 484 cm^2 Amersham Hybond-N membranes on ura^-/trp^- plates containing 2% glucose (~4x 10^4 colonies/plate). These plates were incubated at 30°C until the colonies were just visible (usually 26-30 hours) and the membranes transferred to selective plates which were 10^{-6} M oestradiol and 2% galactose to induce the expression of the SRF-ER and VP16/cDNA. After 18 hours the membranes were removed and processed for the colony colour assay.

Colony colour assay.

To assay colonies for β-galactosidase activity the colony colour assay was used (Breeden and Nasmyth, 1987). Membranes were allowed to dry for a few minutes at room temperature, immersed in liquid nitrogen for around 5 seconds to permeabalise the cells and air dried. These membranes were then placed on Whatman 3MM paper which had been soaked in Z buffer 1.37% (v/v) in X-gal solution (25 ml/membrane). Yeast containing pSRF-ER alone would appear blue after 30-40 minutes. Colonies which appeared blue before 30-40 minutes were picked, diluted in selective media and plated on Hybond-N membranes on 9 cm selective plates (2% glucose). These plates were incubated at 30°C and the colonies retested in the colony colour assay along side a strain containing pSRF-ER alone. Two to three "positive"

colonies which turned blue before 30-40 minutes were picked and grown for further analysis.

Plasmid curing.

To assess if the blue colour observed was the result of SRF-ER recruiting a VP16 tagged protein to the promoter the plasmids expressing each of these cDNAs were removed individually from the yeast by plasmid curing.

This is accomplished by growing the yeast in the presence of either tryptophan or uracil which removes the selection for pSRF-ER (TRP marker) and pVP16/cDNA (URA marker) respectively. This results in some cells losing the plasmid.

Two small patches of cells from positive colonies were grown overnight at 30°C with vigorous shaking in media lacking either tryptophan or uracil only. These cultures were then diluted, the OD_{660} determined and around 150 cells plated onto nylon membranes on agar lacking tryptophan or uracil (10 cm dishes). These colonies were retested in the colour assay and the presence of a few white colonies on ura^- plates (containing VP16/cDNA) or pale blue colonies on trp^- plates (containing SRF-ER) judged to indicate that the blue colour was the result of an interaction between SRF-ER and a VP16 tagged protein. If this was not the case to assess if a plasmid had been lost a number of colonies were picked and replica plated on to ura^- or trp^- and ura^-/ trp^- plates and analysed for their ability to grow in these conditions.

Liquid assay for β-galactosidase activity.

To determine quantitatively β-galactosidase activity in yeast cultures the following protocol was used (Harshman *et al.*, 1988 modified by B. Amati, Growth Control and Development, ICRF).

To 5 ml of selective media 2% in galactose (or glucose, uninduced) with ligand or 0.01% ethanol (carrier) a single colony was added and incubated for 14 hours with vigorous shaking at 30°C. The cells, collected by centrifugation (3000 rpm, 3 minutes), were washed in water transferred to an Eppendorf tube and spun at 6500 rpm for 10 seconds, resuspended in 0.2 ml of lysis buffer and frozen at -70°C. Eppendorf tubes containing a mixture of 0.5 ml of Z buffer (made 50 mM in β-mercaptoethanol just prior to use) and 0.1 ml of 4 mg/ml ONPG were incubated at 37°C for around 30 minutes to warm the reaction mixture. During this time the cells were thawed on ice and samples vortexed just prior to assaying. To the Z buffer/ONPG mix 0.1 ml of cell suspension was added, the tube vortexed and the time of addition noted upon returning the tube to the 37°C water bath. Samples having high β-galactosidase activity were diluted in lysis buffer. When the mixture appeared light yellow the reaction was stopped by the addition of 0.25 ml of 1 M Na_2CO_3 (with vortexing) and the time recorded. A blank was made with lysis buffer alone. These tubes were then spun for 10 minutes in a microfuge and the supernatant transferred to a polystyrene cuvette and the absorbance at a wavelength of 420 nm

determined. Samples giving OD_{420} readings outside the linear range (0.2-0.4) were reassayed. The enzyme activity was normalised to cell number that was determined by measuring the absorbance of the cell suspension (usually 2-8 µl) at 600 nm diluted in 1 ml of water. Linear range 0.1-0.25. The data was entered into a computer program which calculated the units (U) of activity for each sample, expressed as $U=1000A_{420}(CVt)^{-1}$ where A_{420} is the absorbance at 420 nm, C is the density of the cell suspension (in $A_{600}ml^{-1}$), V is the volume of the cell suspension (µl) and t is the total incubation time (minutes).

Yeast protein extract preparation (for Western blotting).

Large yeast colonies (3 mm in diameter) on 2% glucose selective plates were inoculated into 45 ml of selective media 2% in galactose with 0.01% carrier (ethanol) or oestradiol at 10^{-7}M and incubated at 30°C for 14 hours with vigorous shaking. The cells density was determined by measuring the OD at 660 nm and was between 0.6 and 0.8, equal numbers of cells were pelleted by centrifugation (1500 rpm for 5 minutes). All subsequent steps were performed at 0-4°C. The cells were washed in 1 ml of ice cold yeast lysis buffer transferred to a 2 ml Eppendorf tube repelleted and resuspended in 0.35 ml of lysis buffer with freshly added protease inhibitors. Protease inhibitors were stored in aliquots at -20°C. To the cell suspension an equal volume of acid washed glass beads (0.5 mm size) were added and the tube vortexed for 5 minutes with intermittent cooling on ice. To this suspension 0.2 ml of RIPA buffer (with protease inhibitors) was added, the tube vortexed and the supernatant removed to a fresh tube after spinning for 10 minutes in a microfuge. An additional 0.2 ml of RIPA was added to the suspension containing the glass beads and the tube vortexed and left on ice for 20 minutes before spinning and removal of the supernatant. The pooled supernatants were spun for a further 5 minutes, transferred to a fresh tube and the protein concentration of each extract determined. Samples were then mixed with SDS-PAGE loading buffer.

Cell culture methods.

Maintenance of cell stocks.

Cells were routinely maintained as monolayer cultures on Nunclon 175 cm^2 tissue culture flasks at 37°C in a humidifying atmosphere 10% in CO_2.

COS-1 and NIH 3T3 D4 cells , were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal calf serum (FCS). Chicken embryo fibroblast (CEF) cells were primary cell cultures (provided by ICRF central cell culture services) and maintained in DMEM containing 10% (v/v) foetal calf serum (FCS) and 1% (v/v) chick serum. The stocks were subcultured twice weekly. Prior to passage the growth media was removed and the cell monolayer washed with 25 ml of PBSA. The cells were then incubated at 37°C for 3-5 minutes with 10 ml of a

prewarmed 1: 5 trypsin/versene mix (1 in 10 for CEF cells). When the cells had detached from the flask the trypsin was inhibited by the addition of 10 ml of serum containing culture media. The cells were then subcultured directly into fresh media, dilutions greater than 1 in 15, or pelleted by centrifugation at 1500 rpm for 5 minutes resuspended in 10-20 ml of DMEM containing 10% FCS and subcultured as desired. Apart from CEF cells frozen stocks of each cell line were prepared after the first passage. Cells were treated with trypsin as described and pelleted by centrifugation at 1500 rpm for 5 minutes. The pellet was resuspended in 4.5 ml of DMEM containing 10% FCS and 0.5 ml of DMSO added. The cells were then transferred to 2.5 ml Nunc freezing vials in 1 ml aliquots. The vials were wrapped in tissue and placed in a polystyrene container and frozen at -20°C for a few hours then placed at -70°C overnight before being transferred to liquid nitrogen for long term storage.

Charcoal treatment of serum. (Modified from Page and Parker, 1983).

Foetal calf serum is known to contain endogenous steroids (Challis *et al.*, 1974) that might mask the effects of exogenously added steroids in transfection experiments. Serum used for transient transfection was therefore pre-treated with dextran-coated charcoal which is known to remove small molecules including steroid hormones. Dextran-coated charcoal suspension (200 ml) was divided between two 250 ml disposable centrifuge bottles and the charcoal precipitated by centrifugation at 2, 000 g for 15 minutes at 4°C. The supernatant was then removed and the dextran-coated charcoal in each bottle resuspended in 250 mls of FCS. This was then shaken vigorously for 30 minutes at 55°C and respun. The serum was decanted into bottles containing a fresh dextran-coated charcoal pellet, re-incubated and respun as above. The serum was then filter sterilised using a 0.22 μm Nalgene nitrocellulose filter unit.

Transient transfection.

Calcium phosphate precipitation (Graham and Eb, 1973).

Cells were seeded for transient transfection at 2x 10^5 per 6 cm dish in 4 mls of phenol red free DMEM containing 10% charcoal treated foetal calf serum (FCS), CEF cells were plated in the same medium with the addition of charcoal treated chick serum to 1%. Phenol red has been shown to be a weak oestrogen and all transfection experiments were performed using phenol red free DMEM (Berthois *et al.*, 1986). After 16 hours the cells were transfected by calcium phosphate precipitation with a total of 10 μg of DNA per 6 cm dish. The precipitate was prepared by mixing two freshly prepared solutions:-

Solution A	Solution B
1 ml 2x HBS, pH 7.1	1 ml distilled water
10 μl 70 mM NaH_2PO_4	120 μl 2M $CaCl_2$
10 μl 70 mM Na_2HPO_4	40 μg supercoiled plasmid DNA

Solution B was added to A at a rate of approximately one drop every second while air was continuously bubbled through solution A to aid mixing. The mixture was left standing for 25 minutes to allow the precipitate to form and 0.5 ml added dropwise to each of the quadruplicate dishes. The precipitate was left on the cells for 8 hours at 37°C. The media was then removed and the cells were washed two to three times, 10-15 minutes each wash, with warm DMEM to remove the precipitate and then fed with DMEM containing 10% dextran-charcoal treated FCS containing or lacking ligands. Ligands were dissolved in ethanol at 10^{-2}M to 10^{-4}M and stored at -20°C and added to the medium at 0.01% (v/v). Cells were routinely harvested 48 hours post transfection.

The transfected DNA normally included a reporter plasmid containing the chloramphenicol acetyl transferase (CAT) gene an internal control plasmid pJ3 luciferase the appropriate wild-type or mutant receptor expression vector and carrier DNA (pJ3Ω, Morgenstern and Land, 1990) to a total of 10 μg/6 cm dish. The amounts used are described in the text. The reporter that was used in the majority of the experiments described in this thesis pERE BLCAT contained an oestrogen response element (see the section on band shift assays) from the *Xenopus laevis* vitellogenin A2 promoter (Klein-Hitpass *et al.*, 1986) upstream of the herpes simplex virus thymidine kinase promoter (sequences from -105 to +51) and chloramphenicol acetyl transferase gene (CAT) in pBLCAT 2 (Luckow and Schutz, 1987). The level of chloramphenicol acetyl transferase activity measured in transfected cell extracts was taken to be directly proportional to the level of gene transcription and was measured in cell extracts using a non-chromatographic method. Luciferase activity in the cell extracts was measured and these data used to normalise for transfection efficiency in all experiments.

Electroporation (Neumann *et al.*, 1982).

This transfection method was used to transfect COS-1 cells. COS-1 cells grown to 70% confluence in 175 cm^2 flasks were treated with trypsin as described in general cell culture methods apart from the addition of PBSA to the harvested cells to a final volume of 50 ml prior to centrifugation. Cells were resuspended at $7x\ 10^6$/ml in PBSA and 0.8 ml of cell suspension added to 15 μg of plasmid DNA in a 0.4 cm Biorad Gene Pulser cuvette mixed and incubated on ice for 10 minutes. The electroporation was carried out at 450 V and 250 μF (micro Farads) this giving a time constant between 4.8 and 5.2 milliseconds. The cuvettes were then placed on ice for 10 minutes after which the cells were resuspended in phenol red free DMEM containing 10% charcoal treated foetal calf serum and plated as required. Cells were harvested for whole cell extracts, luciferase assays, or used in whole cell uptake assays 44 hours post transfection.

DEAE-dextran (Sompayrac and Danna, 1981).

NIH 3T3 cells were plated in DMEM 10% in charcoal treated FCS at 5x 10^5 per 6 cm dish and after 14 hours washed twice with warm PBSA and 2 mls of a 0.5 mg/ml solution of DEAE-dextran in PBSA containing 10 μg (unless stated otherwise) of the appropriate plasmid DNAs was added to each dish. These cells were then incubated for one hour at 37°C. This solution was made from mixing equal volumes of a 1 mg/ml solution of DEAE-dextran in PBSA and PBSA containing plasmid DNA. The DEAE-dextran stock was stored at -20°C at 10 mg/ml in PBSA and the plasmid DNA at 0.5 mg/ml in PBSA. After this incubation the DEAE mix was removed and 1 ml/plate of DMEM (without phenol red) 10% in DMSO was added for two minutes. The cells were then washed once with warm PBSA and 2 ml/plate of DMEM 5% in charcoal treated FCS, 0.1 mM in chloroquine added and incubated for four hours at 37°C. The cells were maintained in DMEM 10% in charcoal treated FCS with or without ligand and harvested 48 hours after transfection. For transfections using pSRE TKCAT the cells were maintained in DMEM 0.5% in charcoal treated FCS with or without ligand. For serum stimulation this media was replaced with media 10% in FCS for the last 8 hours of the transfection. In these cases a 6 cm dish was transfected with 5 μg of pSRE TKCAT, 1 μg of pJ3 luciferase and pSGSRF-ER (as indicated) or pJ3Ω to 16 μg. The reporter pSRE TKCAT contains an oligonucleotide identical to the c-*fos* SRE joined to the Herpes simplex virus thymidine kinase gene promoter in pBLCAT2 (Luckow and Schutz, 1987), described in Hill *et al.*, (1993).

Whole cell steroid binding assay.

Transfected COS-1 cells were assessed for ligand binding receptor using a whole cell binding assay based on that of Danielsen *et al.*, (1986). Transfected cells (44 hours post transfection) were washed three times with phenol-red free DMEM (lacking serum) and then incubated in this medium containing 10 nM of ^{3}H oestradiol or 100 nM ^{3}H dexamethasone as required with or without a 500-fold excess of cold ligand for 1 hour at 37°C. Cells were then placed on ice and washed four times (5 minutes each wash) with ice cold PBSA. The dishes were then drained and the cells lysed in 1 M sodium hydroxide for 1 hour. This sample contained the retained ligand which was quantified by liquid scintillation counting. The specific binding was calculated by subtracting the counts obtained in the presence of competitor from those in the absence. In each experiment the assay was performed in duplicate and the specifically bound values corrected for protein levels in duplicate dishes. COS-1 cells transfected with the expression plasmid lacking receptor cDNA were also assayed.

Harvesting cell monolayers.

The procedure used is based on that of S. Goodbourn and P. King, Gene Expression, ICRF which allows luciferase and CAT activities to be determined from the same cell extract. Following 44-48 hours after the addition of hormone the medium was removed from the monolayers and the monolayers washed twice with PBSA and the cells lysed by the addition of 0.1 ml of luciferase lysis buffer to each plate. When only nuclei were visible under a microscope (1-2 minutes) the lysate was transferred to a microfuge tube, on ice, and spun for 1 minute to remove cell debris. The supernatant was transferred to a fresh tube and stored on ice until a luciferase assay could be carried out. Owing to the short half-life of the enzyme in cellular extracts (24 hours) this assay was performed as soon as possible. In contrast CAT remains stable in these extracts for several months at -20°C.

Assay of cytoplasmic luciferase activity.

The relative amounts of luciferase in cell extracts was determined by an assay based on that of De Wet *et al.*,(1987). Cell extract (usually 20 µl) was added to 0.35 ml of luciferase reaction buffer in a luminometer cuvette. These were loaded into an LKB 1251 luminometer that injected 33 µl of the substrate, 3 mM luciferin, and mixed the samples; the peak light emission for each sample was recorded (light units). Controls with extracts from untransfected cells had peak activities of around 0.6 to 0.8 units. Transfected cell extracts routinely gave peak activities of 50-2000 units and COS-1 cell extracts were routinely diluted in lysis buffer for this assay. The assay was linear up to 3000 units (personal observations).

Assay of cytoplasmic CAT activity. (Sleigh, 1986).

The remaining cell extract was heated to 65°C for 5 minutes to denature endogenous deacetylases and stored at -20°C until required. CAT activity was determined by analysis of 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 Co-A is not. The acetylated chloramphenicol products therefore can be separated from the labelled substrate by solvent extraction with ethyl acetate allowing direct quantitation of CAT activity without the use of chromatographic separation techniques. The assay was set up with 30 µl 250 mM Tris-HCL pH 7.8, 20 µl 8 mM chloramphenicol, 20 µl acetyl CoA mix (0.1 mCi of [1-^{14}C] acetyl Co-A in 0.5 mM unlabelled acetyl Co-A), cell extract, the final amounts in the reaction ranged from 0.1 µl, diluted in lysis buffer, to 30 µl. The total reaction volume was 0.1 ml. Routinely the components of this reaction apart from the cell extract were made as a cocktail and then added to cell extracts. This mixture was incubated at 37°C for 1 hour. The reaction was stopped by the addition of 0.11 ml of ice cold ethyl acetate and vortexing. This was then spun for 2 minutes in an microfuge and 80 µl of the upper phase transferred directly to a scintillation vial.

The extraction was repeated with 0.1 ml of ethyl acetate and 0.1 ml of the upper phase was removed. The samples were counted by liquid scintillation after the addition of 5 ml of scintillation fluid. This assay was linear up to 55, 000 cpm (J. Lees, Molecular Endocrinology, ICRF, personal communication) and extracts with higher activities were diluted in lysis buffer and reassayed. Untransfected cell extracts were always assayed and the background of the assay (usually 200-300 cpm) subtracted from the counts obtained for transfected samples before correction for transfection efficiency with luciferase activity.

Whole cell protein extract.

Cells on 6 or 10 cm plates were washed three times with ice cold PBSA and harvested with a rubber policeman in 5 ml of ice cold PBSA. After centrifugation at 1500 rpm for 5 minutes the cell pellet was frozen at -70°C. Cell pellets were thawed on ice in approximately 10 volumes of whole cell extract buffer (with freshly added protease inhibitors) passed 5 times through a 25 gauge needle and insoluble material removed by centrifugation at 4°C (50, 000 g for 15 minutes). The resulting whole cell extracts were stored in aliquots at -70°C (Fawell *et al.*, 1990b).

Indirect immunofluorescence.

To assess the expression of transfected cDNA clones in intact cells indirect immunofluorescence was used. Glass coverslips (22 mm x 22 mm) were coated with 0.5 mg/ml poly-L-lysine and cells plated onto these in 3 cm wells for transfection (NIH 3T3 cells) or post transfection (COS-1 cells) cells as described above. After 44-48 hours the cells were washed with PBSA and each coverslip fixed with 1 ml of a solution of paraformaldehyde for one minute. The solution made in PBSA was 3% (w/v) paraformaldehyde, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ pH 7.4 and stored at -20°C. This solution was removed and the cells washed three times with PBSA and the fixing quenched by the addition of 2 ml of PBSA 50 mM in ammonium chloride for 10 minutes. The cells were washed as described and permeabalised by incubation with 2 ml of PBSA 0.2% Triton X-100 for 4 minutes and washed with PBSA 10 mg/ml in BSA. Antibodies were diluted in PBSA 10 mg/ml in BSA and 30 µl aliquots placed on parafilm that was on top of moist Whatman 3MM paper. Coverslips were inverted onto the antibody and incubated for forty minutes in a humid chamber. The coverslips were removed and washed three times in PBSA 10 mg/ml in BSA (5 minutes/wash) and the above procedure repeated with an appropriate fluorescein (FITC) conjugated antibody. After washing the undersides of the coverslips were rinsed with water, drained and mounted on slides with 90% glycerol in PBSA. Slides were examined using a Zeiss Axiophot fluorescence microscope and photographed using 400 ASA film.

Chapter 3

Functional analysis of a conserved region required for hormone dependent transcriptional activation by the mouse oestrogen and glucocorticoid receptors.

Introduction.

The oestrogen receptor contains at least two separable regions that are able to stimulate transcription. One of these regions, called transcriptional activation function 2 (TAF-2), located in the hormone binding domain, requires the binding of oestrogens for its activity. To try to determine the exact position of this activation domain in the mouse oestrogen receptor Lees *et al.*, (1989) examined the ability of receptor deletion mutants to stimulate transcription in transient transfection assays. The results showed that the level of transcription in the presence of oestradiol was significantly reduced when the receptor was truncated at amino acid 538 whilst a receptor that was truncated at amino acid 552 retained nearly wild-type transcriptional activation in the presence of oestradiol (the wild-type receptor has 599 amino acids). Both of these receptor mutants bound DNA and oestradiol with high affinity (Lees *et al.*, 1989). This suggested that the C-terminal boundary of the hormone dependent transcriptional activation function (TAF-2) was located between amino acids 538 and 552. The aim of the functional analysis described in this chapter was to assess the importance of this region for the ability of the oestrogen receptor to stimulate transcription.

Identification of a conserved sequence.

As other members of the nuclear receptor family stimulate transcription in a hormone dependent manner it was possible that the amino acids involved may be conserved between the members of the nuclear receptor family. Therefore the protein sequences of members of the family were examined for similarities to the amino acid sequence between amino acids 538 and 552 of the mouse oestrogen receptor. With R. White (Molecular Endocrinology, ICRF) it was noted that an amino acid sequence in this region was conserved in members of the nuclear receptor family (Figure 3.1). The conserved sequence was located in a similar position in all these proteins close to the C-terminus of the molecule. The sequence of the human receptors (where cloned) are shown and the sequence was also conserved in the receptors that have been cloned from other species.

The major features included a highly conserved glutamic acid residue, flanked by pairs of bulky hydrophobic (nonpolar) amino acids (Figure 3.1). In many cases the amino acid proline was present within the first four amino acids of the motif shown, also, in some sequences acidic amino acids such as glutamic acid or aspartic acid flanking the pairs of hydrophobic amino acids appeared to be conserved. Secondary structure algorithms predicted that this region of the oestrogen receptor would form an α-helix which because of the positions of the acidic and hydrophobic amino acids would be amphipathic.

539	P	L	Y	D	L	L	L	E	M	L	D	A	H	R	L	H	554	mER
535	P	L	Y	D	L	L	L	E	M	L	D	A	H	R	L	H	550	hER
754	E	F	P	E	M	L	A	E	I	I	T	N	Q	I	P	K	769	mGR
748	E	F	P	E	M	L	A	E	I	I	T	N	Q	I	P	K	763	hGR
904	E	F	P	E	M	M	S	E	V	I	A	A	Q	L	P	K	919	hPR
889	D	F	P	E	M	M	A	E	I	I	S	V	Q	V	P	K	904	hAR
955	E	F	P	A	M	L	V	E	I	I	S	D	Q	L	P	K	970	hMR
413	K	L	T	P	L	V	L	E	V	F	G	N	E	I	S	•	427	hVDR
405	S	M	P	P	L	I	Q	E	M	L	E	N	S	E	G	L	420	hRARα
398	S	M	P	P	L	I	Q	E	M	L	E	N	S	E	G	H	413	hRARβ
407	P	M	P	P	L	I	R	E	M	L	E	N	P	E	M	F	422	hRARγ
446	P	I	D	T	F	L	M	E	M	L	E	A	P	H	Q	M	461	hRXRα
510	P	I	D	T	F	L	M	E	M	L	E	A	P	H	Q	L	525	hRXRβ
447	P	I	D	S	F	L	M	E	M	L	E	T	P	L	Q	I	462	mRXRγ
396	L	F	P	P	L	F	L	E	V	F	E	D	Q	E	V	•	410	hTRα-1
445	L	L	P	P	L	F	L	E	V	F	E	D	•				456	hTRβ
507	P	M	H	K	L	F	L	E	M	L	E	A	M	M	D	•	521	hERR1
420	P	M	H	K	L	F	L	E	M	L	E	A	K	V	•		433	hERR2
356	K	I	D	N	L	L	Q	E	M	L	L	G	G	S	A	S	371	HNF-4
447	P	R	N	N	L	L	I	E	M	L	Q	A	K	Q	T	•	461	Ad4BP
795	A	D	F	N	L	L	M	E	L	L	R	G	E	H	•		808	DHR39

Figure 3.1. Sequence alignment of nuclear receptor proteins.

Sequences of the human (h) or mouse (m) proteins are shown. The conserved hydrophobic residues are boxed and conserved acidic residues shaded. The amino acid numbers are shown and the discs indicate the C-terminus of the protein. The sequences shown are:

The oestrogen receptor, White *et al.*, (1987) and Green *et al.*, (1986); glucocorticoid receptor, Danielsen *et al.*, (1986) and Hollenberg *et al.*, (1985); progesterone receptor, Misrahi *et al.*, (1987); androgen receptor, Chang *et al.*, (1988); mineralocorticoid receptor, Arriza *et al.*, (1987); vitamin D receptor, Baker *et al.*, (1988); retinoic acid receptor type α, Giguére *et al.*, (1987); retinoic acid receptor type β, Benbrook *et al.*, (1988); retinoic acid receptor type γ, Krust *et al.*, (1989); retinoid X receptor type α, Mangelsdorf *et al.*, (1990); retinoid X receptor type β, Leid *et al.*, (1992b); retinoid X receptor type γ, Leid *et al.*, (1992b); thyroid hormone receptor type α-1, Nakai *et al.*, (1988); thyroid hormone receptor type β, Weinberger *et al.*, (1986); oestrogen receptor related proteins 1 and 2, Giguére *et al.*, (1988); hepatocyte nuclear factor-4, Sladek *et al.*, (1990); Ad4 binding protein, Honda *et al.*, (1993); *Drosophila* hormone receptor 39, Ayer *et al.*, (1993).

A number of members of the nuclear receptor family for which no ligand has been identified (these have been called orphan receptors) also contained the conserved region. The proteins hERR1, hERR2, HNF-4, Ad4BP and DHR39 are all orphan receptors, (see Figure 3.1). Other orphan receptors contained similar sequences whilst it was not apparent in others (Figure 6.1, see chapter 6 for details). The conserved sequence in the oestrogen receptor shares little homology to the pattern of hydrophobic and acidic residues proposed by Cress and Triezenberg, (1991) to be important for the transcriptional activation.

Functional analysis of the conserved motif.

Mutagenesis strategy.

As other members of the nuclear receptor family show hormone dependent transcriptional activation the importance of the conserved amino acids for TAF-2 in the oestrogen receptor were tested. This was accomplished by examining the effects of replacing the conserved amino acids with different amino acids upon the ability of the receptor to stimulate transcription in a transfection assay.

This study would require the introduction of several changes into the mouse oestrogen receptor cDNA sequence. To simplify the mutagenesis the cDNA encoding amino acids 538 to 560 was replaced with a pair of annealed oligonucleotides that did not alter the amino acids encoded by the cDNA but introduced an unique restriction enzyme site at codon 552. This recombinant cDNA, pSPMORK TAV (see Appendix A1), contained unique restriction enzyme sites at codons 538 and 552 allowing the replacement of the cDNA between these unique enzyme sites with oligonucleotide "cassettes" that contained specified DNA sequences. All mutant receptor cDNA clones were generated in a plasmid suitable for *in vitro* transcription and transferred into eukaryotic expression vectors for transient transfection experiments (see Appendix sections A2 to A5 and methods). The receptor mutants are described as the amino acid, its position in the wild-type receptor and the amino acid with which it was replaced. For example E-546A is a receptor in which the glutamic acid residue at position 546 was replaced with an alanine residue and Δ 540-552 indicates that these amino acids were deleted.

Besides TAF-2 the oestrogen receptor contains a transcriptional activation function, called TAF-1, located N-terminal to the DNA binding domain that can cooperate with TAF-2 to stimulate transcription in the full-length receptor (see chapter 1). To assess the effects of the mutations in the absence and presence of TAF-1 the mutations were introduced in the full-length receptor (MOR 1-599) and a receptor lacking the N-terminal TAF-1 (MOR 121-599). MOR 121-599 binds oestradiol and DNA with affinities similar to those of the wild-type receptor *in vitro* (Fawell *et al.*, 1990a).

Transient transfection assays.

To test the ability of mutant receptors to stimulate transcription a transient transfection assay was used. For this assay NIH 3T3 cells, that lack oestrogen receptor, were transfected using the calcium phosphate coprecipitation method with a reporter gene plasmid pERE BLCAT (5 μg), an internal control plasmid pJ3 luciferase (1 μg), the appropriate oestrogen receptor expressing plasmid (usually 0.5 μg) and pJ3Ω, the expression vector used (Morgenstern and Land, 1990), to a total of 10 μg/6 cm dish (see methods). Preliminary experiments were performed to assess over what range of input oestrogen receptor expression plasmid the transcriptional response was linear. These results showed that at 0.5 μg/dish of pJ3MOR (the plasmid containing the wild-type mouse oestrogen receptor cDNA, White *et al.*, 1987) the level of chloramphenicol acetyltransferase (CAT) gene transcription stimulated, in the presence of oestradiol, was half maximal (data not shown and Lees *et al.*, 1989). Transcriptional activation was maximal at 3 μg/dish. Therefore 0.5 μg/dish of pJ3MOR (wild-type and mutant) was routinely used ensuring that the response was in the linear range. No receptor or ligand dependent transcriptional activation was seen when the reporter plasmid pERE BLCAT was replaced with pBLCAT 2 (Luckow and Schutz, 1987) that lacks the oestrogen response element (data not shown). In these transfection experiments dishes were transfected in quadruplicate and pairs maintained in the absence or presence of oestradiol. For experiments with the antioestrogen tamoxifen six dishes were transfected. In all cases at least two recombinant clones of each construct were tested.

The wild-type receptor in the transient transfection assay stimulated transcription between 8 and 10-fold in the absence of oestradiol and approximately 90-fold in the presence of 10^{-8}M oestradiol (Figure 3.2). The activity in the absence of added oestradiol was, in part, probably due to residual oestrogens in the charcoal stripped serum (see chapter 5). The fold induction was calculated by dividing the CAT activity (normalised for transfection efficiency with luciferase activity) observed in the presence of receptor by that observed in the absence of receptor (with or without ligand), reporter alone.

Transcriptional activation by mutant receptors.

To assess the importance of the conserved region a mutant was generated that lacked amino acids 540-552 (Δ 540-552) (see Appendix A3). This mutation reduced the oestradiol induced transcriptional activation to less than 5-fold, less than 6% that of the wild-type receptor, confirming the importance of this region (Figure 3.2).

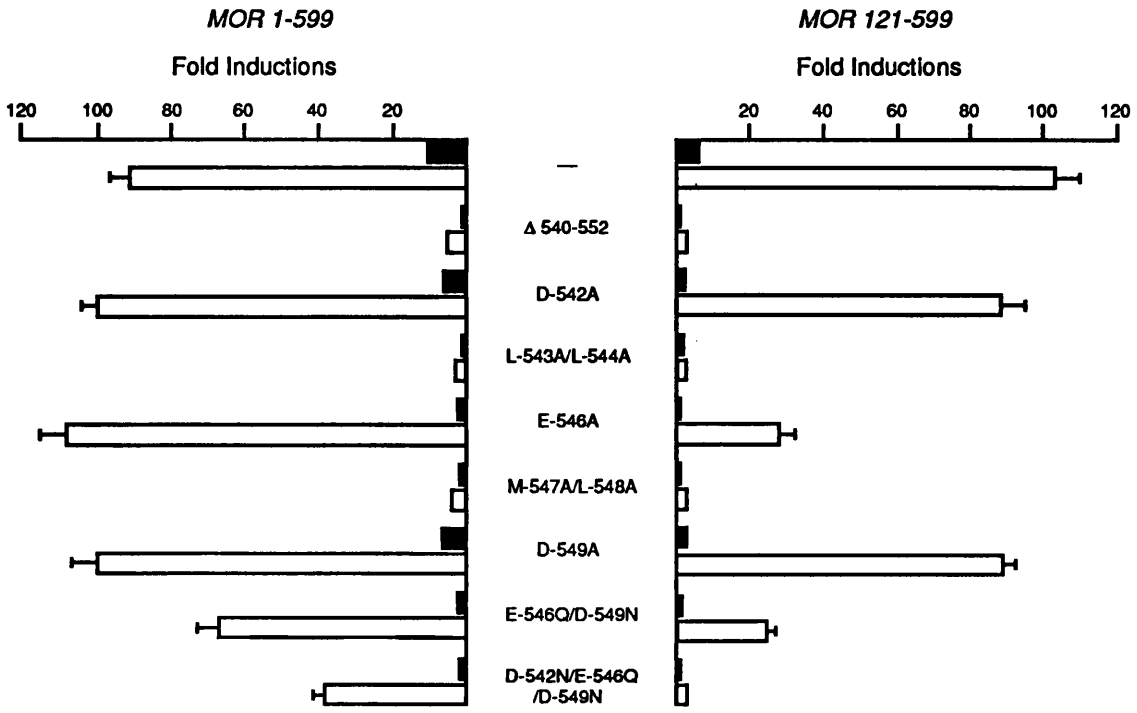


Figure 3.2. Transcriptional activation by mutant oestrogen receptors.
The ability of the mutant mouse oestrogen receptors (MORs) to stimulate transcription from the reporter plasmid pERE BLCAT was tested in transient transfection experiments using NIH 3T3 cells (see methods). In all transfection experiments the CAT activities were corrected for differences in transfection efficiency with luciferase activity derived from a cotransfected internal control plasmid. The level of transcriptional activation is shown as fold inductions over reporter alone in the absence (solid bars) or presence (open bars) of 10^{-8}M oestradiol. The mutations were introduced into the full-length receptor (MOR 1-599) or a deletion mutant that lacked TAF-1 (MOR 121-599). The dash indicates that the conserved region was unaltered. The error bars (that are not apparent in some cases) represent standard errors derived from the results of at least three independent transfection experiments each carried out in duplicate.

To test if the amino acids between 538 and 552 could act as an independent activation domain cDNA clones were generated that encoded the DNA binding domain of the yeast (*Saccharomyces cerevisiae*) transcription factor GAL4 joined to residues 538-552 of the mouse oestrogen receptor. This chimera was tested in a transient transfection assay but failed to stimulate transcription to a level significantly above that of the GAL4 DNA binding domain alone (data not shown). Therefore this peptide may not contain an independent activation domain. Since several functions are conserved between members of the nuclear receptor further mutagenesis of this region focused on the conserved residues indicated in Figure 3.1.

Of the mutations in the full length receptor (MOR 1-599) those which resulted in the replacement of the hydrophobic residues with other amino acids were the most deleterious (Figure 3.2). Both L-543A/L-544A and M-547A/L-548A stimulated transcription less than 4-fold in the presence of 10^{-8}M oestradiol, only 5% of that stimulated by wild-type receptor. These double mutants were made using oligonucleotides that introduced a codon for alanine or aspartic acid at each position (see Appendix A4). The following mutants were also tested and all stimulated transcription less than 5-fold in the presence of oestradiol in several independent transfection experiments: L-543A/L-544D, L-543D/L-544A, M-547D/L-548A and M-547A/L-548D.

The mutants D-542A and D-549A in which the aspartic acid residues had been replaced with alanine residues, in the presence of oestradiol, stimulated transcription to a level similar to that of the wild-type receptor. Replacement of the highly conserved glutamic acid residue (E-546A) with alanine also appeared to have no significant effect on the level of transcription observed in the presence of oestradiol. Further mutations to assess the role of the acidic amino acids which replaced two (E-546Q/D-549N) or all three (D-542N/E-546Q/D-549N) of the acidic amino acids with uncharged residues only slightly reduced transcriptional activation to approximately 75 and 40% that of the wild-type receptor respectively. A reasonably well conserved hydrophobic residue was also located at position 540 (Figure 3.1) but this amino acid was not changed since in other members of the nuclear receptor family it was located N-or C-terminal to proline residues that would be expected to result in very different protein structures in this region.

As indicated previously the mutations were also introduced into a receptor lacking part of the N-terminus (MOR 121-599) to assess the effects of the mutations on TAF-2 in the absence of the residues important for TAF-1. As in the full-length receptor the mutation of the pairs of hydrophobic residues resulted in negligible transcriptional activation, less than 3-fold in the presence of oestradiol, only 3% of the level of transcription stimulated by MOR 121-599. The replacement of the aspartic acid residues with alanines (D-542A or D-549A) had no significant affect

upon transcriptional activation by MOR 121-599. In contrast to the full-length receptor, however, the replacement of the highly conserved glutamic acid residue in E-546A reduced the transcriptional activation in the presence of oestradiol to 25% that of MOR 121-599. The mutant E-546Q/D-549N stimulated transcription 20-fold (20% that of MOR 121-599) whilst replacement of all three acidic residues in D-542N/E-546Q/D-549N reduced the transcriptional activation to approximately 3-fold (only 3%). The presence of the N-terminus therefore appeared to alter the transcriptional activation by three of the mutants in which acidic residues had been replaced with other amino acids. Only one acidic amino acid was present in the mutant E-546Q/D-549N and this appeared to be sufficient for TAF-2 to stimulate transcription in the absence of the N-terminus. In comparison D-542N/E-546Q/D-549N in MOR 121-599 was essentially inactive compared with MOR 121-599. However, D-542N/E-546Q/D-549N in MOR 1-599 stimulated transcription to nearly wild-type levels, Figure 3.2. These results suggested that the N-and C-terminus of the receptor are able to cooperate in the full-length receptor to stimulate transcription. This cooperation is examined in a later section. Transfection experiments using wild-type receptor and L-543A/L-544A (in MOR 1-599) were repeated using a different transfection method, DEAE-dextran (see methods) and similar results were obtained suggesting that the results were not peculiar to one transfection technique (data not shown).

Mutations in the conserved region appear not to affect other receptor functions.

To assess if the results observed were due to the mutations affecting other functions of the receptor rather than transcriptional activation a number of control experiments were performed.

Assessment of wild-type and mutant receptor protein levels in transfected cells.

The expression of the wild-type and mutant receptors in transfected NIH 3T3 cells was assessed by indirect immunofluorescence using the monoclonal antibody antibody H222 raised against the human oestrogen receptor (Greene *et al.*, 1984), the epitope for this antibody has been mapped to between amino acids 463 and 528 in the hormone binding domain (Kumar *et al.*, 1986). Approximately 1% of cells appeared to be expressing receptor and the wild-type and all the mutant receptor proteins were expressed at similar levels and appeared predominantly nuclear (data not shown).

Expression of receptors in COS-1 cells.

Receptor proteins in transfected NIH 3T3 cells were difficult to detect by Western blotting or band shift assays (data not shown). Therefore to assess the relative levels of functional protein expressed in transfected mammalian cells the

COS cell system was chosen. COS cells contain an origin defective mutant of simian virus 40 (SV40) T antigen that allows plasmids, containing a SV40 origin of replication, to be replicated to high copy numbers (Mellon *et al.*, 1981). To assess if this system was suitable COS-1 cells were transfected with wild-type receptor expression plasmid (pJ3MOR) by electroporation using conditions optimised by P. Acland and P. Kiefer (Viral Carcinogenesis, ICRF, see methods). The receptor expressed in COS-1 cells was examined using several techniques. Indirect immuofluorescence (using H222) was used to assess the cellular localisation of the receptor and the proportion of cells transfected. The results indicated that this technique routinely resulted in 30-40% of the cells expressing oestrogen receptor and that the wild-type and mutant receptors were localised to the nucleus (data not shown). Secondly the expression of the receptor protein was monitored by Western blotting using a polyclonal serum (MP16) generated against a peptide containing a sequence identical to that between amino acids 130 and 142 of the mouse oestrogen receptor (Fawell *et al.*, 1990a, b). The receptor protein appeared to migrate at the expected position for a protein having a molecular weight of ~66 kilodaltons (Figure 3.3). No equivalent band was seen when a pre-immune serum was used instead of the immune serum (data not shown). These data indicated that expression was maximal around 44 hours post transfection therefore transfected cells were harvested at this time. Analysis of immunoprecipitated receptor expressed in transfected COS-1 cells that had been incubated with ^{35}S methionine gave similar results (H. Lahooti , Molecular Endocrinology, ICRF, personal communication).

Whole cell extracts from COS-1 cells were then tested for the presence of functional receptor in two assays. Firstly the extracts were tested for the presence of receptor able to bind to DNA in a band-shift assay. These results showed that the receptor bound specifically to an oligonucleotide probe containing an oestrogen response element (ERE) and that this binding was not detected only in the presence of excess unlabelled ERE but not significantly affected by the addition of unlabelled oligonucleotides containing a GAL4 binding site (provided by K. Flint, Gene Regulation, ICRF) or one containing two progesterone receptor binding sites (provided by A. Thomson, Molecular Endocrinology, ICRF) (Figure 3.4). The addition of oestradiol at $10^{-8}M$ resulted in a slightly faster migrating band that is characteristic of oestrogen receptors from breast cancer cell lines as well as those produced *in vitro* or in overexpression systems (see chapters 5 and 6). The addition of the oestrogen receptor specific immune serum MP16 resulted in a band of lower mobility that represents a probe-oestrogen receptor-antibody complex. The addition of pre-immune sera did not induce a shift of the retarded band (see Figure 3.6).

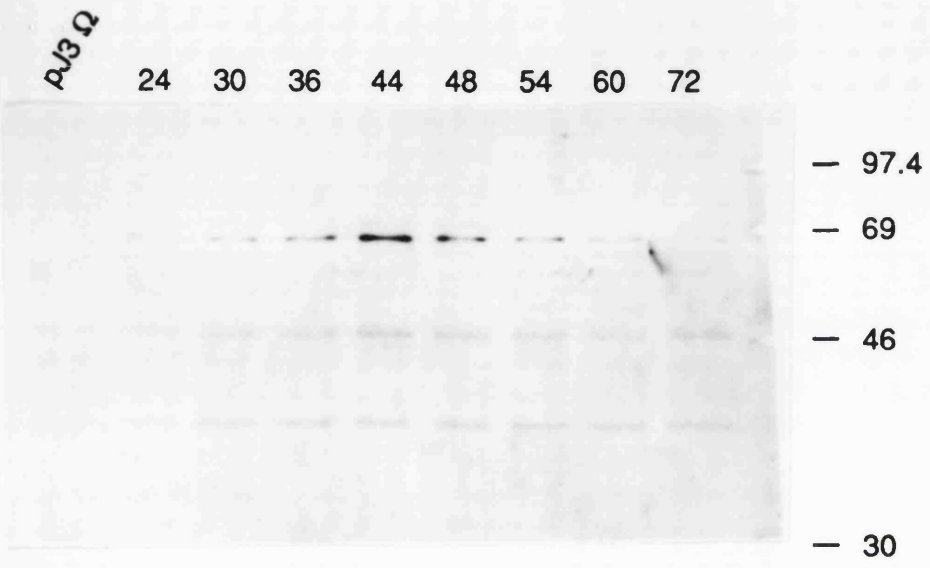


Figure 3.3. Time course of oestrogen receptor expression in transfected COS-1 cells.

COS-1 cells transiently transfected with pJ3MOR were harvested at the times (in hours) indicated after transfection and whole cell extracts prepared. Cells transfected with pJ3Ω (the expression vector lacking the MOR cDNA) were harvested 48 hours after transfection. Equal amounts of the whole cell extracts (2μg of protein) were resolved by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting. The receptor was detected using the oestrogen receptor-specific antiserum MP16 (see methods). The positions of the molecular weight markers of the indicated size (kilodaltons) are marked.

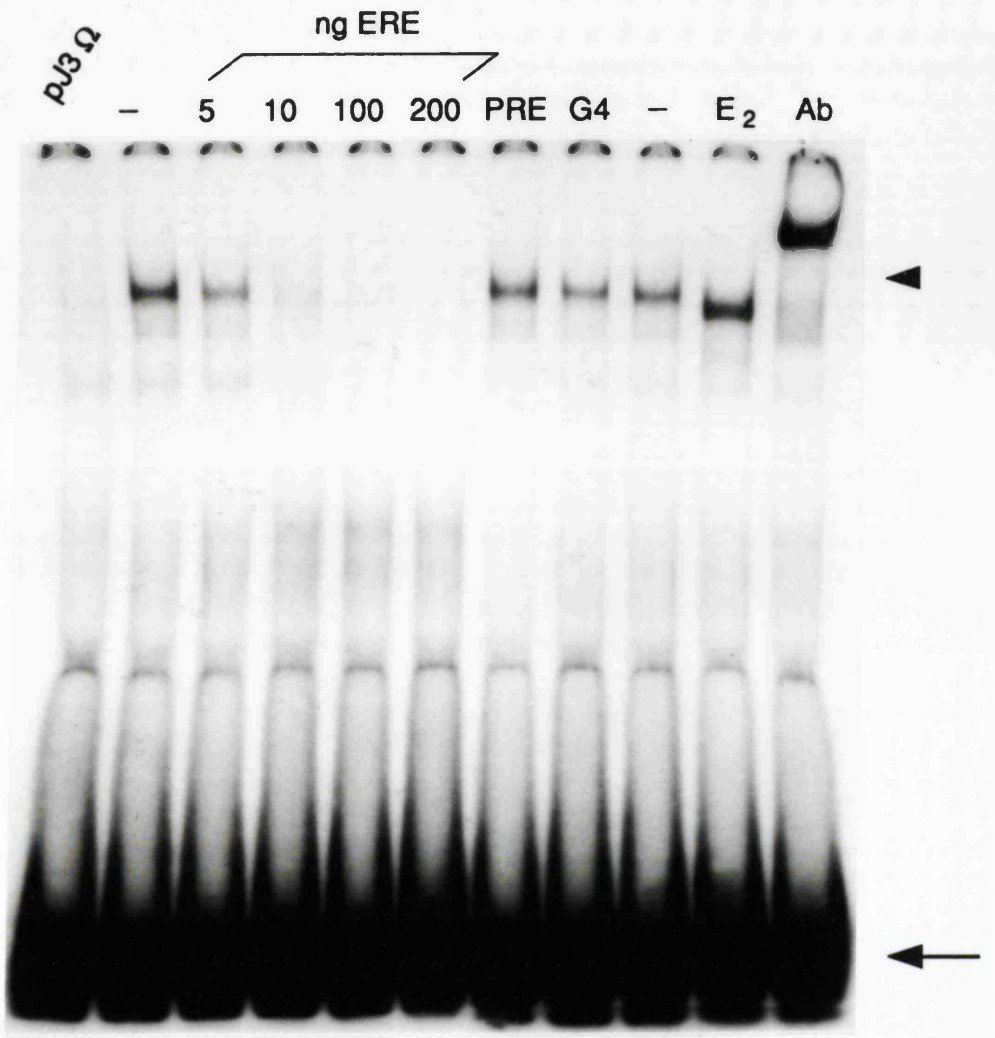


Figure 3.4. DNA binding by COS-1 cell expressed oestrogen receptors.

Equal amounts of whole cell extract (2μg of protein per lane) from COS-1 cells transfected with pJ3MOR or pJ3Ω (first lane only) were tested for the presence of receptor in a band shift assay. The labelled DNA probe was an oligonucleotide containing a consensus ERE (see methods), (-) indicates no addition to the band shift reaction; PRE and G4 indicate the addition of 100ng of unlabelled oligonucleotide containing two progesterone response elements or a GAL4 binding site respectively, the amounts of unlabelled ERE added to the reactions are indicated. E_2 indicates the addition of oestradiol (10^{-8}M) and Ab indicates the addition of 1μl of oestrogen receptor-specific antiserum MP16. The arrow and arrowhead indicate free and retarded probe respectively.

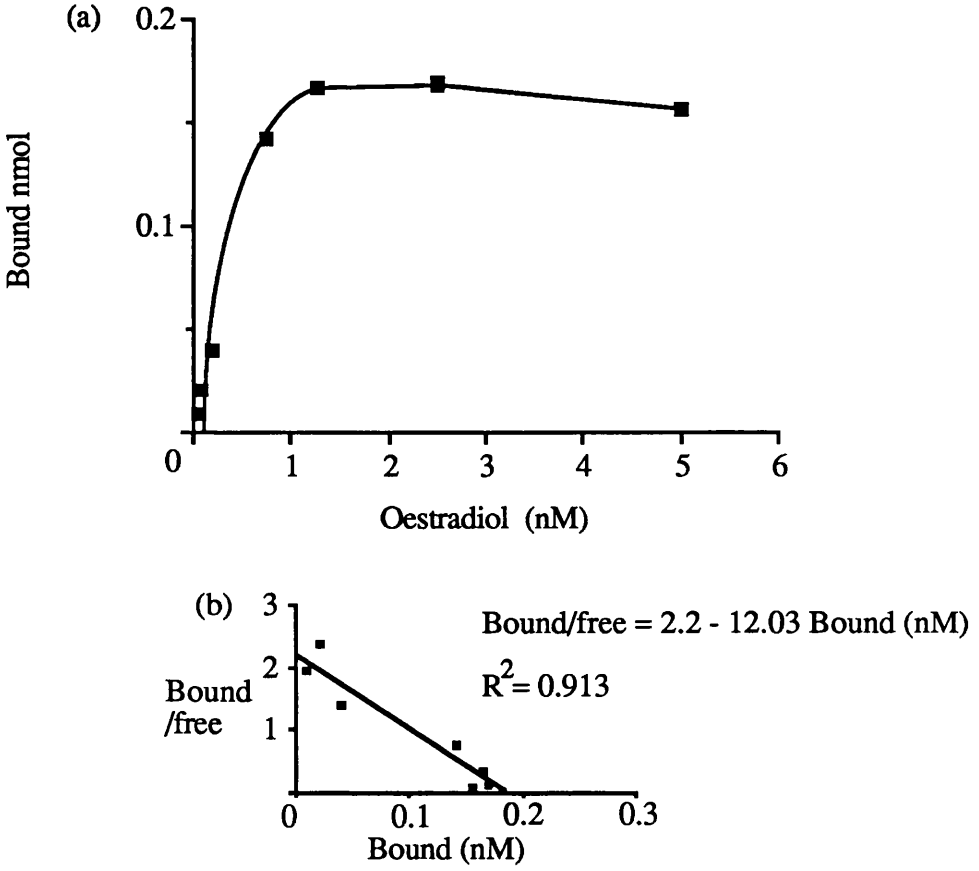


Figure 3.5. Oestradiol binding by the wild-type oestrogen receptor.
Wild-type oestrogen receptors expressed in COS-1 cells were tested for their ability to bind ^{3}H oestradiol over a range of concentrations. Non-specific binding was assessed by the addition of a 500-fold excess of diethylstilbestrol (see methods). A typical binding curve (specific binding) is shown in (a) and the Scatchard analysis of this data in (b).

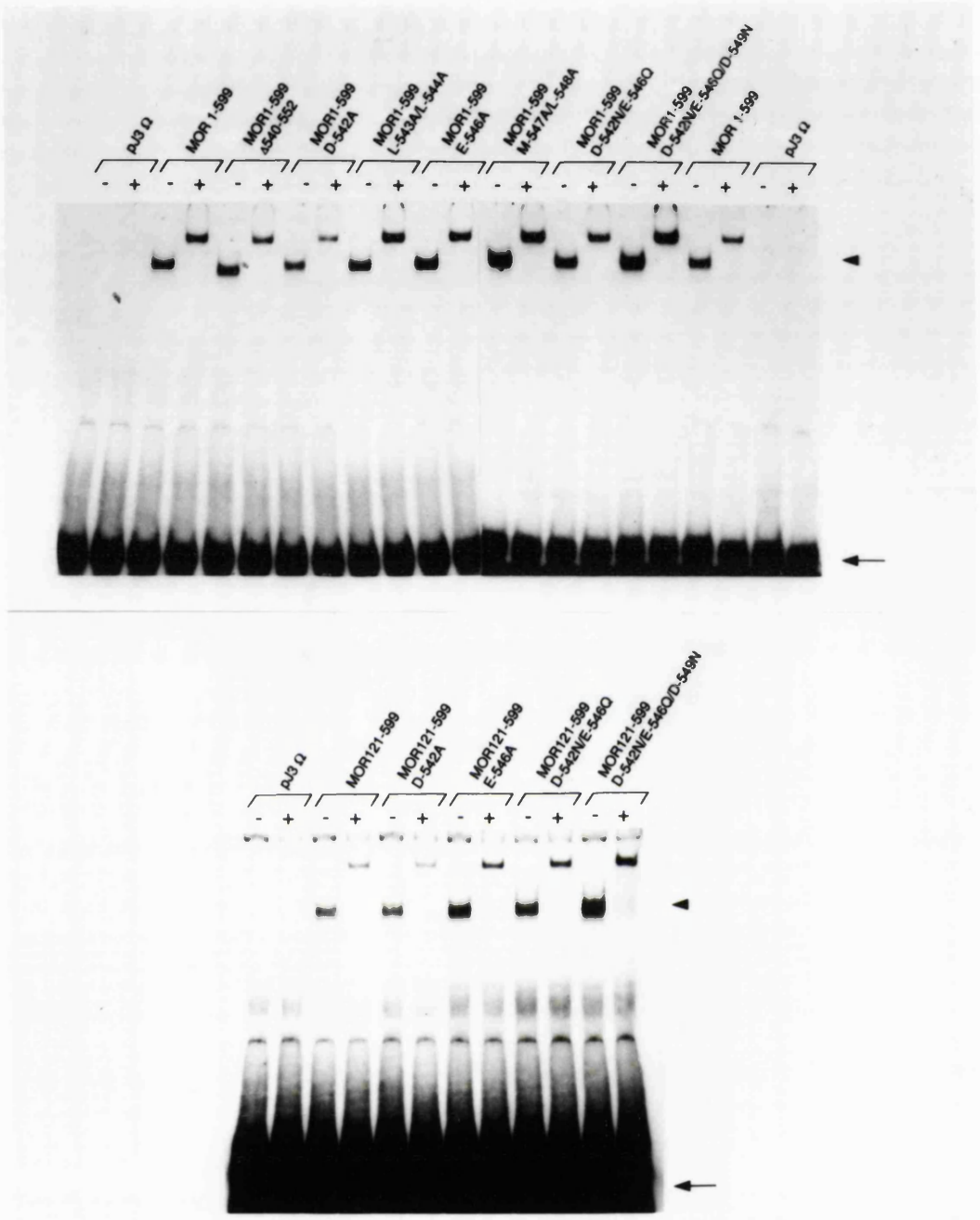


Figure 3.6. DNA binding activity of mutant receptors.
Whole cell extracts (3μg of protein per lane) of COS-1 cells transfected with pJ3Ω or plasmids expressing the indicated receptors were tested for the presence of receptor in a band shift assay. The labelled DNA probe was an oligonucleotide containing an ERE (see methods), (-) indicates the addition of pre-immune sera (1μl) whilst a (+) indicates the addition of 1μl of the oestrogen receptor specific antiserum MP16. The arrow and arrowhead indicate free and retarded probe respectively.

The receptor in COS-1 cells extracts was then tested for its ability to bind oestradiol. The Scatchard analysis, conducted at room temperature, (Figure 3.5) indicated that the receptor bound ^{3}H oestradiol with high affinity having a dissociation constant (Kd) of 0.08 nM, similar to that determined for the human oestrogen receptors from breast cancer cell lines, 0.14 nM (Tora *et al.*, 1989a) or mouse oestrogen receptor synthesised *in vitro* , 0.13 nM (see later). The dissociation constant (Kd) is the concentration of ligand at which binding is half maximal. The Kd is defined from the Scatchard analysis, shown in Figure 3.5 (b), as the reciprocal of the slope of the best fit line. Routinely the concentration of receptors in COS-1 cell extracts was 5-10 pmol/mg protein.

Examination of the mutant receptor proteins in the band shift assay.

To assess if the amounts of functional wild-type and mutant receptor proteins in transfected cells were similar the expression plasmids were transfected into COS-1 cells and extracts from these cells tested for the presence of receptor in a band shift assay. For transfection cells were collected and divided into equal aliquots, using this method the transfection efficiencies, monitored by luciferase activity derived from the internal control plasmid, in any batch were very similar. The protein concentration of each extract was determined and these data used to normalise the amount of extract analysed in the band-shift assay. The results shown in Figure 3.6 indicated that all receptor proteins retained high affinity DNA binding and that alterations in receptor levels were not sufficient to account for the changes in transcriptional activation. The transfections were repeated three times with similar results. The kinetics of DNA binding by the mutant receptors has not been examined but all the mutant receptors retain the part of the protein shown by Fawell *et al.*, (1990a) to be sufficient to bind DNA with nearly wild-type affinity. Similar results were obtained using mutant receptor proteins synthesised *in vitro* (data not shown). These data suggest that the mutant receptor proteins retain the ability to bind DNA with high affinity.

Analysis of the ability of the mutant receptor proteins to bind oestradiol.

As TAF-2 requires the binding of oestradiol for its activity it was essential to determine the affinity of the mutant receptors for oestradiol. The affinities of *in vitro* synthesised wild-type and mutant receptor proteins for 16α-[^{125}I] iodoestradiol was determined by Scatchard analysis. The affinity of the receptor for iodoestradiol is similar to the affinity for [^{3}H] oestradiol (Lippman *et al.*, 1981). The results in Table 3.1 are an average of three independent experiments. The dissociation constants of the mutant receptors were between 0.2 and 0.64 nM therefore at the concentration used in the transient transfection assay, $10^{-8}M$, it would be expected for all the receptor proteins to be saturated with hormone.

Table 3.1 Oestradiol binding by wild-type and mutant receptors.

Receptor	Affinity for Oestradiol Kd (nM)
Wild-type	0.13
D-542A	0.20
L-543A/L-544A	0.28
E-546A	0.20
M-547A/L-548A	0.39
D-549A	0.64
E-546Q/D-549N	0.16
D-542N/E-546Q/D-549N	0.21
Δ 540-552	0.30

The Scatchard analyses shown in Table 3.1 were performed by S. Hoare, Molecular Endocrinology, ICRF. The ability of the wild-type receptor and one of the mutants L-543A/L-544A to bind oestradiol in a whole cell uptake assay was also examined. This assay is performed using intact cells at 37°C and therefore reflects more accurately the conditions in the transient transfection assay (see methods). In this assay transfected COS-1 cells were incubated with [^{3}H] oestradiol (10^{-8}M) with or without cold competitor at 37°C. The specifically retained ligand was expressed /mg protein.

Table 3.2 Oestrogen binding capacity of receptors in the whole cell binding assay.

Transfected Plasmid	Oestradiol binding capacity as a % of wild-type ± s.e.
pJ3MOR	100
pJ3L-543A/L-544A	78.1 ± 11.9
pJ3X	24.5 ± 8.1
pJ3Ω	18.2 ± 3.7

The results shown are from two independent experiments, s.e. indicates standard error. Mutant X will be described in chapter 5. pJ3Ω is the expression vector lacking MOR cDNA.

These results suggest that the wild-type receptor and L-543A/L-544A have a similar capacity to bind oestradiol under transfection assay conditions. In summary these results suggest that mutations in the conserved region do not significantly affect the ability of the receptor to bind DNA and oestradiol.

Mutations in the conserved region affect TAF-2 when attached to a heterologous DNA binding domain.

Previous studies have shown that TAF-2 can act as an independent activation domain when attached to a heterologous DNA binding domain (Webster *et al.*, 1988b; Lees *et al.*, 1989). To assess if the mutations of the conserved amino acids affected TAF-2 when analysed as an independent activation domain the hormone binding domain of the oetrsogen receptor was joined to the DNA binding domain of the yeast, *Saccharomyces cerevisiae*, transcription factor GAL4. To accomplish this the cDNA encoding the hormone binding domain between codons 313 and 599 was amplified using the polymerase chain reaction in order to add restriction enzyme sites at the 5' and 3' ends (see Appendix A6). This fragment was subcloned into the GAL4 1-147 expression vector pSG424 (Sadowski and Ptashne, 1989) to generate GAL4-ER 313-599. GAL4 1-147 is able to dimerise and bind DNA with high affinity (Carey *et al.*, 1989) but fails to stimulate transcription significantly in transfection assays (Kakidani and Ptashne, 1988).

The chimeric receptors were tested in a transient transfection assay for their ability to stimulate transcription of the CAT gene in the reporter pG5E1BCAT. pG5E1BCAT contains five GAL4 binding sites upstream of the E1b TATA box joined to the CAT gene (Lillie and Green, 1989). NIH 3T3 cells were transfected as described with 2 μg of pG5E1BCAT, 1 μg of pJ3 luciferase, 1 μg of pJ3Ω, pSG424 or GAL4-ER expression vector and 6 μg of pJ3Ω per 6 cm dish. In this assay the wild-type chimera (GAL4-ER) stimulated transcription over 1000-fold (represented as 100%) in the presence of oestradiol indicating that TAF-2 could function when attached to a heterologous DNA binding domain (Table 3.3).

The activity in the absence of added oestradiol is probably due to the binding of residual oestradiol in the charcoal stripped serum since this activity was practically abolished in the presence of 10^{-8}M 4-hydroxytamoxifen (data not shown). The antioestrogen 4-hydroxytamoxifen has been shown to bind to the receptor with high affinity, promote DNA binding of the receptor but prevent the formation of TAF-2 (Webster *et al.*, 1988b; Lees *et al.*, 1989; Berry *et al.*, 1990). The mutant GAL4-ER M-547A/L-548A showed negligible transcriptional activation in the presence of oestradiol confirming the importance of the hydrophobic residues for TAF-2 activity. GAL4-ER E-546A was slightly more active than the wild-type chimera in this assay.

Table 3.3 Transcriptional activation by GAL4-ER chimeras

	Transcriptional activation % wild-type ± s.e.	
	-E_2	+E_2
— (pJ3Ω)	<1	<1
GAL4 (1-147)	<1	<1
GAL4-ER	1.8 ± 0.14	100
GAL4-ER M-547A/L-548A	<1	0.2 ± 0.025
GAL4-ER E-546A	<1	160 ± 17

The ability of the chimeric proteins to stimulate transcription from pG5E1BCAT was tested in a transient transfection assay as described in the text. pJ3Ω is a expression vector lacking receptor or GAL4 cDNA. The results are presented as a percentage of activity the wild-type chimera in the presence of 10^{-8}M oestradiol (E_2) and show the average of three independent experiments each carried out in duplicate, s.e. indicates standard error.

The wild-type and mutant chimeras were found to be expressed at similar levels in transfected COS-1 cells as judged by band shift assay (data not shown). Analysis of the receptor proteins in transfected COS-1 cells using indirect immunofluorescence (using H222) indicated that the chimeras were present at similar levels and distributed throughout the cell in the absence of oestradiol but predominantly nuclear in the presence of oestradiol or 4-hydroxytamoxifen at 10^{-8}M. This immunofluorescence was performed by S. Dauvois, Molecular Endocrinology, ICRF.

Analysis of the corresponding glucocorticoid receptor mutants.

To examine if the function of these residues was conserved in another receptor the corresponding mutations were made in the glucocorticoid receptor. The mutations were introduced into the mouse glucocorticoid receptor cDNA (Danielsen *et al.*, 1986) by replacing part of the cDNA with pairs of double stranded oligonucleotides (described in Appendix A7).

The wild-type and mutant receptors generated were analysed for their ability to stimulate transcription from MMTV-CAT which contained the mouse mammary tumour virus long terminal repeat sequences from position -1225 to +268 (Parker *et al.*, 1987). The MMTV LTR contains several potential glucocorticoid response elements and is responsive to dexamethasone in transfected cells (see chapter 1).

These transfections were similar to those described for the oestrogen receptor apart from substituting the reporter pERE BLCAT with MMTV-CAT. The glucocoricoid receptor expression plasmid was titrated and 4 μg/6 cm dish was found to be the amount giving half maximal activation. This amount of wild-type and mutant receptor was therefore used.

In this assay the wild-type receptor stimulated transcription 25-fold relative to reporter alone in the presence of 10^{-6}M dexamethasone, Figure 3.7(a). Replacement of either of the pairs of conserved hydrophobic residues (see Figure 3.1) with alanine residues in M-758A/L-759A and I-762A/I-763A reduced transcriptional activation to less that 2-fold. Replacement of the highly conserved glutamic acid residue with alanine however (E-761A) had little affect on transcriptional activation reducing it to around 15-fold. To examine TAF-2 in the glucocorticoid receptor in the absence of the N-terminus a cDNA was constructed that contained the DNA binding domain of GAL4 (encoding amino acids 1-147) joined to the hormone binding domain of the mouse glucocorticoid receptor (from amino acid 506 to 783) see Appendix A8.

Analysis of GAL4-glucocorticoid receptor chimeras.

The chimeric receptors were tested for their ability to stimulate transcription from pG5E1BCAT in a transient transfection assay. Transfections experiments were performed using NIH 3T3 cells and 2 μg of pG5E1BCAT, 1.2 μg of GAL4-GR expression vector, 1 μg of pJ3 luciferase and 5.8 μg of pJ3Ω per 6 cm dish. In these experiments the wild-type chimera stimulated transcription more than 1000-fold in the presence of 1 μM dexamethasone, Figure 3.7 (b). The mutants GAL4-GR M-758A/L-759A and I-762A/I-763A had negligible transcriptional activity (less than 1% that of the wild-type receptor) confirming the results with the full-length receptor. The replacement of the highly conserved glutamic acid residue with an alanine residue in GAL4-GR E-761A reduced the transcriptional activation to 15-20% that of the wild-type receptor. Similar results were obtained using COS-1 cells (not shown).

Mutation of the conserved amino acids appear not to affect other glucocorticoid receptor functions.

To assess if mutations of the conserved residues affected other receptor functions a number of control experiments were performed. The expression of full-length receptor protein in transfected COS-1 cells was assessed by indirect immunofluorescence using the monoclonal antibody 250 that was raised against the rat glucocorticoid receptor and binds an epitope in the N-terminus (provided by S. Okret and J. A.-Gustafsson; Okret *et al.*, 1984). The results indicated that the wild-type and mutant receptor proteins were expressed at low, but similar levels and detected only in the nucleus in the presence of dexamethasone (data not shown).

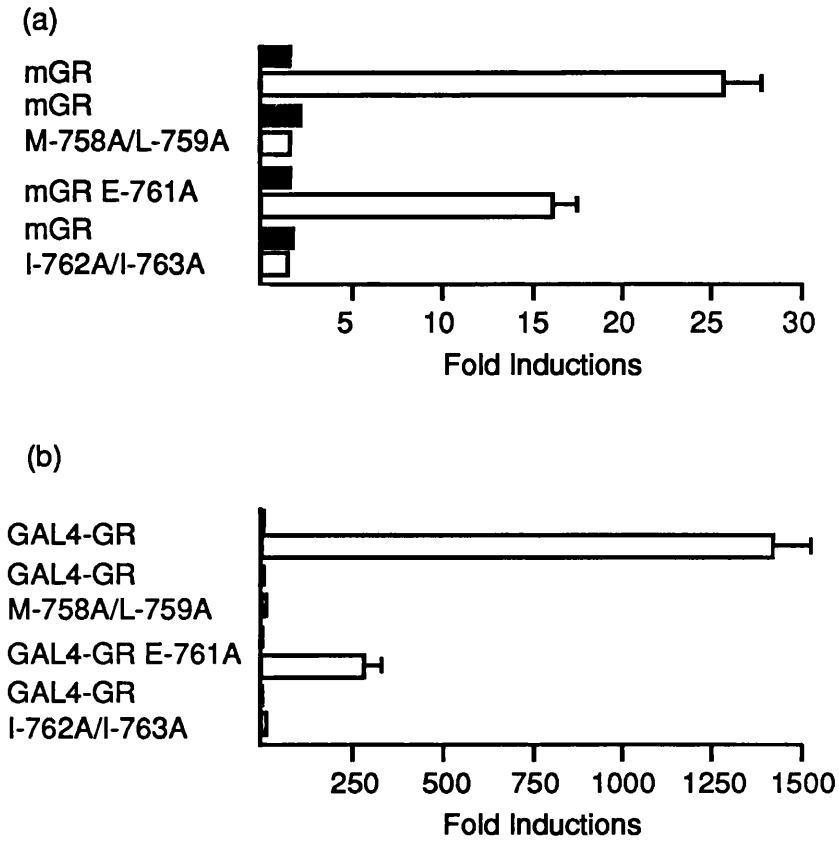


Figure 3.7. Transcriptional activation by wild-type and mutant mouse glucocorticoid receptors.

(a) The ability of the wild-type and mutant glucocorticoid receptors to stimulate transcription from MMTV-CAT was tested in transient transfection experiments. The transcriptional activation is expressed as induction over the reporter alone in the absence (solid bars) or presence (open bars) of 10^{-6}M dexamethasone.

(b) Chimeric GAL4-GR proteins were tested in transfection experiments for their ability to stimulate transcription from pG5E1BCAT as described in (a).

The error bars (that are not always apparent) represent standard errors derived from three to four experiments each carried out in duplicate.

Other attempts to detect the wild-type receptor (either expressed in COS-1 cells or synthesised *in vitro*) by ligand binding, Western blotting and band-shift assay (using oligonucleotides containing GREs or fragments from the MMTV-LTR) were unsuccessful. However, using whole cell binding assays, it was possible to assess the hormone binding capacity of the GAL4-GR chimeras (Table 3.4).

The binding capacity of the mutant E-761A was similar to that of the wild-type receptor whilst that of M-758A/L-759A and I-762A/I-763A were 25-40% that of the wild-type receptor. This assay was carried out at 10^{-7}M dexamethasone, so at 10^{-6}M, the concentration used in the transfection experiments, all the receptor proteins would be expected to be saturated with ligand.

Table 3.4 Dexamethasone binding capacity of chimeric receptors.

Transfected Plasmid	Dexamethasone binding fmol/mg at 10^{-7}M ± s.e.
pGAL4-GR	3806 ± 260
pGAL4-GR M-758A/L-759A	1021 ± 7
pGAL4-GR E-761A	3992 ± 645
pGAL4-GR I-762A/I-763A	1568 ± 360
pSG 424 (GAL4 1-147)	145 ± 22

The results show the average of four independent experiments presented as fmol/mg protein ± standard error.

The GAL4-GR chimeric proteins were not detected in any other assay. This was despite the fact that the GAL4-ER fusions described bound to a GAL4 binding site in a band shift assay (data not shown). To assess if the chimeras containing mutations in the hydrophobic residues were located in the cell nucleus and binding to the GAL4-responsive element in the reporter plasmid (in the presence of dexamethasone) an *in vivo* interference assay was used. In this assay COS-1 cells were transfected with 1 μg of pG5E1BCAT and 1 μg of pJ3 luciferase, 11 μg of pJ3Ω (reporter alone) or 1 μg of GAL4-GR plus a 10-fold excess (10 μg) of one of the following pJ3Ω, pGAL4-GR M-758A/L-759A, pGAL4-GR I-762A/I-763A or pSG424 (GAL4 1-147). The results shown in Figure 3.8 indicate that the GAL4 DNA binding domain and the mutant chimeras were able to interfere with the ability of the wild-type chimera to stimulate transcription from pG5E1BCAT in transfected cells.

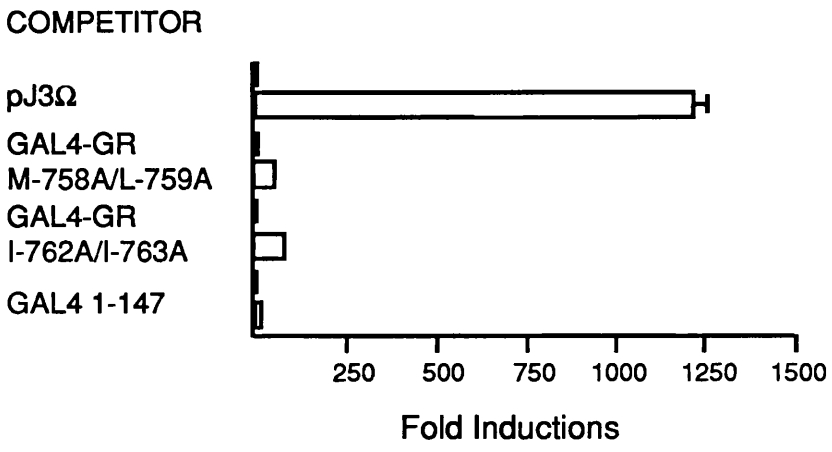


Figure 3.8. ***In vivo*** **interference assay.**

The ability of the transcriptionally inactive GAL4-GR mutants to interfere with the stimulation of transcription by the wild-type chimera was examined by cotransfection of a 10-fold excess of the competitor indicated, pJ3Ω is a control vector. The transcriptional activation is shown as fold inductions over reporter, pG5E1BCAT, alone in the absence (solid bars) or presence (open bars) of 10^{-6}M dexamethasone. The error bars (that are not always apparent) represent the standard errors determined from three independent experiments, each carried out in duplicate.

Cotransfection with GAL4-GR M-758A/L-759A , GAL4-GR I-762A/I-763A or GAL4 1-147 reduced the transcriptional activation by the wild-type chimera by 95%, 93% and 99% respectively. The mutant chimeras therefore appear to interfere with the ability of the wild-type chimera to stimulate transcription suggesting that they retain the ability to bind DNA and compete for the GAL4 binding sites in the reporter plasmid. These results indicated that the conserved residues in the glucocorticoid receptor were also important hormone dependent transcriptional activation.

Analysis of the cooperation between the N-and C-terminus of the mouse oestrogen receptor in stimulating gene transcription.

The results described earlier showed that three mutants of the mouse oestrogen receptor E-546A, E-546Q/D-549N and D-542N/E-546Q/D-549N stimulated transcription more efficiently in the presence of the N-terminus, Figure 3.2. Since the deletion of the N-terminus has not been indicated to affect the ability of the wild-type receptor to bind DNA or oestradiol significantly (Kumar *et al.*, 1986; Lees *et al.*, 1989; Fawell *et al.*, 1990a) it is possible that in these cases the N-terminus may cooperate with the C-terminus in the full length receptor to stimulate transcription. This was proposed because previous studies had indicated that the activity of TAF-1 was low in this system (Lees *et al.*, 1989). To assess the extent of the cooperation the activity of the N-terminal TAF-1 was examined.

Assessment of transcriptional activation by TAF-1.

Initially a deletion mutant MOR 1-339 containing TAF-1 was tested in the transient transfection assay. The results indicated that TAF-1 stimulated transcription only 3-5 fold, Figure 3.9. Whilst MOR 121-599 and wild-type receptor bound DNA with similar affinities (Fawell *et al.*, 1990a) MOR 1-339 had a lower affinity and maximal DNA binding activity was only observed in the presence of a dimerising antibody, MP16 (Fawell *et al.*, 1990b), data not shown. For these reasons the activity of TAF-1 was also assessed by examining the activity of the wild-type receptor in the presence of 10^{-6}M tamoxifen. In this assay the level of transcription was also only 3-5 fold. The antioestrogens tamoxifen and 4-hydroxytamoxifen are thought to promote DNA binding by the receptor but fail to activate TAF-2 (see chapter 6). Deletion of the N-terminus (MOR 121-599) virtually abolishes this activity suggesting that it is derived from TAF-1 (Figure 3.9, Berry *et al.*, 1990).

These results suggest that TAF-1 is only able to stimulate transcription 3-5 fold. The mutant D-542N/E-546Q/D-549N in MOR 121-599 in the presence of oestradiol also stimulated transcription poorly ~3-fold above the basal level. D-542N/E-546Q/D-549N in MOR 1-599, however, stimulated transcription to nearly wild-type levels (40-fold), this may be due to cooperation between the N-and

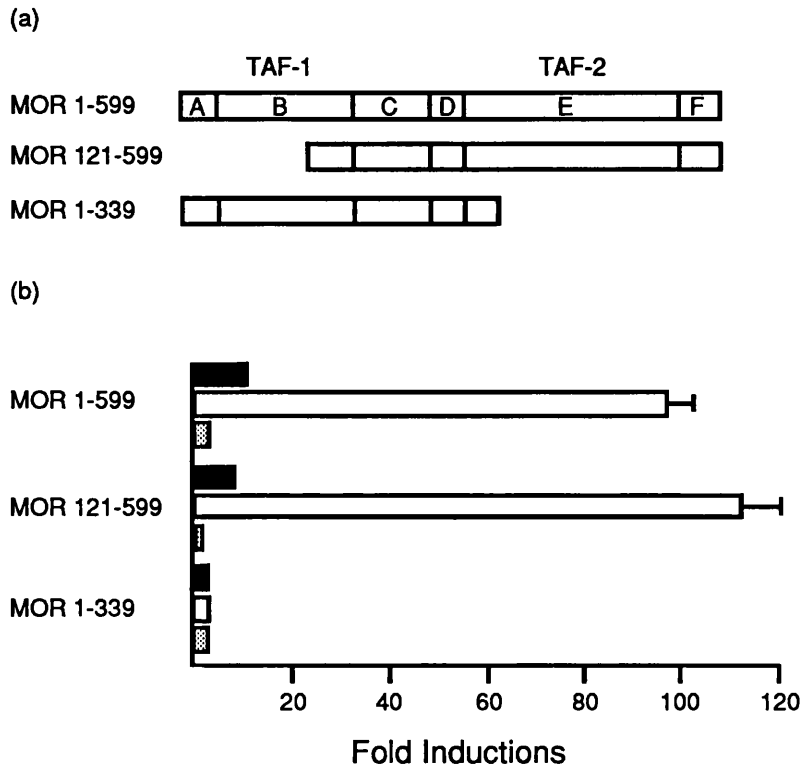


Figure 3.9. Analysis of TAF-1 activity in NIH 3T3 cells.

(a) Schematic organisation of the MOR deletion mutants. The positions of TAF-1 and TAF-2 are indicated and the regions donated A to F shown (Krust *et al.*, 1986).

(b) The mutants shown were tested for their ability to stimulate transcription from pERE BLCAT in the absence of ligand (closed bars) or in the presence of 10^{-8}M oestradiol (open bars) or 10^{-6}M tamoxifen (shaded bars). The error bars represent the standard errors determined from at least three independent experiments each performed in duplicate.

C-terminal domains in the full-length receptor, Figure 3.2. Similar but less marked effects were seen with E-546A and E-546Q/D-549N. In contrast deletion of the N-terminus from the wild-type receptor had little affect on its ability to stimulate transcription in this assay. Compare MOR 1-599 and MOR 121-599 in Figure 3.2.

The cooperation observed with certain of the mutant receptors, however, may be a result of the receptor not folding correctly in the absence of the N-terminus. To try to address this possibility the mutants were tested in a situation where cooperation between the N-and C-terminal domains was necessary for the wild-type receptor to achieve maximal transcriptional activation. This may determine if cooperation is occurring in these mutant receptors. Studies by Tora *et al.*, (1989b) indicated that cooperation between the N-and C-terminal domains in stimulating transcription could occur on some promoters. Preliminary experiments suggested that pERE MLTCAT was suitable for this study.

Cooperation in the wild-type receptor.

The wild type and mutant receptors were therefore tested in transient transfection assays identical to those described earlier apart from the replacement of pERE BLCAT with pERE MLTCAT. pERE MLTCAT contains the same ERE as pERE BLCAT cloned upstream of a "minimal" promoter comprised of the adenovirus major late promoter sequences from position -44 to +11 (Hu and Manley, 1981) that contain a TATA box only. This DNA sequence was linked directly to the CAT gene.

The results of these studies are shown in Figure 3.10. The wild-type receptor in the presence of 10^{-8}M oestradiol stimulated transcription nearly 300-fold in this assay, this is represented as 100%. On this promoter TAF-1 (MOR 1-339) stimulated transcription to approximately 2% that of the wild-type receptor and TAF-2 (MOR 121-599) stimulated transcription to approximately 17% the level of the wild-type receptor in the presence of oestradiol. These results suggest that in the full-length receptor the N-terminus (containing TAF-1) and the C-terminus (containing TAF-2) can cooperate to stimulate transcription. No receptor or oestradiol dependent transcriptional activation was detected in experiments using pMLTCAT, that lacks an ERE (data not shown).

The mutant E-546A in MOR 121-599 stimulated transcription to 7% the level of the wild-type receptor whilst in MOR 1-599 E-546A stimulated transcription to nearly wild-type levels (40% that of the wild-type receptor) indicating that in this mutant the N-terminal domain (that by itself stimulated transcription to only 2% that of the wild-type receptor) may cooperate with the C-terminal domain to stimulate transcription. The mutant D-542N/E-546Q/D-549N stimulated transcription to 1% in MOR 121-599 and 9% that of the wild-type receptor in MOR 1-599 also indicating that some cooperation was occurring.

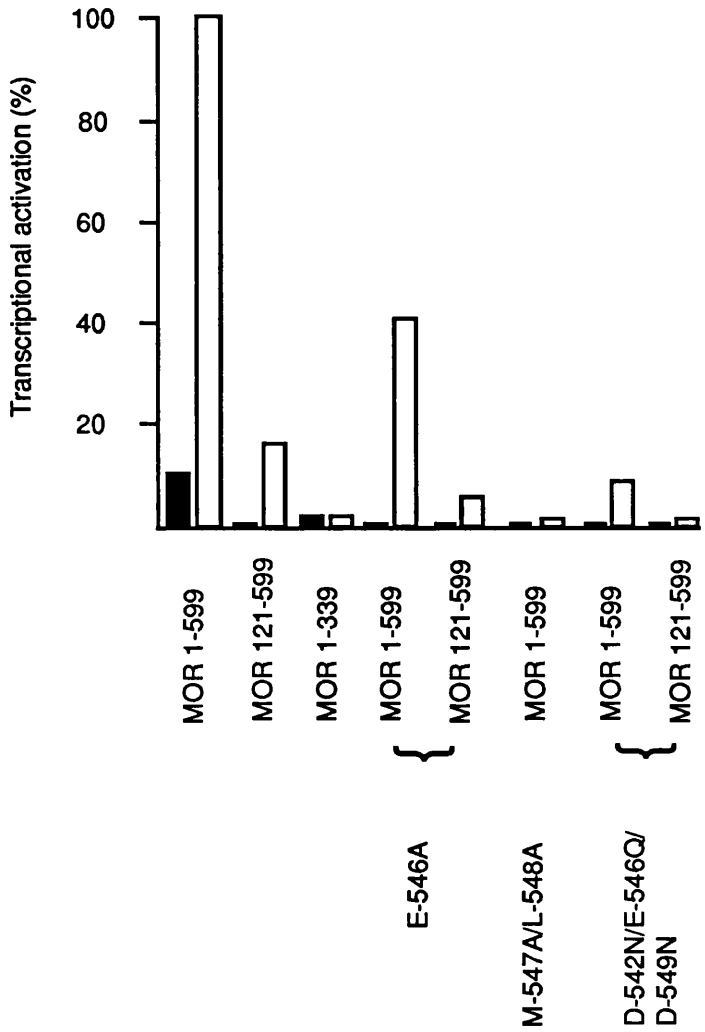


Figure 3.10. Cooperation between the N-and C-terminal domains in transcriptional activation.

The wild-type and mutant receptors were tested for their ability to stimulate transcription from pERE MLTCAT in the absence (solid bars) and presence (open bars) of 10^{-8}M oestradiol. The transcriptional activation is shown as a percentage of the level of transcription stimulated by the wild-type receptor in the presence of oestradiol. The results shown were derived from two independent experiments each carried out in duplicate. The levels of transcription stimulated in each case in these experiments varied less than 15%.

Therefore mutations of the acidic residues appear not to abolish cooperation between the N-and C-terminal domains in stimulating transcription but do affect TAF-2 activity. In contrast the mutant M-547A/L-548A showed negligible transcriptional activation even in MOR 1-599. Mutations that abolish TAF-2 therefore affect the cooperation between the N-and C-terminus suggesting that the cooperating element in the C-terminus may be part of TAF-2. The acidic amino acids appear to be important for TAF-2 in the absence of the N-terminus but less important than the hydrophobic amino acids for cooperation between the N-and C-termini in the full-length receptor.

Mapping the region of the N-terminus important for cooperation.

To try to identify the region of the N-terminus with which the C-terminus cooperates the mutations in D-542N/E-546Q/D-549N were introduced into a series of N-terminal deletion mutants and these mutants analysed for their ability to stimulate transcription of the CAT gene in the reporter plasmid pERE BLCAT, where the greatest cooperation was observed. The N-terminus of the oestrogen receptor has been divided into two regions A and B based on the homology between the sequences of the chicken and human oestrogen receptors, Figure 3.9 (Krust *et al.*, 1986). In region A between the human, chicken, mouse and rat oestrogen receptors 78% of the amino acids are conserved whilst the homology in region B is only 44%. As region A is well conserved a receptor from which region A had been deleted, MOR 38-599 was constructed (see Appendix A9). The deletion mutants containing mutations in TAF-2 were generated by subcloning (Appendix A9). Deletions of 37, 90 or 120 amino acids from the wild-type receptor did not reduce the level of transcriptional stimulation significantly when using pERE BLCAT (Lees *et al.*, 1989; Figure 3.2, data not shown).

Analysis of the transcriptional activation of the mutant D-542N/E-546Q/D-549N in the deletion series (Figure 3.11) indicated that removal of region A did not reduce the transcriptional activation relative to the full-length mutant, and that deletion to amino acid 91 also had little affect (especially considering that this mutant was detected at lower levels in the band shift assay than the full-length mutant, Figure 3.12). These results indicated that residues between amino acid 91 and 121 contain the N-terminal boundary of a region important for cooperation with the C-terminus. The relative levels of these proteins expressed in transfected COS-1 cells was assessed by band-shift analysis (Figure 3.12). The mutant MORs 38-599 and 91-599 (containing D-542N/E-546Q/D-549N) were detected at lower levels than the other receptor proteins suggesting that in NIH 3T3 cells they may bind to the ERE in the reporter plasmid with lower efficiency. This may explain the lower level of transcription stimulated by MOR 91-599, Figure 3.11.

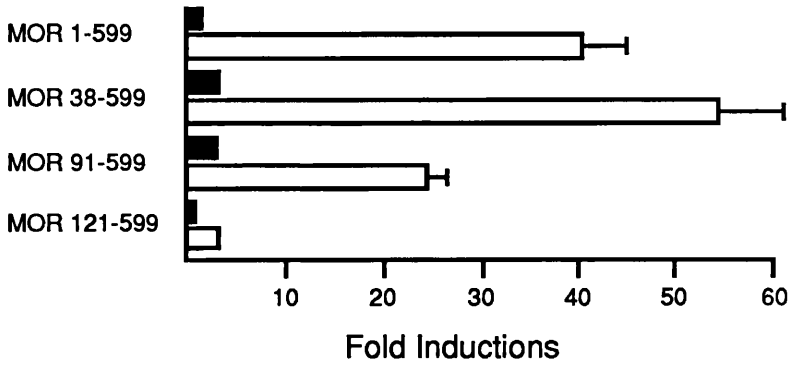


Figure 3.11. Mapping the region in the N-terminus important for cooperation. Deletion mutants of D-542N/E-546Q/D-549N were tested for their ability to stimulate transcription from pERE BLCAT in a transient transfection assay in the absence (closed bars) or presence (open bars) of 10^{-8}M oestradiol. The error bars represent standard errors from at least three experiments each carried out in duplicate.

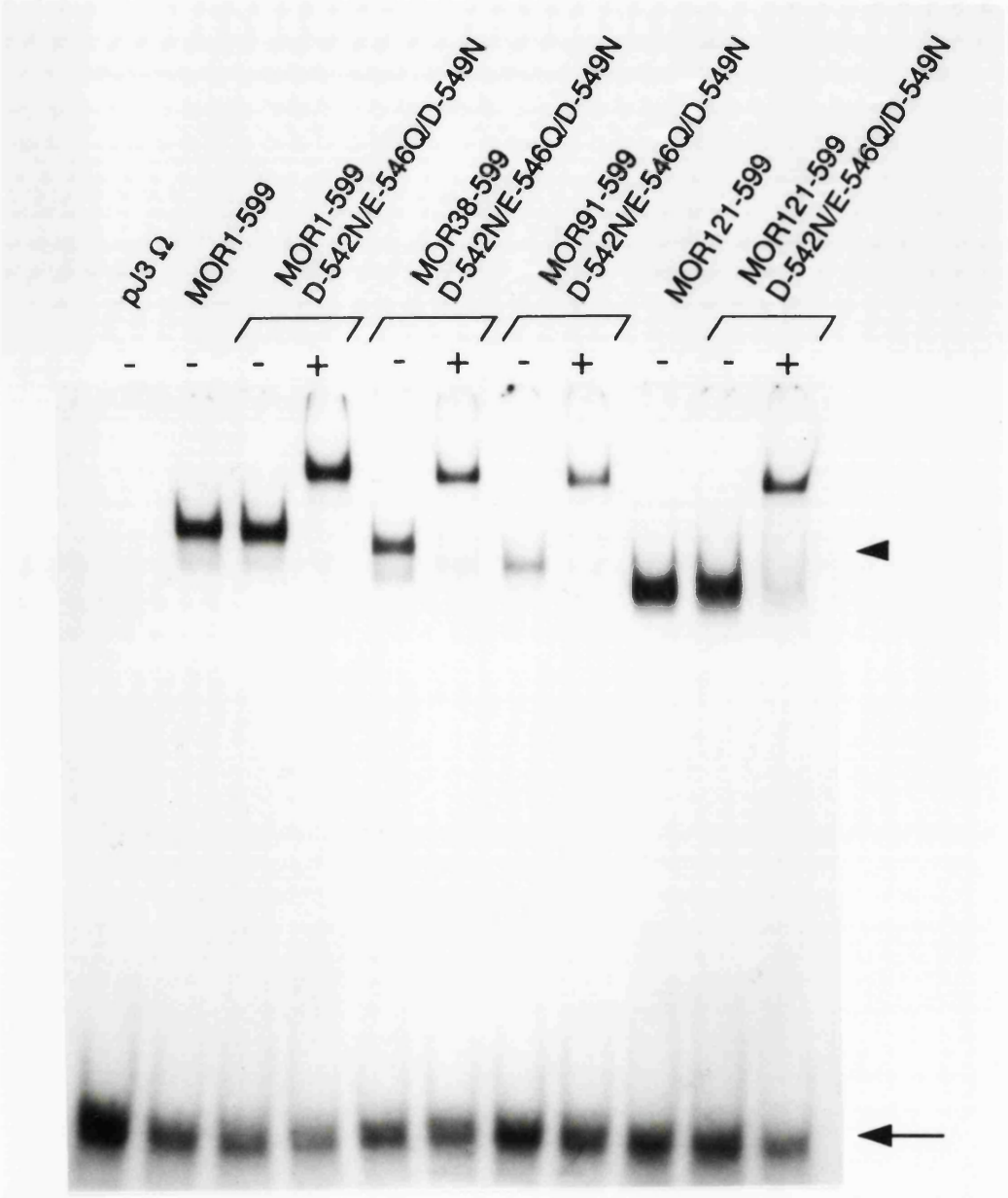


Figure 3.12. DNA binding by N-terminal deletion mutants.
Whole cell extracts (2μg of protein per lane) of COS-1 cells transfected with pJ3Ω or plasmids expressing the indicated receptors were tested for the presence of receptor in a band shift assay. The labelled DNA probe was an oligonucleotide containing an ERE (see methods). (-) indicates the addition of pre-immune sera (1μl) whilst a (+) indicates the addition of 1μl of the oestrogen receptor-specific antiserum MP16. The arrow and arrowhead indicate free and retarded probe respectively.

The deletion mutants were not assessed for hormone binding since deletions N-terminal to the DNA binding domain appear not to affect high affinity hormone binding (Kumar *et al.*, 1986; Fawell *et al.*, 1990a). The wild-type receptor and the deletion mutants MOR 38-599, MOR 91-599 and MOR 121-599 (without mutations in the conserved region) were also assayed for their ability to stimulate transcription from pERE MLTCAT. As described earlier the N-and C-terminus cooperate in the wild-type receptor to stimulate transcription on this promoter (Figure 3.10). The results also showed that the deletion of 120 amino acids reduced the cooperation between the N-and C-terminus to a much greater extent than deletions to residue 38 or 91 (data not shown). These data suggest that the cooperation in mutants in which the acidic amino acids had been replaced may be by a similar mechanism to cooperation in the receptor where the conserved residues are unchanged. These deletion mutants were also tested for their ability to stimulate transcription in chicken embryo fibroblast (CEF) cells in the presence of 4-hydroxytamoxifen. TAF-1 stimulates transcription efficiently in CEF cells and the transcriptional activation observed in the presence of 4-hydroxytamoxifen is thought to be derived from TAF-1 (Berry *et al.*, 1990). These experiments were performed to assess if the deletions that affected cooperation also affected TAF-1 activity. These results suggested that the majority of TAF-1 activity was lost when 120 but not 90 amino acids were deleted. This may indicate that TAF-1 cooperates with TAF-2 in NIH 3T3 cells but since the activity of TAF-1 in CEF cells is cell specific this may not be the case. These CEF cell transfections were performed by S. Hoare, Molecular Endocirnology, ICRF.

To examine the requirements for cooperation further an experiment was performed to assess if a C-terminus could cooperate with the N-terminus of its partner in a receptor dimer. This was examined by transfecting 0.5 μg of the plasmid containing D-542N/E-546Q/D-549N in MOR 121-599 (that stimulates transcription ~3-fold) with an equal amount of L-543A/L-544A in MOR 1-599 or, as a negative control, L-543A/L-544A in MOR 121-599 (both these receptors also stimulate transcription 2 to 4-fold). In a heterodimer between these receptors the N-terminus of the hydrophobic mutant may be able to cooperate with the C-terminus in which the three acidic residues had been replaced. However, no significant cooperation was observed (Figure 3.13). In band shift experiments using extracts from COS-1 cells transfected with equal amounts of plasmids expressing D-542N/E-546Q/D-549N in MOR 121-599 and L-543A/L-544A in MOR 1-599 a retarded band was observed between the bands that migrated at the positions of MOR 1-599 and MOR 121-599 (data not shown). This band of intermediate mobility is indicative of a heterodimer suggesting that the absence of cooperation was probably not due to the lack of heterodimer formation (see chapter 6).

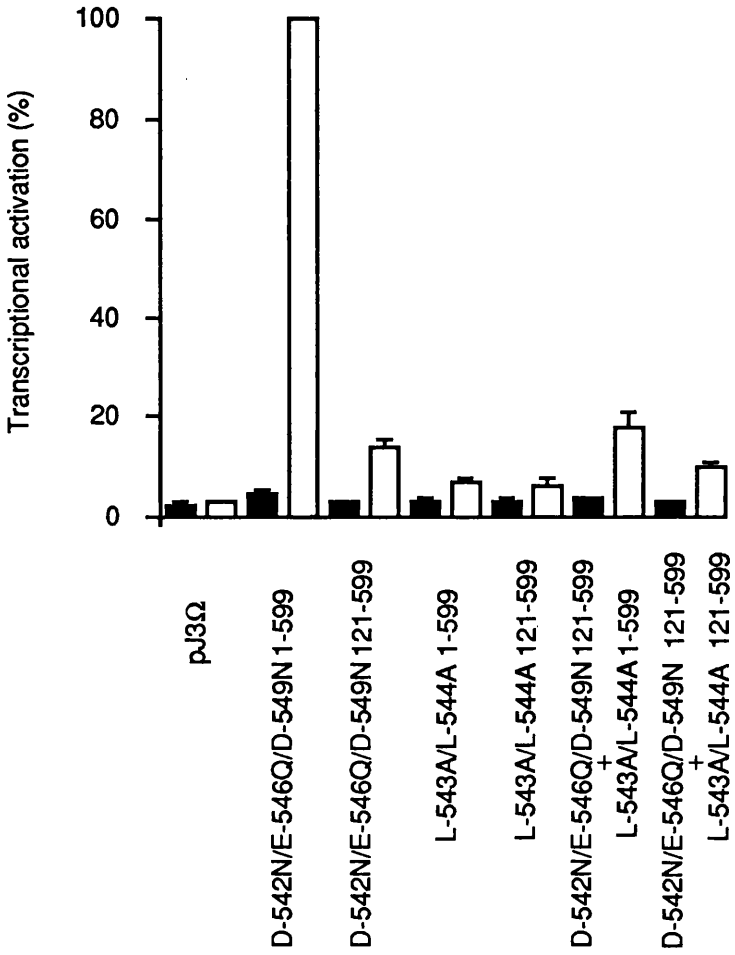


Figure 3.13. Analysis of the requirements for cooperation between the N-and C-terminal activation domains.

The ability of the N-terminal domain of a receptor to cooperate with the C-terminus of another receptor was assessed by cotransfecting D-542N/E-546Q/D-549N 121-599 with a full-length receptor that lacked TAF-2 (L-543A/L-544A). Cells were maintained in medium in the absence of ligand (closed bars) or in the presence of 10^{-8}M oestradiol (open bars). Transcriptional activation is shown as a percentage of the level stimulated by the D-542N/E-546Q/D-549N (1-599) in the presence of 10^{-8}M oestradiol. The error bars (that are not apparent in some cases) represent standard errors derived from two independent experiments each carried out in duplicate.

In another experiment based on a similar strategy MOR 121-599 was cotransfected with L-543A/L-544A in MOR 1-599 using pERE MLTCAT as the reporter plasmid. In this situation MOR 121-599 stimulated transcription to approximately 17% the level of the wild-type receptor whilst L-543A/L-544A in MOR 1-599 had negligible activity (see Figure 3.10). Cotransfection of MOR 121-599 and L-543A/L-544A in MOR 1-599 did not result in a significant increase (or decrease) in transcriptional activation relative to MOR 121-599 alone (data not shown). One explanation for these results is that the presence of only one TAF-1 or TAF-2 in a dimer is not sufficient for cooperation.

Conclusions.

The functional analysis of mutant receptors suggested that amino acids in a region conserved in many members of the nuclear receptor family are important for the ability of the mouse oestrogen and glucocorticoid receptors to stimulate transcription. The mutation of these amino acids did not significantly affect other receptor functions suggesting that they are specifically involved in transcriptional activation. Since these amino acids are conserved it is possible that they are important for transcriptional activation by other members of the nuclear receptor family.

In the oestrogen receptor the mutation of certain conserved acidic residues affected TAF-2 activity but did not abolish the ability of TAF-1 and TAF-2 to cooperate in the full-length receptor. However, no cooperation or TAF-2 activity was detected when the conserved hydrophobic residues were mutated suggesting that these amino acids are important for both these functions. The N-terminal boundary of the region that was important for this cooperation was mapped to between amino acids 91 and 121 in the N-terminus of the receptor.

Chapter 4

A yeast genetic screen for proteins that interact with TAF-2 of the mouse oestrogen receptor.

Introduction.

The oestrogen receptor like other proteins that stimulate gene transcription may make contacts with protein target(s) that mediate this process (for reviews see Ptashne and Gann, 1990; Roeder, 1991; Pugh and Tjian, 1992). To understand the mechanism whereby TAF-2 stimulates transcription the target(s) of TAF-2 need to be identified. This chapter describes the use of a genetic screen in yeast to try to isolate cDNA clones for proteins that contact TAF-2.

Choice of strategy.

Several approaches that could be used to isolate candidate targets for TAF-2, some of these are reviewed here and the reasons for choosing the yeast screen are indicated. One approach, that is possible for some transcription factors, is to purify protein fractions and eventually polypeptides from cell nuclear extracts that support transcription factor mediated transcriptional activation *in vitro*. However, this is currently not possible as TAF-2 activity has not been demonstrated, unequivocally, in *in vitro* transcription assays (see chapter 6). The oestrogen receptor also appeared not to contact TATA binding protein (TBP), a component of TFIID or TFIIB (fractions essential for basal transcription) that have been identified as targets for other transcription factors (A. Thomson, Molecular Endocrinology, ICRF, personal communication). TATA binding protein (TBP) associated factors (described in chapter 1) have also been proposed to be targets for transcription factors, however, the possibility that they may be target (s) for the oestrogen receptor has not been investigated directly in this thesis.

Approaches to identify a potential target for TAF-2 can be divided into those requiring the identification of purified protein(s) and those that may result in the direct isolation of a cDNA clone. The latter approaches assume that the target(s) are, at least, in part composed of protein and that a single polypeptide will be contacted by TAF-2. In all these methods, the point mutants described in the previous chapter that essentially lacked TAF-2 activity but retained oestradiol and DNA binding may be useful controls to assess if a protein that interacted with the receptor was involved in transcriptional activation. This assumes that inactive TAF-2 has a reduced or little affinity for its target(s). Some reports have suggested that this may be the case, for example mutations of the transcriptional activator VP16, from Herpes simplex virus, that reduce its ability to stimulate transcription *in vivo* have been reported to reduce its affinity *in vitro* for TBP (Ingles *et al.*, 1991) and TFIIB (Lin *et al.*, 1991). However in the study of Ingles *et al.*, (1991) indicated that there was not a strict correlation between the transcriptional activation *in vivo* and binding of TBP *in vitro*.

Indirect immuoprecipitation and affinity column chromatography.

Two methods for purification of potential target(s) include indirect immunoprecipitation, with an antibody recognising the oestrogen receptor or affinity column chromatography using a column to which receptor has been attached. These approaches may allow the purification of target proteins from nuclear extracts of cells that support TAF-2 activity that may contain target proteins. Receptor bound proteins may be resolved by SDS-PAGE and visualised by silver staining. Candidate target proteins may be identified by comparing the proteins purified by mutant and wild-type receptors. These proteins could then be partially sequenced and oligonucleotides, based on the amino acid sequence obtained, used to screen cDNA libraries in order to obtain a cDNA clone of the target protein. The purified protein(s) may also be used to immunise rabbits to generate antiserum that may be used to probe cDNA expression libraries.

These approaches could use endogenous oestrogen receptors isolated for example from breast cancer cell lines or, to try to increase the amount of target protein(s) purified, the receptor could be expressed in insect cells (*Spodoptera frugiperda,* using recombinant baculovirus), mammalian cells (using recombinant viruses or COS cells) yeast or *E.coli.* By using these expression systems more receptor protein should be obtainable and the cDNA encoding the receptor can be modified such that the receptor is joined to a polypeptide tag to simplify purification and/or matrix attachment. For example the efficiency of immunoprecipitation could be increased by joining an epitope for a high affinity monoclonal antibody to the receptor or glutathione S-transferase may be linked to the receptor and this fusion protein may then be attached with high efficiency to glutathione sepharose. This type of approach has been used to demonstrate that components of the basal transcription machinery namely TFIID or TFIIB can be purified from HeLa nuclear extract using an affinity column to which the Herpes simplex virus VP16 protein was attached (Stringer *et al.*, 1990; Lin *et al.*, 1991).

To obtain sufficient target protein(s) however may require the use of large amounts of nuclear cell extract that may not contain the target proteins in a suitable form to interact with TAF-2. The interaction between TAF-2 and its target(s) has to be stable for these techniques to work. Additionally the receptors in breast cancer cells are present at low levels that may make isolation of target proteins difficult. Over expression of the oestrogen receptor in other systems may overcome this problem but these receptors may be incorrectly folded and/or modified also the purification of the mouse oestrogen receptor expressed in insect cells or the isolation of stable lines that express high levels of receptor have proved to be difficult (A. Thomson, J. Ham, Molecular Endocrinology, ICRF and A. Coffer, Protein Cloning and Isolation Laboratory, ICRF personal communication).

Screening of λgt11 libraries with labelled protein.

This technique involves probing λgt11 expression libraries with purified protein (e.g. oestrogen receptor) that has been labelled with a radioisotope. Plaques expressing a polypeptide that binds the receptor are therefore indirectly labelled and the cDNA then identified. This technique may involve the over expression, probably in *E.coli*, of a recombinant TAF-2 containing a tag to simplify the purification of the protein (as described above) and a kinase recognition site to enable the labelling of the receptor to a high specific activity for use as a probe, described in Blanar and Rutter, (1992). Although this technique allows the screening of many clones and possibly the direct isolation of cDNA clones it also relies on a stable interaction between TAF-2 and the polypeptide and on over expressed TAF-2 being capable of interacting with *E.coli* expressed target(s) that may be incorrectly folded and/or modified.

A genetic screen in yeast.

The colony colour assay allows the visualisation of *lacZ* expressing yeast colonies as blue compared with non-expressing colonies that appear white. This assay provides the basis for a genetic screen in yeast described by Fields and Song, (1989) (reviewed in Fritz and Green, 1992). This method requires that a yeast strain containing the protein under study bound near the *LacZ* based reporter gene appears white or pale blue in the colony colour assay. This strain is then transformed with a suitable cDNA library in which the cDNAs have been joined to the cDNA encoding a transcriptional activation domain. The interaction of a protein encoded by the library cDNA with the protein under study results in the recruitment of the activation domain to the promoter and the expression of *lacZ*, producing blue colonies in the colony colour assay.

One major advantage of this technique is that it allows the detection of protein-protein interactions occurring *in vivo*, in a cell nucleus, rather than in artificial conditions *in vitro*. This should, in theory, increase the chances of isolating clones of genuine targets especially since TAF-2 may not to function *in vitro* (see chapter 6). The direct isolation of cDNA clones rather than polypeptides is also an advantage. This technique also allows the screening of large numbers of cDNA clones that can be characterised, to a certain extent, in the yeast. For example deletion mutants of the target clone that are tagged with an activation domain can be tested easily in yeast for their ability to interact with the protein under study. It is also possible that this screen may allow the detection of protein-protein interactions that are weaker than those detectable by the other described strategies. As the available reports indicated that TAF-2 was inactive in yeast (White *et al.*, 1988; Berry *et al.*, 1990), it appeared possible to use the yeast screen on the basis that yeast may lack the target of TAF-2

and that the target may be provided by a mammalian cDNA library. Previous studies have indicated that TAF-2 can function attached to a heterologous DNA binding domain (Webster *et al.*, 1988b; Lees *et al.*, 1989 and chapter 3). This may allow the use of a genetic screen in yeast developed with a reporter gene containing the binding site for any transcription factor. For these reasons this screen was chosen.

The genetic screen in yeast.

Using a genetic screen in yeast (*Saccharomyces cerevisiae*) to isolate proteins that interacted with serum response factor (SRF) Dalton and Treisman isolated SRF accessory protein-1 (SAP-1) that bound in a ternary complex to SRF and the SRF binding site (Dalton and Treisman, 1992). SRF fails to stimulate transcription significantly in yeast (Dalton and Treisman, 1992). Since TAF-2 can function when attached to heterologous DNA binding domains (Webster *et al.*, 1988b; Lees *et al.*, 1989 and Chapter 3) a chimera between part of SRF (containing the DNA binding domain) and the region of the oestrogen receptor containing TAF-2 was made to utilise this yeast screen (kindly provided by S. Dalton, Transcription Laboratory, ICRF), described below. The strategy for this screen is illustrated in Figure 4.1, note that this type of screen may result in the isolation of clones encoding polypeptides that are not target(s) for TAF-2 but still contact the oestrogen receptor.

The yeast strain S62L contained the reporter gene composed of a SRE joined to the TATA box of the *CYC1* gene linked to *lacZ*. This reporter gene was integrated into the yeast genome which is preferential to maintaining it on a plasmid since the copy number of plasmids can vary resulting in different levels of β–galactosidase (Futcher and Cox, 1984; Breeden and Nasmyth, 1987). Integration of the reporter may also result in a lower basal level of transcription. The cDNA library was made from HeLa cell RNA and the first cDNA strand was generated using reverse transcriptase and random hexamer primers (Dalton and Treisman, 1992).
The resulting cDNAs were cloned at the 3' end of codons 410-490 of the Herpes simplex virus VP16 gene that had either 0, 1, or 2 nucleotides after the codon 490 to obtain different reading frames. This region of VP16 is a very strong activation domain (Sadowski *et al.*, 1988; Cousens *et al.*, 1989). The low copy number expression plasmids containing SRF-ER or VP16-library cDNA were maintained in the yeast under the appropriate nutrient selection and the expression of the SRF-ER and VP16-library cDNA chimeras was galactose inducible (see methods and Dalton and Treisman, 1992).

For this screen the region of the oestrogen receptor that contained TAF-2 was joined to the DNA binding domain of SRF creating an SRF-ER chimera. To generate a chimera between SRF and the oestrogen receptor it was necessary to introduce unique restriction endonuclease sites into the oestrogen receptor cDNA.

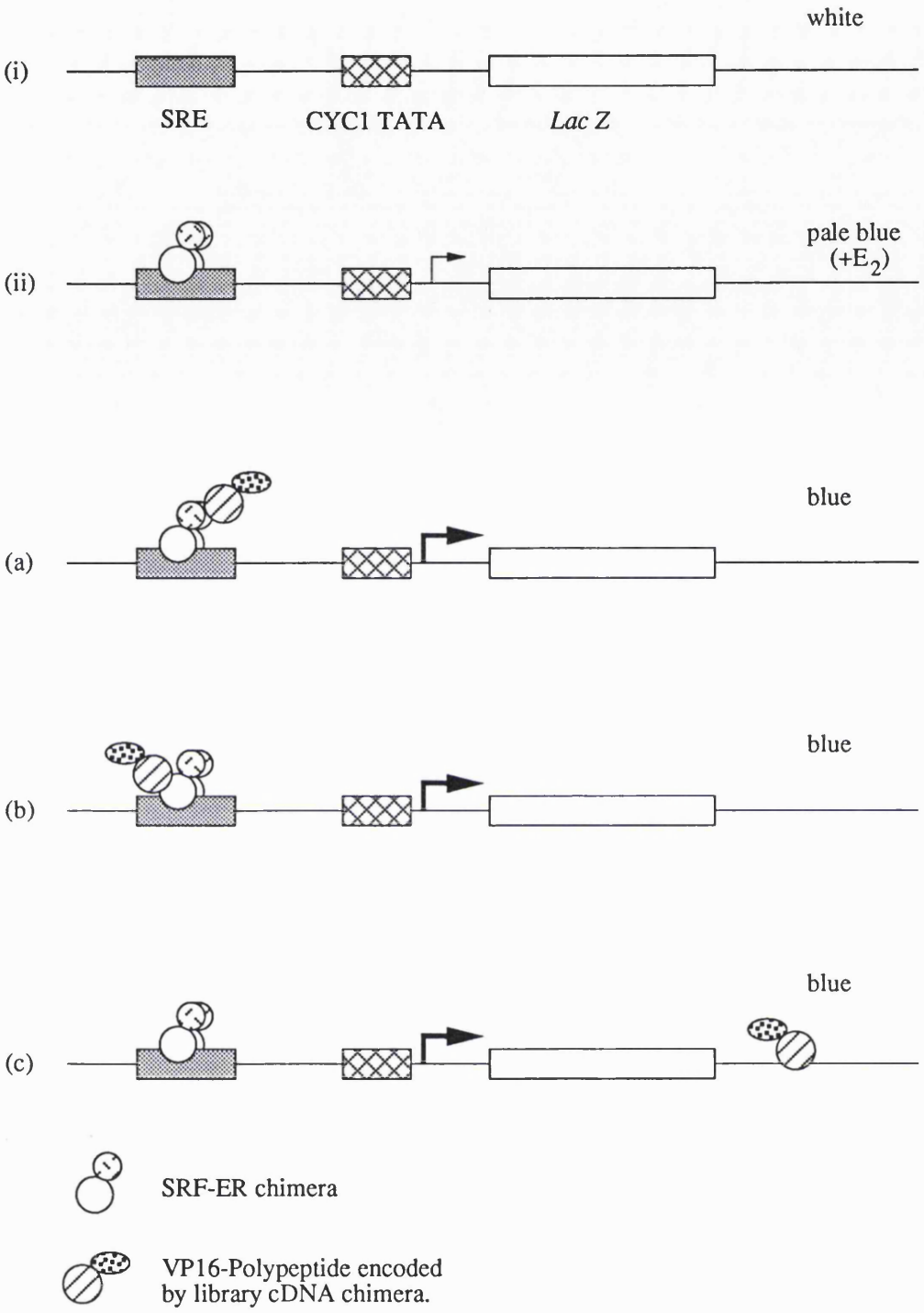


Figure 4.1. Yeast screen strategy.
The outcomes in the colony colour assay are illustrated for the following:
(i) Reporter strain S62L
(ii) S62L containing SRF-ER
(a), (b) and (c) possible outcomes giving blue colonies involving the VP16 tagged cDNA library. Interaction of the cDNA encoded polypeptide with (a) the ER (b) SRF or (c) directly with DNA or an endogenous protein near the reporter gene.

This was accomplished by amplifying the cDNA encoding the C-terminus of the oestrogen receptor, that contains TAF-2, using the polymerase chain reaction and primers designed such that the final product would contain appropriate restriction enzyme sites (see Appendix sections A6 and A10). The product was cloned into a intermediate vector and the 5' and 3' ends were sequenced; to remove any copying errors the bulk of the DNA was excised and replaced with a fragment taken from pJ3MOR or receptor cDNAs containing mutations in the conserved region. Recombinant clones were then cut with the unique enzyme sites in the 5' and 3' ends and cloned into a yeast expression vector containing SRF cDNA. The resulting chimeric protein contained SRF amino acids 1-412 (wild-type SRF has 508 amino acids) joined to amino acids 313-599 of the oestrogen receptor (Appendix A10). A plasmid containing SRF 1-412 alone was also generated (Appendix A11). A chimera containing ER amino acids 281-599 was also made but, because it stimulated transcription of *lacZ* to a slightly higher level in yeast than the SRF-ER 313-599, was not used in the screen (data not shown).

Analysis of SRF-ER in yeast.

The level of *lacZ* transcription stimulated by the SRF-ER chimera in the yeast strain S62L was then tested in the colony colour assay to assess if the level of transcription was low enough to perform the screen. These studies indicated that yeast expressing SRF-ER in the presence of 10^{-6}M oestradiol appeared only pale blue in the colony colour assay after approximately 40 minutes. In the absence of added oestradiol this yeast strain remained white for approximately 90 minutes. Yeast expressing SRF 1-412 appeared pale blue after 50-60 minutes whilst yeast expressing a strong activator e.g. SRF-VP16 (SD08, Dalton and Treisman, 1992) or SRF cotransformed with VP16-SAP-1 routinely appeared blue in approximately 20 minutes (data not shown). The low activity of SRF-ER in the absence of oestradiol may be due to oestrogen receptor in the chimera binding to the yeast homologues of hsp90, hsp82 and hsc82, that may prevent it from binding to the SRE in the absence of ligand (chapter 1 and Picard *et al.*, 1990a; McDonnell *et al.*, 1991). Upon the binding of ligand this complex may be dissociated allowing SRF-ER to bind DNA. However, based on experiments in mammalian cells the part of the oestrogen receptor in the SRF-ER fusion would be insufficient to bind to the hsp90 homologues in yeast (Chambraud *et al.*, 1990; Schlatter *et al.*, 1992). To test if SRF-ER was able to bind to DNA in the absence of ligand yeast containing SRF-ER were cotransformed with VP16 tagged SAP-1 that should bind to the DNA binding domain of SRF and the SRE only when the SRF-ER chimera is bound to DNA (Dalton and Treisman, 1992). These yeast appeared blue in the absence and presence of ligand indicating that the SRF-ER chimera was able to bind to DNA in the absence of ligand, however, from

this result it is difficult to assess what proportion of the SRF-ER protein is bound to DNA in the absence of ligand. These data may suggest that the hormone binding domain of the oestrogen receptor, in the absence of ligand, may repress the activity of the chimera when it is bound to DNA.

The activity of SRF-ER in the presence of oestradiol was slightly greater than that of SRF 1-412 but was judged to be low enough to attempt the screen also co-expression of SRF-ER with the activation domain of VP16 (amino acids 410-490) expressed by the vectors used to make the tagged cDNA library did not significantly increase the activity of SRF-ER in the absence or presence of oestradiol in the colony colour assay or the liquid β-galactosidase assay (data not shown). SRF-ER fusions containing the mutations described in chapter 3 that affected TAF-2 activity were also tested to assess if the low level of transcription stimulated by SRF-ER was stimulated by TAF-2. The mutations that nearly abolished transcriptional activation in mammalian cells had little affect on the transcription stimulated in yeast as judged in the colony colour assay (data not shown). This was assessed quantitatively using a liquid β-galactosidase assay (Figure 4.2).

The results showed that SRF-ER stimulated transcription in a hormone dependent manner but that the activity in the presence of oestradiol was only 2.5-fold greater than that of SRF 1-412. This low level of transcriptional activation was probably not stimulated by TAF-2 since the mutation of the hydrophobic residues (M-547A/L-548A) or deletion of the conserved region (Δ 540-552) that essentially abolished transcriptional activation in mammalian cells had no significant affect on transcriptional activation by SRF-ER in yeast.

The replacement of the conserved glutamic acid residue with an alanine residue (E-546A) resulted in a slight increase in the ability of SRF-ER to stimulate transcription the reason for this is not clear but the corresponding GAL4 fusion also stimulated transcription to a slightly higher level than the wild-type fusion (Table 3.3). The addition of 4-hydroxytamoxifen (which abolishes TAF-2 activity in mammalian cells) also failed to reduce the level of transcriptional activation. The transcriptional activation by SRF-ER was not observed when the plasmids were transformed into a yeast strain containing a mutated SRE which is not bound by SRF *in vitro* (strain S62L* Dalton and Treisman, 1992) indicating that stimulation of transcription observed is probably dependent on direct binding of SRF-ER to the SRE. A SRF-VP16 chimera in these assays gave approximately 300 units. As expected little activity was observed when the yeast strains are maintained in the presence of glucose which repress the expression of the SRF-ER protein.

The ability of the receptor, expressed in yeast, to bind oestradiol was assessed indirectly by measuring the levels of β–galactosidase activity induced over a range of oestradiol concentrations (Figure 4.3).

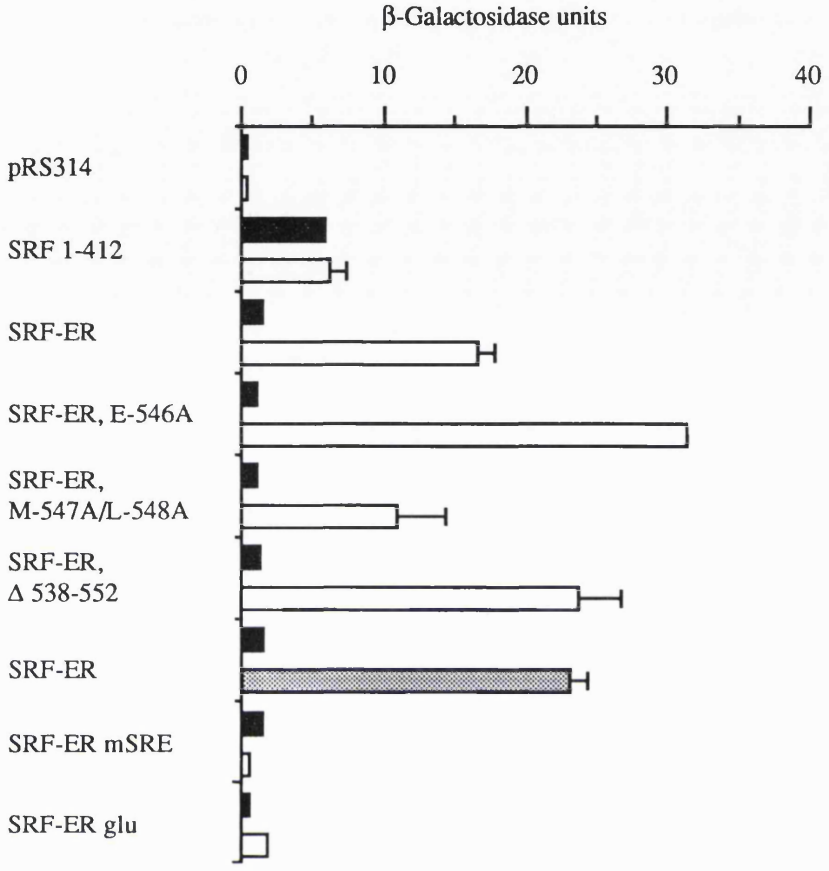


Figure 4.2. Transcriptional activation by SRF-ER chimeric proteins in yeast. The level of transcription stimulated from the SRE-*lacZ* reporter in the yeast strain S62L by SRF and SRF-ER chimeras was assessed using a liquid β-galactosidase assay (see methods). The levels of β–galactosidase were normalised to cell number and the error bars (where apparent) represent standard errors derived from the results of three independent experiments each performed in duplicate. Galactose induction of the expression of the proteins was for 14 hours in the presence of 0.01% ethanol carrier (solid bars), 10^{-7}M oestradiol (open bars) or 10^{-5}M 4-hydroxytamoxifen (shaded bar). pRS314 is the expression vector lacking the SRF-ER cDNA, mSRE indicates identical assays using the yeast strain S62L* that contains a mutated SRE and glu indicates that the cells were maintained in glucose (uninduced).

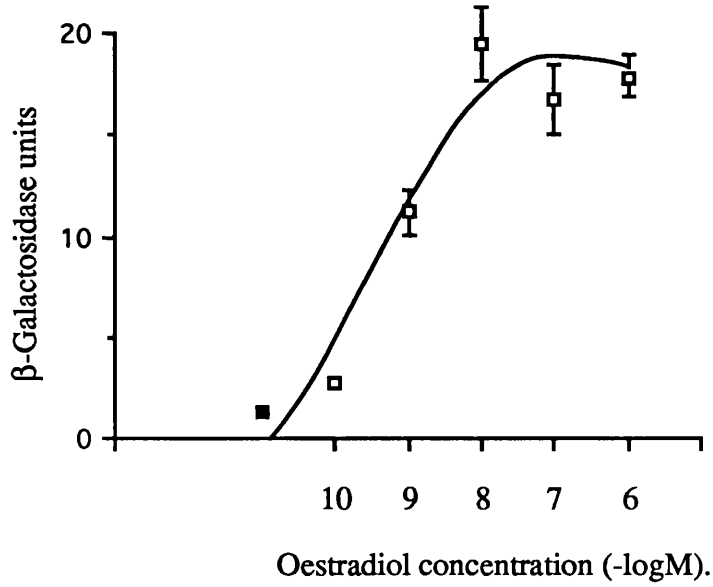


Figure 4.3. Transcriptional activation by SRF-ER in yeast is oestradiol dependent.

The SRF-ER chimera was tested for its ability to stimulate transcription of the *lacZ* gene in the yeast strain S62L in the presence of a range of oestradiol concentrations. β-galactosidase activity was assessed using a liquid assay (see methods). The filled square indicates the level of transcriptional activation in the absence of oestradiol. The error bars (that were not apparent in some cases) were derived from the results of three independent experiments each carried out in duplicate.

These data show that the stimulation of transcription is dose responsive and that 10^{-9}M oestradiol is sufficient to obtain approximately 50% of the maximal transcriptional activation suggesting that the affinity of the receptor for oestradiol is slightly lower than that of the wild-type receptor expressed in mammalian cells. Maximal transcriptional activation was obtained at 10^{-8}M. To ensure that all the receptor proteins were saturated with oestradiol 10^{-7}M oestradiol was used in the liquid β-galactosidase assays in Figure 4.2.

One possible explanation for the lack of TAF-2 activity was that the wild-type SRF-ER fusion protein and possibly SRF-ER E-546A were present at lower levels in yeast than those SRF-ER fusions containing mutations that abolished TAF-2. The relative levels of SRF-ER fusions in yeast was assessed by Western blotting using the antibody H222 raised against the human oestrogen receptor (Greene *et al.*, 1984) , the epitope for this antibody has been mapped to between amino acids 463 and 528 in the hormone binding domain of the human receptor (Kumar *et al.*, 1986). These results showed that SRF-ER and SRF-ER E-546A were expressed at slightly higher levels than SRF-ER M-547A/L-548A or Δ 540-552 suggesting that the TAF-2 characterised in mammalian cells is inactive in yeast (Figure 4.4). The explanation for the changes in the amount of receptor detected in the presence of oestradiol for SRF-ER and SRF-ER E-546A but not the other chimeras is unknown. From the Western blotting it appears that the molecular weight of the chimera is ~90 kD (kilodaltons) whilst the predicted molecular weight is ~77 kD this may be due to the SRF part of the SRF-ER fusion since SRF from mammalian cells runs at ~68 kD whilst having a predicted molecular weight of ~56 kD (R. Treisman, Transcription Laboratory, ICRF, personal communication).

These studies showed that the SRF-ER fusion was able to bind oestradiol and DNA in the yeast suggesting that is was, at least partially, correctly folded. The lack of TAF-2 activity in the SRF-ER chimera may be because the protein target(s) that mediate transcription in mammalian cells are absent from or not sufficiently conserved in yeast. In this screen the hope is that target(s) will be provided by the VP16 tagged cDNA library. The library was made from HeLa cells that in transfection experiments support transcriptional stimulation by TAF-2 and therefore may contain cDNAs encoding for target proteins (for an example see Tora *et al.*, 1989b).

Screening the library.

The screening was performed by transforming the yeast strain containing the SRE-*CYC1 /lacZ* reporter (S62L) and the plasmid pSRF-ER (TRP selectable marker) with the VP16 tagged cDNA plasmid library (URA selectable marker) and plating these yeast onto nylon membranes on uracil$^-$/tryptophan$^-$ plates (containing glucose)

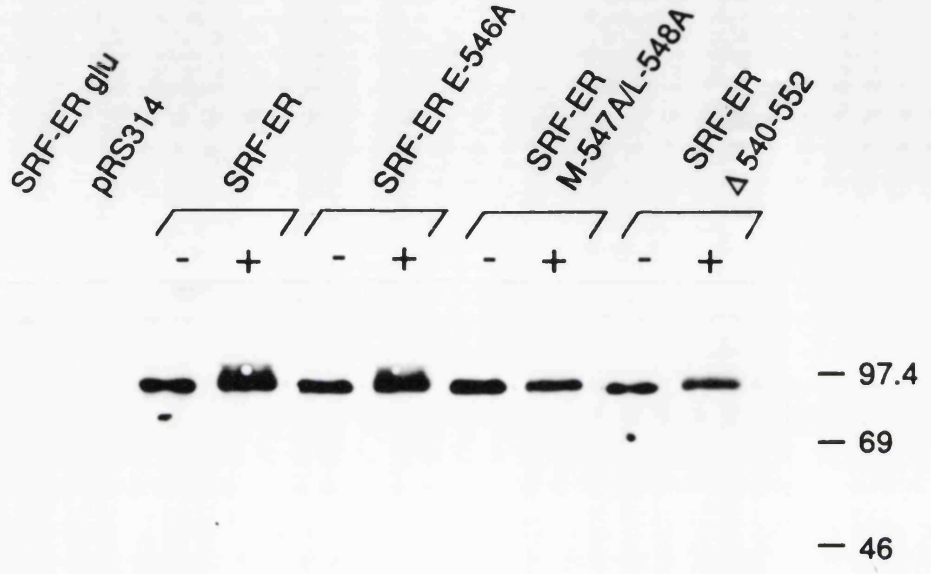


Figure 4.4. Expression of SRF-ER chimeras in yeast.
The expression of the SRF-ER chimeras in the yeast strain S62L was assessed by Western blotting. Yeast were grown as for β-galactosidase assays harvested and whole cell extracts prepared. 35μg of protein from each extract was resolved by SDS-PAGE and transferred to nitrocellulose for Western blotting (see methods). The SRF-ER chimeras were detected using the oestrogen receptor-specific monoclonal antibody H222. Yeast were maintained in the absence (-) or presence (+) of 10^{-7}M oestradiol, SRF-ER glu indicates yeast maintained in the presence of glucose where the expression of SRF-ER is repressed and pRS314 is the expression vector lacking the SRF-ER cDNA. The positions of the molecular weight markers (sizes in kilodaltons) are indicated.

to select for yeast containing both pSRF-ER and the VP16 tagged cDNA (see methods) . These colonies were grown for 26-30 hours after which the membranes transferred to selective plates which were 10^{-6}M in oestradiol and contained galactose to induce the expression of the SRF-ER and VP16/cDNA fusions. After a further 18 hours the membranes were removed and processed for the colony colour assay. Routinely 2-4x 10^4 colonies per plate were obtained.

This technique was used to screen approximately 1.2x 10^6 colonies and those that appeared blue before 40 minutes (when yeast containing SRF-ER appeared pale blue) were picked and colony purified. From this screen 41 colonies were picked, upon re-testing 35 of these appeared to be pale blue after 40 minutes in the colony colour assay and were discarded. The remaining 6 clones turned blue within 15-30 minutes in the presence or absence of oestradiol. These strains may therefore not contain a target for TAF-2, assuming that TAF-2 only contacts its target(s) in the presence of oestradiol. To assess if the blue colour observed was the result of an interaction between SRF-ER and the VP16 fusion an attempt was made to remove the plasmids containing SRF-ER and the VP16/cDNA from the yeast strains (see methods). This was successful with only 2 of the clones. The 4 remaining strains could not be cured of the SRF-ER containing plasmid (that contained the TRP selectable marker). These results are possibly due to the integration of the *GAL 1-10* promoter (containing binding sites for GAL4) and the TRP marker from the plasmid pSRF-ER near the *lacZ* gene that would result in the constitutive transcription of the gene in the presence of galactose. One explanation for this is that integration occurred by homologous recombination between the *CYC1* promoter sequences in front of *lacZ* and the *CYC1* promoter sequences in the *CYC1 /GAL 1-10* promoter in front of the SRF-ER cDNA. The remaining 2 clones could be cured of both plasmids but surprisingly the pSRF-ER plasmid alone was responsible for the blue colour one possibility for this result is that the SRF-ER chimera contains a mutation such that transcription is stimulated very efficiently in the absence or presence of oestradiol, this is currently being examined.

Testing SRF-ER in mammalian cells.

Whilst the yeast system was being established the ability of TAF-2 to function in the context of the SRF-ER chimera was tested in transient transfection experiments using NIH 3T3 cells. For these assays the cDNAs encoding SRF-ER chimeras were excised from the yeast expression vectors by digestion with Bam HI and Spe I (Appendix A10) and subcloned into the eukaryotic expression vector pSG424 (Appendix A6) that had been digested with Bgl II and Xba I. Initially transfections were performed as described for the oestrogen receptor using the calcium phosphate co-precipitation method but with 0.5% stripped serum (see chapter 3 and methods).

Low serum concentrations were used to avoid transcriptional activation by endogenous and transfected SRF. These results indicated that SRF-ER stimulated transcription from pSRE TKCAT in a hormone dependent manner. The level of transcription stimulated by SRF-ER increased approximately 6-fold upon the addition of 10^{-8}M oestradiol whilst the transcription stimulated by SRF-ER M-547A/L-548A only increased, approximately, 1.5-fold upon the addition of oestradiol (data not shown). In these experiments, however, the basal level from pSRE TKCAT alone was quite high, this may be due to the introduction of a second messenger, calcium ions, into transfected cells that may activate second messenger pathways that subsequently activate endogenous SRF resulting in transcriptional activation of the reporter gene (R. Treisman, Transcription Laboratory, ICRF personal communication). Therefore these experiments were repeated using the DEAE-dextran method of transfection and increasing amounts of chimera expression plasmid (Figure 4.5). At 10 μg/dish in the presence of 10^{-8}M oestradiol SRF-ER stimulated transcription approximately 13-fold whilst SRF-ER M-547A/L-548A stimulated transcription approximately 2-fold. These results were similar to those obtained with the calcium phosphate method and suggested that TAF-2 could function in mammalian cells when attached to SRF and that TAF-2 activity was affected by mutations in the conserved region. The induction level achieved by SRF-ER in the presence of oestradiol was similar to that achieved by the activation of endogenous SRF by the addition of media containing 10% foetal calf serum. This level of induction is maximal for this system and cannot be increased, for example by transfecting SRF (C. Hill, Transcription Laboratory, ICRF, personal communication). The cellular localisation of the chimeric protein in transfected COS-1 was assessed by indirect immunofluorescence (using the monoclonal antibody H222). These results indicated that in the absence of ligand the proteins were distributed throughout the cell whilst in the presence of 10^{-8}M oestradiol the proteins appeared predominantly nuclear. This immunofluoresence was performed by S. Dauvois, Molecular Endocrinology, ICRF.

To assess if the chimeric proteins were present at similar levels in COS-1 cells COS-1 cell extracts were tested for the presence of SRF-ER in a band shift assay using a probe containing a serum response element (SRE). SRF-ER and SRF-ER M-547A/L-548A were detected at similar levels Figure 4.6, however, in both cases a faster migrating band was observed. This was probably due to a proteolytic fragment of the chimera containing the DNA binding domain of SRF only since this band was not shifted by the addition of the monoclonal antibody H222 that recognises the oestrogen receptor. Proteolysis may have occurred *in vivo* or during the preparation of the whole cell extract. This band was not observed when extracts from COS-1 cells expressing SRF 1-412 were examined also less COS-1 cell extract was used in this case suggesting that SRF 1-412 is either expressed at higher levels than the SRF-ER

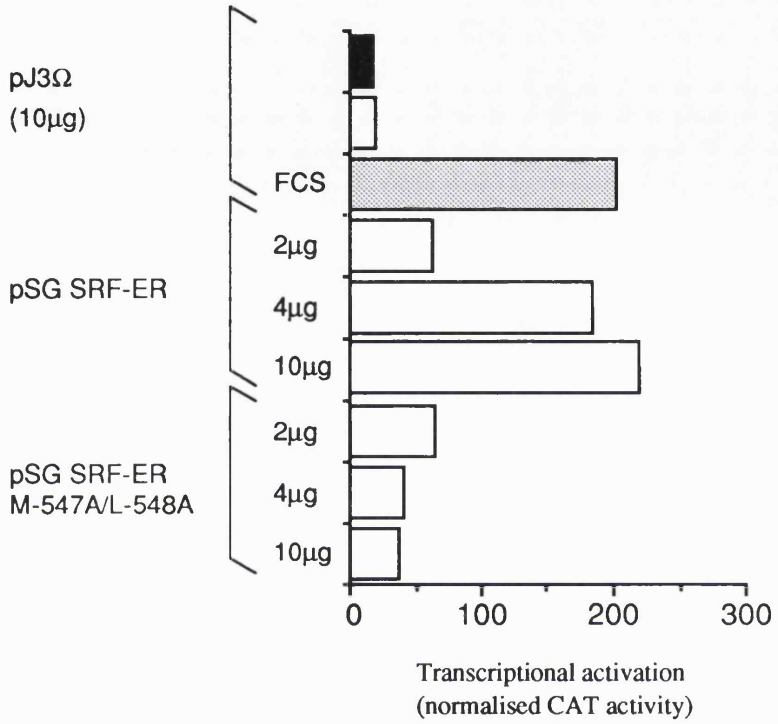


Figure 4.5. Transcriptional activation by SRF-ER chimeras in NIH 3T3 cells.

The ability of the SRF-ER chimeras to stimulate transcription from pSRE TKCAT in mammalian cells was assessed in transient transfection experiments in the presence of 10^{-8}M oestradiol and in media 0.5% in foetal calf serum (open bars). The solid bar indicates the absence of oestradiol and the shaded bar indicates the addition of media containing 10% foetal calf serum 8 hours before harvesting the cells. Transfections were performed using the DEAE-dextran technique (see methods). The figure shows a representative result from several independent experiments.

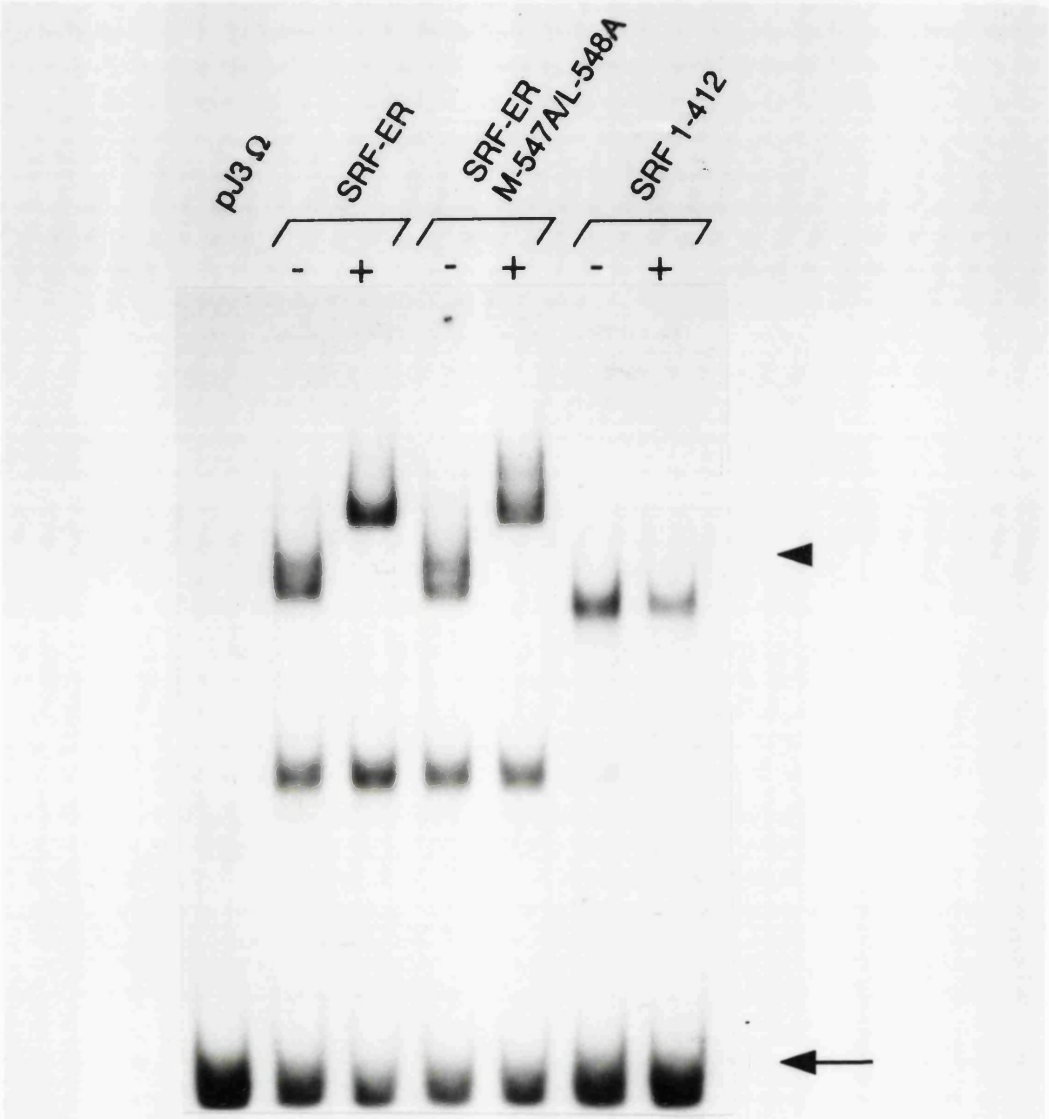


Figure 4.6. DNA binding activity of SRF-ER chimeras.
The SRF-ER chimeras indicated and SRF 1-412 that had been expressed in COS-1 cells were tested for their ability to bind DNA in a band shift assay. The probe was a labelled DNA fragment equivalent to the sequences in the c-*fos* gene promoter from position -387 to -250 that contain an SRE. Equal amounts (2μg of protein) of whole cell extract was used in each lane apart from those labelled SRF 1-412 where 0.2μg of protein was added. The oestrogen receptor-specific monoclonal antibody H222 (0.5μg) was added to the lanes marked (+) whilst (-) indicates no addition. The arrow and arrowhead indicate free and retarded probe respectively.

chimeras or that SRF 1-412 has a higher affinity for DNA than the SRF-ER chimeras. One explanation is that the chimeric protein has a lower affinity for DNA and may also be unstable in COS-1 cells. To assess which of these is the case the affinity of these proteins for a SRE needs to be measured (as no antibody to SRF 1-412 was available).

Conclusions.

In this study a yeast genetic screen was used to try to identify proteins that interact with TAF-2, however, no candidate clones were identified. The possible reasons for this are discussed in chapter 6. Analysis of the chimeric receptors containing point mutations in the conserved region indicated that the mechanism whereby TAF-2 stimulates transcription in mammalian cells may not be conserved in yeast.

Chapter 5

Examination of mutant mouse oestrogen receptors with altered ligand specificity.

Introduction.

Analysis of a region in the hormone binding of the mouse oestrogen receptor identified point mutations that prevented receptor dimerisation and DNA binding *in vitro* but did not abolish oestradiol binding (Fawell *et al.*, 1990a). To examine if these mutations abolished these functions completely mutant receptors were tested for their ability to stimulate transcription in transient transfection experiments. Surprisingly the mutant receptors stimulated transcription to nearly wild-type levels in the presence of oestradiol (data not shown). The analysis of several other mutants containing mutations in the same region of the hormone binding domain suggested that this region was particularly important for the binding of oestradiol.

Functional analysis of receptors containing mutations in the hormone binding domain.

Analysis of oestradiol binding by the mutant receptors.

Since the mutations were located within the hormone binding domain the mutant receptors were first tested for their ability to bind oestradiol. To accomplish this the cDNAs encoding the wild-type and mutant receptors were transcribed and translated *in vitro* and the affinity of these receptor proteins for 16α-[^{125}I] iodoestradiol determined by Scatchard analysis. The dissociation constants (Kd) are shown in Table 5.1. The Scatchard analyses were performed by S. Hoare, Molecular Endocrinology, ICRF.

The mutants are described as the amino acid, its position in the wild-type protein and the amino acid with which it was replaced, for example G-525R is encoded by a cDNA in which a codon for glycine at position 525 was replaced with a codon for arginine; H-520, A, M-521 indicates that a codon for alanine was inserted between codons 520 and 521 in the cDNA and Δ M-521, S-522 indicates that the codon for methionine at position 521 and serine at position 522 were deleted. All the mutant receptor cDNAs in vectors containing the promoter for SP6 RNA polymerase were provided by R. White, Molecular Endocrinology, ICRF (described in Fawell *et al.*, 1990a).

The results shown in Table 5.1 indicate that the affinities of I-518R, G-525R and Δ M-521, S-522 for oestradiol are, approximately, 1000-fold lower than that of the wild-type receptor in this assay. The remaining three mutants were judged to have affinities for oestradiol approximately 4 to 10-fold lower than that of the wild-type receptor.

Table 5.1 Oestradiol binding by wild-type and mutant receptors

Receptor	Affinity for oestradiol Kd (nM) ± standard error.
Wild-type	0.15 ± 0.01
I-518R	>30*
G-525R	>30*
M-532R	0.56 ± 0.15
H-520, A, M-521	1.2 ± 0.5
Δ M-521, S-522	>30†
L-529A/M-532A/C-534A/V-537A	0.85 ± 0.4

The affinity of each receptor for oestradiol was determined in three independent experiments and is shown as an average dissociation constant (Kd) ± the standard error, * indicates that weak binding was detected at 30 nM whilst † indicates no detectable binding at 30 nM.

Transcriptional activation by the mutant receptors in NIH 3T3 cells.

To assess the affects of these mutations further the mutant receptors were then tested for their ability to stimulate transcription from pERE BLCAT in a transient transfection assay using NIH 3T3 cells (described in chapter 3 and methods). For transient transfection assays the mutations in the cDNA were transferred into receptor cDNA in a eukaryotic expression vector (see Appendix A2). In these transfections the ability of the mutants to stimulate transcription in the presence of 10^{-8}M oestradiol and 10^{-8}M 4-hydroxytamoxifen was tested. The antioestrogen 4-hydroxytamoxifen appears to bind to the receptor, promote dimerisation and DNA binding but fails to activate TAF-2, any transcriptional activation observed in the presence of this ligand may be derived from the N-terminal TAF-1 (Webster *et al.*, 1988b; Lees *et al.*, 1989; Berry *et al.*, 1990).

The results indicated that the wild-type receptor stimulated transcription approximately 90-fold in the presence of 10^{-8}M oestradiol, this is represented as 100% in Figure 5.1(a). In the absence of added oestradiol the receptor stimulated transcription to 9-11% that in the presence of oestradiol. This could, in part, be due to the binding of residual oestrogens in the medium since the activity could be reduced to 3-5% by the addition of the antioestrogen 4-hydroxytamoxifen that would block TAF-2 activity. Alternatively this activity may be due to the receptor binding to DNA and stimulating transcription in the absence of oestradiol. To assess if the receptor was able to stimulate transcription in the absence of ligand the charcoal stripped

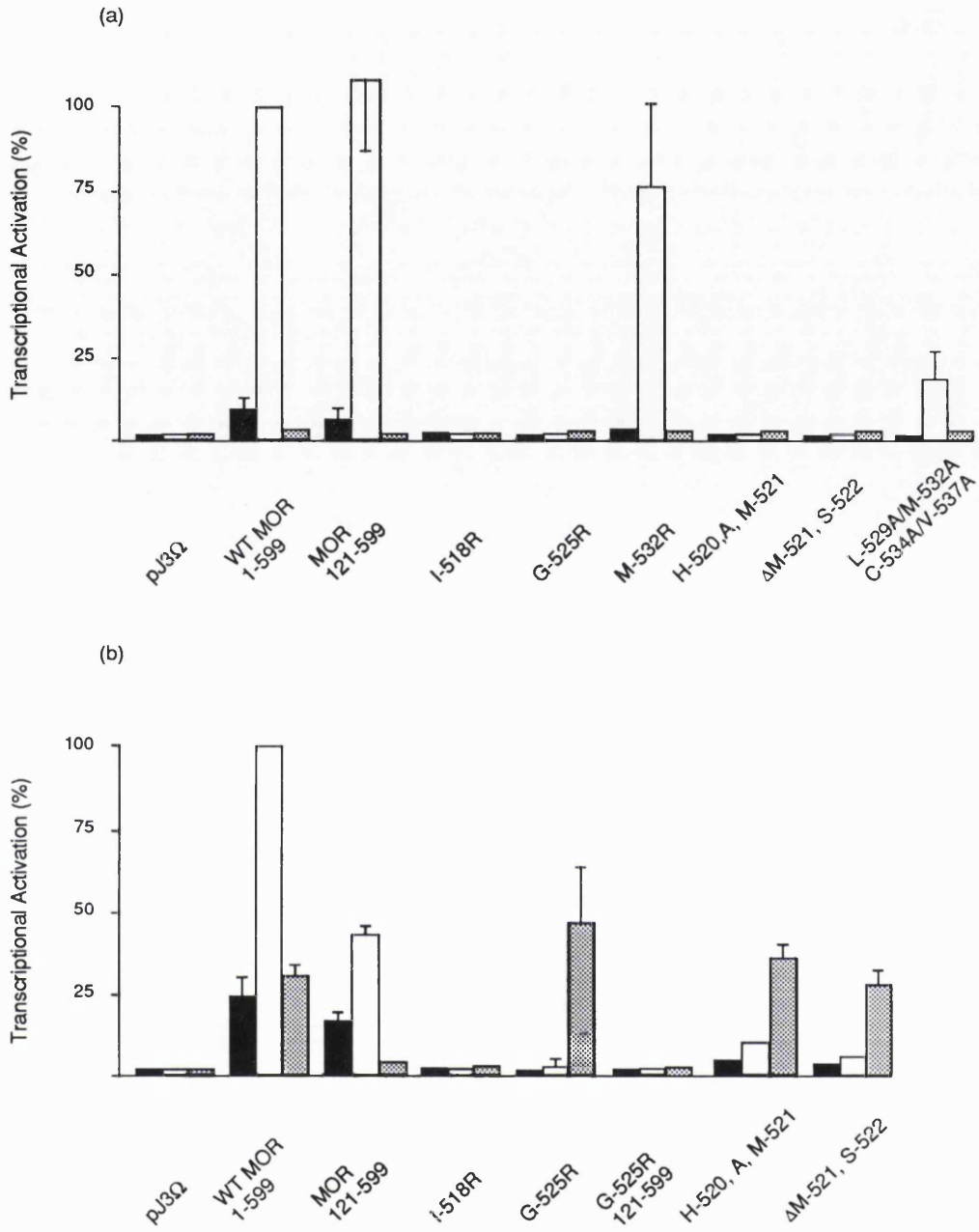


Figure 5.1. Transcriptional activation by mutant oestrogen receptors.
The ability of the mutant mouse oestrogen receptors (MORs) to stimulate transcription from the reporter plasmid pERE BLCAT was tested in transient transfection experiments using NIH 3T3 cells (a) and CEF cells (b), see methods. The level of transcriptional activation is shown as percentage of that stimulated by the wild-type receptor in the presence of 10^{-8}M oestradiol. Transfected cells were maintained in the absence of added ligand (solid bars) or in the presence of 10^{-8}M oestradiol (open bars) or in the presence of 10^{-8}M 4-hydroxytamoxifen (shaded bars). The error bars (that are not apparent in some cases) represent standard errors derived from the results of at least three independent transfection experiments each carried out in duplicate. pJ3Ω is the expression vector lacking receptor cDNA.

serum was replaced with a chemically defined serum substitute lacking any known oestrogen. This approach was, however, unsuccessful since little luciferase or CAT activity was detected in extracts from transfected cells possibly because the transfected cells did not survive in the serum substitute tested (S. Hoare, Molecular Endocrinology, ICRF, personal communication).

The analysis of the mutant receptors in the transient transfection assay showed that the mutant receptors with very low affinities for oestradiol I-518R, G-525R and Δ M-521, S-522 failed to stimulate of transcription significantly in the presence of 10^{-8}M oestradiol, Figure 5.1(a). Similar results were obtained when the amount of expression plasmid transfected was increased 8-fold (data not shown). These data suggest that oestradiol binding is essential for transcriptional activation. Two of the mutant receptors M-532R and one in which four amino acids had been replaced with alanines (L-529A/M-532A/C-534A/V-537A) that had reduced affinities for oestradiol, compared with the wild-type receptor, were able to stimulate gene transcription to 75% and 20% respectively that of the wild-type receptor in the presence of 10^{-8}M oestradiol suggesting that the amino acids mutated are not essential for transcriptional activation. These two mutants in MOR 121-599 stimulated transcription to 20-30% that of the wild-type receptor in the presence of oestradiol suggesting that TAF-2 was active and that some of the transcriptional activation stimulated by the full-length receptors may be due to cooperation with the N-terminal TAF-1 (data not shown). The insertion mutant H-520, A, M-521 failed to stimulate transcription of the reporter gene significantly above background levels in the presence of oestradiol raising the possibility that this mutation has affected TAF-2 activity, Figure 5.1(a).

In the presence of 10^{-8}M 4-hydroxytamoxifen the wild-type receptor stimulated transcription only 3-5% that in the presence of oestradiol, this transcriptional activation may be derived from TAF-1 since the mutant MOR 121-599, lacking the majority of the region N-terminal to the DNA binding domain, essentially fails to stimulates transcription in the presence of this ligand. All the mutant receptors apart from I-518R also stimulated transcription to a low level, 3-5% that of the wild-type receptor, in the presence of 4-hydroxytamoxifen. Analysis of the mutants in MOR 121-599 suggested that this activity was derived from TAF-1 (data not shown). If this is the case the data suggested that 4-hydroxytamoxifen may be capable of promoting DNA binding and transcriptional activation (by means of TAF-1) by those receptors that failed to bind oestradiol.

The mutant I-518R showed negligible transcriptional activity in the absence or presence of ligand this is probably due to its inability to bind to DNA (see later and Fawell *et al.*, 1990a). In the presence of 4-hydroxytamoxifen the level of transcription stimulated in NIH 3T3 cells by the wild-type and mutant receptors was low therefore

to examine these mutants further the transient transfection experiments were repeated using cells in which TAF-1 stimulates transcription efficiently.

Transcriptional activation by the mutant receptors in chicken embryo fibroblast cells.

The reports of Tora *et al.*, (1989b) and Berry *et al.*, (1990) indicated that TAF-1 of the human oestrogen receptor was able to stimulate transcription efficiently in chicken embryo fibroblast (CEF) primary cell cultures. Therefore to assess if the receptors that failed to stimulate transcription in the presence of oestradiol were able to stimulate transcription by means of TAF-1 in the presence of 4-hydroxytamoxifen transient transfection experiments were performed using CEF cells (see methods). The wild-type receptor in this assay stimulated transcription approximately 55-fold in the presence of 10^{-8}M oestradiol, this is represented as 100% in Figure 5.1 (b). In the presence of 10^{-8}M 4-hydroxytamoxifen the receptor stimulated transcription to approximately 30% that in the presence of oestradiol. This activity is, in part, probably due to TAF-1 as deletion of the majority of the N-terminal region in MOR 121-599 reduces the transcriptional activation to approximately 5%. Additionally transfection of a mutant receptor MOR 1-339 (that contains TAF-1 but not TAF-2) stimulated transcription to 25-30% that of the wild-type receptor (data not shown). The high levels of activity in the absence of added ligand may be due to residual oestrogens in the medium that promote the DNA binding of the receptor allowing transcriptional activation by TAF-1 and TAF-2.

The mutant G-525R failed to stimulate transcription in the absence of added hormone or in the presence of 10^{-8}M oestradiol and the mutants H-520, A, M-521 and Δ M-521, S-522 also stimulated transcription poorly in the absence or presence of 10^{-8}M oestradiol, Figure 5.1 (b). These results are similar to those obtained from experiments using NIH 3T3 cells. In the presence of 10^{-8}M 4-hydroxytamoxifen, however, all of these mutant receptors, apart from I-518R, stimulated transcription to at least 25% the level stimulated by the wild-type receptor in the presence of oestradiol. This activity may be derived from the N-terminal TAF-1 as deletion of the N-terminus of G-525R (in G-525R 121-599) reduces the transcriptional activation in the presence of 4-hydroxytamoxifen to less than 5%, Figure 5.1 (b).

Therefore mutants that essentially failed to stimulate transcription in the presence of oestradiol are still capable of stimulating transcription in the presence of 4-hydroxytamoxifen. To try to determine the affinity of these mutants for 4-hydroxytamoxifen the wild-type and G-525R receptor proteins were expressed in COS-1 cells and the whole cell extracts used in ligand binding assays with ^{3}H 4-hydroxytamoxifen. Scatchard analysis of the data generated using the wild-type receptor indicated that the dissociation constant (Kd) was approximately 0.4 nM (data not shown) similar to that reported by other groups, ~0.15 nM (Coezy *et al.*, 1982;

Eckert and Katzenellenbogen, 1982). The affinity of G-525R for 4-hydroxytamoxifen, however, was not accurately determined since upon plotting the data for Scatchard analysis a straight line was not obtained preventing the calculation of a Kd. At a rough estimate the affinity of G-525R was 5-10 fold lower than that of the wild-type receptor. Whole cell binding assays and assays using *in vitro* translated receptors were also unsuccessful (data not shown).

To obtain an indirect indication of the ability of G-525R to bind 4-hydroxytamoxifen its ability to stimulate transcription in the presence of this ligand was assessed in a dose response experiment. This assay measures the sensitivity of G-525R to 4-hydroxytamoxifen. For this experiment CEF cells were transfected with G-525R or the wild-type receptor and maintained in the absence of added ligand or in the presence of oestradiol or 4-hydroxytamoxifen at concentrations between 10^{-10} and 10^{-6}M. The transcriptional activation by the receptors is shown as normalised CAT activity, Figure 5.2 (see methods). These results indicate that half maximal transcriptional activation by G-525R occurs between 10^{-9} and 10^{-8}M 4-hydroxytamoxifen. In contrast significant transcriptional activation in the presence of oestradiol was only apparent at 10^{-6}M. The activity of the wild-type receptor in the absence of added ligand is probably in part due to the binding of residual oestrogens in the medium. A high level of transcription stimulated in the absence of added ligand (~30% that in the presence of oestradiol) was also observed in experiments using the wild-type human oestrogen receptor in CEF cells (Berry *et al.*, 1990). The activity observed was reduced slightly by the addition of 4-hydroxytamoxifen to the medium, Figure 5.1(b) and 5.2. For the wild-type receptor the addition of oestradiol to 10^{-9}M was sufficient to achieve nearly maximal rates of transcription. These data indicate that the replacement of a single amino acid results in a receptor that only stimulates transcription at an oestradiol concentration approximately 1000-fold higher than that of the wild-type receptor whilst, in contrast, a transcriptional response is observed at a 100-fold lower concentration of 4-hydroxytamoxifen (10^{-8}M).

One explanation for this result is that the glycine residue at position 525 is important for discriminating between oestradiol and 4-hydroxytamoxifen binding. One other possibility is that residues in this region are not required for high affinity 4-hydroxytamoxifen binding and that the C-terminal end of the binding site for 4-hydroxytamoxifen is N-terminal to that for oestradiol. The C-terminal boundary for the oestradiol binding domain has been mapped to between amino acids 522 and 538 (Lees *et al.*, 1989; Fawell *et al.*, 1990a). To assess if the binding site for 4-hydroxytamoxifen is N-terminal to this deletion mutants need to be examined. This, however, is difficult since the deletion to amino acid 522 and other deletions further N-terminal reduce the ability of the receptor to bind DNA *in vitro* (Fawell *et al.*, 1990a) so if these receptors failed to stimulate transcription in the presence of

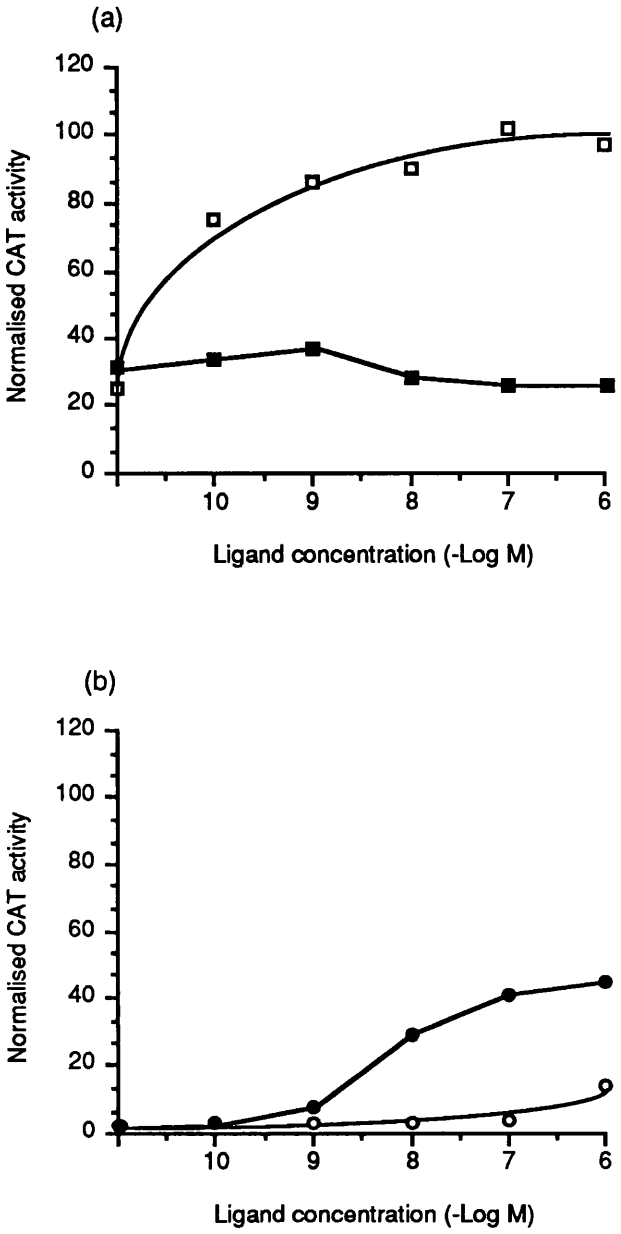


Figure 5.2. Analysis of transcriptional activation by G-525R in the presence of oestradiol and 4-hydroxytamoxifen.

CEF cells were transiently transfected with the reporter plasmid pERE BLCAT, the wild-type receptor (a) or G-525R (b) in the presence of the indicated concentrations of oestradiol (open symbols) or 4-hydroxytamoxifen (solid symbols). The CAT activities were normalised for differences in transfection efficiency with luciferase activity derived from an internal control plasmid (see methods). Typical values are shown; the experiment was repeated twice in duplicate.

4-hydroxytamoxifen in CEF cells (the only assay for binding) it would be impossible to conclude if they are able to bind 4-hydroxytamoxifen or not. However, since other mutants, not described in this thesis, that failed to bind DNA efficiently *in vitro* stimulated transcription in the presence of ligand *in vivo* some deletion mutants (MOR 1-538, 1-522, 1-507, 1-498) were tested. Apart from MOR 1-538 that stimulated transcription in the presence of oestradiol and 4-hydroxytamoxifen, by means of TAF-1, all these mutants failed to stimulate transcription in the presence of either ligand and thus it was not possible to conclude if the binding site of 4-hydroxytamoxifen is N-terminal to that for oestradiol.

Oestradiol binding by H-520, A, M-521 appears to be temperature sensitive.

The mutant H-520, A, M-521 was judged to have a dissociation constant (Kd) of 1.2 nM, Table 5.1, therefore in the transient transfection experiments where 10^{-8}M oestradiol was used ~80% of the receptors would be expected to have bound ligand. As this receptor bound DNA with high affinity *in vitro* (see later) in the presence of oestradiol the receptor should bind to DNA in transfected cells and stimulate transcription efficiently by means of TAF-1 in CEF cells this, however, was not the case, Figure 5.1(b). One explanation for this result was that this receptor failed to bind oestradiol in the transfection experiments. To test if this receptor was able to bind oestradiol under transfection conditions whole cell binding assays were performed. In this assay transfected COS-1 cells were incubated (at 37°C) with phenol red free DMEM containing ^{3}H oestradiol with or without the addition of an excess of cold competitor (see methods). The results in Table 3.2 indicate that H-520, A, M-521 (pJ3X) bound only slightly more oestradiol than COS-1 cells transfected with a plasmid lacking receptor cDNA (pJ3Ω). This result was not due to the degradation of the mutant receptor protein in transfected cells (see later) suggesting that the ability of this receptor to bind oestradiol may be temperature sensitive. This was tested directly by examining the ability of the wild-type and mutant receptor proteins that had been expressed in COS-1 cells to bind ^{3}H oestradiol at 4 and 37°C. However, in this experiment the number of counts bound by H-520, A, M-521 at 4°C was such that a reduction of greater than two-fold would be close to background levels. No specific binding was detected at 37°C suggesting that the change in temperature causes at least a two-fold reduction in the amount of oestradiol bound. In contrast at 37°C the wild-type receptor bound only 15% less counts than it bound at 4°C (data not shown).

Examination of receptor proteins in transfected cells.

To assess if the differences in transcriptional activity of the mutant receptors compared with the wild-type receptor were attributable to alterations in the levels of

receptor protein or the ability of the receptors to bind DNA a number of control experiments were performed. The expression of the receptor proteins in transfected NIH 3T3 cells was examined by indirect immunofluorescence using the monoclonal antibody H222 that binds to an epitope in the hormone binding domain (Greene *et al.*, 1984; Kumar *et al.*, 1986). Transfected cells appeared to contain similar amounts of wild-type and mutant receptors and all receptor proteins were predominantly nuclear (data not shown). The relative levels of receptor proteins expressed in transfected COS-1 cells were assessed by Western blotting using the polyclonal antiserum MP16. This serum was raised against a peptide corresponding to amino-acids 130-142 of the mouse oestrogen receptor (Fawell *et al.*, 1990a, b). The results indicated that all the receptor proteins were of the expected size and expressed at similar levels, Figure 5.3(a). A duplicate membrane probed with pre-immune serum instead of the immune serum did not result in an equivalent band (data not shown). All Western analysis were repeated using extracts from at least two transfections. These data suggest that none of the mutant receptor proteins was significantly less abundant than the wild-type receptor and that they may be present at similar levels in transfected NIH 3T3 cells. Maintaining COS-1 cells transfected with the wild-type receptor in the presence of 10^{-8}M oestradiol rather than in the absence of ligand made no significant difference to the amount of receptor or to the amount that bound DNA in a band shift assay (data not shown).

The activity of some of the mutants in the presence of 4-hydroxytamoxifen in CEF cells may be a result of this ligand increasing the amount of protein in transfected cells. To assess if this was the case the relative levels of wild-type and G-525R protein in transfected CEF cells was assessed by Western blotting using the monoclonal antibody H222. For this assay 10 cm dishes of CEF cells were transfected with 1 μg of pJ3 luciferase and 9 μg of one of the following pJ3Ω, pJ3MOR (WT) or pJ3MOR G-525R. The dishes were maintained in media containing 0.01% ethanol (carrier), 10^{-8}M oestradiol or 10^{-8}M 4-hydroxytamoxifen. The results of Western blotting analysis of whole cell extracts from these cells indicated that G-525R was expressed at similar levels to that of the wild-type receptor in the presence or absence of ligand, Figure 5.3 (b). In the presence of oestradiol slightly more wild-type and mutant receptor proteins were detected.

The expression of functional receptor proteins in transfected cells was assessed by band shift assay. Whole cell extracts made from transfected COS-1 cells (described above) were tested for the presence of receptor in a band shift assay using a pair of annealed and labelled oligonucleotides containing an ERE identical to that in pERE BLCAT (see methods). These results indicated that I-518R failed to bind to DNA with high affinity (Figure 5.4) as described in Fawell *et al.*, (1990a). The other mutant receptor proteins bound DNA with high affinity.

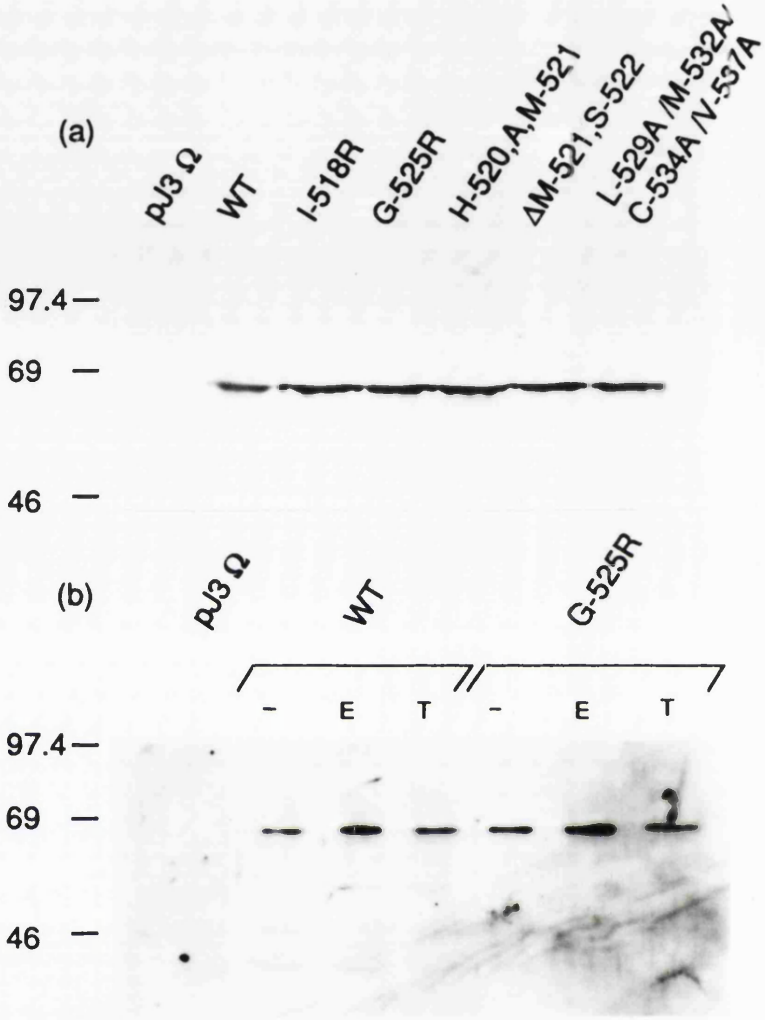


Figure 5.3. Analysis of the relative levels of transiently expressed wild-type and mutant receptors.

(a) Transiently transfected COS-1 cells expressing the indicated receptors were harvested and whole cell extracts prepared. Equal amounts (10µg of protein) of each extract were resolved by SDS-PAGE and transferred to nitrocelulose for Western blotting (see methods). The receptor was detected using the oestrogen-receptor specific antiserum MP16. (b) Transiently transfeced CEF cells maintained in the absence of ligand (-) or in the presence of 10^{-8}M oestradiol (E) or 10^{-8}M 4-hydroxytamoxifen (T) were harvested and normalised amounts of cell extract were resolved by SDS-PAGE and transferred to nitrocellulose for Western blotting. The receptor was detected using the monoclonal antibody H222. WT indicates the wild-type receptor and pJ3Ω is the expression vector lacking receptor cDNA. The positions of the molecular weight markers (sizes in kilodaltons) are shown.

There was some variation in the amounts of probe bound by the receptors but these appeared insufficient to account for the lack of transcriptional activation. One exception may be H-520, A, M-521 that bound less probe than the wild-type receptor, note however, that this mutant stimulated transcription in the presence of 4-hydroxytamoxifen in CEF cells. The relative amounts of the N-terminally truncated mutants (121-599) expressed in COS-1 or CEF cells has not been investigated but the deletion of the N-terminus has not been found to alter the level of expression or the affinity for DNA of the wild-type receptor or other mutants tested (chapter 3 and Fawell *et al.*, 1990a).

Analysis of DNA binding by receptors in the presence of ligands.

To investigate if ligand was able to alter the ability of the receptor to bind DNA oestradiol or 4-hydroxytamoxifen (both at 10^{-8}M) or carrier (0.01% ethanol) were added to the band shift reactions for the 30 minute incubation before loading the gel. The mobility of the wild-type mouse oestrogen receptor-probe complex increased in the presence of 10^{-8}M oestradiol relative to the complex in the absence of ligand or that in the presence of 10^{-8}M, 4-hydroxytamoxifen Figure 5.5 (Lees *et al.*, 1989; Fawell *et al.*, 1990b, see chapter 6).

This change in mobility of the probe-receptor complex may be due to a conformational change and/or posttranslational modification and since it was observed when agonists (oestrogens) but not antagonists were bound was suggested to be indicative of the formation of an active TAF-2 (Figure 5.5 and see chapter 6). None of the mutant receptor-probe complexes exhibited an increase in mobility in the presence of oestradiol. This was expected for G-525R and Δ M-521, S-522 that had very low affinities for oestradiol but was unexpected for M-532R and L-529A/M-532A/C-534A/V-537A that bound oestradiol *in vitro* and in transfected cells (since they stimulated transcription). At 10^{-8}M oestradiol ~90% of these receptors would be expected to be occupied with ligand. To try to ensure that nearly all the receptors were saturated with ligand the experiment was repeated using 10^{-7}M oestradiol (a 100-fold greater concentration than the dissociation constant) but no mobility shift was observed (data not shown). Since these mutants stimulated transcription in the presence of oestradiol implying that TAF-2 was active, Figure 5.1 (a) these data suggest that the mobility shift may not be indicative of oestradiol binding or an active TAF-2. The mobility of the mutant M-547A/L-548A-probe complex increased slightly in the presence of 10^{-8}M oestradiol but not to the same extent as the wild-type receptor(data not shown). This mutant has negligible TAF-2 activity. The mutant D-542N/E-546Q/D-549N in MOR 1-599 or MOR 121-599 exhibited an oestradiol induced increase in mobility that was indistinguishable from that of the wild-type receptor or MOR 121-599 (data not shown).

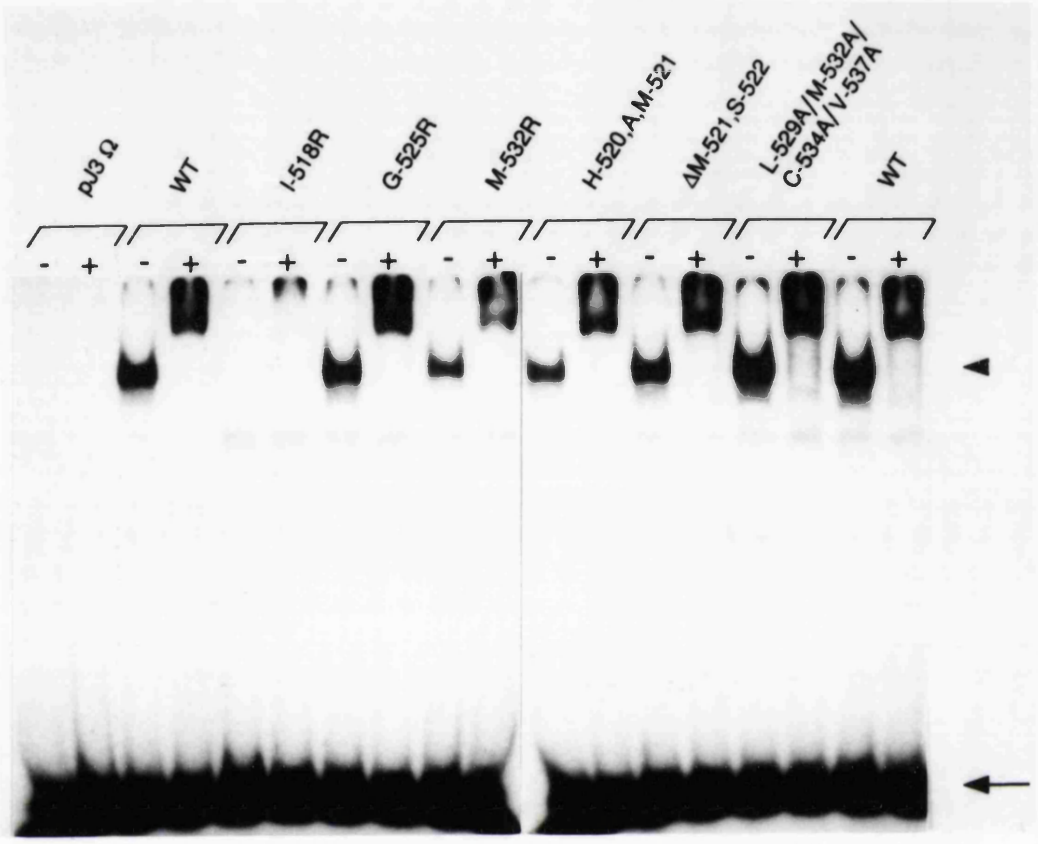


Figure 5.4. DNA binding activity of the wild-type and mutant receptors expressed in COS-1 cells.

The ability of the mutant receptor proteins to bind to DNA was assessed in a band shift assay. Equal amounts (4μg of protein) of each COS-1 cell extract were incubated with a labelled DNA probe containing an ERE (see methods). WT indicates the wild-type receptor. The oestrogen receptor-probe complexes were identified using a oestrogen receptor-specific antiserum MP16 (+), a (-) indicates the addition of pre-immune serum. pJ3Ω is the expression vector lacking receptor cDNA. The arrow and arrowhead indicate free and retarded probe respectively.

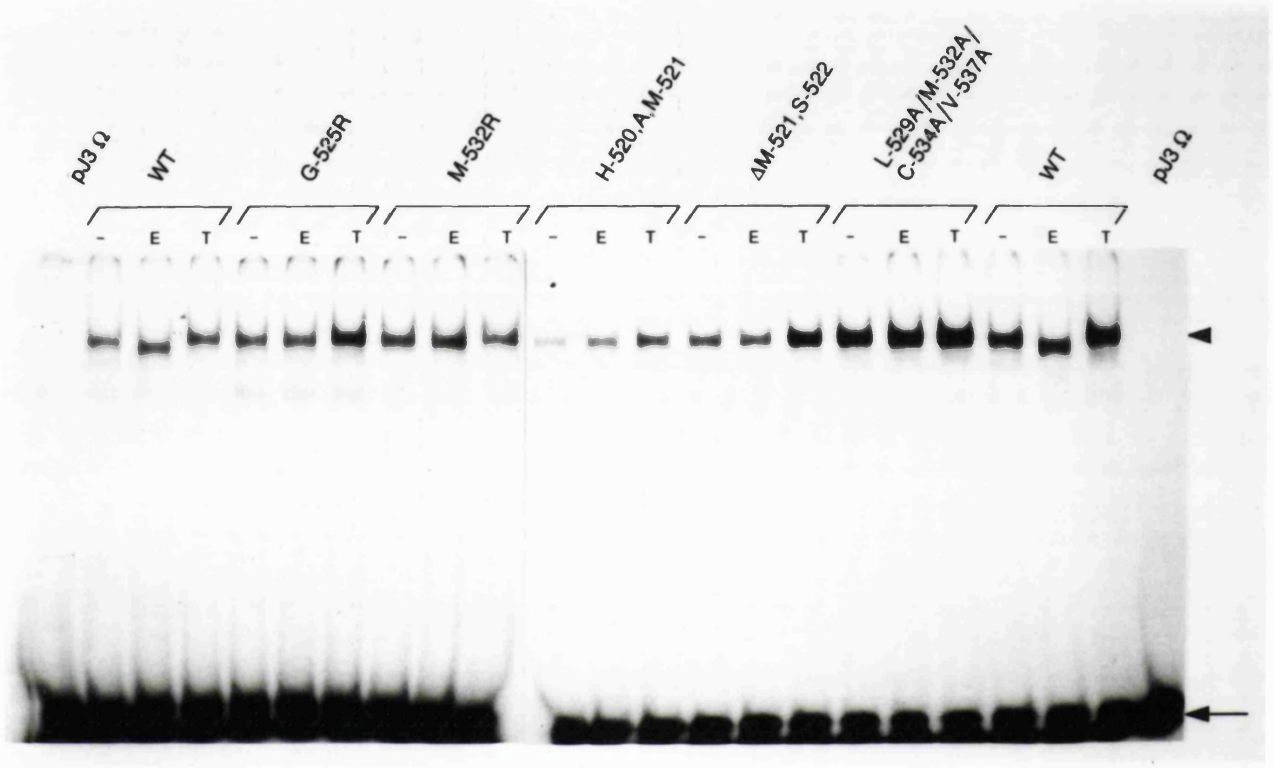


Figure 5.5. The effects of ligand on the ability of the wild-type and mutant receptors to bind DNA.

Equal amounts (2μg of protein) of each COS -1 cell extract were incubated with a labelled DNA probe containing an ERE (see methods) in the presence of 0.01% ethanol (-), 10^{-8}M oestradiol (E) or 10^{-8}M 4-hydroxytamoxifen (T). WT indicates the wild-type receptor. The arrow and arrowhead indicate free and retarded probe respectively.

These mutations also practically abolished TAF-2 activity but not cooperation between the N-and C-terminal domains (chapter 3). These data also suggest that the mobility shift may not be indicative of the formation of an active TAF-2.

In some cases the amount of receptor that bound DNA increased upon the addition of ligand. For example the amount of probe bound by H-520, A, M-521 increased upon the addition of oestradiol and 4-hydroxytamoxifen. These data may suggest that at room temperature H-520, A, M-521 can bind oestradiol whilst it appears to bind oestradiol with a very low affinity in transfected cells. The mutants G-525R and Δ M-521, S-522 that had very low affinities for oestradiol bound more probe in the presence of 4-hydroxytamoxifen only. These results suggest that ligand binding may aid DNA binding by oestrogen receptors. Since these mutations were in a region of the hormone binding domain important for receptor dimerisation (Fawell *et al.*, 1990a) one possibility is that ligand binding may promote receptor dimerisation.

Conclusions.

The results indicate that mutation of amino acids 520 to 522 (in Δ M-521, S-522 and H-520, A, M-521) or 525 (in G-525R) causes a dramatic decrease in the ability of the receptor to stimulate transcription in the presence of oestradiol whilst not significantly affecting transcriptional activation, by TAF-1, in the presence of 4-hydroxytamoxifen. Therefore these residues may be important for discriminating between oestradiol and 4-hydroxytamoxifen. The results also suggest that the binding sites for these ligands can be distinguished at the amino acid level. The lack of transcriptional activation in the absence of ligand (in CEF cells where TAF-1 is active) by those receptors that failed to bind oestradiol but bound DNA *in vitro* suggest that ligand binding may be required for DNA binding in transfected cells. The ability of one mutant H-520, A, M-521 to bind oestradiol may be temperature sensitive and finally the mobility change of the oestrogen receptor-probe complex observed in a band shift assay upon the addition of oestradiol is probably not indicative of the formation of an active TAF-2.

Chapter 6

Discussion

The hormone dependent transcriptional activation function in nuclear receptors.

All members of the nuclear receptor family contain a similar DNA binding domain, composed of two zinc fingers, that is conserved at the sequence, structural and functional level (chapter 1). Additionally the conservation of protein sequence suggests that they all have a similar ligand binding domain. Since some members of the nuclear receptor family act as ligand inducible transcription factors regions of the protein involved in hormone dependent transcriptional activation may also be conserved.

A "TAF-2 like" activity in steroid hormone receptors.

The first section of this thesis described the identification of amino acids important for the hormone dependent transcriptional activation function, TAF-2, of the mouse oestrogen receptor. These amino acids were located in a region conserved in many members of the nuclear receptor family and were also shown to be important for ligand dependent transcription by the mouse glucocorticoid receptor. These results suggested that many members of the nuclear receptor family may contain a "TAF-2 like" activity that requires these conserved amino acids.

A transcriptional activation domain similar to TAF-2 of the oestrogen receptor was proposed to exist in the glucocorticoid receptor by Tasset *et al.*, (1990). This conclusion was based on assays that examined a phenomenon called squelching. Squelching involves a transcriptional activation domain binding specific limiting factors, target(s), that are normally involved in its ability to stimulate transcription thus possibly preventing their use by transcription factors bound near the target gene (Ptashne, 1988 and see chapter 1). These assays tested the ability of the hormone dependent activation functions of the glucocorticoid and oestrogen receptors, the transcriptional activation domains located in the N-terminal regions of the oestrogen and glucocorticoid receptors and the acidic activation domains of the Herpes simplex virus protein VP16 and the yeast transcription factor GAL4 to interfere with transcription stimulated by each of these proteins. The results of these experiments indicated that the C-terminal activation domains of the oestrogen receptor and the glucocorticoid receptor required common factors to stimulate transcription suggesting that the glucocorticoid C-terminal domain contained a "TAF-2 like" activation function (Tasset *et al.*, 1990). Additionally these factors were different from those required by acidic activation domains to stimulate transcription. In these experiments the transcription from a basal promoter was unaffected suggesting that these target(s), factors, were not involved in basal transcription. Oehler and Angel, (1992) also suggested, on the basis of similar assays, that the oestrogen receptor contained transcriptional activation domains that required target(s) different from acidic

activation domains to stimulate transcription. In addition to the "TAF-2 like" activation domain, Tasset *et al.*, (1990) proposed that a weak acidic activation domain was present in the C-terminal activation domain of the glucocorticoid receptor and that this could be τ2 described by Hollenberg and Evans, (1988). Other studies of the glucocorticoid, oestrogen and progesterone receptors indicated that they required a similar pool of limiting target(s) to stimulate transcription (Meyer *et al.*, 1989) this in combination with the results of Tasset *et al.*, (1990) may suggest that a "TAF-2 like" activity may also be present in the progesterone receptor. Whether this activity is dependent upon the conserved region has yet to be examined.

In the oestrogen receptor loss of TAF-2 activity reduces transcriptional activation from natural or artificial promoters to less than 10% that of the wild-type receptor in a number of cell lines suggesting that it plays a major role in oestrogen receptor function (Kumar *et al.*, 1987; Bocquel *et al.*, 1989; Lees *et al.*, 1989; Tora *et al.*, 1989b; Danielian *et al.*, 1992). TAF-2 also appears to be important for oestrogen receptor function *in vivo* since ligands that are thought to block TAF-2 activity whilst not significantly affecting receptor DNA binding and TAF-1 activity, such as tamoxifen and 4-hydroxytamoxifen (Kumar and Chambon, 1988; Webster *et al.*, 1988b; Lees *et al.*, 1989; Berry *et al.*, 1990), can act as antioestrogens *in vivo* and fail to significantly stimulate the transcription of some endogenous oestrogen responsive genes (for examples see Westley *et al.*, 1984; Katzenellenbogen *et al.*, 1985; May and Westley, 1987; May *et al.*, 1989; Jordan and Murphy, 1990).

In the case of the glucocorticoid receptor, however, as described in chapter 1, a strong acidic transcriptional activation domain is located N-terminal to the DNA binding domain that in some cases appears to be responsible for the majority of the transcriptional activation stimulated by the receptor. Only a few reports identified a "TAF-2 like" activity in the glucocorticoid receptor (Hollenberg and Evans 1988; Webster *et al.*, 1988b; Danielian *et al.*, 1992) and in most studies no "TAF-2 like" activity was detected suggesting that it may not contribute significantly to the ability of the receptor to stimulate transcription. In chapter 3 mutation of the conserved residues in the full-length mouse glucocorticoid receptor abolished transcriptional activation suggesting that a mutant TAF-2 may repress the activity of the N-terminal activation domain. The analysis of these receptors indicated that they bound steroid and DNA *in vivo* suggesting that the C-terminal activation domain may be important for transcriptional activation of the full-length receptor. Other point mutations located within the hormone binding domain of the rat glucocorticoid receptor have been reported to nearly abolish transcriptional activation by the full-length receptor in transfected cells but not significantly affect at least one other receptor function, namely hormone binding (Garabedian and Yamamoto, 1992). Despite these results the consensus view is that the N-terminal activation domain is

the major transcriptional activation domain in the glucocorticoid receptor and possibly the major role of the C-terminal region containing a "TAF-2 like" activity is to confer ligand inducibility.

Several different conclusions have been reached following the analysis of progesterone receptors mutants. For example the N-terminal domain (Carson *et al.*, 1987; Guiochon-Mantel *et al.*, 1988), N-and C-terminal domains (Gronemeyer *et al.*, 1987; Meyer *et al.*, 1990) or a small region encompassing the DNA binding domain (Bradshaw *et al.*, 1991) have been proposed to be important for transcriptional activation. Despite these results the C-terminal domain of the human and chicken progesterone receptors stimulate transcription in a hormone dependent manner attached to a heterologous DNA binding domain suggesting the existence of a "TAF-2 like" activity (Meyer *et al.*, 1990). This transcriptional activation, in the case of the human receptor, was blocked by the hormone antagonist RU486 that appears to promote DNA binding by the receptor but prevent the formation of the "TAF-2 like" activity (Guiochon-Mantel, *et al.*, 1988; Meyer *et al.*, 1990). RU486 is not bound by the chicken receptor (see later). Analysis of the ability of the full-length human receptor to stimulate transcription from a MMTV based reporter or a PRE/GRE-tk-CAT reporter in the presence of RU486 suggests that the "TAF-2 like" activity was responsible for the majority of the transcriptional activation in the presence of agonist (Meyer *et al.*, 1990). In these studies the authors showed that transcriptional activation observed in the presence of RU486 was derived from the constitutively active N-terminal activation domain suggesting that the receptor bound the response element in this assay. This result is in contrast to other assays that suggest that RU486 may prevent steroid receptors from binding to DNA *in vivo* (Becker *et al.*, 1986; Reik *et al.*, 1991). When a minimal promoter containing only a GRE and a TATA box was used a mutant lacking the C-terminal region (the "TAF-2 like" activity) was able to stimulate transcription nearly as efficiently as the full-length receptor (Meyer *et al.*, 1992). These data suggest that the contribution of the "TAF-2 like" activity to transcriptional activation by the full-length progesterone receptor may be promoter dependent.

The removal of the region N-terminal to the DNA binding domain of the androgen receptor reduces the transcriptional activation to basal levels and no "TAF-2 like" activity has been reported, also the major transcriptional activation domain appears to be located in the N-terminus (Rundlett *et al.*, 1990; Jenster *et al.*, 1991; Simental *et al.*, 1991). In the vitamin D receptor one mutant that lacked the majority of hormone binding domain stimulated transcription constitutively, at a level slightly lower than that of the ligand bound full-length receptor (McDonnell *et al.*, 1989). Again no evidence for a "TAF-2 like" activity was presented. Whether the androgen, vitamin D or the mineralocorticoid receptors have a "TAF-2 like" activity is, at the

moment, unclear. As suggested for the glucocorticoid receptor from these experiments a major role of the C-terminus may be to confer hormone inducibility. The importance of a C-terminal activation domain could be assessed by the mutation of the conserved hydrophobic amino acids that abolished hormone dependent transcriptional activation in the oestrogen and glucocorticoid receptors.

Thyroid hormone, retinoic acid and retinoid X receptors.

These receptors function as ligand inducible transcription factors but differ in several respects from steroid hormone receptors. Firstly these receptors bind to DNA response elements without binding a ligand (Damm *et al.*, 1989; Graupner *et al.*, 1989) secondly many natural response elements bound by these receptors are not inverted repeats like those bound by steroid receptors but direct repeats of similar sequences (Naar *et al.*, 1991; Umesono *et al.*, 1991; Mader *et al.*, 1993a; see chapter 1) and thirdly in contrast to the steroid receptors, that bind DNA as homodimers, the thyroid hormone and retinoic acid receptors can preferentially bind response elements as heterodimers with retinoid X receptors (Yu *et al.*, 1991; Bugge *et al.*, 1992; Kliewer *et al.*, 1992b; Leid *et al.*, 1992b; Marks *et al.*, 1992; Zhang *et al.*, 1992).

The functional analysis of deletion mutants of the thyroid hormone receptor (type β) have shown that the region N-terminal to the DNA binding domain can be removed without significantly affecting transcriptional activation in the presence of hormone, T_3 (Thompson and Evans, 1989). This suggests that a "TAF-2 like" activity is responsible for the majority of the transcriptional activation. In addition the hormone binding domain of the thyroid hormone receptor (type α) is able to stimulate transcription when attached to a heterologous DNA binding domain (Baniahmad *et al.*, 1992). Other analysis of the thyroid hormone receptors suggested amino acids located near the N-terminal end of the hormone binding domain, are important for hormone dependent transcriptional activation. O'Donnell and Koenig, (1990) identified three amino acids in the thyroid hormone receptor (type β-1) that were important for transcriptional activation but not for the ability of the receptor to bind hormone and DNA. The amino acids were also not important for nuclear localisation of receptors. These amino acids were in a sequence conserved in other nuclear receptors raising the possibility that they are another conserved part of a "TAF-2 like" activity present in nuclear receptors. In another study Lee and Mahdavi, (1993) identified point mutations in the thyroid hormone receptor type α–1 (also near the N-terminus of the hormone binding domain) that reduced the ligand dependent transcriptional activation to less than 10% the level stimulated by the wild-type receptor. This mutant was correctly located in the nucleus and retained high affinity hormone and DNA binding. The analysis of v-erbA, reviewed below, suggests the thyroid hormone receptor (type α) in addition to the amino acids indicated above

requires amino acids in the conserved region described in chapter 3 for transcriptional activation.

Like the thyroid hormone receptor analysis of deletion mutants of the retinoic acid receptor (RAR), type α, indicated that a ligand dependent transcriptional activation domain may be responsible for the majority of the transcriptional activation of the full-length receptor (de The *et al.*, 1991; Kakizuka *et al.*, 1991) and that the hormone binding domain stimulates transcription in a hormone dependent manner when attached to a heterologous DNA binding domain suggesting the presence of a "TAF-2 like" activity (Baniahmad *et al.*, 1992).

Futher analysis of mutant retinoic acid and retinoid X receptors lacking the region N-terminal to the DNA binding domain have shown that a ligand dependent transcriptional activation domain is present but the level of transcription stimulated, relative to the wild-type receptor, varies depending on the response element and its promoter context (Nagpal *et al.*, 1992). The regions N-terminal to the DNA binding domain were not reported to stimulate transcription alone but appeared to modulate the "TAF-2 like" activity. Recent studies have shown that a deletion mutant of RAR type α that lacks the conserved region is transcriptionally inactive but retains the ability to bind DNA and retinoic acid with high affinity supporting the possibility that the conserved region is important for hormone dependent transcriptional activation (Damm *et al.*, 1993).

The deletion of the conserved region in v-erbA abolishes transcriptional activation.

The viral oncogene *v-erbA* encodes a mutated thyroid hormone receptor (type α) that may act by repressing normally T_3 inducible genes (for a review see Beug and Vennstrom, 1991). The mutations include a N-terminal deletion, two point mutations in the DNA binding domain, nine in the hormone binding domain and a nine amino acid deletion in the C-terminus of the protein. These mutations abolish high affinity T_3 binding but do not significantly affect the ability to bind DNA (Sap *et al.*, 1986; Munoz *et al.*, 1988; Boucher *et al.*, 1988).

To locate the mutations important for the oncogenic activity of v-erbA sequences containing the different mutations were replaced with the corresponding sequences of the thyroid hormone receptor and the resulting chimeric proteins tested in a number of assays. These experiments showed that the nine amino acid deletion practically abolished hormone dependent stimulation of transcription but did not abolish ligand binding by the receptor and contributed, in part, to the oncogenic activity of v-erbA (Zenke *et al.*, 1990). This deletion removed both pairs of hydrophobic residues in the conserved motif, amino acids 399 to 407 in the human thyroid hormone receptor (type α-1) shown in Figure 3.1. The authors also suggested that the protein sequence in this region was conserved, in the thyroid hormone and

retinoic acid receptors and predicted that it may form an amphipathic α-helix in which the polar face was composed of acidic amino acids. Further studies showed that the replacement of the conserved acidic or hydrophobic residues in the conserved region of the thyroid hormone receptor (type α) with other amino acids essentially abolished transcriptional activation but not ligand binding reinforcing the possibility that the conserved region may be important for a "TAF-2 like" activity in many members of the nuclear receptor family (Saatcioglu *et al.*, 1993).

The ecdysone receptor and orphan receptors.

The sequence alignment in Figure 3.1 shows members of the nuclear receptor family in which the conserved motif (loosely defined as a glutamic acid residue flanked by pairs of bulky hydrophobic residues) was present. In Figure 6.1 less well conserved sequences found in other members of the nuclear receptor family are shown. The *knirps* encoded subfamily from *Drosophila* has been omitted because the sequence 3' to that which encodes the putative DNA binding domain in these genes has no significant similarity to those encoding a "nuclear receptor ligand binding domain" (see Oro *et al.*, 1992). A conserved sequence was also, apparently, absent from Rev-ErbAα (Lazar *et al.*, 1989), TR2 and variants (Chang and Kokontis, 1988; Chang *et al.*, 1989), ELP/SF-1 (Lala *et al.*, 1992; Tsukiyama *et al.*, 1992) and the *Drosophila* proteins E75A and B (Seagraves and Hogness, 1990) and FTZ-F1 (Lavorgna *et al.*, 1991). Some members are not shown as it was difficult to assess unambiguously if an amino acid sequence constituted a sequence similar to the conserved motif.

Of the receptors represented in Figure 6.1 only the mouse oestrogen receptor (included to indicate the position of the conserved residues) and the ecdysone receptor, EcR, bind a ligand (Koelle *et al.*, 1991) the others are orphan receptors. Note that all other members of the nuclear receptor family known to bind a ligand are represented in Figure 3.1. The ecdysone receptor has been shown to be capable of ligand dependent transcriptional activation in ecdysone-resistant S2 cells but from this report it is not possible to assess if this is due to a "TAF-2 like" activity (Koelle *et al.*, 1991). Unlike most nuclear receptors the ecdysone receptor contains a long proline and glutamine amino acid rich sequence C-terminal to the conserved region potentially important for a "TAF-2 like" activity (Koelle *et al.*, 1991). Proline and glutamine rich regions have been found in transactivation domains of several transcription factors (see chapter 1). This region may therefore function as an transcriptional activation domain, possibly in place of a "TAF-2 like" activity. Similarly the hepatocyte nuclear factor-4 (HNF-4, represented in Figure 3.1) contains a sequence located C-terminal to the conserved residues that contains a high percentage of proline residues (Sladek *et al.*, 1990).

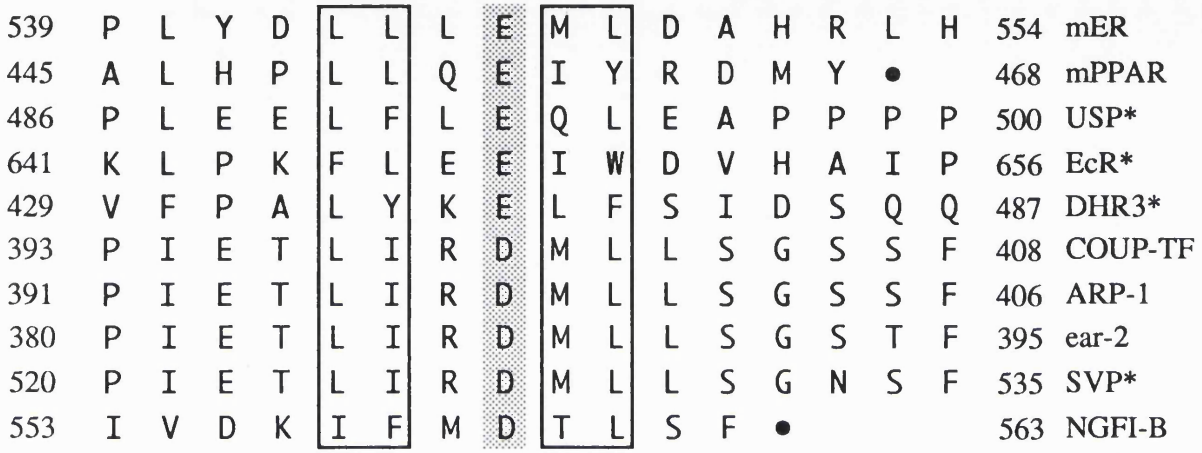


Figure 6.1. Sequence alignment of nuclear receptor proteins.

The sequences shown are: the mouse oestrogen receptor, White *et al.*, (1987); mouse peroxisome proliferator-activated receptor, Issemann and Green, (1990); ultraspiracle, Oro *et al.*, (1990); ecdysone receptor, Koelle *et al.*, (1991); *Drosophila* hormone receptor 3, Koelle *et al.*, (1992); chicken ovalbumin upstream promoter transcription factor, Wang *et al.*, (1989); apolipoprotein AI regulatory protein, Ladias and Karathanasis, (1991); v-erbA-related protein 2, Miyajima *et al.*, (1988); seven-up (type 1), Mlodzik *et al.*, (1990); nerve growth factor induced clone B, Milbrandt, (1988). The "conserved" hydrophobic residues are boxed and the acidic residue shaded. The amino acid numbers are shown and the discs indicate the C-terminus of the protein, the asterisks indicate *Drosophila* proteins .

In one orphan receptor NGFI-B, also called Nur77 or N10, encoded by an early response gene, a major transcriptional activation domain was identified in the N-terminus raising the possibility that it lacks a "TAF-2 like" activity (Davis *et al.*, 1991; Paulsen *et al.*, 1992).

Some of the receptors cloned from *Drosophila* are considered to be homologues of mammalian nuclear receptors. For example ultraspiracle (Oro *et al.*, 1990) and seven-up, type 1, (Mlodzik *et al.*, 1990) appear to be *Drosophila* homologues of the retinoid X receptor and chicken ovalbumin upstream promoter transcription factor (COUP-TF, Wang *et al.*, 1989) respectively. The functional analysis of orphan receptors has shown that in transfection experiments they can repress or stimulate transcription but the regions of the protein responsible for transcriptional activation or repression have not been located in most cases. For example FTZ-F1 (that lacks the conserved region) stimulates transcription of the *Drosophila* alcohol dehydrogenase gene whilst another member of the nuclear receptor family expressed in *Drosophila*, DHR39 (represented in Figure 3.1) that binds to a similar DNA sequence represses transcription of this gene (Ayer *et al.*, 1993). Similarly COUP-TF and ARP-1 have been shown to repress transcription stimulated by several members of the nuclear receptor family including HNF-4, retinoic acid and retinoid X receptors (Cooney *et al.*, 1992; Kliewer *et al.*, 1992a; Ladias *et al.*, 1992; Mietus-Snyder *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992 and see chapter 1). The C-terminus of ARP-1 is nearly identical to that of COUP-TF (Ladias and Karathanasis, 1991) this is also the case for ear-2 (Miyajima *et al.*, 1988), Figure 6.1. These results might suggest that these receptors lack a "TAF-2 like" activity or that a ligand was not bound by the receptor in these experiments.

In one instance a chimera of COUP-TF (containing the DNA binding domain of the progesterone receptor in place of its own DNA binding domain) has been reported to function, in cells, as an inducible transcription factor (Power *et al.*, 1991). The inducer, dopamine, was reported not to be bound by COUP-TF with high affinity suggesting that the activation was not dependent on the binding of dopamine.

The peroxisome proliferator-activated receptors (PPARs) are able to mediate an increase in the transcription of specific genes in cells upon the addition of a wide variety of chemicals (including some fatty acids) to the culture medium that, as yet, have not been shown to be bound with high affinity by PPARs (for examples see Isseman and Green, 1990; Dreyer *et al.*, 1992; Gottlicher *et al.*, 1992; Tugwood *et al.*, 1992; Keller *et al.*, 1993). Chemicals that induce PPARs could act by causing the binding of a ligand and/or induce transcriptional activation by means of posttranslational modifications. Recent studies have shown that NGFI-B, Ad4BP and ELP/SF-1 can regulate transcription of genes encoding steroidogenic enzymes (Lala *et al.*, 1992; Honda *et al.*, 1993; Wilson *et al.*, 1993) whilst HNF-4, COUP-TF,

ARP-1 and PPARs regulate genes that are important for cholesterol and/or lipid metabolism (Ladias *et al.*, 1992; Mietus-Snyder *et al.*, 1992; Tugwood *et al.*, 1992; Keller *et al.*, 1993). One possibility is that metabolites of steroids, cholesterol or lipids are ligands for these receptors and thus regulate the expression of these genes by a feedback mechanism.

In conclusion the conserved region contains amino acids important for transcriptional activation by the oestrogen, glucocorticoid and thyroid hormone (type α) receptors that may also be important for ligand dependent transcriptional activation by many members of the nuclear receptor family. The importance of the less well conserved sequences in some orphan receptors that may not bind a ligand is, as yet, unclear.

Cooperation between the N-and C-terminal domains in stimulating transcription.

The results described in chapter 3 indicated that the region N-terminal to the DNA binding domain of mouse oestrogen receptor, containing TAF-1 was important for maximal transcriptional activation in some situations. In these cases the level of transcription stimulated by the full-length receptor was greater than that stimulated by the N-and C-terminal activation domains, when tested individually, added together. This suggested that the two domains could cooperate, or synergise, to stimulate transcription in the full-length receptor. Similar observations for the mouse and human oestrogen receptors have been reported previously (Kumar *et al.*, 1987; Lees *et al.*, 1989; Tora *et al.*, 1989b; Tasset *et al.*, 1990).

Another explanation for these results is that the N-terminus is important for the structure of the C-terminus of the protein and consequently the activity of TAF-2. This, however, is unlikely since the protein appears to fold such that TAF-2 and TAF-1 can function independently (Webster *et al.*, 1988b; Lees *et al.*, 1989; Tora *et al.*, 1989b; Berry *et al.*, 1990; chapter 3) and cooperate in stimulating transcription (Tora *et al.*, 1989b; Tasset *et al.*, 1990) attached to heterologous DNA binding domains. Additionally deleting the N-terminus from the wild-type oestrogen receptor does not significantly alter its affinity, *in vitro*, for DNA or oestradiol or its ability to dimerise suggesting that the protein must be at least partially folded correctly (Kumar *et al.*, 1986; Kumar and Chambon, 1988; Fawell *et al.*, 1990a).

Analysis of the mutant receptors in which conserved amino acids had been replaced with different residues showed that cooperation occurred when the highly conserved glutamic acidic residue had been changed but not when the conserved hydrophobic amino acids had been replaced by alanine residues. The different effects of mutating the hydrophobic and acidic residues may be because the mutation of the hydrophobic residues affected TAF-2 activity more severely. However, mutation of

all three acidic amino acids in the conserved region (in D-542N/E-546Q/D-549N) practically abolished TAF-2 activity but this mutant still stimulated transcription in the full-length receptor suggesting that cooperation was occurring (chapter 3). This may suggest that the acidic residues are not essential for cooperation whilst the hydrophobic amino acids are important for TAF-2 activity and cooperation. Analysis of mutant human oestrogen receptors corresponding to D-542N/E-546Q/D-549N also indicated that cooperation occurs between the N-and C-terminal domains in stimulating transcription (D. McDonnell, Ligand Pharmaceuticals Inc, San Diego, personal communication).

The analysis of the N-terminal deletion mutants indicated that the removing 120 amino acids of the mouse oestrogen receptor significantly reduced cooperation between the N-and C-terminal domains. Based on the results of Ali *et al.*, (1993) this may, in part, be due to the loss of phosphorylation of a serine at position 122 in the mouse oestrogen receptor (H. Lahooti, Molecular Endocrinology, ICRF personal communication). In the human oestrogen receptor phosphorylation of the corresponding serine residue was shown to influence the cooperation between TAF-1 and TAF-2 (Ali *et al.*, 1993). Interestingly in the sequence that contained the N-terminal boundary of the region important for cooperation in NIH 3T3 cells and TAF-1 activity in CEF cells (between amino acids 91 and 121 of the mouse oestrogen receptor) 20% of the amino acids are proline residues. Proline rich regions have been found in transcriptional activation domains of other transcription factors including a N-terminal activation domain of the progesterone receptor (Meyer *et al.*, 1992).

A possible model for cooperation between the N-and C-terminal domains involves TAF-1 and TAF-2 contacting target(s) that mediate an increase in the level of transcription (reviewed in Roeder, 1991; Pugh and Tjian, 1992; Herschlag and Johnson, 1993). For convenience the level of transcription stimulated might be assumed to be directly proportional to the strength of these contacts. The targets for TAF-1 and TAF-2 may be different or identical. The ability of, for example, TAF-1 to stimulate transcription efficiently in CEF cells may be due to the presence of a target that is absent from NIH 3T3 cells or to differences in the strengths of the contacts with identical target proteins. In NIH 3T3 cells it is possible that TAF-1 contacts targets weakly and that TAF-2 (MOR 121-599) makes slightly stronger contacts with its target(s) whilst in the full-length receptor the simultaneous contact of a single or multiple targets by TAF-1 and TAF-2 results in a much higher level of transcription (see Figure 3.10). The C-terminus may also interact directly with the N-terminus increasing the activity of TAF-1 and/or TAF-2. Cooperation may also occur if agonist bound TAF-2 is able to negate the affects of a repressor of TAF-1. This possibility has been suggested from studies of *SSN6*, a yeast gene encoding a repressor of

transcription, whose affects, in yeast, were proposed to be negated by active TAF-2 (McDonnell *et al.*, 1992).

The mutations in the conserved region therefore may weaken the contacts between TAF-2 and its target(s) or possibly affect an earlier step required for transcriptional activation that has not been examined. The conserved amino acids identified as being important for transcriptional activation may contact the target(s) of TAF-2 directly or be important for the structure of the interacting surface either directly or by allowing posttranslational modifications. In the wild-type receptor the removal of the N-terminus (in the mutant MOR 121-599) had no significant effect on the ability of the oestrogen receptor to stimulate transcription from pERE BLCAT whilst it reduced the level of transcriptional activation, approximately 5-fold, when pERE MLTCAT was used. This may be due to the presence of different endogenous factors binding to the promoter, differences in the TATA box sequence or differences in the positions of factor binding sites. With regards to the model it is possible that a factor binding to the promoter of pERE BLCAT is able to substitute for TAF-1 in cooperating with TAF-2 when TAF-1 is removed. pERE MLTCAT being a minimal promoter may lack a binding site for a suitable factor. Mutations in TAF-2, however, affect the ability to cooperate with the endogenous transcription factors bound to pERE BLCAT resulting in a decrease in the level of transcription.

Cooperation between the N-and C-terminal domains of the glucocorticoid and progesterone receptors (Godowski *et al.*, 1988; Bocquel *et al.*, 1989) in stimulating transcription has been reported and the analysis of the retinoic acid and retinoid X receptors have indicated that the N-and C-terminal domains can cooperate in a promoter dependent fashion (Nagpal *et al.*, 1992). Cooperation between the N-and C-terminal domains of these receptors may also be affected by mutations in the conserved region. The presence of (at least) two transcriptional activation domains in some members of the nuclear receptor family could dramatically increase the diversity of genes regulated.

The cooperation of activation domains in stimulating transcription also occurs in other transcription factors. For example in the mammalian transcription factor Oct-2 a proline, serine and threonine rich activation domain C-terminal to the DNA binding domain is able to cooperate with a N-terminal glutamine rich activation domain to stimulate transcription from mRNA promoters (Tanaka and Herr, 1990). This study also showed that the C-terminal domain of Oct-2 in combination with the glutamine rich N-terminal region of Oct-1 (a closely related protein that stimulates transcription from small nuclear RNA promoters) confers upon Oct-1 the ability to stimulate transcription efficiently from mRNA promoters. In the transcription factor CREB an activation region called α was reported to cooperate with the activation

induced by the phosphorylation of CREB by protein kinase A (Yamamoto *et al.*, 1990). Additionally domains located in the N-and C-terminal regions of the muscle-specific transcription factor Myf-5, that are able to stimulate transcription independently when attached to a heterologous DNA binding domain, cooperate to stimulate transcription in the full-length protein (Winter *et al.*, 1992).

In the last section of chapter 3 an attempt was made to assess if in a dimer the C-terminus of 121-599 could cooperate with the N-terminus of 1-599. The full-length receptor contained mutations that abolished TAF-2 activity. In these experiments, however, no cooperation was detected, one possibility is that direct intramolecular interactions between the N-and C-terminal activation functions required for cooperation were not possible in this experiment. Another explanation is that the mutations that abolish TAF-2 may also affect TAF-1 activity and thus prevent cooperation. However, the mutations that abolished TAF-2 did not abolish the phosphorylation of the serine residue in the N-terminus which had been indicated by Ali *et al.*, (1993) to be important for maximal cooperation between TAF-1 and TAF-2 (H. Lahooti, Molecular Endocrinology, ICRF personal communication). In CEF cells TAF-1 of this mutant was as active as that of the wild-type receptor (S. Hoare, Molecular Endocirnology, ICRF, personal communication) but this activity, being cell specific may not be relevant in NIH 3T3 cells. The the lack of cooperation may therefore not be due to a defect in TAF-1. A further explanation for the lack of cooperation is that the presence of a single TAF-2 and/or TAF-1 may not be sufficient for cooperation. Finally it is also possible that the mutation of the hydrophobic residues may result in a mutant that is able to interfere with the activity of a partner. This is difficult to assess, experimentally, in the case of the oestrogen receptor since an inactive mutant will compete with the receptor for DNA binding sites as well as possibly affecting the TAF-2 or TAF-1 activity of a partner in a dimer.

The possibility that the activity of a member of a dimer is affected by its partner has been addressed by analysing the activity of nuclear receptors that are suspected to heterodimerise rather than homodimerise *in vivo*. In some of these cases transcription is stimulated if a ligand that is bound by one member of a heterodimer is present indicating that it may be possible for a "TAF-2 like" activity to function when the "TAF-2 like" activity of the partner is inactive (for examples see Durand *et al.*, 1992; Rosen *et al.*, 1992; Carlberg *et al.*, 1993). The ability of an inactive mutant to affect the activity of a partner was examined directly in the following experiment by Durand *et al.*, (1992). The retinod X receptor α (RXRα) was cotransfected with a truncated retinoic acid receptor α (RARα) that lacked part of the hormone binding domain (including the conserved region), RAR and RXR have been proposed to form heterodimers on DNA in preference to homodimers (see chapter 1). This truncated receptor failed to bind ligand and stimulate transcription but retained the ability to

bind DNA and heterodimerise with RXR. Transcriptional activation was only observed in the presence of the ligand for RXR (9-*cis* retinoic acid) but not in the presence of 50 nM all-*trans* retinoic acid (which does not activate RXR but will activate RAR) suggesting that the "TAF-2 like" activity of RXR was functional in a heterodimer with the mutant RAR. The reciprocal experiment with a mutant RXR gave similar results (Durand *et al.*, 1992).

These data suggest that an inactive receptor may not interfere with the "TAF-2 like" activity of its partner in a dimer. Considering these results it is possible that the mutant in which the hydrophobic amino acids had been replaced may not interfere with the activity of the TAF-2 in its partner in a dimer making one of the other explanations described for the lack of cooperation more likely. Since the conserved region described in this thesis was located next to a region important for receptor dimerisation, that may be part of the dimer interface (Fawell *et al.*, 1990a; Lees *et al.*, 1990), one possibility was that TAF-2 was formed upon dimerisation of the conserved sequence, however, the above data suggest that this may not be the case.

Ligand and the activity of TAF-2.

Studies using hormone antagonists such as 4-hydroxytamoxifen and RU486 indicate that ligand plays an important role in determining the activity of the hormone dependent transcriptional activation function of the oestrogen, progesterone and glucocorticoid receptors (Webster *et al.*, 1988b; Lees *et al.*, 1989; Berry *et al.*, 1990; Meyer *et al.*, 1990). Initial studies of TAF-2 of the oestrogen receptor indicated that it was probably composed of elements throughout the hormone binding domain that form TAF-2 upon the binding of oestradiol (Webster *et al.*, 1989). This proposal was based on the fact that none of the five exons that encode the hormone binding domain were able to stimulate transcription significantly when attached to the DNA binding domain of GAL4. This is in agreement with the observation that the conserved region containing amino acids important for TAF-2 activity failed to stimulate transcription in a similar assay. Alternatively TAF-2 may be active in the absence of ligand but blocked by hsp90, in this model oestrogen binding may only required to dissociate hsp90 and antioestrogens such as tamoxifen may inhibit TAF-2. The binding of ligands such as 4-hydroxytamoxifen appear to promote DNA binding but prevent TAF-2 activity *in vivo* even when TAF-2 is attached to a heterologous DNA binding domain (Webster *et al.*, 1988b; Berry *et al.*, 1990). The study of Webster *et al.*, (1988b) also showed that one GAL4-hormone binding domain (TAF-2) chimera generated, as judged in an *in vivo* interference assay, bound DNA without ligand but failed to stimulate transcription suggesting that TAF-2 is inactive in the absence of oestrogens.

A different view of the formation of TAF-2 was proposed by Vegeto *et al.*, (1992). Using a screen in yeast a mutant progesterone receptor was identified that in mammalian cells, in contrast to the wild-type receptor, stimulated transcription to wild-type levels in the presence of antagonists including RU486. The mutation resulted in the loss of the C-terminal 54 amino acids that contained the conserved amino acids described in chapter 3. The authors suggested that the C-terminal region repressed transcriptional activation and that this repression is normally relieved by agonists or, alternatively, could be relieved by deletion. The authors did not, however, determine if the transcriptional activation observed was derived from TAF-1 or the C-terminus of the receptor (TAF-2).

As this mutant receptor lacks the conserved region the activity may be derived from TAF-1 or, as described in earlier studies, a region close to the DNA binding domain (Bradshaw *et al.*, 1991) although it is not possible to rule out the fact that the remaining amino acids in the C-terminus of the mutant receptor may form an activation domain in the presence of RU486 that does not require the conserved region. These results raise the possibility that point mutations in the conserved region described in chapter 3 that abolished transcriptional activation may in fact prevent oestrogen induced de-repression. However, the functional analysis of several C-terminal deletion mutants of the oestrogen receptor have shown that in the presence of 4-hydroxytamoxifen transcription is stimulated at a low level, by means of TAF-1, suggesting that the C-terminus of the mouse oestrogen receptor may not contain sequences involved in repression as suggested for the progesterone receptor by Vegeto *et al.*, (1992) (Lees *et al.*, 1989 and personal observations).

The importance of the ligand for TAF-2 activity was examined by analysing the ability of several progestins that differed at only one position of the molecule to induce transcriptional activation by the "TAF-2 like" activity of the progesterone receptor (Garcia *et al.*, 1992). The results showed that the level of transcriptional activation did not correlate with the affinity with which the ligand was bound and some ligands, that were bound with high affinity, induced intermediate levels of transcriptional activation indicating the partial formation of TAF-2. These results suggest that the ligand determines the activity of TAF-2. One chimeric receptor containing 15 point mutations (introduced by replacing sequence in the human receptor with the corresponding sequence in the chicken receptor) was cited to stimulate transcription to a level similar to that of the wild-type receptor in the presence of an agonist and an antagonist but stimulated transcription to nearly wild-type levels in the presence of a progestin that was an antagonist for the wild-type receptor. As this mutant retains TAF-2 activity and a wild-type response to other progestins the authors suggested that the position of the ligand in the hormone binding domain influenced the formation of TAF-2. Whether ligand alters the protein

structure directly or induces a posttranslational modification(s), such as phosphorylation, is as yet unknown.

The analysis of the oestrogen receptor, in band shift assays, has shown that the addition of agonists such as oestradiol and diethylstilbestrol causes an increase in the mobility of the receptor-probe complex relative to untreated samples or samples treated with antagonists, such as 4-hydroxytamoxifen, tamoxifen, RU39411, LY117018 and ICI 164, 384 (Kumar and Chambon, 1988; Lees *et al.*, 1989; Martinez and Wahli, 1989; Brown and Sharp, 1990; Curtis and Korach, 1990; Fawell *et al.*, 1990b; Tzukerman *et al.*, 1990; Reese and Katzenellenbogen, 1991a; Sabbah *et al.*, 1991). This change in mobility appears to be associated with a conformational change and/or posttranslational modification near the hormone binding domain as judged by biochemical studies (Fritsch *et al.*, 1992) and is observed when oestrogen receptor mutants that lack the region N-terminal to the DNA binding domain or chimeric proteins containing a heterologous DNA binding domain joined to the hormone binding domain of the receptor are tested in a band shift assay (Brou *et al.*, 1993, personal observations). In addition the induction of this mobility shift is not restricted to a class of ligands having a similar molecular structure since for example oestradiol and ICI 164, 384 are steroidal ligands whilst diethylstilbestrol and tamoxifen are non-steroidal ligands (see Jordan and Murphy, 1990). The mobility shift was proposed to be the result of a ligand induced conformational change and/or posttranslational modification and since it was agonist induced was suggested to be indicative of TAF-2 formation (Lees *et al.*, 1989; Berry *et al.*, 1990; Brown and Sharp, 1990). However, two mutants of the mouse oestrogen receptor M-532R and L-529A/M-532A/C-534A/V-537A (described in chapter 5) and two mutants of the human oestrogen receptor (Pakdel and Katzenellenbogen, 1992), that contained substitutions of residues in the corresponding region of the human receptor, retained the ability to bind oestradiol and stimulate transcription in transient transfection experiments but failed to show a mobility shift in the presence of oestradiol. These data suggest that the mobility shift is not indicative of the formation of an active TAF-2. Based on these results the mobility shift may involve a conformational change or posttranslational modification dependent on sequences between amino acids 529 to 537 in the mouse receptor. Structural differences between oestrogen receptors bound to oestradiol or antagonists have been detected in some biochemical studies using limited proteolysis (Attardi and Happe, 1986) or antibodies (Giambiagi and Pasqualini, 1988; Martin *et al.*, 1988) suggesting that these ligands do induce different structural changes .

In contrast to the oestrogen receptor the progesterone receptor, in a band shift assay, shows an increase in mobility upon binding an antagonist (RU486) that prevents the formation of TAF-2 relative to samples treated with the agonist R5020

(El-Ashry *et al.*, 1989; Meyer *et al.*, 1990; Vegeto *et al.*, 1992). In the progesterone receptor the differences in the conformation of the protein induced by agonist and antagonist appear to involve the last 30-40 amino acids that, interestingly, contain the conserved region described in chapter 3 (Allan *et al.*, 1992a; Vegeto *et al.*, 1992; Weigel *et al.*, 1992). Antibodies to the progesterone receptor that induce DNA binding *in vitro* fail to cause a conformational change and fail to induce transcriptional activation *in vitro* whilst the addition of an agonist to this mixture induces receptor mediated stimulation of transcription (Allan *et al.*, 1992b). The authors suggested that the conformational change was important for transcriptional activation and that DNA binding alone was insufficient. However, one explanation for these results, that was not discounted, was that the bound antibodies inhibited transcriptional activation by the receptor and were displaced upon the binding of ligand. The retinoic acid and thyroid receptors when bound to DNA, possibly as heterodimers, also show a mobility shift in the presence of ligand (Andersson *et al.*, 1992; Mader *et al.*, 1993a).

Ligand binding and receptor function.

The ligand binding domain, located C-terminal to the DNA binding domain, in most nuclear receptors consists of approximately 250 amino acids. In artificial systems the hormone binding domain is able to fold correctly as an independent domain (Kumar *et al.*, 1986; Rusconi and Yamamoto, 1987; Eul *et al.*, 1989) and can also confer hormone inducible "activation" of the biological function of a wide variety of proteins. For example attaching the hormone binding domain of the human oestrogen receptor to the oncoprotein myc can result in oestradiol induced cell transformation (Eilers *et al.*, 1989). The amino acids in the hormone binding domain that are conserved between members of the nuclear receptor family may be involved in a number of functions such as forming the ligand binding site, receptor dimerisation, transcriptional activation and possibly ligand dependent nuclear localisation.

The role of ligand binding in steroid receptor function as indicated above has been investigated by assessing the affects of synthetic ligands and by analysing mutant receptors. Functional analysis of mutant receptors has identified the approximate boundaries of the hormone binding domain and mutations that alter the affinity and/or specificity for particular ligands. Point mutations that reduce the affinity of the receptor significantly may identify critical amino acids involved in forming the ligand binding pocket. This type of mutation has been found to occur naturally and leads to hormone resistance. For example the analysis of complete androgen insensitivity patients has identified more than 9 point mutations throughout

the hormone binding domain of the androgen receptor that practically abolish ligand binding and transcriptional activation (Brown *et al.*, 1990; McPhaul *et al.*, 1991; Ris *et al.*, 1991; Brinkmann and Trapman, 1992). Point mutants of the mouse glucocorticoid receptor have been found in mouse lymphoma cell lines that are not growth inhibited by glucocorticoids (Danielsen *et al.*, 1986; Byravan *et al.*, 1991). These receptors have very low affinities for dexamethasone and are practically transcriptionally inactive. For the oestrogen receptor, however, no naturally occurring mutations have been identified suggesting that they may be fatal early in development. Some mutations introduced *in vitro* reduce the affinity for oestradiol dramatically. For example the mutations at positions 521/522 and 525 in the mouse oestrogen receptor reduced the affinity for oestradiol approximately 1000-fold and practically also abolish oestradiol dependent transcriptional activation (described in chapter 5). In comparison further mutations of the mouse oestrogen receptor, of each amino acid between 503 and 519, have identified only one additional mutant receptor with significantly (greater than 10-fold) reduced affinity for oestradiol (S. Hoare and C. Emmas, Molecular Endocrinology, ICRF personal communication). These results suggest that the binding of a particular ligand may be dependent on a small number of critical amino acids.

The mouse oestrogen receptor mutants G-525R and Δ M-521, S-522 failed to stimulate transcription significantly in transfection experiments at 10^{-8}M oestradiol but stimulated transcription in the presence of 10^{-8}M 4-hydroxytamoxifen (Danielian *et al.*, 1993). The mutated amino acids may therefore be important for discriminating between oestradiol and 4-hydroxytamoxifen (chapter 5). Similarly in the human oestrogen receptor the mutation of two or three amino acids close to amino acid 530 (534 of the mouse receptor) reduced the affinity for oestradiol 5- to 10-fold but did not significantly reduce the affinity for 4-hydroxytamoxifen (Pakdel and Katzenellenbogen, 1992). Therefore although the binding sites for these two ligands overlap they can be distinguished at the amino acid level. Amino acids important for discriminating between different ligands have also been identified in other receptors.

The human but not the chicken progesterone receptor binds the antagonist RU486 and functional analysis of mutant progesterone receptors has shown that replacement of a glycine residue at position 722 in the human receptor with a cysteine residue (the residue present at the corresponding position in the chicken receptor) abolished the binding of an antagonist RU486 but not of an agonist (Benhamou *et al.*, 1992). However, the corresponding mutation in the glucocorticoid receptor (that also has a glycine at this position) abolished RU486 and dexamethasone binding. The amino acids at positions 661 or 664 of the rat glucocorticoid receptor appear to be important for discriminating between ligands as their replacement with other amino acids abolished the binding of one agonist whilst not to significantly affecting the

binding of three other agonists (Garabedian and Yamamoto, 1992). These results also suggest that the ability of a receptor to bind a particular ligand may depend upon a few amino acids.

Conversely some mutations reduce the ability of a receptor to discriminate between different ligands. For example the androgen receptor present in LNCaP prostate tumour cells contains a mutation that results in the replacement of a threonine with an alanine at position 868, this receptor stimulates transcription to nearly wild-type levels in the presence of progesterone, oestradiol and cyproterone acetate (an antiandrogen) at concentrations 100-fold lower than those which barely induce transcription by the wild-type receptor (Veldscholte *et al.*, 1990).

The mutant H-520, A, M-521 described in chapter 5 bound oestradiol at 4°C with an affinity approximately 10-fold lower than that of the wild-type receptor, however, under transfection conditions (at 37°C) the affinity for oestradiol appears to very low suggesting that the binding of oestradiol was temperature sensitive. The receptor protein in transfected cells was stable and present at levels similar to that of the wild-type receptor and also retained sensitivity to 4-hydroxytamoxifen. These results verify the importance of the region between amino acids 520 and 525 for discriminating between oestradiol and 4-hydroxytamoxifen. Other temperature sensitive mutations have been identified, for example the original clone of the human oestrogen receptor was found to contain a point mutation that resulted in a codon for a valine rather than a glycine at position 400 (404 in the mouse receptor) (Tora *et al.*, 1989a). The mutant receptor containing a valine at position 400 bound oestradiol with an affinity similar to that of the wild-type receptor at 4°C but at 25°C a Kd was not determinable and binding was only detected at saturating concentrations of oestradiol (Tora *et al.*, 1989a). A different mutant receptor that contained an alanine at position 447 instead of a cysteine residue stimulated transcription to half the maximal rate at an oestradiol concentration 25-fold higher than that required for the wild-type receptor at 37°C whilst at 25°C the difference was only 2.5-fold suggesting the oestradiol binding by this mutant was temperature sensitive (Reese and Katzenellenbogen, 1992a). These mutations may result in thermal denaturation of the protein structure resulting in a reduction of the affinity for oestradiol.

The requirement of ligand for DNA binding.

In the absence of ligand steroid hormone receptors may be associated with a number of proteins, one of which is probably hsp90, that preclude the receptor from binding to DNA. Upon the binding of ligand this complex is dissociated allowing receptor dimerisation, DNA binding and transcriptional activation (see chapter 1). This model suggests that ligand binding is a requirement for DNA binding. In the case of the oestrogen receptor, however, this proposal has been questioned because

significant transcriptional activation is observed in the absence of added ligand in transient transfection assays where phenol red free medium containing charcoal treated serum has been used (White *et al.*, 1987; Lees *et al.*, 1989; Tora *et al.*, 1989b; Berry *et al.*, 1990; Tzurkman *et al.*, 1990; chapters 3 and 5). The transcriptional activation in the absence of ligand may not be due to the overexpression of receptors in transiently transfected cells since it is also observed in stable cell lines that contain concentrations of oestrogen receptors that are similar to those found in breast cancer cell lines (A. Thomson, Molecular Endocrinology, ICRF, personal communication). Although in cell lines containing endogenous receptors relatively low levels of oestrogen responsive gene transcription can be observed in the absence of oestradiol it is not known if this is due to the oestrogen receptor binding DNA in the absence of ligand (for examples see Westley and May 1987; May and Westley 1987). In the case of the oestrogen responsive cathepsin D gene, for example, transcription is stimulated at a low level by other transcription factors even in the presence of a pure antioestrogen (Cavaillès *et al.*, 1993).

The transcriptional activation observed in transfected cells in the absence of added ligand could be, in part, due to the presence of residual oestrogens in the medium. This has been suggested since the addition of 4-hydroxytamoxifen (in cell lines where TAF-1 stimulates transcription poorly) reduces the level of transcriptional activation to below that observed in the absence of added ligand (Berry *et al.*, 1990; and chapters 3 and 5). This is presumed to be due to 4-hydroxytamoxifen binding to the receptor and preventing the activation of TAF-2. The activity observed in the presence of 4-hydroxytamoxifen appears to be derived from TAF-1 since the deletion of the N-terminus reduces the level of transcriptional activation significantly (Berry *et al.*, 1990, chapters 3 and 5). These data therefore suggest that the transcriptional activation observed in the absence of oestradiol is mainly due to the presence of residual oestrogens that are bound by receptors that stimulate transcription by means of TAF-1 and TAF-2.

In support of this hypothesis the mutant receptor M-532R that has a slightly lower affinity for the oestradiol than the wild-type receptor fails to stimulate transcription in the absence of added ligand possibly because it is unable to bind residual oestrogens. In the presence of oestradiol this receptor stimulates transcription to nearly wild-type levels suggesting that it is capable of transcriptional activation (chapter 5). The ability of a chimeric protein, containing the DNA binding domain of GAL4 joined to the hormone binding domain of the original clone of the oestrogen receptor (described above), to bind to DNA in the absence of ligand was assessed in an *in vivo* interference assay (Webster *et al.*, 1988b). In this assay transcriptional activation by GAL4 was blocked by cotransfected GAL4-ER in the presence of 4-hydroxytamoxifen but not in the absence of ligand. The authors concluded that

4-hydroxytamoxifen promoted DNA binding by the GAL4-ER that competed with GAL4 for GAL4 response elements and prevented it from stimulating transcription whilst in the absence of ligand GAL4-ER (that did not bind residual oestrogens) was not bound or bound weakly to DNA. Note, however, that a different GAL4-ER fusion in this study did bind DNA in the absence of ligand this may be due to additional amino acids that had been placed between the GAL4 DNA binding domain and the ER hormone binding domain that prevented the hormone binding domain of the ER repressing DNA binding in the absence of ligand (Webster *et al.*, 1988b).

The possibility that ligand binding is required for DNA binding is also supported by the fact that G-525R only stimulates transcription significantly, by means of TAF-1 in CEF cells, in the presence of 4-hydroxytamoxifen. If this receptor was able to bind DNA in the absence of ligand it would be expected to stimulate transcription by means of TAF-1. A similar result was reported for the original clone of the oestrogen receptor that was unable to stimulate transcription in CEF cells in the absence of ligand suggesting that the receptor is probably not bound to DNA *in vivo* (Berry *et al.*, 1990). This, however, may not be the case if in the context of the full-length receptor DNA binding alone is insufficient for TAF-1 activity. *In vivo* footprinting of oestrogen responsive genes suggests that the receptor is not bound or bound very weakly to DNA in the absence of oestradiol and DNase I hypersensitive sites that indicate changes in chromatin structure following oestradiol treatment are also not observed in the absence of ligand suggesting that the receptor may not be bound to DNA (Bakker *et al.*, 1988; Philipsen *et al.*, 1988; Wijnholds *et al.*, 1988).

Studies of the oestrogen receptor in yeast have provided further evidence that ligand binding is required for DNA binding. Some studies have indicated that ligand binding is required to induce changes in chromatin structure near the ERE in reporter genes (Pham *et al.*, 1991a, b; Gilbert *et al.*, 1992). Whilst in another study using an *in vivo* interference assay it appeared that in the absence of hormone the oestrogen receptor failed to interfere with the DNA binding and transcriptional activation by GAL4 (McDonnell *et al.*, 1991). The reporter gene contained an ERE located close to a GAL4 binding site such that only one of these molecules could bind at any one time. In the presence of oestradiol or an antioestrogen transcriptional activation by GAL4 was inhibited.

In contrast some reports have suggested that in mammalian cells the oestrogen receptor can bind to DNA *in vivo* in the absence of ligand. The study of Tzukerman *et al.*, (1990) suggested that the oestrogen receptor was able to bind to DNA in the absence of ligand since it was able to repress the level of transcription stimulated by a chimeric transcription factor that was bound to an ERE, presumably by competing for the binding site. In this study, however, the authors did not discount the possibility that this was due to the presence of residual oestrogens and/or ligands in the medium.

The study of Reese and Katzenellenbogen, (1992b) examined directly the ability of the receptor to bind DNA *in vivo* using a different type of interference assay. The assay was based on the observation that oestrogen receptor bound to DNA between the TATA box and the site of initiation of transcription reduced the transcription of the reporter gene (CAT) that was stimulated by a constitutively active enhancer. The experiments were performed with cells transiently transfected with oestrogen receptor cDNA and cells containing endogenous oestrogen receptors. Both these studies showed that level of transcription was reduced in the absence of ligand suggesting that the receptor was binding to DNA. In the experiments using transfected receptors no transcriptional activation from an ERE reporter plasmid was observed in the absence of ligand suggesting that the DNA binding detected was not due to residual oestrogens. In either of these experiments, however, it is not possible to rule out the presence of residual ligands that promote DNA binding but not transcriptional activation, or in the case of the cells containing endogenous receptor the presence of other protein(s) that bind to an ERE with high affinity.

The possibility that the oestrogen receptor bound to DNA in the absence of ligand *in vivo* was also suggested to be the case on the basis that the receptor could bind to DNA *in vitro* efficiently in the absence or presence of ligand, however, the *in vitro* system may not be representative of what occurs *in vivo* (Lees *et al.*, 1989; Brown and Sharp, 1990; Fawell *et al.*, 1990b; Tzukerman *et al.*, 1990; Reese and Katzenellenbogen, 1991a and chapter 5). The ability of oestrogen receptors, extracted from cells, to bind DNA in the absence of ligand *in vitro* may be due to dissociation of the receptor from a non-DNA binding complex during the extraction procedure. This possibility is supported by studies that show that the progesterone and glucocorticoid receptors can be extracted from cells still associated with hsp90 and need to be dissociated from this complex by the binding of hormone, heating and/or altering the ionic conditions to bind DNA *in vitro* (Denis *et al.*, 1988; DeMarzo *et al.*, 1991). Oestrogen receptors synthesised *in vitro* appear not be efficiently recruited into non-DNA binding complexes after translation. In conclusion, the current reports which argue that the oestrogen receptor can bind to DNA in the absence of ligand are difficult to assess due to the possibility that the DNA binding observed in the "absence" of ligand is a result of the binding of residual oestrogens and/or ligands.

However, Denner *et al.*, (1990) reported that in CV-1 cells progesterone receptor mediated transcription can be observed in the absence of ligand upon the activation of protein kinase A and Power *et al.*, (1991) showed, also in CV-1 cells, that dopamine was able to stimulate transcriptional activation by the progesterone and oestrogen receptors also in the absence of ligand. These results suggest that in some cases ligand independent transcription may occur. However, other studies have not observed significant hormone independent transcription by the progesterone

(Beck *et al.*, 1992), oestrogen (Cho and Katzenellenbogen, 1993) or glucocorticoid (Rangarajan *et al.*, 1992) receptors upon activation of protein kinase A. The dopamine induced transcriptional activation by the progesterone receptor was prevented by the replacement of a serine residue in the hormone binding domain with a threonine residue (Power *et al.*, 1991). Interestingly this serine residue is only a few amino acids N-terminal to the conserved region identified in chapter 3. As this residue was cited to be phosphorylated one possibility is that the phosphorylation may induce the formation of TAF-2 in the progesterone receptor.

Although the ability of the wild-type oestrogen receptor to bind DNA *in vitro* appears not to be affected by the addition of oestradiol or 4-hydroxytamoxifen (Lees *et al.*, 1989; Curtis and Korach, 1990; Tzukerman *et al.*, 1990 and chapter 5) the ability of several mutant receptors to bind DNA appeared to be increased upon ligand binding. For example the mutant human receptor, containing a valine at position 400 binds DNA more efficiently in the presence of ligand (Kumar and Chambon, 1988; Martinez and Wahli, 1989; Tzukerman *et al.*, 1990). The mutants G-525R and Δ M-521, S-522 described in chapter 5 also bind DNA with greater efficiency in the presence of 4-hydroxytamoxifen whilst H-520, A, M-521 binds more efficiently in the presence of oestradiol and 4-hydroxytamoxifen. The studies of two other mutant mouse oestrogen receptors that failed to dimerise and bind DNA *in vitro* but retained the ability to bind oestradiol with high affinity (Fawell *et al.*, 1990a) showed that they bound DNA inefficiently *in vitro* in the presence of ligand and stimulated transcription to nearly wild-type levels in transfection experiments in the presence of oestradiol or tamoxifen (personal observations). These data suggest that in certain cases the binding of ligand can increase the ability of the oestrogen receptor to bind to DNA possibly by aiding receptor dimerisation, whether this is true for the wild-type receptor *in vivo* is unclear.

The genetic screen in yeast.

Since the oestrogen receptor may stimulate transcription by contacting protein targets a genetic screen in yeast was used to try to identify targets of TAF-2. This type of screen is based on the fact that interactions occurring *in vivo* can be visualised in the colony colour β-galactosidase assay. As described in chapter 4 the screen was unsuccessful. The possible reasons for this are discussed here in conjunction with studies of the oestrogen receptor and other transcription factors in yeast.

In 1988 several groups reported that yeast transcription factors could function in mammalian cells and vice versa suggesting that the basic mechanism of transcriptional activation may be conserved between these eukaryotes (for a review see Guarente and Bermingham-McDonogh, 1992). For example, the yeast transcription factor GAL4 stimulated transcription in mammalian cells (Kakidani and

Ptashne, 1988; Webster *et al.*, 1988a) and the mammalian transcription factors Jun and a deletion mutant of the glucocorticoid receptor stimulated transcription in yeast (Schena and Yamamoto, 1988; Struhl, 1988). In the same year the human oestrogen receptor was also reported to function, in yeast, as a ligand dependent transcription factor (Metzger *et al.*, 1988). Subsequent studies, of deletion mutants, suggested that the majority of the transcriptional activation by the wild-type receptor in yeast was stimulated by TAF-1 whilst TAF-2 activity was not detected (White *et al.*, 1988; Berry *et al.*, 1990). As described in chapter 4 this result suggested that TAF-2 could be used in the genetic screen in yeast where the DNA bound protein is required to stimulate transcription poorly so that an increase in transcription, upon the interaction of the DNA bound protein with a VP16 tagged protein (encoded by a library cDNA clone) should be detectable. In agreement with these results the analysis of the SRF-oestrogen receptor (ER) chimera showed that this region of the oestrogen receptor that contained TAF-2 stimulated transcription poorly in yeast additionally this low level of transcriptional activation was not significantly affected by the mutations that reduced the level of transcription stimulated in mammalian cells suggesting that in this system the TAF-2 characterised in mammalian cells was inactive in yeast. Despite the fact that the level of transcription stimulated by SRF-ER was only sufficient to produce light blue colonies in the colony colour assay this may have restricted the positive colonies detectable to those cases where the interaction between the SRF-ER chimera and the VP16 tagged polypeptide was strong. This "background" may have contributed to the lack of success with the screen as weakly interacting proteins would not have been detected.

In the screen described in this thesis approximately one million colonies were examined this, however, was not sufficient to cover the library thus reducing the chances of obtaining a positive clone, the library was reported to contain approximately ten million individual clones (Dalton and Treisman, 1992). Note that it should be possible to recover clones of SAP-1 (Dalton and Treisman, 1992) in this screen but none were isolated. In the tagged cDNA library less than one sixth of the clones contains the cDNA from the HeLa cell library in frame with VP16. This also reduces the chances of obtaining a clone for a target clone. The cDNAs were inserted 3' to that encoding VP16 (to ensure that all the clones were tagged) and may be inserted either way round and in any reading frame, also the 5' untranslated sequence of a library cDNA may contain a stop codon in frame with the ATG. These problems may be reduced by the directional cloning of the library or by screening an additional library in which the cDNAs have been cloned at the 5' end of the sequence encoding VP16. The number of "genuine" tagged polypeptides generated by cloning the cDNA at the 5' end of VP16 would also be small since an in frame ATG would have to be present. The screen may also be improved by the addition of a nuclear localisation

signal to the activation domain that would target the tagged polypeptides to the nucleus.

It is also possible that in yeast the SRF-ER and/or the VP16-cDNA encoded polypeptide chimera may be incorrectly folded and/or modified. The interaction domains may also be masked in the chimeras preventing an interaction. The target proteins may also be unstable in yeast and rapidly degraded. Other possibilities are that an endogenous yeast protein is bound to SRF-ER or the target protein preventing them from interacting also the interaction of TAF-2 with a target may be too weak and/or transient to detect in this screen.

Clones for target proteins will also not be isolated if the target is composed of several different proteins as each yeast clone contains one cDNA. Additionally the target protein with or without a VP16 activation domain may be toxic to yeast. For example if the VP16 tagged target of TAF-2 was able to interact with yeast basal transcription factors this would almost certainly be lethal. The presence of partial cDNAs that encode only the region of the target protein that interacts with TAF-2 may avoid this type of event. It is difficult to assess if this type of screen is suitable for isolating cDNA clones of transcription factors target(s), however, several transcription factors have been used in this type of screen and the clones isolated have encoded proteins other than target(s) involved in transcriptional activation.

To improve this type of screen a selection system has recently been developed using a SRE-*HIS3* reporter in conjunction with a competitive inhibitor of the *HIS3* gene product (N. Jones, Gene Regulation, ICRF). In this screen the competitive inhibitor would be added to the selective plates to prevent the growth of yeast containing SRF-ER and VP16 alone. In the screen growing colonies may therefore contain cDNA clones encoding VP16 tagged polypeptides that have been recruited to the promoter (resulting in a higher level of *HIS3* transcription) and would be tested for this in the colony colour assay and subsequent analysis. The presence of two reporter genes (SRE-*HIS3* and SRE-*lacZ*) should also reduce the number of false positives analysed that may result from the integration of the galactose inducible promoter near the *lacZ* gene (described in chapter 4) since it is unlikely that integration would occur into both reporters in the same yeast cell. This strategy should improve the efficiency of this type of screen greatly that, in conjunction with other techniques such as affinity chromatography, could be used to isolate target(s) for TAF-2.

TAF-2 of the human oestrogen receptor in yeast.

Whilst this screen was in progress three reports indicated that deletion mutants of the oestrogen receptor that contained TAF-2 only were able to stimulate transcription in a hormone dependent manner in yeast (Metzger *et al.*, 1992; Pham

et al., 1992; Pierrat *et al.*, 1992). These results raised the possibility that the inactivity of TAF-2 in the SRF-ER protein may have been due to an artefact of the chimera. Analysis of these reports revealed that TAF-2 activity was only detected when a "complex" promoter, one having binding sites for other transcription factors, was used. Metzger *et al.*, (1992) suggested that this may be due to the "complex" but not the "simple" promoter containing binding sites for endogenous transcription factors that were able to cooperate with the receptor. The reporter used in the genetic screen, based on the *CYC 1* gene promoter, lacks known transcription factor binding sites and may therefore be classified as a "simple" promoter. This may explain why little TAF-2 activity was observed and may therefore exclude the possibility that TAF-2 was inactive in yeast, in this thesis, because it was in a chimeric protein. These three reports also showed that in the wild-type receptor TAF-2 appeared to cooperate with TAF-1 to stimulate transcription in yeast.

Recent studies of a mutant mouse oestrogen receptor (MOR 121-599), that lacks the majority of TAF-1, have shown that it stimulates gene transcription efficiently in yeast and that transcriptional activation is not significantly reduced by mutation of the conserved hydrophobic residues or by the binding of 4-hydroxytamoxifen (V. Cavaillès, Molecular Endocrinology, ICRF, personal communication). These results suggest that TAF-2 may not function in yeast. The observation that 4-hydroxytamoxifen has little effect upon transcriptional activation by this mutant in yeast is, however, contrary to the results using the human oestrogen receptor (Metzger *et al.*, 1992). This may reflect differences in the reporter or yeast strain.

Point mutagenesis has suggested that some non-yeast transcription factors do stimulate transcription by similar mechanisms in yeast and mammalian cells. For example point mutations in the activation domain of p53 (Scharer and Iggo, 1992) and that of the helix-loop-helix protein E2A (Quong *et al.*, 1993) reduce their ability to stimulate transcription when assayed in yeast and mammalian cells. Analysis of the glucocorticoid receptor has shown that the region N-terminal to the DNA binding domain and the C-terminal region containing the hormone binding domain stimulate gene transcription in yeast (Schena and Yamamoto, 1988; Wright *et al.*, 1990). This is consistent with the presence of activation domains in these regions (described in chapter 1). In contrast the mammalian transcription factors Sp1, CTF/NFI (cited in Pugh and Tjian, 1992) and CREB 1 (N. Jones, Gene Regulation, ICRF personal communication) fail to stimulate transcription in yeast. Although several other members of the nuclear receptor family act as ligand dependent transcription factors in yeast it is unclear if the mechanism of transcriptional activation is the same mechanism as that used in mammalian cells. One cautionary example is v-erbA.

V-erbA, as described earlier, is a mutated version of the thyroid hormone receptor type α, that fails to bind hormone or stimulate transcription in mammalian cells but, surprisingly, stimulates transcription in yeast (Privalsky *et al.*, 1990). The transcriptional activation by v-erbA in yeast was shown to be dependent on the hormone binding domain and was also increased by ligand. These data suggest that not all non-yeast transcription factors stimulate transcription in yeast by a mechanism similar to that used in mammalian cells.

Possible target(s) for the glucocorticoid receptor in yeast.

Genetic analysis of yeast has identified a number of genes important for transcriptional activation by yeast transcription factors (for a review see Winston and Carlson, 1992). Three of these genes, *SWI1*, *SWI2* and *SWI3* are also important for the ability of the glucocorticoid receptor to stimulate transcription in yeast (Yoshinaga *et al.*, 1992). This study showed that *SWI3* may also be important for the ability of the oestrogen receptor to stimulate transcription in yeast. Further analysis indicated that the DNA binding domain of the glucocorticoid receptor with or without the N-terminal activation domain could co-immunoprecipitate SWI3 protein from yeast extract suggesting that the recruitment of SWI3 to the promoter by the receptor may be important for transcriptional activation.

Mutations in the genes *SIN1* and *SIN2* that alleviated the effects of the *swi*⁻ mutations in yeast allowed transcriptional activation by the glucocorticoid receptor reinforcing the possibility that glucocorticoid receptor required SWI3 to stimulate transcription (Yoshinaga *et al.*, 1992). Interestingly *SIN1* and *SIN2* encode components of chromatin, *SIN1* encodes a protein that is similar to high mobility group protein 1 (HMG 1) (Kruger and Herskowitz, 1991) whilst *SIN2* was cited to encode histone H3. The authors suggested that SWI proteins were recruited to the promoter by the glucocorticoid receptor and acted by relieving chromatin mediated repression of transcription (see chapter 1). These suggestions are supported by studies that show that the glucocorticoid receptor can alter the chromatin structure of responsive genes *in vivo* (described in chapter 1). In contrast to this model the authors also indicated that the *Drosophila* protein, similar to SWI3, was important for glucocorticoid receptor mediated transcription *in vitro* where it is unlikely for the template DNA to be associated with nucleosomes, components of chromatin. This, however, does not rule out the possibility that the potential mammalian homologues of the SWI proteins may be target(s) for the glucocorticoid and oestrogen receptors.

Possible target(s) for nuclear receptors identified *in vitro*.

Analysis of the orphan receptor, COUP-TF, has suggested it requires a protein called S300-II to stimulate transcription *in vitro* (Tsai *et al.*, 1987). This protein was

also important for transcription of the lysozyme and a MMTV gene but not SV40 early genes. The cloning of S300-II indicated that it was the basal transcription factor TFIIB (Ing *et al.*, 1992). Purified COUP-TF was reported to bind *E. coli* expressed TFIIB suggesting that the interaction was direct. To assess if steroid receptors also contacted TFIIB HeLa nuclear extract was passed over affinity columns containing an *E. coli* expressed progesterone receptor mutant, that was transcriptionally active in transfected cells (Bradshaw *et al.*, 1991), or *E. coli* expressed TAF-2 or TAF-1 of the oestrogen receptor. TFIIB appeared to bind to columns containing the progesterone receptor mutant or TAF-2 but not TAF-1, as judged by immunoblotting of the flowthrough fractions (Ing *et al.*, 1992). TFIIB was eluted from the columns containing the progesterone receptor or TAF-2 of the oestrogen receptor after the application of a salt gradient. Further studies are required to assess if the interaction is direct and whether this interaction is affected by ligand.

Previous studies of the ability of the oestrogen receptor to stimulate transcription *in vitro* have used the full-length receptor making it difficult to assess if the activity observed was stimulated by TAF-1 and/or TAF-2. Recent studies of a chimeric protein containing only TAF-2 have suggested that TAF-2, in contrast to the studies described above, may interact with TATA binding protein (TBP) associated factors (see chapter 1) (Brou *et al.*, 1993). Brou *et al.*, (1993) reported that the factors required by TAF-2 were different from those required by VP16 in agreement with *in vivo* studies (Tora *et al.*, 1989b; Tasset *et al.*, 1990). However, in this study a GAL4-ER chimera (containing TAF-2 only) was shown to stimulate transcription 5-7 fold irrespective of the presence of oestradiol, 4-hydroxytamoxifen or a pure antioestrogen ICI 164, 384. These results contradicted observations *in vivo* where oestradiol binding is required for transcriptional activation and 4-hydroxytamoxifen and ICI 164, 384 failed to stimulate transcriptional activation by an identical chimera (Webster *et al.*, 1988b). The authors suggested several possibilities for these results. One explanation was that in this assay TAF-2 was constitutively active and could not be affected by the binding of the antioestrogens. A second possibility was that ligand binding is only required for chromatin associated templates *in vivo* and is not required *in vitro*. However, some studies suggest that transiently transfected DNA may not be associated correctly with nucleosomes (Bresnick *et al.*, 1990; Archer *et al.*, 1992 see chapter 1). In support of the *in vitro* transcription results the possibility that the binding of 4-hydroxytamoxifen to the oestrogen receptor does not abolish the interaction of TAF-2 with its target proteins was suggested by the fact that the binding of 4-hydroxytamoxifen did not completely abolish its ability to interfere with (squelch) transcription stimulated by the progesterone receptor in transfection experiments suggesting that the receptor was still able to bind target protein(s) (Meyer *et al.*, 1989). From the *in vitro* transcription results, however, it is difficult to assess if

the transcriptional activation observed *in vitro* is achieved by a mechanism similar to that *in vivo* especially since no ligand dependent TAF-2 activity was observed. If the mutations in the conserved region that practically abolished transcriptional activation by TAF-2 *in vivo* had a similar affect *in vitro* this would provide good evidence that the mechanism of transcriptional activation was authentic. A negative result would be inconclusive since the mutations in the conserved region could affect a different step required for TAF-2 activity *in vivo* that may be bypassed *in vitro.*

The acidic activation domain in the N-terminal region of the glucocorticoid receptor (τ1) also stimulates transcription *in vitro* (Freedman *et al.*, 1989) and recent studies have suggested that it may interact with a basal transcription factor present in yeast nuclear extract (McEwan *et al.*, 1993). This result was based on the ability of τ1 to interfere with (squelch) basal transcription, in this a case analysis of a mutant showed that transcriptional interference correlated with the ability to stimulate transcription. *In vitro* transcription studies have suggested the activation domain N-terminal to the DNA binding domain of the progesterone receptor interacts with factors distinct from those involved in basal transcription (Shemshedini *et al.*, 1992). One study has indicated that, in transfected cells, the retinoic acid receptor stimulates transcription efficiently when the adenovirus transcription factor E1a is cotransfected with TBP suggesting that an E1a like activity present in some cells may assist transcriptional activation by the retinoic acid receptor (Berkenstam *et al.*, 1992). Whether the E1a activity is a target for the retinoic acid receptor is not yet clear. The possibility that any of the targets described above are those used *in vivo* is not yet established. In summary these results raise the possibility that steroid receptors may contact target(s) that are involved in the alteration of chromatin structure as well as target(s) involved directly in increasing the efficiency of transcription both of which may lead to an increase in gene expression (see chapter 1 for details). Many nuclear receptors appear to regulate gene transcription in a species, tissue and promoter specific manner. This may reflect the presence of different cooperating transcription factors and/or different target proteins in specific cells. The isolation of factors responsible for these phenomena may help to understand how nuclear receptors regulate gene transcription *in vivo.*

Appendix

Key to Appendix.


pBR322 origin of replication.


pUC12 origin of replication.


β-lactamase coding sequence (Amp^r).


SV40 origin of replication and early promoter.


SV40 small t intervening sequence.


SV40 large T polyadenylation signal.


mouse oestrogen receptor coding sequence.


mouse oestrogen receptor non-coding sequence.


SP6 or T7 RNA polymerase promoter.


mouse glucocorticoid receptor coding sequence.


mouse glucocorticoid receptor non-coding sequence.


GAL 4 coding sequence.


serum response factor (SRF) coding sequence.


VP16 coding sequence.


GAL1-10/CYC 1 hybrid promoter.

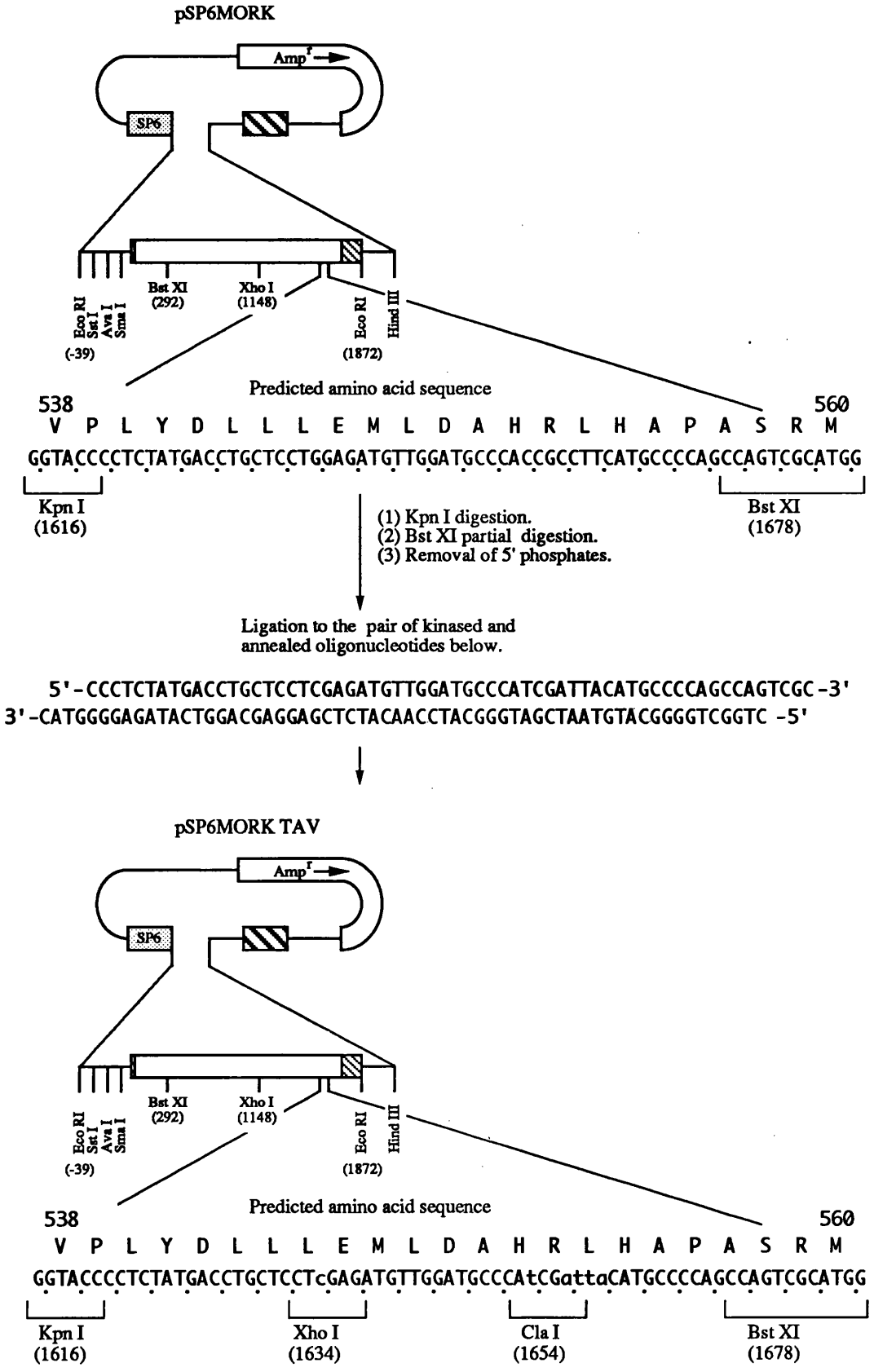

stop codons /CYC 1 polyadenylation signal.


TRP 1 selectable marker.


CEN and ARS sequences.


M13 phage f1 region.

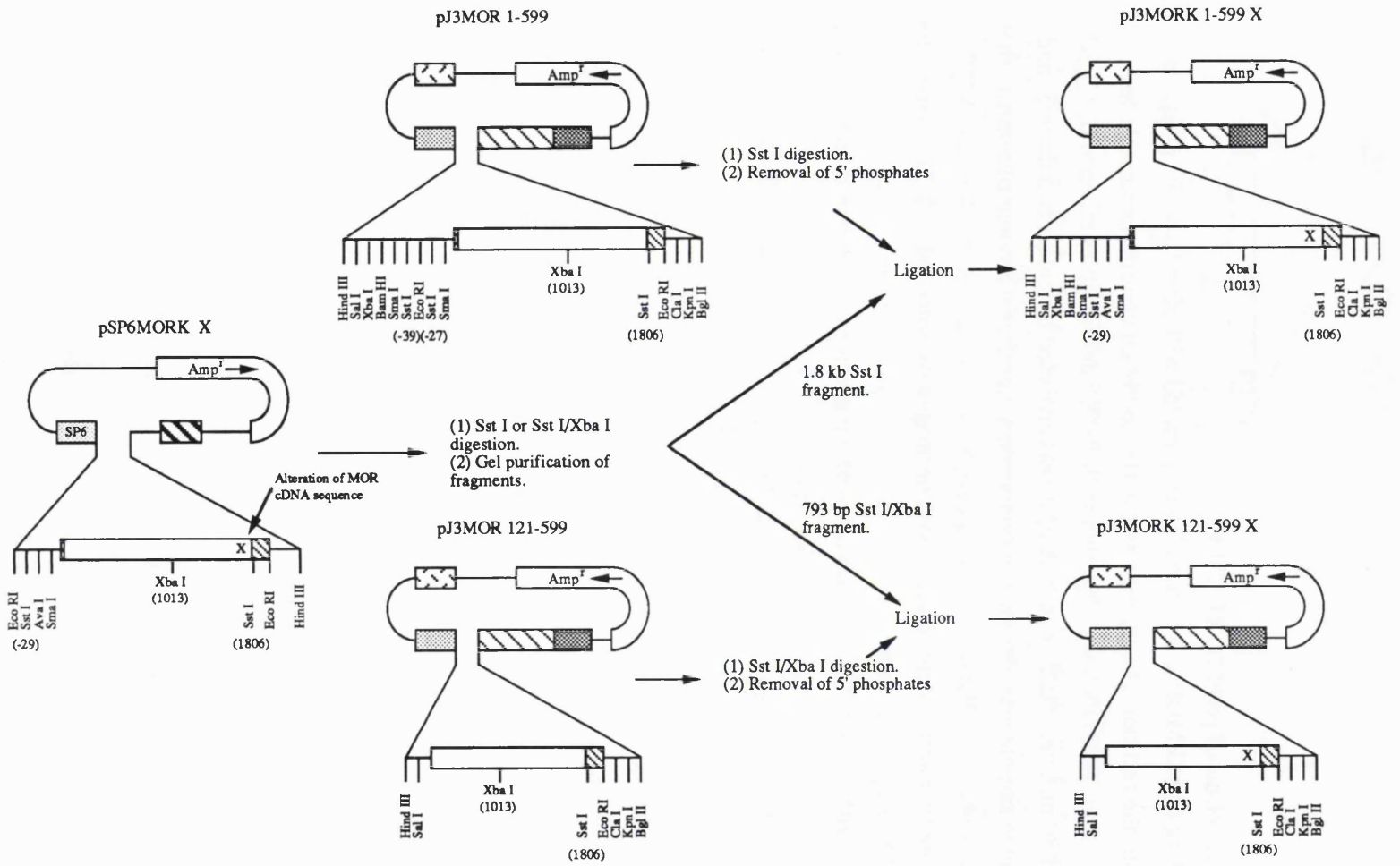
A1. Construction of pSPMORK TAV.



A1. Construction of pSPMORK TAV.

20 μg of pSPMORK was completely digested with Kpn I and the products diluted with the addition of very high salt buffer to obtain conditions suitable for BstXI digestion. This DNA was digested with BstX I and samples taken at 0, 15, 30, 40, 60 and 80 minutes. Half of each sample was analysed by gel electrophoresis and the 60 minute sample was chosen as it contained a significant amount of plasmid that had been cut once with BstX I, at position 1678. Nucleotide 1 is the A of the ATG encoding the first amino acid of the mouse oestrogen receptor (MOR). The remainder of this DNA sample was treated with phenol/chloroform, precipitated with ethanol and treated with calf intestinal alkaline phosphatase. The pair of kinased and annealed oligonucleotides illustrated were subcloned into this vector and the recombinants identified by the presence of a 486 bp Xho I fragment. The recombinant clones were cut with the enzymes used for their construction to assess the reformation of the site(s) and the DNA sequence assessed by sequencing. The nucleotides different to those in the MORK cDNA are in lower case. This cloning introduces the indicated sites without altering the predicted amino acid sequence which is shown using the one letter code.

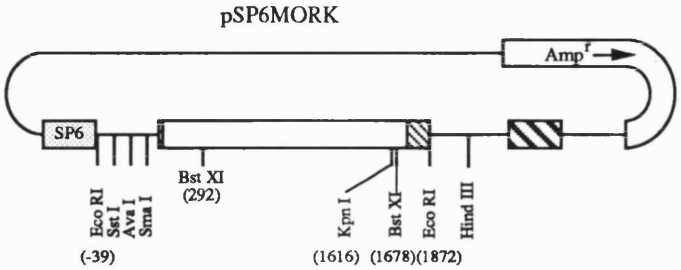
A2. Transfer of mutations into pJ3MOR and pJ3MOR 121-599.



A2. Transfer of mutations into pJ3MOR 1-599 and pJ3MOR 121-599.

2 μg of pSPMORK containing a mutation (indicated as X in the figure) was digested with either Sst I (for transfer into pJ3MOR 1-599) or Sst I and Xba I (for transfer into pJ3MOR 121-599) and the products separated on a 1% agarose gel. The 1.8 kb Sst I fragment was isolated using NA 45 paper and subcloned into pJ3MOR 1-599 that had been digested with Sst I and treated with calf intestinal alkaline phosphatase. The 793 bp Sst I/Xba I fragment was isolated as described and subcloned into pJ3MOR 121-599 that had been digested with Sst I and Xba I and treated with calf intestinal alkaline phosphatase. Correct recombinant colonies were identified by the presence of a 272 bp Kpn I fragment. The recombinant clones were cut with the enzymes used for their construction to assess the reformation of the site(s) and the mutations verified by DNA sequencing.

A3 Construction of pSPMORK Δ 540-552 and D-542N/E-546Q/D-549N.



(1) Kpn I digestion.
(2) Bst XI partial digestion.
(3) Removal of 5' phosphates.
(4) Ligation to the pairs of kinased and annealed oligonucleotides below.

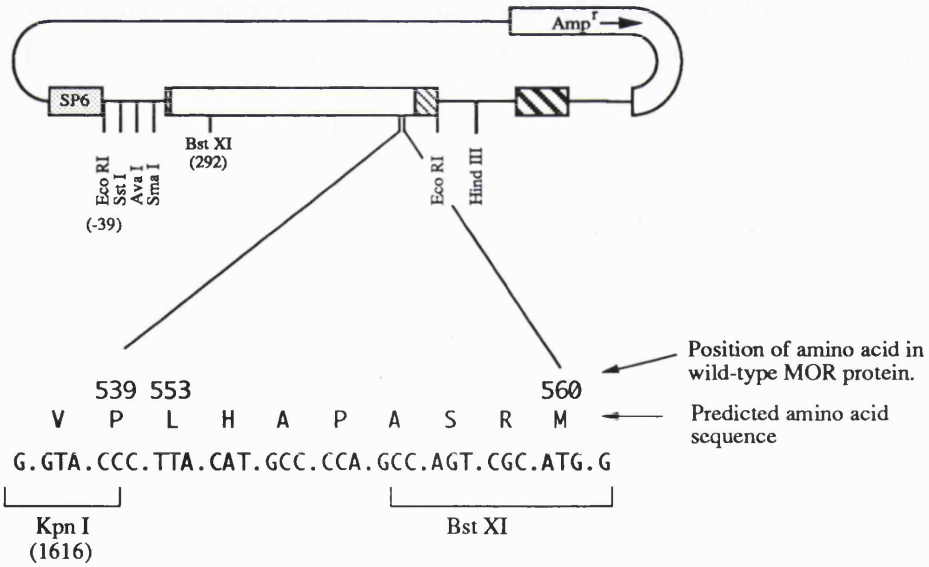
(1)

5'-CCTTACATGCCCCAGCCAGTCGC-3'
3'-CATGGGAATGTACGGGGTCGGTC-5'

(2)

5'- CCCTCTATAACCTCCTCCTGCAGATGTTGAATGCCCATCGATTACATGCCCCAGCCAGTCGC-3'
3'-CATGGGGAGATATTGGAGGAGGAGGTCTACAACTTACGGGTAGCTAATGTACGGGGTCGGTC -5'

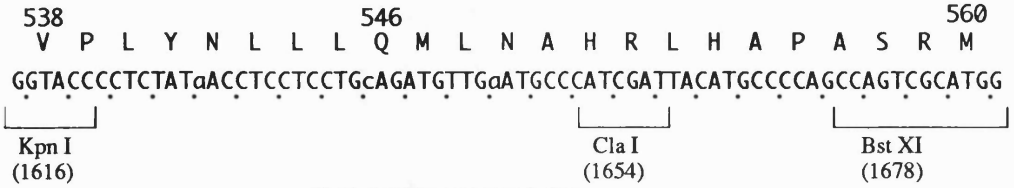
pSP6MORK (1) or (2)



Position of amino acid in wild-type MOR protein.

Predicted amino acid sequence

(1) Δ 540-552

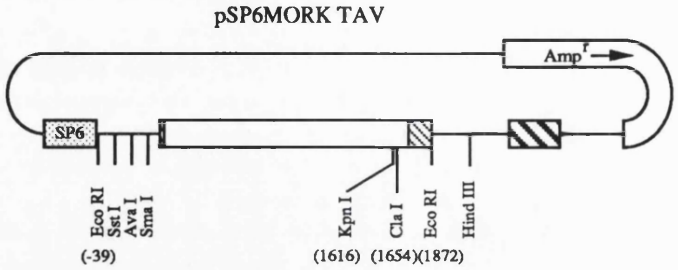


(2) D-542N/E-546Q/D-549N

A3. Construction of pSPMORK Δ 540-552 and D-542N/E-546Q/D-549N.

The vector prepared in A1 was ligated to the kinased and annealed oligonucleotides shown. Recombinants were identified by DNA sequencing. The recombinant clones were cut with the enzymes used for their construction to assess the reformation of the site(s). The nucleotides different to those in the MORK TAV cDNA are in lower case and the predicted amino acid sequence shown using the one letter code. The original positions of the codons in MORK TAV cDNA are shown above the sequence of Δ 540-552 to illustrate the deletion.

A4. Replacement of the amino acids at positions 543 and 544 or 547 and 548.

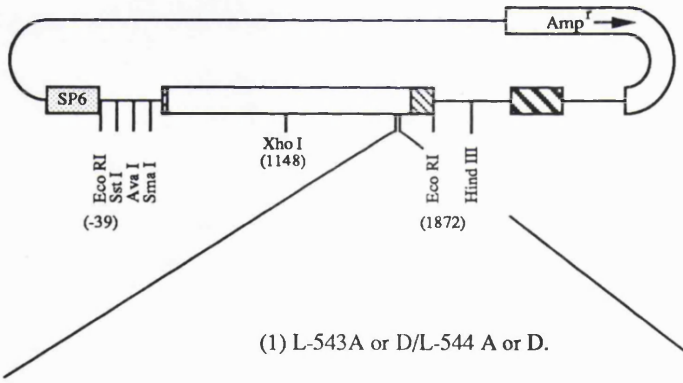


(1) Kpn I/Cla I digestion.
(2) Removal of 5' phosphates.
(3) Ligation to the pairs of kinased and annealed oligonucleotides below.

(1) 5'-CCCTCTATGACGMCGMCCTCGAGATGTTGGATGCCCAT-3'
3'-CATGGGGAGATACTGCKGCKGGAGCTCTACAACCTACGGGTAGC-5'

(2) 5'-CCCTCTATGACCTGCTCCTCGAGGMCGMCGATGCCCAT-3'
3'-CATGGGGAGATACTGGACGAGGAGCTCCKGCKGCTACGGGTAGC-5'

pSP6MORK (1) or (2).



(1) L-543A or D/L-544 A or D.

538								546						552
	V	P	L	Y	D	A/D	A/D	L	E	M	L	D	A	H	R
(1)	G.GTA	CCC.	CTC.	TAT.	GAC.	gmC.	gmC.	CTC.	GAG.	ATG.	TTG.	GAT.	GCC.	CAT.	CGA.T

Kpn I (1616) Xho I (1634) Cla I (1654)

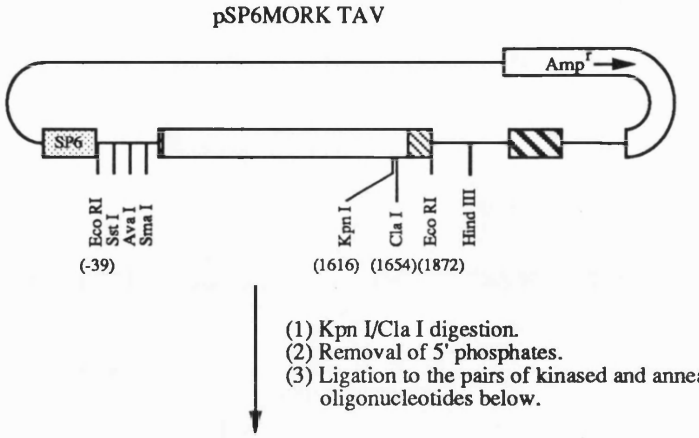
(2) M-547 A or D/L-548 Aor D.

538								546						552
	V	P	L	Y	D	L	L	L	E	A/D	A/D	D	A	H	R
(2)	G.GTA	CCC.	CTC.	TAT.	GAC.	CTG.	CTC.	CTC.	GAG.	gmc.	gmc.	GAT.	GCC.	CAT.	CGA.T

A4. Alteration of the codons at positions 543 and 544 or 547 and 548.

2 μg of pSPMORK TAV was digested with Kpn I and Cla I and treated with calf intestinal alkaline phosphatase. The kinased and annealed oligonucleotides shown were subcloned into this vector and the recombinants identified by DNA sequencing. M indicates A or C and K indicates T or G bases. This results in the possibility of obtaining codons for alanine or aspartic acid at the indicated positions. The recombinant clones were cut with the enzymes used for their construction to assess the reformation of the site(s). The nucleotides different to those in the MORK TAV cDNA are in lower case. The predicted amino acid sequence is shown using the one letter code.

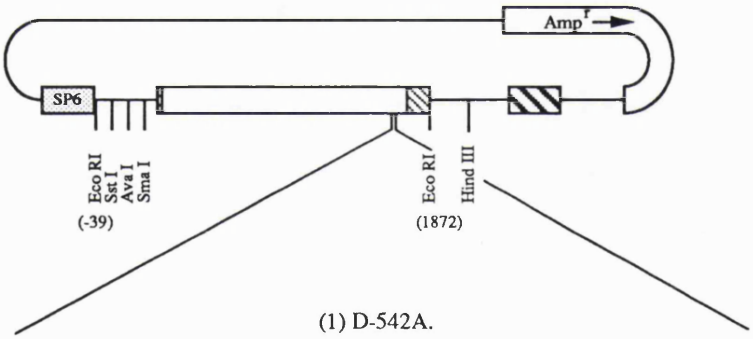
A5. Construction of pSPMORK D-542A, E-546A and D-549A.



(1) 5'-CCCTCTATGCCCTGCTCCTCGAGATGTTGGATGCCCAT-3'
3'-CATGGGGAGATACGGGACGAGGAGCTCTACAACCTACGGGTAGC-5'

(2) 5'-CCCTCTATGACCTGCTCCTCGCCATGTTGGATGCCCAT-3'
3'-CATGGGGAGATACTGGACGAGGAGCGGTACAACCTACGGGTAGC-5'

(3) 5'-CCCTCTATGACCTCCTCCTCCAGATGTTGAATGCCCAT-3'
3'-CATGGGGAGATACTGGAGGAGGAGGTCTACAACTTACGGGTAGC-5'



(1) D-542A.

538 546 552

V P L Y A L L L E M L D A H R

(1) G.GTA.CCC.CTC.TAT.gcC.CTG.CTC.CTC.GAG.ATG.TTG.GAT.GCC.CAT.CGA.T

Kpn I (1616) Xho I (1634) Cla I (1654)

(2) E-546A.

538 546 552

V P L Y D L L L A M L D A H R

(2) G.GTA.CCC.CTC.TAT.GAC.CTG.CTC.CTC.Gcc.ATG.TTG.GAT.GCC.CAT.CGA.T

(3) D-549A.

538 546 552

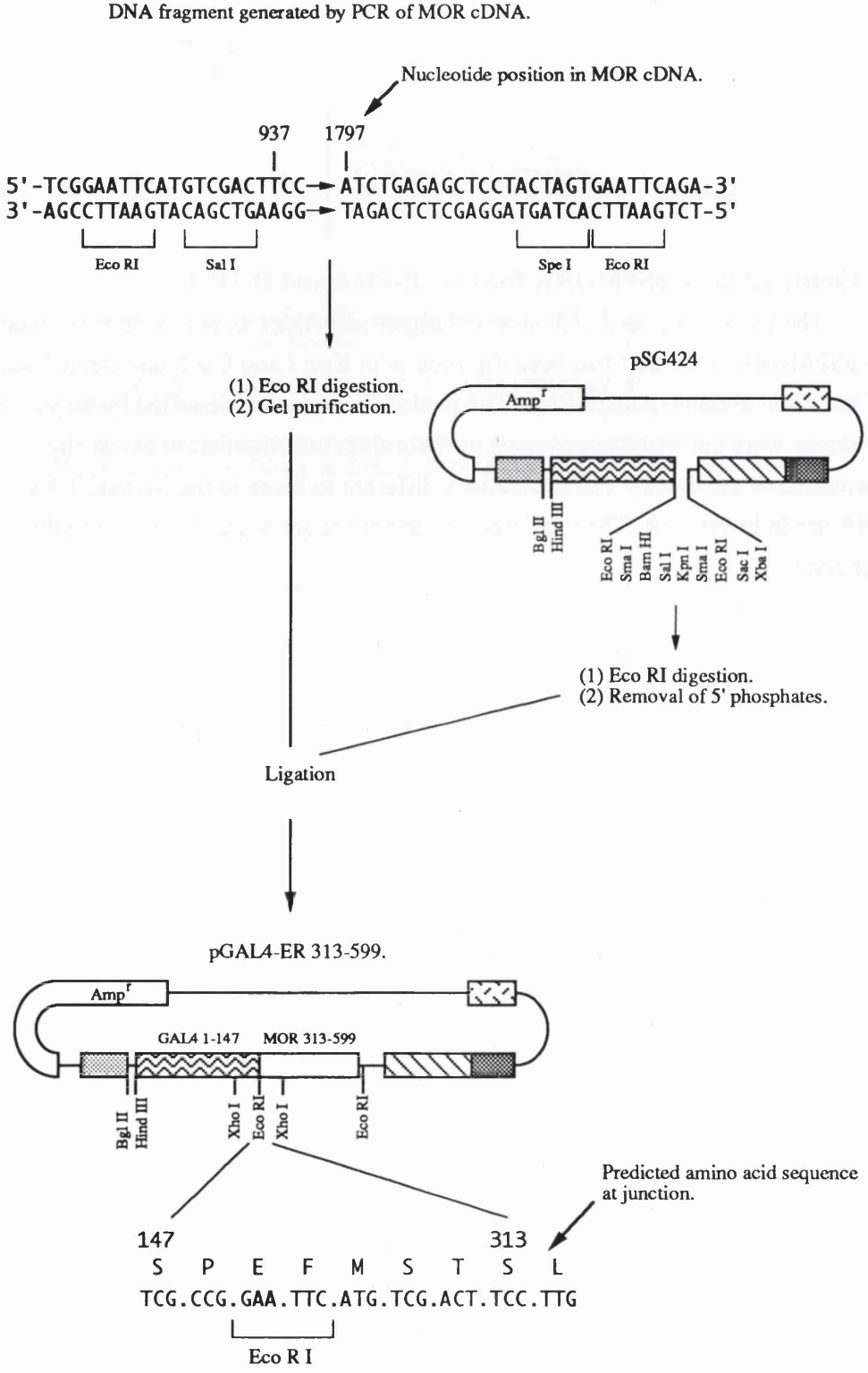
V P L Y D L L L E M L A A H R

(3) G.GTA.CCC.CTC.TAT.GAC.CTG.CTC.CTC.GAG.ATG.TTG.Gcc.GCC.CAT.CGA.T

A5. Construction of pSPMORK D-542A, E-546A and D-549A.

The pairs of kinased and annealed oligonucleotides shown were subcloned into pSPMORK TAV that had been digested with Kpn I and Cla I, and treated with calf intestinal alkaline phosphatase. The recombinants were identified by sequencing and clones were cut with the enzymes used for their construction to assess the reformation of the site(s). The nucleotides different to those in the MORK TAV cDNA are in lower case. The predicted amino acid sequence is shown using the one letter code.

A6. Construction of GAL4-ER 313-599.



A6. Construction of GAL4-ER 313-599.

To generate GAL4-ER and SRF-ER chimeras which contained TAF-2 of the ER it was necessary to introduce unique restriction endonuclease sites at the 5' and 3' ends of the region to be transferred. This was accomplished by amplifying the pJ3MOR cDNA in a polymerase chain reaction (PCR) using the primers illustrated below.

The numbers above the spaced nucleotides indicate the codon position at which the oligonucleotide is expected to hybridise in the PCR reaction and the restriction enzyme sites which should be formed by the PCR reaction underlined, see methods. The 5' and 3' primers contain sequences corresponding to the MOR cDNA coding and non-coding sequences respectively.

5' primer

313 319

5'-TCGGAATTCATGTCGACT.TCC.TTG.ACA.GCT.GAC.CAG.ATG.G-3'

Eco R I Sal I

3' primer

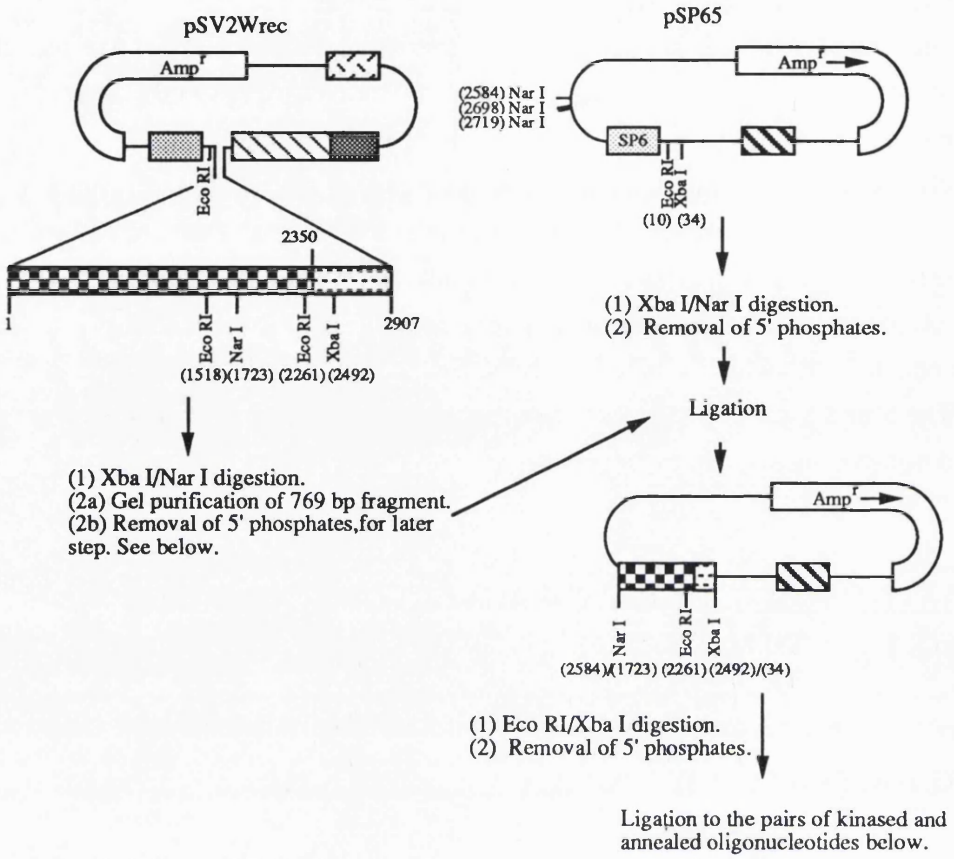
597 599 stop

3'-GG.TTG.TGC.TAG.ACTCTCGAGGATGATCACTTAAGTCT-5'

Spe I EcoR I

One third of the PCR product was digested with EcoR I and the products separated on a 1% agarose gel. The 0.9 kb fragment was isolated using NA 45 paper and subcloned into pGEM3 (Promega Limited, Southampton, U.K.) that had been digested with EcoR I and treated with calf intestinal alkaline phosphatase. The sequence at the 5' and 3' of the cDNA was determined by DNA sequencing using primers to the SP6 and T7 RNA polymerase promoters in this vector (see A9). To reduce the amount of sequencing necessary the wild-type cDNA or that containing mutations in the conserved region were subcloned from the pJ3MOR clones into this cDNA by transferral of 538 bp fragments generated by digestion with Xho I (1148)and Bst XI (1678). The sites were cut to verify reformation and the insertion of mutant cDNA verified by digestion with Kpn I. Insertion of the wild-type sequence was assessed by DNA sequencing. These clones were digested with EcoR I and subcloned into pSG424 (Sadowski and Ptashne, 1989) that had been digested with EcoR I and treated with calf intestinal alkaline phosphatase. Correct recombinants were identified by the presence of a 451 bp Xho I fragment and the sequence at the junction of the two cDNAs determined by sequencing.

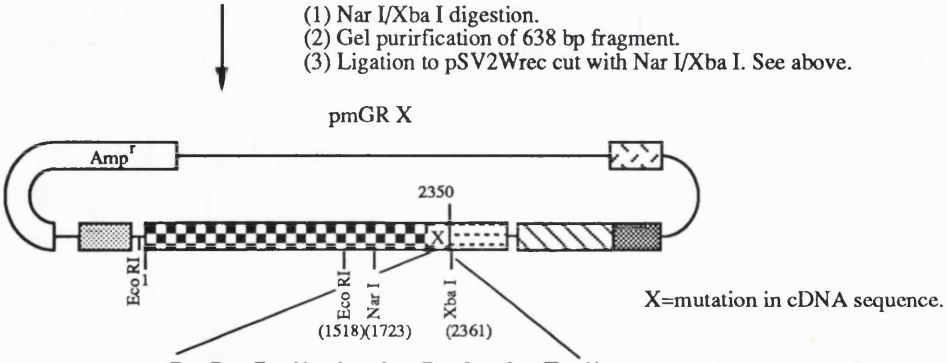
A7 Introduction of mutations into the mouse glucocorticoid receptor cDNA.



(1)
```
5'- AATTTCCGGAGATGTTAGCTGAGGCAGCTACTAATCAGATACCAAAATACTCAAATGGAAATATCAA
    3'- AGGCCTCTACAATCGACTCCGTCGACGATTAGTCTATGGTTTTATGAGTTTACCTTTATAGTT
    AAAGCTACTGTTTCATCAGAAATGACTGCCTTT -3'
    TTTCGATGACAAAGTAGTCTTTACTGACGGAAAGATC-5'
```

(2)
```
5'- AATTTCCGGAGGCCGCGGCTGAAATCATCACTAAT
    3'-AGGCCTCCGGCGCCGACTTTAGTAGTGATTA
```
As above. →

(3)
```
5'- AATTTCCGGAGATGTTAGCTGCGATCATCACTAAT
    3'-AGGCCTCTACAATCGACTCTAGTAGTGATTA
```
As above. →



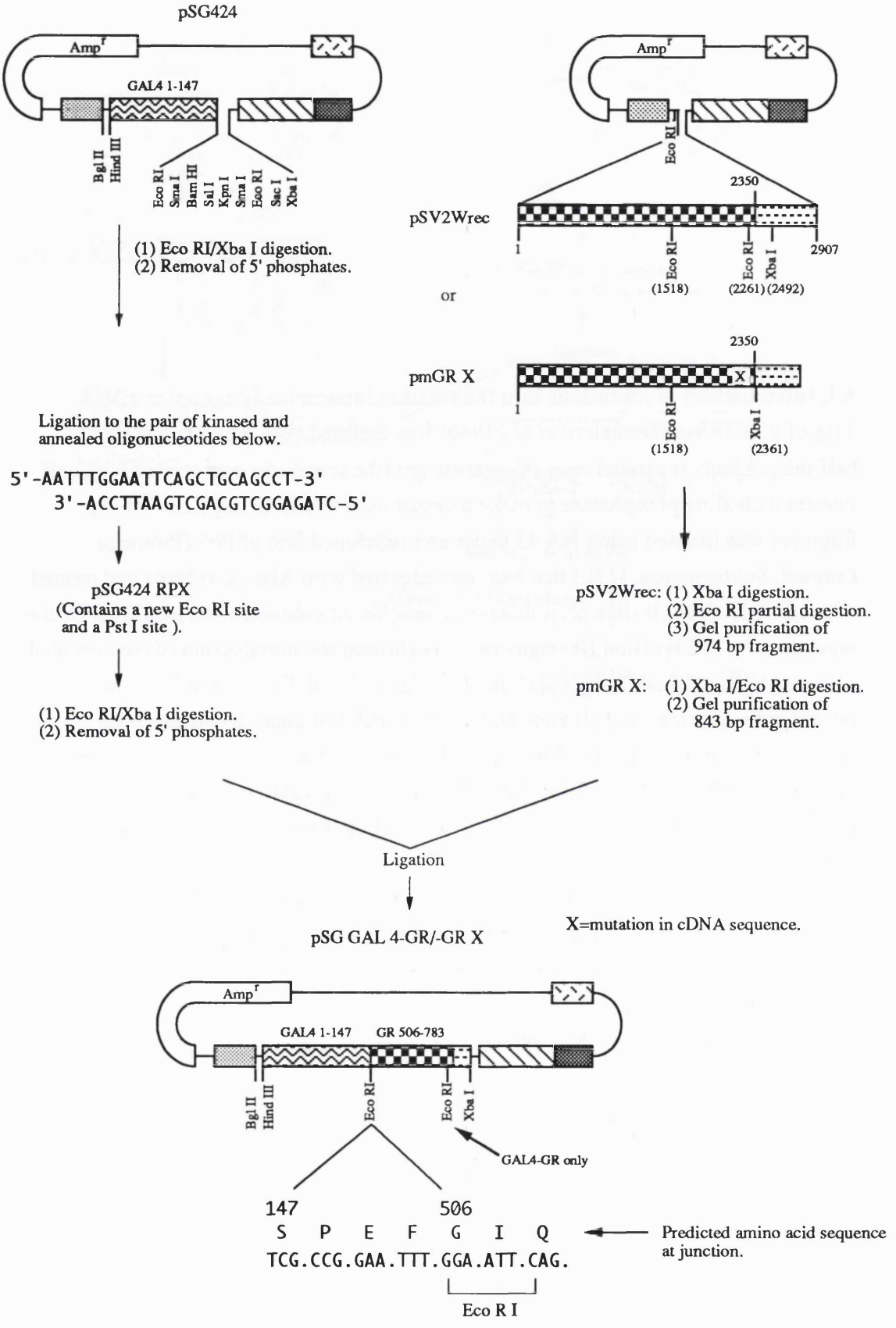
(1) mGR I-762A/I-763A.
```
F P E M L A E A A T N
AATTTCCGGAGATGTTAGCTGAGGCAGCTACTAAT
```
← Predicted amino acid sequence

(2) mGR M-758A/L-759A.
```
F P E A A A E I I T N
AATTTCCGGAGGCCGCGGCTGAAATCATCACTAAT
```

(3) mGR E-761A.
```
F P E M L A A I I T N
AATTTCCGGAGATGTTAGCTGCGATCATCACTAAT
```

A7. Introduction of mutations into the mouse glucocorticoid receptor cDNA.
4 μg of pSV2Wrec (Danielsen *et al.*, 1986) was digested with Xba I and Nar I and half the products separated on a 1% agarose gel (the remainder was treated with calf intestinal alkaline phosphatase to make a vector used in a later step).The 769 bp fragment was isolated using NA 45 paper and subcloned into pSP65 (Promega Limited, Southampton, U.K.) that had been digested with Xba I and Nar I and treated with calf intestinal alkaline phosphatase. Recombinant colonies were identified by the presence of a 212 bp Hind III fragment. This allowed the introduction of the annealed pairs of oligonucleotides. This plasmid was digested with EcoR I and Xba I and treated with calf intestinal alkaline phosphatase. Pairs of kinased and annealed oligonucleotides that had overhanging ends complementary to those produced on digestion of DNA with EcoR I and Xba I were subcloned into the above vector. Recombinant clones were identified by the loss of the Eco R I site and the sequence in this region verified by DNA sequencing. These recombinants were then digested with Nar I and Xba I and the products separated on a 1% agarose gel. The 638 bp fragments were isolated using NA 45 paper and subcloned into pSV2Wrec vector described above. Recombinants were identified by the loss of an EcoR I site and cut with the enzymes used for their construction to assess the reformation of the sites and the DNA sequence verified by sequencing.

A8. Construction of GAL4 -GR 506-783.

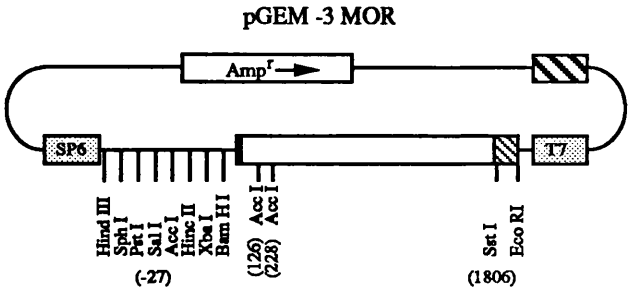


A8. Construction of GAL4-GR 506-783.

The EcoR I site at position 1518 of the glucocorticoid receptor cDNA is just 5' of the sequences that encode the hormone binding domain and 3' to those encoding the DNA binding domain. To transfer the DNA sequence encoding the hormone binding domain 20 μg of pSV2Wrec was completely digested with Xba I and the products diluted with the addition of very high salt buffer to obtain conditions suitable for EcoR I digestion. This DNA was digested with EcoR I and samples taken at 0, 1, 2, 5, 10, 20, 35 and 80 minutes. Samples were then analysed by gel electrophoresis and the 974 bp fragment isolated using NA 45 paper from the 5 minute sample. Receptor mutants described in A7 were digested with Xba I and EcoR I and the 843 bp fragment isolated as described.

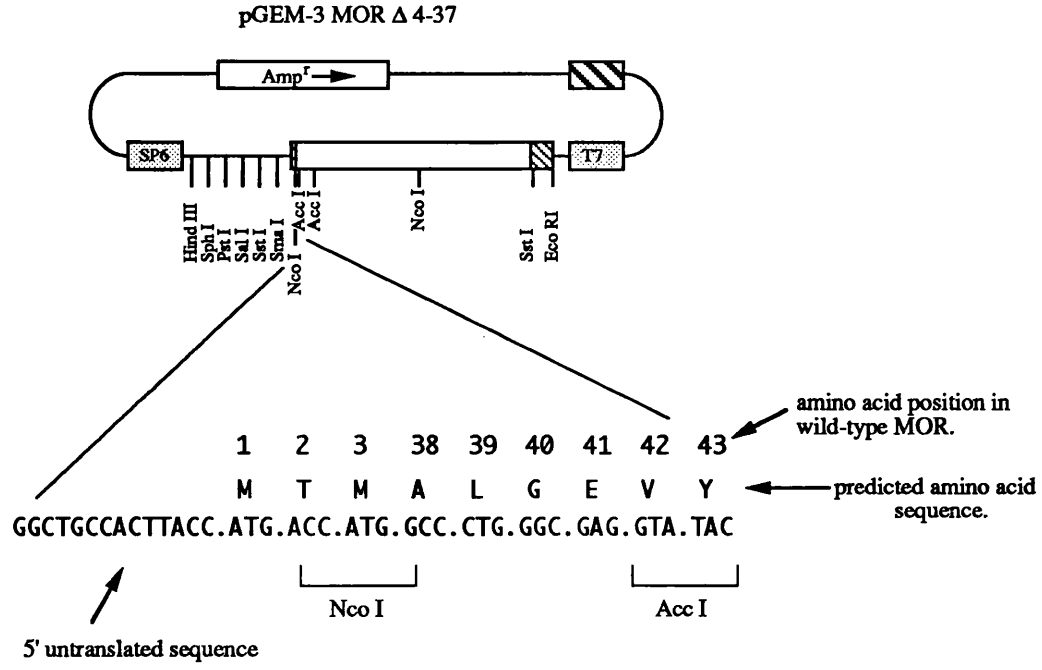
The EcoR I sites in pSG424 are not in the same position, relative to the reading frame, to the site at position 1518 in the receptor cDNA. To alter the position of the EcoR I site in pSG424 the pair of oligonucleotides illustrated in the figure were kinased and annealed and subcloned into pSG424 that had been digested with EcoR I and Xba I and treated with calf intestinal alkaline phosphatase. Recombinants were identified by the presence of a Pst I fragment and the DNA sequence of the altered region determined by sequencing. 2 μg of pSG424 RPX was digested with EcoR I and Xba I and treated with calf intestinal alkaline phosphatase and ligated to the fragments described above. Recombinants were identified by Hind III/Xba I digestion and the sequence at the junction of the two cDNAs determined by DNA sequencing.

A9. Construction of pGEM3 MOR Δ 4-37.



(1) Sal I digestion.
(2) Acc I partial digestion.
(3) Removal of 5' phosphates.
(4) Ligation to the pairs of kinased and annealed oligonucleotides below.

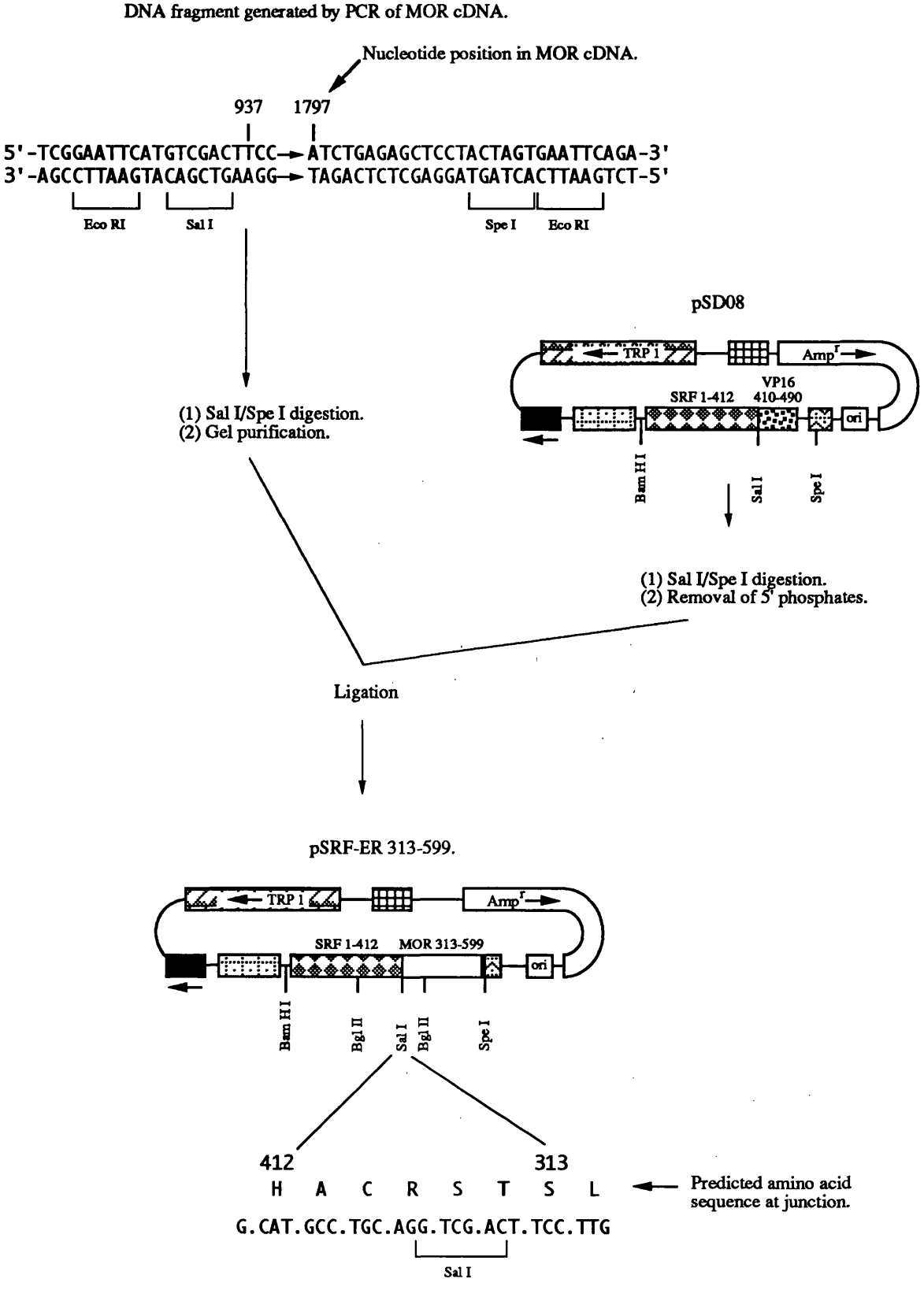
```
5'-TCGACGAGCTCGCCCGGGGATCGGCTGCCACTTACCATGACCATGGCCCTGGGCGAGGT-3'
    3'-GCTCGAGCGGGCCCCTAGCCGACGGTGAATGGTACTGGTACCGGGACCCGCTCCATA-5'
```



A9. Construction of pGEM3 MOR Δ 4-37.

20 μg of pGEM3 MOR was digested completely with Sal I and the DNA extracted with phenol/chloroform and ethanol precipitated. This DNA was then digested with Acc I and samples taken at 0, 1, 5, 10, 15, 30, 60, 90 and 140 minutes. Half of each sample was analysed by gel electrophoresis and the 90 minute sample was chosen as it contained a significant amount of plasmid that had been cut once with Acc I, at position 126. The Acc I site in the polylinker was destroyed upon Sal I digestion. The remainder of this DNA sample was treated with phenol/chloroform, precipitated with ethanol and treated with calf intestinal alkaline phosphatase. The pair of kinased and annealed oligonucleotides illustrated were subcloned into this vector and the recombinants identified by the presence of a 1.1 kb Nco I fragment. The oligonucleotides contained the Sst I site and 5' untranslated sequence found in pJ3MOR 1-599. The recombinant clones were cut with the enzymes used for their construction to assess the reformation of the site(s) and the DNA sequence verified by sequencing. To construct pJ3 38-599, pGEM-3 MOR Δ 4-37 was digested with Sst 1 and transferred as described in A2. pJ3 38-599 D-542N/E-546Q/D-549N was then generated by subcloning a Bam H1 to Xho 1 fragment from pJ3 38-599 into pJ3 D-542N/E-546Q/D-549N in MOR 1-599. pJ3 MOR 91-599 D-542N/E-546Q/D-549N was generated by transferring a 859 bp Xba I to Eco R I fragment from pJ3 MOR D-542N/E-546Q/D-549N into pJ3 MOR 91-599 (sites are indicated on A2).

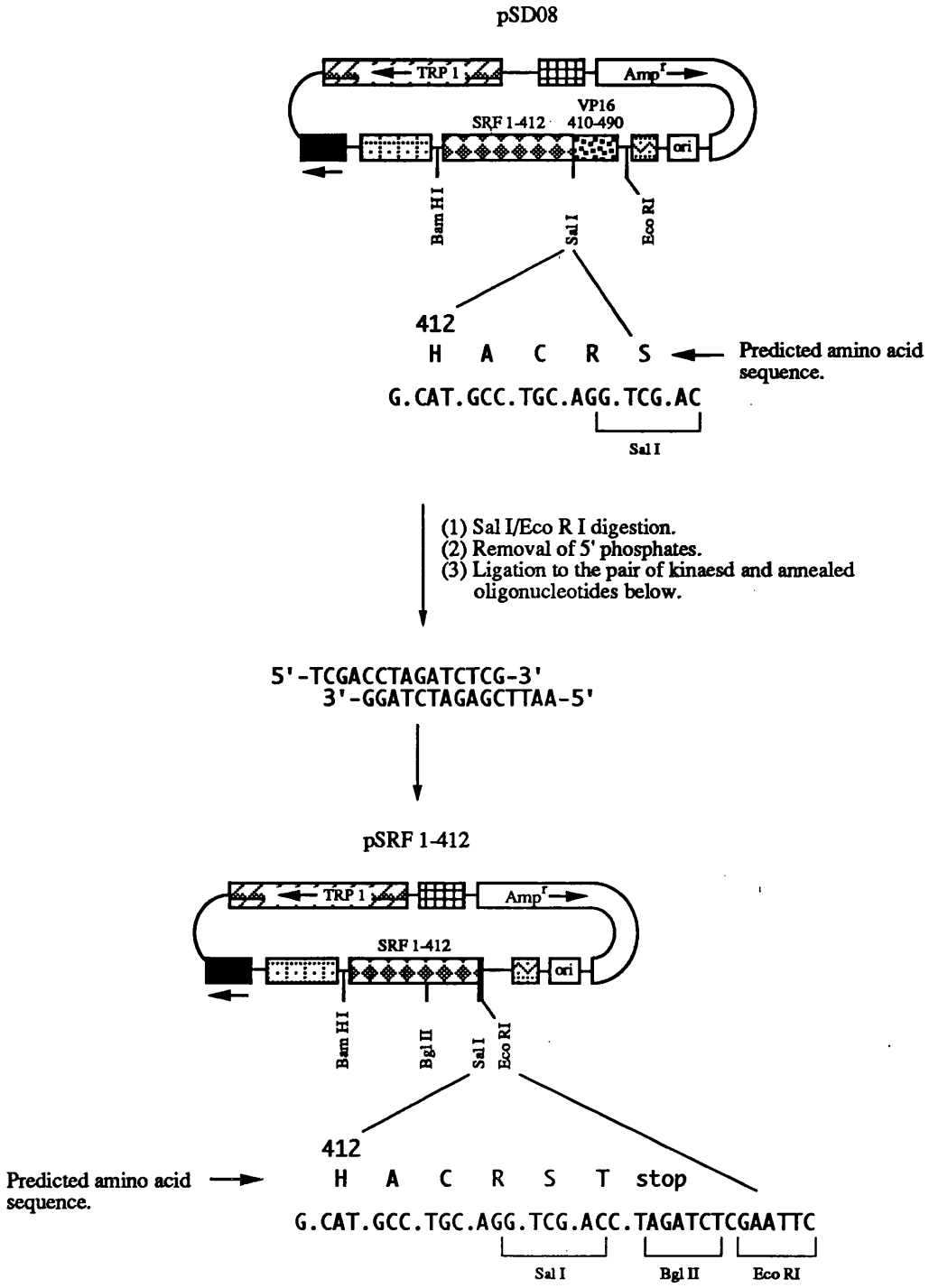
A10. Construction of pSRF-ER 313-599.



A10. Construction of pSRF-ER 313-599.

One third of the PCR product containing the hormone binding domain of the mouse oestrogen receptor (described in A6) was digested with Sal I and Spe I and the products separated on a 1% agarose gel. The 0.9 kb fragment was isolated using NA 45 paper and subcloned into pSDO8 (Dalton and Treisman, 1992) that had been digested with Sal I and Spe I and treated with calf intestinal alkaline phosphatase. Recombinants were identified by the presence of a 0.8 kb Bgl II fragment and also cut with the enzymes used for their construction to assess the reformation of the site(s). The DNA sequence was assessed by sequencing. The hatched box in pSDO8 (see key) contains centromere (CEN) and autonomously replicating sequences (ARS).

A11 Construction of pSRF 1-412.



A11. Construction of SRF 1-412.

2 μg of pSDO8 (Dalton and Treisman, 1992) was digested with Sal I and EcoR I and the products treated with calf intestinal alkaline phosphatase. The kinased and annealed oligonucleotides shown were subcloned into this vector and recombinants identified by the presence of a 0.5 kb Bgl II fragment. The DNA sequence was determined by sequencing.

Bibliography

Abmayr, S. M., Workman, J. L. and Roeder, R. G. (1988). Genes Dev, **2**, 542-53.

Adler, A. J., Danielsen, M. and Robins, D. M. (1992). Proc Natl Acad Sci U S A, **89**, 11660-3.

Ali, S., Metzger, D., Bornert, J.-M. and Chambon, P. (1993). EMBO J, **12**, 1153-60.

Allan, G. F., Leng, X., Tsai, S. Y., Weigel, N. L., Edwards, D. P., Tsai, M.-J. and O'Malley, B. W. (1992a). J Biol Chem, **267**, 19513-20.

Allan, G. F., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1992b). Proc Natl Acad Sci U S A, **89**, 11750-4.

Allison, L. A. and Ingles, C. J. (1989). Proc Natl Acad Sci U S A, **86**, 2794-8.

Amero, S. A., Kretsinger, R. H., Moncrief, N. D., Yamamoto, K. R. and Pearson, W. R. (1992). Mol Endocrinol, **6**, 3-7.

Andersson, M. L., Nordstrom, K., Demczuk, S., Harbers, M. and Vennstrom, B. (1992). Nucleic Acids Res, **20**, 4803-10.

Andres, A. J. and Thummel, C. S. (1992). Trends Genet, **8**, 132-8.

Angel, P. and Karin, M. (1991). Biochim Biophys Acta, **1072**, 129-57.

Ankenbauer, W., Strahle, U. and Schutz, G. (1988). Proc Natl Acad Sci U S A, **85**, 7526-30.

Arbuckle, N. D., Dauvois, S. and Parker, M. G. (1992). Nucleic Acids Res, **20**, 3839-44.

Archer, T. K., Cordingley, M. G., Wolford, R. G. and Hager, G. L. (1991). Mol Cell Biol, **11**, 688-98.

Archer, T. K., Lefebvre, P., Wolford, R. G. and Hager, G. L. (1992). Science, **255**, 1573-6.

Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E. and Evans, R. M. (1987). Science, **237**, 268-75.

Attardi, B. and Happe, H. K. (1986). Endocrinology, **119**, 904-15.

Ayer, S., Walker, N., Mosammaparast, M., Nelson, J. P., Shilo, B. Z. and Benyajati, C. (1993). Nucleic Acids Res, **21**, 1619-27.

Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J. and O'Malley, B. W. (1988). Proc Natl Acad Sci U S A, **85**, 3294-98.

Bakker, O., Philipsen, J. N., Hennis, B. C. and Ab, G. (1988). Mol Cell Biol, **8**, 4557-60.

Baniahmad, A., Kohne, A. C. and Renkawitz, R. (1992). EMBO J, **11**, 1015-23.

Beato, M. (1989). Cell, **56**, 335-44.

Beck, C. A., Weigel, N. L. and Edwards, D. P. (1992). Mol Endocrinol, **6**, 607-20.

Becker, P. B., Gloss, B., Schmid, W., Strähle, U. and Schütz, G. (1986). Nature, **324**, 686-91.

Benbrook, D., Lernhardt, E. and Pfahl, M. (1988). Nature, **333**, 669-72.

Benhamou, B., Garcia, T., Lerouge, T., Vergezac, A., Gofflo, D., Bigogne, C., Chambon, P. and Gronemeyer, H. (1992). Science, **255**, 206-9.

Berg, J. M. (1988). Proc Natl Acad Sci U S A, **85**, 99-102.

Berger, S. L., Cress, W. D., Cress, A., Triezenberg, S. J. and Guarente, L. (1990). Cell, **61**, 1199-208.

Berger, S. L., Pina, B., Silverman, N., Marcus, G. A., Agapite, J., Regier, J. L., Triezenberg, S. J. and Guarente, L. (1992). Cell, **70**, 251-65.

Berkenstam, A., Vivanco Ruiz, M. de M., Barettino, D., Horikoshi, M. and Stunnenberg, H. G. (1992). Cell, **69**, 401-12.

Berrodin, T. J., Marks, M. S., Ozato, K., Linney, E. and Lazar, M. A. (1992). Mol Endocrinol, **6**, 1468-78.

Berry, M., Nunez, A. M. and Chambon, P. (1989). Proc Natl Acad Sci U S A, **86**, 1218-22.

Berry, M., Metzger, D. and Chambon, P. (1990). EMBO J, **9**, 2811-18.

Berthois, Y., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (1986). Proc Natl Acad Sci U S A, **83**, 2496-500.

Beug, H. and Vennstrom, B. (1991). In Nuclear hormone receptors. Ed. M.G. Parker. Academic Press.

Blanar, M. A. and Rutter, W. J. (1992). Science, **256**, 1014-18.

Bocquel, M. T., Kumar, V., Stricker, C., Chambon, P. and Gronemeyer, H. (1989). Nucleic Acids Res, **17**, 2581-95.

Boucher, P., Koning, A. and Privalsky, M. L. (1988). J Virol, **62**, 534-44.

Bradford, M. M. (1976). Anal Biochem, **72**, 248-54.

Bradshaw, M. S., Tsai, M. J. and O'Malley, B. W. (1988). Mol Endocrinol, **2**, 1286-93.

Bradshaw, M. S., Tsai, S. Y., Leng, X. H., Dobson, A. D., Conneely, O. M., O'Malley, B. W. and Tsai, M. J. (1991). J Biol Chem, **266**, 16684-90.

Breathnach, R. and Chambon, P. (1981). Annu Rev Biochem, **50**, 349-83.

Breeden, L. and Nasmyth, K. (1987). Cell, **48**, 389-97.

Bresnick, E. H., Dalman, F. C., Sanchez, E. R. and Pratt, W. B. (1989). J Biol Chem, **264**, 4992-7.

Bresnick, E. H., John, S., Berard, D. S., LeFebvre, P. and Hager, G. L. (1990). Proc Natl Acad Sci U S A, **87**, 3977-81.

Bresnick, E. H., Bustin, M., Marsaud, V., Richard, F. H. and Hager, G. L. (1992a). Nucleic Acids Res, **20**, 273-8.

Bresnick, E. H., Rories, C. and Hager, G. L. (1992b). Nucleic Acids Res, **20**, 865-70.

Brinkmann, A.O. and Trapman, J. (1992). In Growth regulation by nuclear hormone receptors. Ed. M.G. Parker. Cold Spring Harbour Laboratory Press.

Brou, C., Wu, J., Ali, S., Scheer, E., Lang, C., Davidson, I., Chambon, P. and Tora, L. (1993). Nucleic Acids Res, **21**, 5-12.

Brown, M. and Sharp, P. A. (1990). J Biol Chem, **265**, 11238-43.

Brown, T. R., Lubahn, D. B., Wilson, E. M., French, F. S., Migeon, C. J. and Corden, J. L. (1990). Mol Endocrinol, **4**, 1759-72.

Bruggemeier, U., Rogge, L., Winnacker, E. L. and Beato, M. (1990). EMBO J., **9**, 2233-9.

Bruggemeier, U., Kalff, M., Franke, S., Scheidereit, C. and Beato, M. (1991). Cell, **64**, 565-72.

Buetti, E. and Diggelmann, H. (1981). Cell, **23**, 335-45.

Buetti, E. and Kuhnel, B. (1986). J Mol Biol, **190**, 379-89.

Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992). EMBO J, **11**, 1409-18.

Buratowski, S., Hahn, S., Guarente, L. and Sharp, P. A. (1989). Cell, **56**, 549-61.

Buratowski, S., Sopta, M., Greenblatt, J. and Sharp, P. A. (1991). Proc Natl Acad Sci U S A, **88**, 7509-13.

Byravan, S., Milhon, J., Rabindran, S. K., Olinger, B., Garabedian, M. J., Danielsen, M. and Stallcup, M. R. (1991). Mol Endocrinol, **5**, 752-8.

Carey, M., Kakidani, H., Leatherwood, J., Mostashari, F. and Ptashne, M. (1989). J Mol Biol, **209**, 423-32.

Carey, M., Lin, Y. S., Green, M. R. and Ptashne, M. (1990). Nature, **345**, 361-4.

Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L. J., Grippo, J. F. and Hunziker, W. (1993). Nature, **361**, 657-60.

Carson, M. A., Tsai, M. J., Conneely, O. M., Maxwell, B. L., Clark, J. H., Dobson, A. D., Elbrecht, A., Toft, D. O., Schrader, W. T. and O'Malley, B. W. (1987). Mol Endocrinol, **1**, 791-801.

Catelli, M. G., Binart, N., Jung, T. I., Renoir, J. M., Baulieu, E. E., Feramisco, J. R. and Welch, W. J. (1985). EMBO J, **4**, 3131-5.

Cato, A. C., Geisse, S., Wenz, M., Westphal, H. M. and Beato, M. (1984). EMBO J, **3**, 2771-8.

Cato, A. C., Miksicek, R., Schutz, G., Arnemann, J. and Beato, M. (1986). EMBO J, **5**, 2237-40.

Cato, A. C., Skroch, P., Weinmann, J., Butkeraitis, P. and Ponta, H. (1988a). EMBO J, **7**, 1403-10.

Cato, A. C., Heitlinger, E., Ponta, H., Klein, H. L., Ryffel, G. U., Bailly, A., Rauch, C. and Milgrom, E. (1988b). Mol Cell Biol, **8**, 5323-30.

Cato, A. C. and Ponta, H. (1989). Mol Cell Biol, **9**, 5324-30.

Cavaillès, V., Augereau, P. and Rochefort, H. (1993). Proc Natl Acad Sci U S A, **90**, 203-7.

Chalepakis, G., Arnemann, J. Slater, E. Bruller, H-J., Gross, B. and Beato, M. (1988). Cell, **53**, 371-82.

Challis, J. R., Kim, C. K., Naftolin, F., Judd, H. L., Yen, S. S. and Benirschke, K. (1974). J Endocrinol, **60**, 107-15.

Chatterjee, V.K.K. and Tata, J.R. (1992). In Growth regulation by nuclear hormone receptors. Ed. M.G. Parker. Cold Spring Harbour Laboratory Press.

Chambraud, B., Berry, M., Redeuilh, G., Chambon, P. and Baulieu, E. E. (1990). J Biol Chem, **265**, 20686-91.

Chandler, V. L., Maler, B. A. and Yamamoto, K. R. (1983). Cell, **33**, 489-99.

Chang, C., Kokontis, J. and Liao, S. (1988). Proc Natl Acad Sci U S A, **85**, 7211-15.

Chang, C. and Kokontis, J. (1988). Biochem Biophys Res Commun, **155**, 971-7.

Chang, C., Kokontis, J., Acakpo, S. L., Liao, S., Takeda, H. and Chang, Y. (1989). Biochem Biophys Res Commun, **165**, 735-41.

Cho, H. and Katzenellenbogen, B. S. (1993). Mol Endocrinol, **7**, 441-52.

Coezy, E., Borgna, J. L. and Rochefort, H. (1982). Cancer Res, **42**, 317-23.

Coffer, A.I., King, R.J.B. and Brockas, A.J. (1980). Biochem. Int. **1**: 126-32.

Cooney, A. J., Tsai, S. Y., O'Malley, B. W. and Tsai, M.-J. (1992). Mol. Cell. Biol., **12**, 4153-63.

Corden, J.L. (1993). Curr Opin Genet Dev, **3**, 213-8.

Cordingley, M. G., Riegel, A. T. and Hager, G. L. (1987). Cell, **48**, 261-70.

Cousens, D. J., Greaves, R., Goding, C. R. and O'Hare, P. (1989). EMBO J, **8**, 2337-42.

Cress, W. D. and Triezenberg, S. J. (1991). Science, **251**, 87-90.

Croston, G. E., Laybourn, P. J., Paranjape, S. M. and Kadonaga, J. T. (1992). Genes Dev, **6**, 2270-81.

Cuhna, G.R., Cooke, P.S., Bigsby, R. and Brody, J. R. (1991). In Nuclear hormone receptors. Ed. M.G. Parker. Academic Press.

Curtis, S. W. and Korach, K. S. (1990). Mol Endocrinol, **4**, 276-86.

Dahlman-Wright, K., Wright, A., Gustafsson, J. A. and Carlstedt-Duke, J. (1991). J Biol Chem, **266**, 3107-12.

Dalton, S. and Treisman, R. (1992). Cell, **68**, 597-612.

Damm, K., Thompson, C. C. and Evans, R. M. (1989). Nature, **339**, 593-7.

Damm, K., Heyman, R. A., Umesono, K. and Evans, R. M. (1993). Proc Natl Acad Sci U S A, **90**, 2989-93.

Danesch, U., Gloss, B., Schmid, W., Schutz, G., Schule, R. and Renkawitz, R. (1987). EMBO J, **6**, 625-30.

Danielian, P. S., White, R., Lees, J. A. and Parker, M. G. (1992). EMBO J, **11**, 1025-33.

Danielian, P. S., White, R., Hoare, S. A., Fawell, S. E. and Parker, M. G. (1993). Mol Endocrinol, **7**, 232-40.

Danielsen, M., Northrop, J. P. and Ringold, G. M. (1986). EMBO J, **5**, 2513-22.

Danielsen, M., Northrop, J. P., Jonklaas, J. and Ringold, G. M. (1987). Mol Endocrinol, **1**, 816-22.

Danielsen, M., Hinck, L. and Ringold, G. M. (1989). Cell, **57**, 1131-8.

Darbre, P., Page, M. and King, R. J. B. (1986). Mol Cell Biol, **6**, 2847-54.

Dauvois, S., Danielian, P. S., White, R. and Parker, M. G. (1992). Proc Natl Acad Sci U S A, **89**, 4037-41.

Davis, I. J., Hazel, T. G. and Lau, L. F. (1991). Mol Endocrinol, **5**, 854-9.

de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. and Dejean, A. (1991). Cell, **66**, 675-84.

De Wet, J. R., Wood K.V., Deluca, M., Helinski, D. R. and Subramani, S. (1987). Mol Cell Biol, **7**, 725-37.

DeMarzo, A. M., Beck, C. A., Onate, S. A. and Edwards, D. P. (1991). Proc Natl Acad Sci U S A, **88**, 72-6.

Denis, M., Wikstrom, A. C. and Gustafsson, J. A. (1987). J Biol Chem, **262**, 11803-6.

Denis, M., Poellinger, L., Wikstom, A. C. and Gustafsson, J. A. (1988). Nature, **333**, 686-8.

Denner, L. A., Weigel, N. L., Maxwell, B. L., Schrader, W. T. and O'Malley, B. W. (1990). Science, **250**, 1740-3.

Diamond, M. I., Miner, J. N., Yoshinaga, S. K. and Yamamoto, K. R. (1990). Science, **249**, 1266-72.

Dingwall, C. and Laskey, R. A. (1991). Trends, Biochem Sci., **16**, 478-81.

Dretzen, G., Bellard, M., Sassone, C. P. and Chambon, P. (1981). Anal Biochem, **112**, 295-8.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992). Cell, **68**, 879-87.

Durand, B., Saunders, M., Leroy, P., Leid, M. and Chambon, P. (1992). Cell, **71**, 73-85.

Durrin, L. K., Mann, R. K., Kayne, P. S. and Grunstein, M. (1991). Cell, **65**, 1023-31.

Durrin, L. K., Mann, R. K. and Grunstein, M. (1992). Mol Cell Biol, **12**, 1621-9.

Dynlacht, B. D., Hoey, T. and Tjian, R. (1991). Cell, **66**, 563-76.

Eckert, R. L. and Katzenellenbogen, B. S. (1982). J Biol Chem, **257**, 8840-6.

Eilers, M., Picard, D., Yamamoto, K. R. and Bishop, J. M. (1989). Nature, **340**, 66-8.

Eisen, L. P., Reichman, M. E., Thompson, E. B., Gametchu, B., Harrison, R. W. and Eisen, H. J. (1985). J Biol Chem, **260**, 11805-10.

El-Ashry. D., Onate, S. A., Nordeen, S. K. and Edwards, D. P. (1989). Mol Endocrinol, **3**, 1545-58.

Emmens, C.W., Cox, R.I. and Martin, L. (1962). Recent Progr. Hormone Res, **18**, 415-66.

Eul, J., Meyer, M. E., Tora, L., Bocquel, M. T., Quirin, S. C., Chambon, P. and Gronemeyer, H. (1989). EMBO J, **8**, 83-90.

Evans, R. M. (1988). Science, **240**, 889-95.

Fawell, S. E., Lees, J. A., White, R. and Parker, M. G. (1990a). Cell, **60**, 953-62.

Fawell, S. E., White, R., Hoare, S., Sydenham, M., Page, M. and Parker, M. G. (1990b). Proc Natl Acad Sci U S A, **87**, 6883-7.

Fedor, M. J. (1992). Curr Opin Cell Biol, **4**, 436-43.

Felsenfeld, G. (1992). Nature, **355**, 219-24.

Fields, S. and Song, O. (1989). Nature, **340**, 245-6.

Flanagan, P. M., Kelleher, R. 3., Sayre, M. H., Tschochner, H. and Kornberg, R. D. (1991). Nature, **350**, 436-8.

Flores, O., Lu, H., Killeen, M., Greenblatt, J., Burton, Z. F. and Reinberg, D. (1991). Proc Natl Acad Sci U S A, **88**, 9999-10003.

Flores, O., Lu, H. and Reinberg, D. (1992). J Biol Chem, **267**, 2786-93.

Forman, B. M., Yang, C., Au, M., Casanova, J., Ghysdael, J. and Samuels, H. H. (1989). Mol Endocrinol, **3**, 1610-26.

Frankel, A. D. and Kim, P. S. (1991). Cell, **65**, 717-9.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B. and Yamamoto, K. R. (1988). Nature, **334**, 543-6.

Freedman, L. P., Yoshinaga, S. K., Vanderbilt, J. N. and Yamamoto, K. R. (1989). Science, **245**, 298-301.

Fritsch, M., Leary, C. M., Furlow, J. D., Ahrens, H., Schuh, T. J., Mueller, G. C. and Gorski, J. (1992). Biochemistry, **31**, 5303-11.

Fritz, C.C. and Green, M. (1992). Current Biology, **2**, 403-5.

Futcher, A. B. and Cox, B. S. (1984). J Bacteriol, **157**, 283-90.

Garabedian, M. J. and Yamamoto, K. R. (1992). Mol Biol Cell, **3**, 1245-57.

Garcia, T., Benhamou, B., Gofflo, D., Vergezac, A., Philibert, D., Chambon, P. and Gronemeyer, H. (1992). Mol Endocrinol, **6**, 2071-8.

Gearing, K. L., Gottlicher, M., Teboul, M., Widmark, E. and Gustafsson, J. A. (1993). Proc Natl Acad Sci U S A, **90**, 1440-4.

Geisse, S., Scheidereit, C., Westphal, H. M., Hynes, N. E., Groner, B. and Beato, M. (1982). EMBO J, **1**, 1613-9.

Giambiagi, N. and Pasqualini, J. R. (1988). J Steroid Biochem, **30**, 213-7.

Gibson, M. K., Nemmers, L. A., Beckman Jr, W. C., Davis, V. L., Curtis, S. W. and Korach, K. S. (1991). Endocrinology, **129**, 2000-10.

Giguére, V., Hollenberg, S. M., Rosenfeld, M. G. and Evans, R. M. (1986). Cell, **46**, 645-52.

Giguére, V., Ong, E. S., Segui, P. and Evans, R. M. (1987). Nature, **330**, 624-9.

Giguére, V., Yang, N., Segui, P. and Evans, R. M. (1988). Nature, **331**, 91-4.

Gilbert, D. M., Losson, R. and Chambon, P. (1992). Nucleic Acids Res, **20**, 4525-31.

Gill, G. and Ptashne, M. (1987). Cell, **51**, 121-6.

Gill, G. and Ptashne, M. (1988). Nature, **334**, 721-4.

Gill, G., Sadowski, I. and Ptashne, M. (1990). Proc Natl Acad Sci U S A, **87**, 2127-31.

Giniger, E. and Ptashne, M. (1987). Nature, **330**, 670-2.

Glascock, R.F. and Hoekstra, W.G. (1959). Biochem J, **72**, 673-82.

Glass, C. K., Lipkin, S. M., Devary, O. V. and Rosenfeld, M. G. (1989). Cell, **59**, 697-708.

Godowski, P. J., Rusconi, S., Miesfeld, R. and Yamamoto, K. R. (1987). Nature, **325**, 365-8.

Godowski, P. J., Picard, D. and Yamamoto, K. R. (1988). Science, **24**, 812-6.

Gorski, J., Toft, D., Shyamala, G., Smith, D. and Notides, A. (1968). Recent Progr. Hormone Res, **24**, 45-80.

Gottlicher, M., Widmark, E., Li, Q. and Gustafsson, J. A. (1992). Proc Natl Acad Sci U S A, **89**, 4653-7.

Govindan, M. V. (1980). Exp Cell Res, **127**, 293-7.

Govindan, M. V., Spiess, E. and Majors, J. (1982). Proc Natl Acad Sci U S A, **79**, 5157-61.

Graham, F. L. and Eb, A. v. d. (1973). Virology, **54**, 536-9.

Graupner, G., Wills, K. N., Tzukerman, M., Zhang, X. K. and Pfahl, M. (1989). Nature, **340**, 653-6.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P. and Chambon, P. (1986). Nature, **320**, 134-9.

Green, S. and Chambon, P. (1987). Nature, **325**, 75-8.

Green, S. and Chambon, P. (1988). Trends Genet, **4**, 309-14.

Green, S., Kumar, V., Theulaz, I., Wahli, W. and Chambon, P. (1988). EMBO J, **7**, 3037-44.

Greene, G. L., Sobel, N. B., King, W. J. and Jensen, E. V. (1984). J Steroid Biochem, **20**, 51-6.

Gronemeyer, H., Turcotte, B., Quirin, S. C., Bocquel, M. T., Meyer, M. E., Krozowski, Z., Jeltsch, J. M., Lerouge, T., Garnier, J. M. and Chambon, P. (1987). EMBO J, **6**, 3985-94.

Grove, J. R., Dieckmann, B. S., Schroer, T. A. and Ringold, G. M. (1980). Cell, **21**, 47-56.

Grunstein, M. (1990). Annu Rev Cell Biol, **6**, 643-78.

Guarente, L. and Bermingham-McDonogh, O. (1992). Trends. Genet, **8**, 27-32.

Guiochon-Mantel, A., Loosfelt, H., Ragot, T., Bailly, A., Atger, M., Misrahi, M., Perricaudet, M. and Milgrom, E. (1988). Nature, **336**, 695-8.

Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M. and Milgrom, E. (1989). Cell, **57**, 1147-54.

Guiochon-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat, M. and Milgrom, E. (1991). EMBO J, **10**, 3851-9.

Hagemeier, C., Bannister, A. J., Cook, A. and Kouzarides, T. (1993). Proc Natl Acad Sci U S A, **90**, 1580-4.

Hager, G.L. and Archer, T.K. (1991). In Nuclear hormone receptors. Ed. M.G. Parker. Academic Press.

Ham, J., Thomson, A., Needham, M., Webb, P. and Parker, M. G. (1988). Nucleic Acids Res, **16**, 5263-76.

Ham, J. and Parker, M. G. (1989). Current Opinion in Cell Biology, **1**, 503-11.

Han, M. and Grunstein, M. (1988). Cell, **55**, 1137-45.

Han, M., Kim, U. J., Kayne, P. and Grunstein, M. (1988). EMBO J, **7**, 2221-8.

Hanahan, D. (1983). J Mol Biol, **166**, 557-80.

Hard, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., Carlstedt, D. J., Yamamoto, K. R., Gustafsson, J. A. and Kaptein, R. (1990). Science, **249**, 157-60.

Harding, H. P. and Lazar, M. A. (1993). Mol Cell Biol, **13**, 3113-21.

Harlow, K. W., Smith, D. N., Katzenellenbogen, J. A., Greene, G. L. and Katzenellenbogen, B. S. (1989). J Biol Chem, **264**, 17476-85.

Harshman, K. D., Moye, R. W. and Parker, C. S. (1988). Cell, **53**, 321-30.

Herschlag, D. and Johnson, F. B. (1993). Genes Dev, **7**, 173-9.

Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. and Thaller, C. (1992). Cell, **68**, 397-406.

Hill, C.S., Marais, R., John, S., Wynne, J., Dalton, S. and Treisman, R. (1993). Cell, **73**, 395-406.

Hirschhorn, J. N., Brown, S. A., Clark, C. D. and Winston, F. (1992). Genes Dev, **6**, 2288-98.

Hirst, M. A., Hinck, L., Danielsen, M. and Ringold, G. M. (1992). Proc Natl Acad Sci U S A, **89**, 5527-31.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G. and Evans, R. M. (1985). Nature, **318**, 635-41.

Hollenberg, S. M., Giguere, V., Segui, P. and Evans, R. M. (1987). Cell, **49**, 39-46.

Hollenberg, S. M. and Evans, R. M. (1988). Cell, **55**, 899-906.

Holloway, J. M., Glass, C. K., Adler, S., Nelson, C. A. and Rosenfeld, M. G. (1990). Proc Natl Acad Sci U S A, **87**, 8160-4.

Honda, S., Morohashi, K., Nomura, M., Takeya, H., Kitajima, M. and Omura, T. (1993). J Biol Chem, **268**, 7494-7502.

Horikoshi, M., Carey, M. F., Kakidani, H. and Roeder, R. G. (1988a). Cell, **54**, 665-9.

Horikoshi, M., Hai, T., Lin, Y. S., Green, M. R. and Roeder, R. G. (1988b). Cell, **54**, 1033-42.

Housley, P. R., Sanchez, E. R., Danielsen, M., Ringold, G. M. and Pratt, W. B. (1990). J Biol Chem, **265**, 12778-81.

Howard, K. J. and Distelhorst, C. W. (1988). J Biol Chem, **263**, 3474-81.

Howard, K. J., Holley, S. J., Yamamoto, K. R. and Distelhorst, C. W. (1990). J Biol Chem, **265**, 11928-35.

Hu, S. L. and Manley, J. L. (1981). Proc Natl Acad Sci U S A, **78**, 820-4.

Hughes, M.R. and O'Malley, B.W. (1991). In Nuclear hormone receptors. Ed. M.G. Parker. Academic Press.

Hynes, N., van, O. A., Kennedy, N., Herrlich, P., Ponta, H. and Groner, B. (1983). Proc Natl Acad Sci U S A, **80**, 3637-41.

Ing, N. H., Beekman, J. M., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1992). J Biol Chem, **267**, 17617-23.

Ingles, C. J., Shales, M., Cress, W. D., Triezenberg, S. J. and Greenblatt, J. (1991). Nature, **351**, 588-90.

Issemann, I. and Green, S. (1990). Nature, **347**, 645-50.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). J Bacteriol, **153**, 163-8.

Jantzen, H. M., Strahle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R. and Schutz, G. (1987). Cell, **49**, 29-38.

Jensen, E.V. and Jacobson, H.I. (1962). Recent Progr. Hormone Res., **18**, 387-414.

Jensen, E.V., Suzuki T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. and DeSombre, E.R. (1968). Proc Natl Acad Sci U S A, **59**, 632-38.

Jenster, G., van, d. Korput, H. A. G. M. , van, Vroohoven, C., van, d. Kwast, T. H., Trapman, J. and Brinkmann, A. O. (1991). Mol Endocrinol, **5**, 1396-404.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M. G., Binart, N., Mester, J. and Baulieu, E. E. (1984). Nature, **308**, 850-3.

Johnson, P. F. and McKnight, S. L. (1989). Annu Rev Biochem, **58**, 799-839.

Johnson, L. M., Kayne, P. S. Kahn, E. S. and Grunstein, M. Proc Natl Acad Sci U S A , **87**, 6286-90.

Jordan, V. C. and Murphy, C. S. (1990). Endocr Rev, **11**, 578-610.

Kadowaki, Y., Toyoshima, K. and Yamamoto, T. (1992). Biochem Biophys Res Commun, **183**, 492-8.

Kakidani, H. and Ptashne, M. (1988). Cell, **52**, 161-7.

Kakizuka, A., Miller, W. J., Umesono, K., Warrell, R. J., Frankel, S. R., Murty, V. V., Dmitrovsky, E. and Evans, R. M. (1991). Cell, **66**, 663-74.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R. I., Krauter, P., Westphal, H. M. and Beato, M. (1984). Nature, **308**, 513-9.

Kato, S., Tora, L., Yamauchi, J., Masushige, S., Bellard, M. and Chambon, P. (1992). Cell, **68**, 731-42.

Katzenellenbogen, B. S., Miller, M. A., Mullick, A. and Sheen, Y. Y. (1985). Breast Cancer Res Treat, **5**, 231-43.

Katzenellenbogen, B. S., Elliston, J. F., Monsma, F. J., Springer, P. A., Ziegler, Y. S. and Greene, G. L. (1987). Biochemistry, **26**, 2364-73.

Kelleher, R. 3., Flanagan, P. M. and Kornberg, R. D. (1990). Cell, **61**, 1209-15.

Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. and Wahli, W. (1993). Proc Natl Acad Sci U S A, **90**, 2160-4.

Killeen, M. T. and Greenblatt, J. F. (1992). Mol Cell Biol, **12**, 30-7.

Kim, H. S., Crone, D. E., Sprung, C. N., Tillman, J. B., Force, W. R., Crew, M. D., Mote, P. L. and Spindler, S. R. (1992). Mol Endocrinol, **6**, 1489-501.

King, R.J.B. and Mainwaring, W.I.P. (1974). Steroid-cell interactions. Butterworths.

King, W. J. and Greene, G. L. (1984). Nature, **307**, 745-7.

Klein-Hitpass, L., Schorpp, M., Wagner, U. and Ryffel, G. U. (1986). Cell, **46**, 1053-61.

Klein-Hitpass, L., Ryffel, G. U., Heitlinger, E. and Cato, A. C. (1988). Nucleic Acids Res, **16**, 647-63.

Kliewer, S. A., Umesono, K., Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A. and Evans, R. M. (1992a). Proc Natl Acad Sci U S A, **89**, 1448-52.

Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. and Evans, R. M. (1992b). Nature, **355**, 446-9.

Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. and Evans, R. M. (1992c). Nature, **358**, 771-4.

Klock, G., Strahle, U. and Schutz, G. (1987). Nature, **329**, 734-6.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P. and Hogness, D. S. (1991). Cell, **67**, 59-77.

Koelle, M. R., Segraves, W. A. and Hogness, D. S. (1992). Proc Natl Acad Sci U S A, **89**, 6167-71.

Kruger, W. and Herskowitz, I. (1991). Mol Cell Biol, **11**, 4135-46.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J. M. and Chambon, P. (1986). EMBO J, **5**, 891-7.

Krust, A., Kastner, P., Petkovich, M., Zelent, A. and Chambon, P. (1989). Proc Natl Acad Sci U S A, **86**, 5310-4.

Kuhnel, B., Buetti, E. and Diggelmann, H. (1986). J Mol Biol, **190**, 367-78.

Kumar, V., Green, S., Staub, A. and Chambon, P. (1986). EMBO J, **5**, 2231-6.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R. and Chambon, P. (1987). Cell, **51**, 941-51.

Kumar, V. and Chambon, P. (1988). Cell, **55**, 145-56.

Ladias, J. A. and Karathanasis, S. K. (1991). Science, **251**, 561-5.

Ladias, J. A., Hadzopoulou, C. M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V. and Cladaras, C. (1992). J Biol Chem, **267**, 15849-60.

Laemmli, U. K. (1970). Nature, **227**, 680-5.

Lala, D. S., Rice, D. A. and Parker, K. L. (1992). Mol Endocrinol, **6**, 1249-58.

Laudet, V., Hanni, C., Coll, J., Catzeflis, F. and Stehelin, D. (1992). EMBO J, **11**, 1003-13.

Lavorgna, G., Ueda, H., Clos, J. and Wu, C. (1991). Science, **252**, 848-51.

Laybourn, P. J. and Dahmus, M. E. (1989). J Biol Chem, **264**, 6693-8.

Laybourn, P. J. and Kadonaga, J. T. (1991). Science, **254**, 238-45.

Lazar, M. A., Hodin, R. A., Darling, D. S. and Chin, W. W. (1989). Mol Cell Biol, **9**, 1128-36.

Lee, F., Mulligan, R., Berg, P. and Ringold, G. (1981). Nature, **294**, 228-32.

Lee, Y. and Mahdavi, V. (1993). J Biol Chem, **268**, 2021-8.

Lees, J. A., Fawell, S. E. and Parker, M. G. (1989). Nucleic Acids Res, **17**, 5477-88.

Lees, J. A., Fawell, S. E., White, R. and Parker, M. G. (1990). Mol Cell Biol, **10**, 5529-31.

Leid, M., Kastner, P. and Chambon, P. (1992a). Trends Biochem Sci, **17**, 427-33.

Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J. Y., Staub, A., Garnier, J. M., Mader, S. and Chambon, P. (1992b). Cell, **68**, 377-95.

Leuther, K. K., Salmeron, J. H. and Johnston, S. A. (1993). Cell, **72**, 575-85.

Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J. F. (1992). Nature, **355**, 359-61.

Lillie, J. W. and Green, M. R. (1989). Nature, **338**, 39-44.

Lin, Y. S., Carey, M., Ptashne, M. and Green, M. R. (1990). Nature, **345**, 359-61.

Lin, Y. S. and Green, M. R. (1991). Cell, **64**, 971-81.

Lin, Y. S., Ha, I., Maldonado, E., Reinberg, D. and Green, M. R. (1991). Nature, **353**, 569-71.

Lippman, M. E., Do, H. M. and Hochberg, R. B. (1981). Cancer Res, **41**, 3150-4.

Lu, H., Flores, O., Weinmann, R. and Reinberg, D. (1991). Proc Natl Acad Sci U S A, **88**, 10004-8.

Lu, H., Zawel, L., Fisher, L., Egly, J. M. and Reinberg, D. (1992). Nature, **358**, 641-5.

Lucibello, F. C., Slater, E. P., Jooss, K. U., Beato, M. and Muller, R. (1990). EMBO J, **9**, 2827-34.

Luckow, B. and Schutz, G. (1987). Nucleic Acids Res, **15**, 5490.

Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R. and Sigler, P. B. (1991). Nature, **352**, 497-505.

Ma, J. and Ptashne, M. (1987). Cell, **51**, 113-9.

Madan, A. P. and Defranco, D. B. (1993). Proc Natl Acad Sci U S A, **90**, 3588-92.

Maden, M. and Holder, N. (1992). Bioessays, **14**, 431-8.

Mader, S., Kumar, V., de Verneuil, H. and Chambon, P. (1989). Nature, **338**, 271-4.

Mader, S., Leroy, P., Chen, J. Y. and Chambon, P. (1993a). J Biol Chem, **268**, 591-600.

Mader, S., Chambon, P. and White, J. H. (1993b). Nucleic Acids Res, **21**, 1125-32.

Majors, J. and Varmus, H. E. (1983). Proc Natl Acad Sci U S A, **80**, 5866-70.

Maldonado, E., Ha, I., Cortes, P., Weis, L. and Reinberg, D. (1990). Mol Cell Biol, **10**, 6335-47.

Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. and Evans, R. M. (1990). Nature, **345**, 224-9.

Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S. and Evans, R. M. (1991). Cell, **66**, 555-61.

Marks, M. S., Hallenbeck, P. L., Nagata, T., Segars, J. H., Appella, E., Nikodem, V. M. and Ozato, K. (1992). EMBO J, **11**, 1419-35.

Martin, P. M., Berthois, Y. and Jensen, E. V. (1988). Proc Natl Acad Sci U S A, **85**, 2533-7.

Martin, K. J., Lillie, J. W. and Green, M. R. (1990). Nature, **346**, 147-52.

Martinez, E., Givel, F. and Wahli, W. (1987). EMBO J, **6**, 3719-27.

Martinez, E. and Wahli, W. (1989). EMBO J, **8**, 3781-91.

Martinez, E., Dusserre, Y., Wahli, W. and Mermod, N. (1991). Mol Cell Biol, **11**, 2937-45.

May, F. E. and Westley, B. R. (1987). J Biol Chem, **262**, 15894-9.

May, F. E., Johnson, M. D., Wiseman, L. R., Wakeling, A. E., Kastner, P. and Westley, B. R. (1989). J Steroid Biochem, **33**, 1035-41.

McDonell, M. W., Simon, M. N. and Studier, F. W. (1977). J Mol Biol, **110**, 119-46.

McDonnell, D. P., Scott, R. A., Kerner, S. A., O'Malley, B. W. and Pike, J. W. (1989). Mol Endocrinol, **3**, 635-44.

McDonnell, D. P., Nawaz, Z. and O'Malley, B. W. (1991). Mol Cell Biol, **11**, 4350-5.

McDonnell, D. P., Vegeto, E. and O'Malley, B. W. (1992). Proc Natl Acad Sci U S A, **89**, 10563-7.

McEwan, I. J., Wright, A. P., Dahlman, W. K., Carlstedt, D. J. and Gustafsson, J. A. (1993). Mol Cell Biol, **13**, 399-407.

McPhaul, M. J., Marcelli, M., Tilley, W. D., Griffin, J. E., Isidro, G. R. and Wilson, J. D. (1991). J Clin Invest, **87**, 1413-21.

Mellon, P., Parker, V., Gluzman, Y. and Maniatis, T. (1981). Cell, **27**, 279-88.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984). Nucleic Acids Res, **12**, 7035-56.

Mermod, N., O'Neill, E. A., Kelly, T. J. and Tjian, R. (1989). Cell, **58**, 741-53.

Metzger, D., White, J. H. and Chambon, P. (1988). Nature, **334**, 31-6.

Metzger, D., Losson, R., Bornert, J. M., Lemoine, Y. and Chambon, P. (1992). Nucleic Acids Res, **20**, 2813-7.

Meyer, M. E., Gronemeyer, H., Turcotte, B., Bocquel, M. T., Tasset, D. and Chambon, P. (1989). Cell, **57**, 433-42.

Meyer, M. E., Pornon, A., Ji, J., Bocquel, M.-T., Chambon, P. and Gronemeyer, H. (1990). EMBO J, **9**, 3923-32.

Meyer, M. E., Quirin, S. C., Lerouge, T., Bocquel, M. T. and Gronemeyer, H. (1992). J Biol Chem, **267**, 10882-7.

Miesfeld, R., Godowski, P. J., Maler, B. A. and Yamamoto, K. R. (1987). Science, **236**, 423-7.

Mietus-Snyder, M., Sladek, F. M., Ginsburg, G. S., Kuo, C. F., Ladias, J. A. A., Darnell Jr, J. E. and Karathanasis, S. K. (1992). Mol Cell Biol, **12**, 1708-18.

Milbrandt, J. (1988). Neuron, **1**, 183-8.

Miller, J., McLachlan, A. D. and Klug, A. (1985a). EMBO J, **4**, 1609-14.

Miller, M. A., Mullick, A., Greene, G. L. and Katzenellenbogen, B. S. (1985b). Endocrinology, **117**, 515-22.

Miner, J. N. and Yamamoto, K. R. (1991). Trends Biochem Sci, **16**, 423-6.

Miner, J. N. and Yamamoto, K. R. (1992). Genes Dev, **6**, 2491-2501.

Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Meriel, C., Fridlansky, F., Guiochon, M. A., Galibert, F. and Milgrom, E. (1987). Biochem Biophys Res Commun, **143**, 740-8.

Mitchell, P. J. and Tjian, R. (1989). Science, **245**, 371-8.

Miyajima, N., Kadowaki, Y., Fukushige, S., Shimizu, Semba, K., Yamanashi, Y., Matsubara, K., Toyoshima, K. and Yamamoto, T. (1988). Nucleic Acids Res, **16**, 11057-74.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C. S. and Rubin, G. M. (1990). Cell, **60**, 211-24.

Moore, D. D., Marks, A. R., Buckley, D. I., Kapler, G., Payvar, F. and Goodman, H. M. (1985). Proc Natl Acad Sci U S A, **82**, 699-702.

Morgenstern, J. P. and Land, H. (1990). Nucleic Acids Res, **18**, 1068.

Moyle, M., Lee, J. S. Anderson, W. F. and Ingles, C. J. (1989). Mol Cell Biol, **9**, 5750-3.

Muller, M. M., Gerster, T. and Schaffner, W. (1988). Eur J Biochem, **176**, 485-95.

Munoz, A., Zenke, M., Gehring, U., Sap, J., Beug, H. and Vennstrom, B. (1988). EMBO J, **7**, 155-9.

Naar, A. M., Boutin, J. M., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K. and Rosenfeld, M. G. (1991). Cell, **65**, 1267-79.

Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H. and Chambon, P. (1992). Cell, **70**, 1007-19.

Nakai, A., Sakurai, A., Bell, G. I. and DeGroot, L. J. (1988). Mol Endocrinol, **2**, 1087-92.

Nakajima, N., Horikoshi, M. and Roeder, R. G. (1988). Mol Cell Biol, **8**, 4028-40.

Nemoto, T., Ohara, N. Y., Denis, M. and Gustafsson, J. A. (1990). Biochemistry, **29**, 1880-6.

Neumann, E., Schaefer, R. M., Wang, Y. and Hofschneider, P. H. (1982). EMBO J, **1**, 841-5.

Nitsch, D., Ruppert, S., Kelsey, G., Schedl, A., Wein, F., Stewart, A.G., Strahle, U., Schmid, W., de Vack, C., Reik, A., Boshart, M. and Schutz, G. (1991). In The hormonal control of gene expression. Ed. P. Cohen and J.G. Foulkes. Elsevier.

Notides, A. C., Lerner, N. and Hamilton, D. E. (1981). Proc Natl Acad Sci U S A, **78**, 4926-30.

O'Donnell, A. L. and Koenig, R. J. (1990). Mol Endocrinol, **4**, 715-20.

Oehler, T. and Angel, P. (1992). Mol Cell Biol, **12**, 5508-15.

Okret, S., Wikstrom, A. C., Wrange, O. andersson, B. and Gustafsson, J. A. (1984). Proc Natl Acad Sci U S A, **81**, 1609-13.

Oro, A. E., McKeown, M. and Evans, R. M. (1990). Nature, **347**, 298-301.

Oro, A. E., McKeown, M. and Evans, R. M. (1992). Curr Opin Genet Dev, **2**, 269-74.

Page, M. J. and Parker, M. G. (1983). Cell, **32**, 495-502.

Pakdel, F. and Katzenellenbogen, B. S. (1992). J Biol Chem, **267**, 3429-37.

Papamichail, M., Tsokos, G., Tsawdaroglou, N. and Sekeris, C. E. (1980). Exp Cell Res, **125**, 490-3.

Parker, M. G., Webb, P., Needham, M., White, R. and Ham, J. (1987). J Cell Biochem, **35**, 285-92.

Paulsen, R. E., Weaver, C. A., Fahrner, T. J. and Milbrandt, J. (1992). J Biol Chem, **267**, 16491-6.

Payvar, F., Wrange, O., Carlstedt, D. J., Okret, S., Gustafsson, J. A. and Yamamoto, K. R. (1981). Proc Natl Acad Sci U S A, **78**, 6628-32.

Payvar, F., DeFranco, D., Firestone, G. L., Edgar, B., Wrange, O., Okret, S., Gustafsson, J. A. and Yamamoto, K. R. (1983). Cell, **35**, 381-92.

Pearce, D. and Yamamoto, K. R. (1993). Science, **259**, 1161-5.

Perrot-Applanat, M., Logeat, F., Groyer-Picard, M. T. and Milgrom, E. (1985). Endocrinology, **116**, 1473-84.

Pfahl, M. (1982). Cell, **31**, 475-82.

Pham, T. A., Elliston, J. F., Nawaz, Z., McDonnell, D. P., Tsai, M. J. and O'Malley, B. W. (1991a). Proc Natl Acad Sci U S A, **88**, 3125-9.

Pham, T. A., Hwung, Y. P., McDonnell, D. P. and O'Malley, B. W. (1991b). J Biol Chem, **266**, 18179-87.

Pham, T. A., Hwung, Y. P., Santiso, M. D., McDonnell, D. P. and O'Malley, B. W. (1992). Mol Endocrinol, **6**, 1043-50.

Philipsen, J. N., Hennis, B. C. and Ab, G. (1988). Nucleic Acids Res, **16**, 9663-76.

Picard, D. and Yamamoto, K. R. (1987). EMBO J, **6**, 3333-40.

Picard, D., Khursheed, B., Garabedian, M. J., Fortin, M. G., Lindquist, S. and Yamamoto, K. R. (1990a). Nature, **348**, 166-8.

Picard, D., Kumar, V., Chambon, P. and Yamamoto, K. R. (1990b). Cell Regul, **1**, 291-9.

Pierrat, B., Heery, D. M., Lemoine, Y. and Losson, R. (1992). Gene, **119**, 237-45.

Pina, B., Bruggemeier, U. and Beato, M. (1990). Cell, **60**, 719-31.

Ponglikitmongkol, M., White, J. H. and Chambon, P. (1990). EMBO J, **9**, 2221-32.

Ponta, H., Kennedy, N., Skroch, P., Hynes, N. E. and Groner, B. (1985). Proc Natl Acad Sci U S A, **82**, 1020-4.

Power, R. F., Mani, S. K., Codina, J., Conneely, O. M. and O'Malley, B. W. (1991). Science, **254**, 1636-9.

Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer, G. G., Catelli, M. G., Evans, R. M. and Baulieu, E. E. (1988). J Biol Chem, **263**, 267-73.

Privalsky, M. L., Sharif, M. and Yamamoto, K. R. (1990). Cell, **63**, 1277-86.

Ptashne, M. (1988). Nature, **335**, 683-9.

Ptashne, M. and Gann, A. A. (1990). Nature, **346**, 329-31.

Pugh, B. F. and Tjian, R. (1992). J Biol Chem, **267**, 679-82.

Quong, M. W., Massari, M. E., Zwart, R. and Murre, C. (1993). Mol Cell Biol, **13**, 792-800.

Ragsdale, C.W. and Brockes, J.P. (1991). In Nuclear hormone receptors. Ed. M.G. Parker. Academic Press.

Rangarajan, P. N., Umesono, K. and Evans, R. M. (1992). Mol Endocrinol, **6**, 1451-7.

Redeuilh, G., Moncharmont, B., Secco, C. and Baulieu, E.-E. (1987). J Biol Chem, **262**, 6969-75.

Reese, J. C. and Katzenellenbogen, B. S. (1991a). Nucleic Acids Res, **19**, 6595-602.

Reese, J. C. and Katzenellenbogen, B. S. (1991b). J Biol Chem, **266**, 10880-7.

Reese, J. C. and Katzenellenbogen, B. S. (1992a). J Biol Chem, **267**, 9868-73.

Reese, J. C. and Katzenellenbogen, B. S. (1992b). Mol Cell Biol, **12**, 4531-8.

Regier, J. L., Shen, F. and Triezenberg, S. J. (1993). Proc Natl Acad Sci U S A, **90**, 883-7.

Reik, A., Schutz, G. and Stewart, A. F. (1991). EMBO J, **10**, 2569-76.

Renkawitz, R., Schutz, G., von, d. A. D. and Beato, M. (1984). Cell, **37**, 503-10.

Rexin, M., Busch, W., Segnitz, B. and Gehring, U. (1988). Febs Lett, **241**, 234-8

Rexin, M., Busch, W., Segnitz, B. and Gehring, U. (1992). J Biol Chem, **267**, 9619-21.

Rigaud, G., Roux, J., Pictet, R. and Grange, T. (1991). Cell, **67**, 977-86.

Ringold, G. M., Yamamoto, K. R., Bishop, J. M. and Varmus, H. E. (1977). Proc Natl Acad Sci U S A, **74**, 2879-83.

Ris, S. C., Trifiro, M. A., Kuiper, G. G., Jenster, G., Romalo, G., Sai, T., van, R. H., Kaufman, M., Rosenfield, R. L., Liao, S. and et, a. l. (1991). Mol Endocrinol, **5**, 1562-9.

Ritchie, H. H., Wang, L. H., Tsai, S., O'Malley, B. W. and Tsai, M. J. (1990). Nucleic Acids Res, **18**, 6857-62.

Roeder, R. G. (1991). Trends Biochem Sci, **16**, 402-8.

Rosen, E. D., O'Donnell, A. L. and Koenig, R. J. (1992). J Biol Chem, **267**, 22010-3.

Ruden, D. M., Ma, J., Li, Y., Wood, K. and Ptashne, M. (1991). Nature, **350**, 250-2.

Rundlett, S. E., Wu, X. P. and Miesfeld, R. L. (1990). Mol Endocrinol, **4**, 708-14.

Rusconi, S. and Yamamoto, K. R. (1987). EMBO J, **6**, 1309-15.

Saatcioglu, F., Bartunek, P., Deng, T., Zenke, M. and Karin, M. (1993). Mol Cell Biol, **13**, 3675-85.

Sabbah, M., Gouilleux, F., Sola, B., Redeuilh, G. and Baulieu, E.-E. (1991a). Proc Natl Acad Sci U S A, **88**, 390-4.

Sadowski, I., Ma, J., Triezenberg, S. and Ptashne, M. (1988). Nature, **335**, 563-4.

Sadowski, I. and Ptashne, M. (1989). Nucleic Acids Res, **17**, 7539.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press.

Sanchez, E. R., Toft, D. O., Schlesinger, M. J. and Pratt, W. B. (1985). J Biol Chem, **260**, 12398-401.

Sanchez, E. R., Meshinchi, S., Tienrungroj, W., Schlesinger, M. J., Toft, D. O. and Pratt, W. B. (1987). J Biol Chem, **262**, 6986-91.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). Proc Natl Acad Sci U S A, **74**, 5463-7.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H. and Vennstrom, B. (1986). Nature, **324**, 635-40.

Sap, J., de, M. L., Stunnenberg, H. and Vennstrom, B. (1990). EMBO J, **9**, 887-96.

Sawadogo, M. and Roeder, R. G. (1985). Cell, **43**, 165-75.

Sawadogo, M. and Sentenac, A. (1990). Annu Rev Biochem, **59**, 711-54.

Scatchard, G. (1949). Ann N Y Acad Sci, **51**, 660-72.

Scharer, E. and Iggo, R. (1992). Nucleic Acids Res, **20**, 1539-45.

Scheidereit, C., Geisse, S., Westphal, H. M. and Beato, M. (1983). Nature, **304**, 749-52.

Scheidereit, C. and Beato, M. (1984). Proc Natl Acad Sci U S A, **81**, 3029-33.

Scheidereit, C., Westphal, H. M., Carlson, C., Bosshard, H. and Beato, M. (1986). DNA, **5**, 383-91.
Schena, M. and Yamamoto, K. R. (1988). Science, **241**, 965-7.

Schena, M., Freedman, L. P. and Yamamoto, K. R. (1989). Genes Dev, **3**, 1590-601.

Schlatter, L. K., Howard, J. K., Parker, M. G. and Distelhorst, C. W. (1992). Mol Endocrinol, **6**, 132-40.

Schmidt, A., Endo, N., Rutledge, S. J., Vogel, R., Shinar, D. and Rodan, G. A. (1992). Mol Endocrinol, **6**, 1634-41.

Schuh, S., Yonemoto, W., Brugge, J., Bauer, V. J., Riehl, R. M., Sullivan, W. P. and Toft, D. O. (1985). J Biol Chem, **260**, 14292-6.

Schule, R., Muller, M., Kaltschmidt, C. and Renkawitz, R. (1988a). Science, **242**, 1418-20.

Schule, R., Muller, M., Otsuka, M. H. and Renkawitz, R. (1988b). Nature, **332**, 87-90.

Schule, R. and Evans, R. M. (1991). Trends Genet, **7**, 377-81.

Schwabe, J. W., Neuhaus, D. and Rhodes, D. (1990). Nature, **348**, 458-61.

Segraves, W. A. and Hogness, D. S. (1990). Genes Dev, **4**, 204-19.

Seiler, T. A., Walker, P., Martinez, E., Merillat, A. M., Givel, F. and Wahli, W. (1986). Nucleic Acids Res, **14**, 8755-70.

Seipel, K., Georgiev, O. and Schaffner, W. (1992). EMBO J, **11**, 4961-8.

Selmi, S. and Samuels, H. H. (1991). J Biol Chem, **266**, 11589-93.

Severne, Y., Wieland, S., Schaffner, W. and Rusconi, S. (1988). EMBO J, **7**, 2503-8.

Shemshedini, L., Knauthe, R., Sassone-Corsi, P., Pornon, A. and Gronemeyer, H. (1991). EMBO J, **10**, 3839-49.

Shemshedini, L., Ji, J. W., Brou, C., Chambon, P. and Gronemeyer, H. (1992). J Biol Chem, **267**, 1834-9.

Sigler, P. B. (1988). Nature, **333**, 210-2.

Simental, J. A., Sar, M., Lane, M. V., French, F. S. and Wilson, E. M. (1991). J Biol Chem, **266**, 510-18.

Sladek, F. M., Zhong, W. M., Lai, E. and Darnell Jr, J. E. (1990). Genes Dev, **4**, 2353-65.

Sleigh, M. J. (1986). Anal Biochem, **156**, 251-6.

Smith, D. F. and Toft, D. O. (1993). Mol Endocrinol, **7**, 4-11.

Sompayrac, L. M. and Danna, K. J. (1981). Proc Natl Acad Sci U S A, **78**, 7575-8.

Sopta, M., Burton, Z. F. and Greenblatt, J. (1989). Nature, **341**, 410-4.

Spanjaard, R. A., Darling, D. S. and Chin, W. W. (1991). Proc Natl Acad Sci U S A, **88**, 8587-91.

Strahle, U., Klock, G. and Schutz, G. (1987). Proc Natl Acad Sci U S A, **84**, 7871-5.

Strahle, U., Schmid, W. and Schutz, G. (1988). EMBO J, **7**, 3389-95.

Strahle, U., Boshart, M., Klock, G., Stewart, F. and Schutz, G. (1989). Nature, **339**, 629-32.

Stringer, K. F., Ingles, C. J. and Greenblatt, J. (1990). Nature, **345**, 783-6.

Struhl, K. (1988). Nature, **332**, 649-50.

Svaren, J. and Hörz, W. (1993). Curr Opin Genet Dev, **3**, 219-25.

Tanaka, M. and Herr, W. (1990). Cell, **60**, 375-86.

Tanese, N., Pugh, B. F. and Tjian, R. (1991). Genes Dev, **5**, 2212-24.

Tasset, D., Tora, L., Fromental, C., Scheer, E. and Chambon, P. (1990). Cell, **62**, 1177-87.

Thompson, C. C. and Evans, R. M. (1989). Proc Natl Acad Sci U S A, **86**, 3494-8.

Thompson, N. E., Steinberg, T. H., Aronson, D. B. and Burgess, R. R. (1989). J Biol Chem, **264**, 11511-20.

Thomson, A. A., Ham, J., Bakker, O. and Parker, M. G. (1990). J Biol Chem, **265**, 16709-12.

Tora, L., Gaub, M.-P., Mader, S., Dierich, A., Bellard, M. and Chambon, P. (1988a). EMBO J, **7**, 3771-8.

Tora, L., Gronemeyer, H., Turcotte, B., Gaub, M. P. and Chambon, P. (1988b). Nature, **333**, 185-8.

Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I. and Chambon, P. (1989a). EMBO J, **8**, 1981-6.

Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E. and Chambon, P. (1989b). Cell, **59**, 477-87.

Tran, P., Zhang, X.-K., Salbert, G., Hermann, T., Lehmann, J. M. and Pfahl, M. (1992). Mol. Cell. Biol., **12**, 4666-76.

Tsai, S. Y., Sagami, I., Wang, H., Tsai, M.-J. and O'Malley, B. W. (1987). Cell, **50**, 701-9.

Tsai, S. Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J.-A., Tsai, M.-J. and O'Malley, B. W. (1988). Cell, **55**, 361-9.

Tsukiyama, T., Ueda, H., Hirose, S. and Niwa, O. (1992). Mol Cell Biol, **12**, 1286-91.

Tugwood, J. D., Issemann, I. anderson, R. G., Bundell, K. R., McPheat, W. L. and Green, S. (1992). EMBO J, **11**, 433-9.

Turner, B. M. (1991). J Cell Sci, **99**, 13-20.

Tzukerman, M., Zhang, X., Hermann, T., Wills, K. N., Graupner, G. and Pfahl, M. (1990). New Biologist, **2**, 613-620.

Ucker, D. S. and Yamamoto, K. R. (1984). J Biol Chem, **259**, 7416-20.

Ueda, H., Sun, G.-C., Murata, T. and Hirose, S. (1992). Mol Cell Biol, **12**, 5667-72.

Umesono, K. and Evans, R. M. (1989). Cell, **57**, 1139-46.

Umesono, K., Murakami, K. K., Thompson, C. C. and Evans, R. M. (1991). Cell, **65**, 1255-66.

Usheva, A., Maldonado, E., Goldring, A., Lu, H., Houbavi, C., Reinberg, D. and Aloni, Y. (1992). Cell, **69**, 871-81.

van, der Leede. B., Folkers, G. E., Kruyt, F. A. and van, d. S. P. (1992). Biochem Biophys Res Commun, **188**, 695-702.

Van Dyke, M., Roeder, R. G. and Sawadogo, M. (1988). Science, **241**, 1335-8.

Vegeto, E., Allan, G. F., Schrader, W. T., Tsai, M.-J., McDonnell, D. P. and O'Malley, B. W. (1992). Cell, **69**, 703-13.

Veldscholte, J., Berrevoets, C. A., Ris-Stalpers, C., Kuiper, G. G. J. M., Jenster, G., Trapman, J., Brinkmann, A. O. and Mulder, E. (1992). J Steroid Biochem Molec Biol, **41**, 665-9.

von, der Ahe. D., Janich, S., Scheidereit, C., Renkawitz, R., Schutz, G. and Beato, M. (1985). Nature, **313**, 706-9.

Wahli, W. (1988). Trends Genet, **4**, 227-32.

Walker, P., Germond, J. E., Brown, L. M., Givel, F. and Wahli, W. (1984). Nucleic Acids Res, **12**, 8611-26.

Walters, M. R. (1985). Endocr Rev, **6**, 512-43.

Wang, L.-H., Tsai, S. Y., Cook, R. G., Beattie, W. G., Tsai, M.-J. and O'Malley, B. W. (1989). Nature, **340**, 163-6.

Waterman, M. L., Adler, S., Nelson, C., Greene, G. L., Evans, R. M. and Rosenfeld, M. G. (1988). Mol Endocrinol, **2**, 14-21.

Webster, N., Jin, J. R., Green, S., Hollis, M. and Chambon, P. (1988a). Cell, **52**, 169-78.

Webster, N. J. G., Green, S., Jin, J. R. and Chambon, P. (1988b). Cell, **54**, 199-207.

Webster, N. J., Green, S., Tasset, D., Ponglikitmongkol, M. and Chambon, P. (1989). EMBO J, **8**, 1441-6.

Weigel, N. L., Beck, C. A., Estes, P. A., Prendergast, P., Altmann, M., Christensen, K. and Edwards, D. P. (1992). Mol Endocrinol, **6**, 1585-97.

Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J. and Evans, R. M. (1986). Nature, **324**, 641-6.

Weinzierl, R., Dynlacht, B. D. and Tjian, R. (1993). Nature, **362**, 511-7.

Westley, B., May, F. E., Brown, A. M., Krust, A., Chambon, P., Lippman, M. E. and Rochefort, H. (1984). J Biol Chem, **259**, 10030-5.

Westley, B. R. and May, F. E. (1987). Nucleic Acids Res, **15**, 3773-86.

White, R., Lees, J. A., Needham, M., Ham, J. and Parker, M. (1987). Mol Endocrinol, **1**, 735-44.

White, J. H., Metzger, D. and Chambon, P. (1988). Cold Spring Harb Symp Quant Biol, **2**, 819-28.

White, J. H., Brou, C., Wu, J., Burton, N., Egly, J. M. and Chambon, P. (1991). Proc Natl Acad Sci U S A, **88**, 7674-8.

Wickström, A. C., Bakke, O., Okret, S., Brönnegard, M. and Gustafsson, J. A. (1987). Endocrinology, **120**, 1232-42.

Widom, R. L., Rhee, M. and Karathanasis, S. K. (1992). Mol Cell Biol, **12**, 3380-9.

Wijnholds, J., Philipsen, J. N. and Ab, G. (1988). EMBO J, **7**, 2757-63.

Wijnholds, J., Muller, E. and Ab, G. (1991). Nucleic Acids Res, **19**, 33-41.

Williams, G. R., Harney, J. W., Moore, D. D., Larsen, P. R. and Brent, G. A. (1992). Mol Endocrinol, **6**, 1527-37.

Wilson, T. E., Fahrner, T. J., Johnston, M. and Milbrandt, J. (1991). Science, **252**, 1296-1300.

Wilson, T. E., Paulsen, R. E., Padgett, K. A. and Milbrandt, J. (1992). Science, **256**, 107-10.

Wilson, T. E., Mouw, A. R., Weaver, C. A., Milbrandt, J. and Parker, K. L. (1993). Mol Cell Biol, **13**, 861-8.

Winston, F. and Carlson, M. (1992). Trends Genet, **8**, 387-91.

Winter, B., Braun, T. and Arnold, H. H. (1992). EMBO J, **11**, 1843-55.

Workman, J. L. and Roeder, R. G. (1987). Cell, **51**, 613-22.

Workman, J. L., Abmayr, S. M., Cromlish, W. A. and Roeder, R. G. (1988). Cell, **55**, 211-9.

Workman, J. L., Roeder, R. G. and Kingston, R. E. (1990). EMBO J, **9**, 1299-308.

Workman, J. L., Taylor, I. C. and Kingston, R. E. (1991). Cell, **64** , 533-44.

Wright, A. P., Carlstedt, D. J. and Gustafsson, J. A. (1990). J Biol Chem, **265**, 14763-9.

Yamamoto, K. K., Gonzalez, G. A., Menzel, P., Rivier, J. and Montminy, M. R. (1990). Cell, **60**, 611-7.

Ylikomi, T., Bocquel, M. T., Berry, M., Gronemeyer, H. and Chambon, P. (1992). EMBO J, **11**, 1-14.

Yoshinaga, S. K., Peterson, C. L., Herskowitz, I. and Yamamoto, K. R. (1992). Science, **258**, 1598-1604.

Young, H. A., Shih, T. Y., Scolnick, E. M. and Parks, W. P. (1977). J Virol, **21**, 139-46.

Yu, V. C., Delsert, C. andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K. and Rosenfeld, M. G. (1991). Cell, **67**, 1251-66.

Zandi, E., Galli, I., Dobbeling, U. and Rusconi, S. (1993). J Mol Biol, **230**, 124-36.

Zawel, L. and Reinberg, D. (1992). Curr Opin Cell Biol, **4**, 488-95.

Zenke, M., Munoz, A., Sap, J., Vennstrom, B. and Beug, H. (1990). Cell, **61**, 1035-49.

Zhang, X.-K., Hoffmann, B., Tran, P. B.-V., Graupner, G. and Pfahl, M. (1992). Nature, **355**, 441-6.

Zilliacus, J., Wright, A. P., Norinder, U., Gustafsson, J. A. and Carlstedt, D. J. (1992). J Biol Chem, **267**, 24941-7.