# STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE MOUSE OESTROGEN RECEPTOR

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**To Adrian**

All experiments included in this thesis were performed by myself, unless I specifically indicate otherwise

# ABSTRACT

Steroid receptors are members of a family of ligand-inducible transcription factors which regulate the expression of hormone responsive genes. The mouse oestrogen receptor has been used as a model system to study the structure and function of sequences within the C-terminus.

A conserved region has been identified which contains sequences required for steroid binding and dimerisation. The identification of amino acids involved in these two functions was carried out by analysing the functions of an extensive series of point mutations in this region. Dimerisation was affected by mutations between residues 507 and 519. The sequences involved in ligand binding and dimerisation overlap but the two functions are not entirely coincident. Mutant receptors in which amino acids were replaced with hydrophobic or neutral amino acids tended to retain activity whereas charged residues often affected one or more functions quite markedly. The dimerisation interface is rich in hydrophobic residues and does not appear to form an α helix. The effects of point mutations in the oestrogen receptor supported a model for dimerisation based on the structure of uteroglobin, but no direct evidence to support this model was obtained. Oestrogen and the antioestrogens 4-hydroxytamoxifen and ICI 164,384 restored DNA binding activity to mutants defective in dimerisation. This suggested that ligands stabilise dimerisation, possibly by hydrophobic shielding of the charged residues introduced. The controversy surrounding the agonist effects of ICI 164,384 has also been investigated using sequences from the promoter of the oestrogen regulated creatine kinase gene. Although ICI 164,384 induces transcription from this gene in cells, it appears to inhibit DNA binding in a cell-free system.

A number of mutant oestrogen receptors were tested for their ability to activate transcription in transient transfections. Mutations which had no effect upon either hormone binding or dimerisation did not interfere with transcriptional activity. Whilst some mutations which disrupt dimerisation *in vitro* appear to inhibit transcriptional activity *in vivo,* others do not. Variations in the extent to which oestrogen stabilises dimerisation by mutant receptors *in vivo* appear to account for this difference.

The structure of the oestrogen receptor bound to different hormonal ligands or different DNA binding sites was investigated in a series of proteolytic gel retardation assays. It has been observed that in a gel

retardation assay the oestrogen receptor/DNA complex migrates with different mobilities when bound to various hormonal ligands. However, similar proteolytic patterns were generated with no hormone, oestrogen, ICI 164,384 and 4-hydroxytamoxifen. Unfortunately it appears that the entire hormone binding domain is removed before any changes in the cleavage profile can be detected by this method. The oestrogen receptor is capable of binding to a palindromic thyroid response element (TRE), but is unable to induce transcription through this response element, presumably due to the altered conformation it is forced to adopt. Proteolysis was used to compare the conformation of receptor on an ERE and TRE. Differences detected appeared to reflect an inability of oestrogen receptor lacking the ligand binding domain to dimerise on this response element.

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## Abbreviations

| | |
|---|---|
| AMPS | ammonium persulphate |
| ATP | adenosine 5' triphosphate |
| bp | base pair |
| BPV | bovine papilloma virus |
| BSA | bovine serum albumin |
| BV | baculovirus |
| CAT | chloramphenicol acetyl transferase |
| CTAB | cetyl trimethyl ammonium bromide |
| dATP | 2'deoxyadenosine 5' triphosphate |
| dCTP | 2'deoxycytosine 5' triphosphate |
| dGTP | 2'deoxyguanosine 5' triphosphate |
| dTTP | thymidine 5' triphosphate |
| DCC | dextran coated charcoal |
| DCFCS | dextran coated charcoal-treated foetal calf serum |
| DEAE | diethylaminoethylamine |
| DEPC | diethyl pyrocarbonate |
| DMEM | Dulbecco's modified Eagles media |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| cDNA | complementary deoxyribonucleic acid |
| DPM | disintegrations per minute |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetracetic acid |
| ERE | oestrogen response element |
| FCS | foetal calf serum |
| GR | glucocorticoid receptor (hGR = human, rGR = rat) |
| GRE | glucocorticoid response element |
| HEPES | N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| hsp 90 | 90 kD heat shock protein |
| kb | kilobase |
| kD | kilodalton |
| $K_d$ | dissociation constant |
| LTR | long terminal repeat |
| MMTV | mouse mammary tumor virus |
| NMR | nuclear magnetic resonance |

| | |
|---|---|
| $OD_x$ | optical density at wavelength x |
| OR | oestrogen receptor (hOR = human, cOR = chicken and MOR = mouse) |
| PAGE | polyacrylamide gel electrophoresis |
| PBSA | phosphate buffered saline A |
| PR | progesterone receptor |
| PRE | progesterone response element |
| RNA | ribonucleic acid |
| mRNA | messenger RNA |
| RNase | ribonuclease |
| S | sedimentation coefficient |
| SDS | sodium dodecyl sulphate |
| SV40 | simian virus 40 |
| TEMED | Tetramethylethylenediamine |
| tk | thymidine kinase |
| Tris | Tris (hydroxymethyl) aminomethane |
| UV | ultra violet |

# CHAPTER 1

# Introduction

The purpose of this introduction is to give an overview of the nuclear hormone receptors and the genes they regulate. Since this thesis describes investigations into the structure and function of the oestrogen receptor, particular reference will be made to this transcription factor. After a brief overview of some of the physiological processes mediated by nuclear receptors and their ligands, some of the initial biochemical work which identified specific receptors and evidence that they act as transcription factors is described. This is followed by a section on hormone responsive genes and the identification of DNA sequences which confer this response to hormones. The cloning of cDNAs for receptors and the mapping and characterisation of different functions are reviewed in some detail. Finally the activities of competitive inhibitors of oestrogen action are described.

## 1.1 Physiological roles of nuclear hormone receptors

The oestrogen receptor is a member of the nuclear hormone receptor superfamily. This group of transcription factors are dependent on the binding of specific ligands for their activity. Members of the nuclear receptor family are active in both vertebrate and invertebrate species, with steroid receptors having been identified in mammals, birds, reptiles, amphibians, fish and insects. A wide range of physiological processes in a variety of tissues are regulated by the receptors in response to their ligands. Oestrogen and progestin receptors mediate growth, differentiation and function of female reproductive and accessory sex tissues (King and Mainwaring, 1974). In oviparous species oestrogen receptors are involved in induction of the synthesis of egg proteins by the liver (Wahli, 1988). The androgen receptor has a similar role in the regulation of the male reproductive system (Cunha *et al.*, 1987). The gonadal steroids also regulate other tissues including skin, hair, bone and the brain. Adrenal corticosteroid receptors are involved in general homeostasis. Glucocorticoids are involved in carbohydrate regulation, and the immune and nervous systems, whereas mineralocorticoids and vitamin D control ion transport. The thyroid hormone receptor mediates the regulation of development and metabolism (Oppenheimer, 1983) and amphibian metamorphosis (Baker and Tata, 1990). The growth and development of epithelial cells and embryonic limb buds are controlled by retinoic acid receptors in response to retinoids (Brockes, 1989). In insect

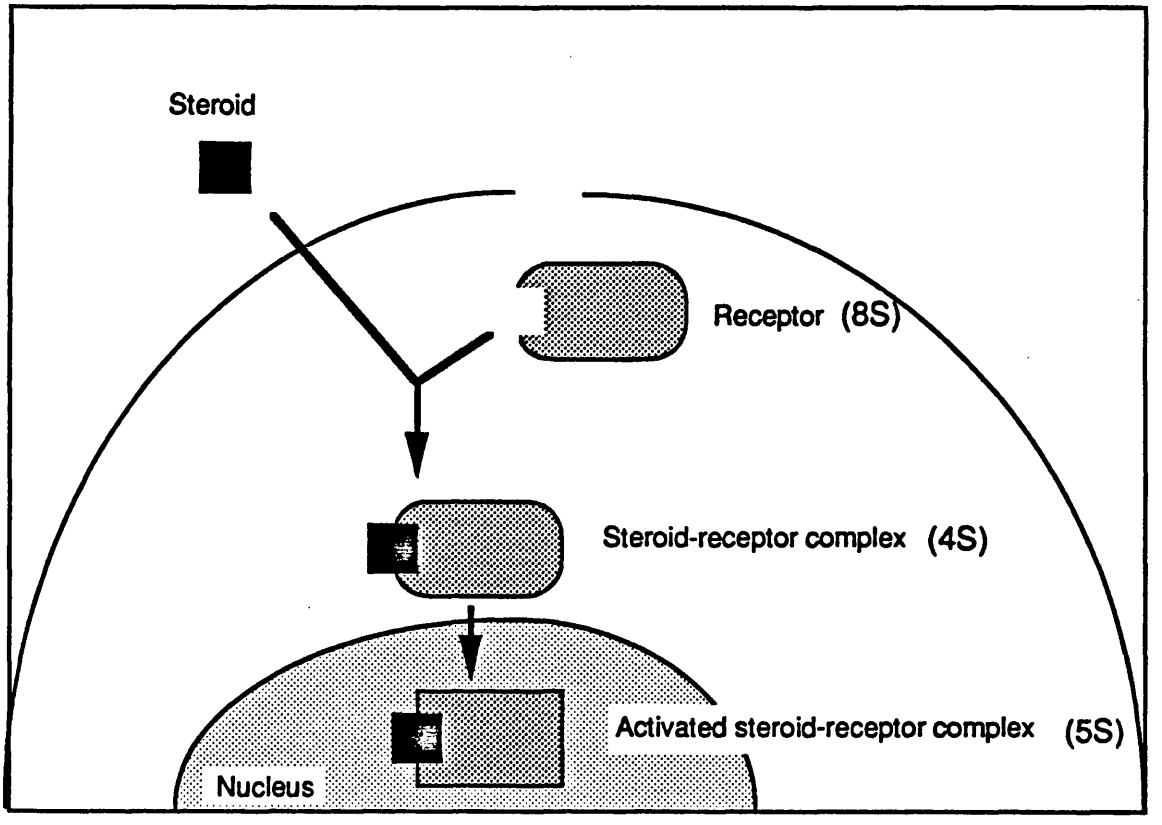
cells gene expression and metamorphosis are regulated by ecdysteroids through the ecdysone receptor (Ashburner, 1990).

## 1.2 Models of steroid receptor action

The physiological effects of steroid hormones such as oestrogens were known for many years before the identification of specific receptors. The first evidence of a hormone binding component in oestrogen responsive tissues was obtained using the newly synthesised radiolabelled oestrogen, [$^3$H]oestradiol. This compound was taken up into the uterus, vagina and anterior pituitary of immature rats (Jensen, 1960), goats and sheep (Glascock and Hoekstra, 1959) across a large concentration gradient. Metabolism in the blood gives rise to a variety of oestradiol metabolites but only oestradiol was taken up by the uterus (Jensen and Jacobson, 1962). The uptake of oestradiol could be blocked by compounds which blocked uterine growth which indicated that the uterotrophic activity of oestradiol depended on its binding to receptors (Jensen, 1962).

These observations on the binding of tritiated oestradiol suggested that target cells were hormone responsive because they contained specific intracellular receptors. Early evidence which supported this hypothesis was gained from cell fractionation studies which identified a cytoplasmic 9.5S species of oestrogen receptor (Toft and Gorski, 1966) and a nuclear 5S species (Jensen *et al.*, 1967). Oestradiol was shown to be initially associated with the 8S species and as this species gradually disappeared a labelled 5S species appeared (Jensen *et al.*, 1968). Based on these observations Jensen proposed that oestrogens act by a two-step process in which the cytoplasmic 8S receptor binds hormonal ligand which causes it to undergo a transformation to the 5S nuclear species (Figure 1.1). The liganded 8S oestrogen receptor proved to be very unstable *in vitro* and at physiological ionic strengths it dissociated to form a 4S species (Jenson and Desombre, 1973). This 4S species has a molecular weight (61-75 kD) and isoelectric point (6.4) corresponding closely to those calculated for the cloned monomeric oestrogen receptor. The nuclear 5S form can be formed from either the 8S or 4S liganded species via a temperature dependent process termed transformation. Chemical crosslinking and dense amino acid labelling experiments suggested that this process involved receptor dimerisation (Miller *et al.*, 1985a). The 5S species is the active form of the oestrogen receptor and has high affinity for DNA. In

Figure 1.1 - Jensen model of steroid hormone action



similar fractionation studies on other classes of steroid receptor only two forms of receptor corresponding to the cytoplasmic 8S and nuclear 5S were found to occur.

It was shown that the 8-9S forms of the progesterone, oestrogen, androgen and glucocorticoid receptors contained a common 90kD component which was absent from the nuclear receptor species (Joab *et al.*, 1984). Subsequently this protein has been identified as the 90kD heat shock protein (hsp90) (Sanchez *et al.*, 1985; Schuh *et al.*, 1985 ; Redeuilh *et al.*, 1987). Hsp90 can be dissociated from the 9S glucocorticoid receptor and 8S oestrogen receptor to give rise to the active DNA binding species under conditions similar to those used to transform receptors from 8-9S to 5S form (Sanchez *et al.*, 1987; Dennis *et al* ., 1988; Baulieu 1987). A number of groups suggested that in view of the high intracellular abundance of hsp90 (1-2% total protein) it may become nonspecifically associated with the receptor during preparation of extracts. The best evidence that hsp90 is associated with the 8-9S species but not the 5S species was obtained *in vivo* using pulse-chase (Howard and Distelhorst, 1988) and chemical crosslinking experiments (Rexin *et al.*, 1988). In addition to hsp90 there is now evidence that two other proteins, p56 and hsp 70 are involved in the receptor/heat shock protein complex (Tai *et al.*, 1986; Estes *et al.*, 1987; Sanchez *et al.*, 1990).

Initially it was thought that 8-9S steroid receptors were cytoplasmic in the absence of ligand and only moved to the nucleus as the 5S form on hormone binding (Jensen *et al.*, 1967). Differences in the subcellular localisation of the steroid receptors were identified by whole cell immunofluorescence. These experiments (Govindan, 1980 ; Papamichail *et al.*, 1980; Antakly and Eisen 1984; Fuxe *et al.*, 1985) confirmed the ligand-dependent translocation of glucocorticoid receptor from cytoplasm to nucleus. However, similar experiments suggested that both the oestrogen receptor (King and Greene 1984; Welshons *et al.*, 1984) and progesterone receptor (Perrot-Applaat *et al.*, 1985; Bailly *et al.*, 1986) remain loosely associated with the nucleus even in the absence of hormone. Since ligand increases the affinity of the oestrogen and progesterone receptors for the nucleus the difference between the receptor classes appears to be in the relative proportions of receptor in the cytoplasmic and nuclear compartments.

The first direct evidence that receptors act as ligand-inducible transcription factors was obtained from studies on the action of ecdysone.

When this steroid hormone was injected into midge larvae, it induced the formation of polytene puffs, associated with high levels of transcription, normally seen later in development (Clever and Karlson, 1960). Similar effects of ecdysone were seen in an *in vitro* culture of Drosophila larval salivary glands (Ashburner, 1972). In this system it could be clearly seen that a small number of genes formed puffs within minutes of hormone addition but the majority reacted only after several hours. The formation of the later puffs required protein synthesis. Hormone initially repressed late genes and this repression was lifted by the products of the early genes (Ashburner, 1974). Ashburner therefore proposed that a complex of ecdysone and receptor protein induced the early genes and inhibited the later genes. This was confirmed by immunofluorescence studies in which the ecdysone receptor was seen to cluster around the induced polytene puffs (Gronemeyer and Pongs, 1980). Two of the early gene products have now been cloned and these are also members of the steroid receptor superfamily (Burtis *et al.*, 1990; Thummel *et al.*, 1990; Seagraves and Hogness, 1990), supporting Ashburner's hypothesis.

## 1.3 Hormone responsive genes

### 1.3 - 1 Glucocorticoid responsive genes

Sequences which conferred responsiveness to steroid hormones were first identified within the glucocorticoid responsive mouse mammary tumour virus (MMTV) promoter. This system was used for much of the early work which helped elucidate the general mechanisms of steroid hormone action. The MMTV is a Class B retrovirus, transmitted to offspring either as endogenous proviruses in the parental genome (vertical transmission) or in the mothers milk (horizontal transmission). Carcinomas of the alveolar epithelial cells of the mammary gland develop 6-9 months after infection with the virus. The alveolar nodules frequently become hyperplastic before this time but tumour induction is not seen until the late stages of pregnancy. In some strains of mice the tumour regresses after pregnancy, appearing again at subsequent pregnancies, until eventually the tumour becomes pregnancy/lactation independent and fails to regress. The correlation of growth with pregnancy suggested that MMTV-induced tumours were hormone dependent.

The first evidence that MMTV was hormone responsive came from experiments which demonstrated that the level of MMTV particles could be increased by incubating primary explants of mouse mammary tumours with glucocorticoids (McGrath *et al.*, 1971). Similar results have subsequently been obtained in cell lines derived from mouse mammary tissue, and the increase in virus production found to correlate with a rise in levels of virus-specific RNA (Parks *et al.*, 1974; Ringold *et al.*, 1975). This increase could be blocked by inhibitors of RNA synthesis, but was unaffected by inhibitors of protein synthesis, demonstrating that this was a primary hormonal effect. Nuclear run-off assays confirmed that the hormone was increasing RNA levels by increasing the rate of transcriptional initiation (Ucker and Yamamoto, 1984).

A number of different groups used a combination of electron microscopy, filter binding and DNA-cellulose competition assays to identify multiple glucocorticoid receptor binding sites between -400 and -100 base pairs upstream of the transcriptional start site of the MMTV long terminal repeat (LTR) (Payver *et al.*, 1981; 1983; Geisse *et al.*, 1982; Govindan *et al.*, 1982; Pfahl, 1982; Pfahl *et al.*, 1983). The LTR sequences -452 to -109 were shown to confer glucocorticoid responsiveness upon the thymidine kinase promoter. They remained active irrespective of orientation and up to 1.1kb from the transcriptional start site indicating that they acted as a classical enhancer element (Chandler *et al.*, 1983).

Studies with deletion mutants established that the 200 bases upstream of the transcription initiation site were sufficient for hormone responsiveness (Majors and Varmus, 1983; Hynes *et al.*, 1983; Lee *et al.*, 1984). Deletions from the 3' end showed that sequences downstream of -53 were not required for hormone responsiveness (Ponta *et al.*, 1985).These results correlated with the *in vitro* binding data. Receptor binding remained unaltered by deletion of MMTV sequences between -450 and -202 but binding activity was reduced by deletion to -137 and was completely abolished by deletion to -50 (Scheidereit *et al.*, 1983). This data also suggested that there were at least two distinct glucocorticoid receptor binding sites between -202 and -50 bp upstream of the LTR start site of transcription. DNAse I footprinting confirmed that the glucocorticoid receptor contacted two discrete regions of the LTR (Scheidereit *et al.*, 1983). The distal region (-192 to -163) contained a single binding site and the larger proximal region (-124 to -72) contained a cluster of three footprints. Comparison of the sequences involved revealed little

homology but all four footprints encompassed the hexanucleotide motif TGTTCT. Subsequent protection and interference assays showed that the receptor is in intimate contact with the guanadine residues of the four repeats and that methylation abolished receptor binding (Scheidereit and Beato 1984).

Direct binding studies *in vitro* using partially purified hormone receptors have found similar results with other hormone responsive genes including human metallothionein IIA (Karin *et al.*, 1984), rabbit uteroglobin (Cato *et al.*, 1984), chicken lysosome (Renkawitz *et al.*, 1984; Hecht *et al.*, 1988), human growth hormone (Slater *et al.*, 1985), and rat tyrosine oxygenase genes (Danesch *et al.*, 1987). Comparison of all the glucocorticoid response elements identified a consensus sequence present in all the genes studied (Scheidereit *et al.*,1986; Jantzen *et al.*, 1987). This consisted of a 15 base pair imperfect palindrome based on a TGTTCT motif found in the earlier studies on MMTV (Figure 1.2).

Expression from MMTV LTR is controlled by a number of different hormones, progestins, androgens and mineralocorticoids (von der Ahe., 1985; Dabre *et al.*, 1986; Arriza *et al.*, 1987; Cato *et al.*, 1987; Strahle *et al.*, 1987; Cato and Weinmann 1988; Ham *et al.*, 1988). It was found that the consensus 15mer glucocorticoid response element was also capable of mediating induction by progesterone, androgens and mineralocorticoids (Strahle *et al.*, 1987; Ham *et al.*, 1988). Binding studies have revealed that whilst glucocorticoid and progesterone receptors make very similar contacts with one consensus GRE (Chalepakis *et al.*, 1988; Slater *et al.*, 1988), there are clear differences in methylation interference patterns and size of footprint on another (von der Ahe *et al.*, 1986; Cato *et al.*, 1988; Chalepakis *et al.*, 1988). Mutations in the GRE which affect the binding of one receptor do not necessarily prevent binding by the other. Thus, in addition to the availability of steroid receptors, differences in binding may account for why some genes are responsive to glucocorticoid and not progesterone or vice versa.

In most cases, regulation of transcription mediated through steroid receptors is believed to be via interactions with other transcription factors. However, the glucocorticoid receptor has also been shown to influence the levels of transcription of MMTV by altering the structure of the chromatin *in vivo*. Changes in the positioning of nucleosomes on the MMTV LTR were observed on addition of hormone (Richard-Foy and Hager, 1987). The binding of the factor NF-1, required for efficient

transcription from this promoter (Miksicek *et al.*, 1987; Buetti and Kuhnel, 1986), is only detected after hormone administration (Cordingley *et al.*, 1987) and yet there is no indication of cooperativity or synergism in their DNA binding. The NF-1 factor was also incapable of binding to an MMTV minichromosome which had nucleosomes positioned in the same pattern as the intact gene. Addition of hormone allowed the glucocorticoid receptor to bind to the MMTV LTR and increased the accessibility of the NF-1 binding site to exonuclease III and, presumably, NF-1 by altering nucleosome arrangement (Pina *et al.*, 1990). It has been proposed that the receptor need not remain on the DNA once the change in chromatin structure has occurred and that the binding of NF-1 may lead to dissociation of the glucocorticoid receptor from the steroid response element. This is supported by the competition for DNA binding observed between the glucocorticoid receptor and NF-1 (Bruggemeier *et al.*, 1990).

### 1.3 - 2 Oestrogen responsive genes

A variety of oestrogen regulated genes have been identified. Much of the early work which investigated the regulation of oestrogen responsive genes was performed using egg protein genes as model systems. One of the first to be studied was vitellogenin, a yolk protein precursor produced in both invertebrates and vertebrates (review - Wahli, 1988). In Drosophila, vitellogenin synthesis is under the control of ecdysone, whereas in chicken and Xenopus vitellogenins are synthesised in the liver in response to oestrogens. In vertebrates liver-specific vitellogenin synthesis is regulated by oestrogens at both transcriptional and post-transcriptional levels (Wahli and Ryffel, 1985). The two egg-white proteins conalbumin and ovalbumin are regulated by a number of steroid hormones, including oestrogens, in chicken oviduct tubular gland cells (Palmiter, 1978). The oestrogen regulation of these two genes is not identical as conalbumin is expressed in chicken parenchymal liver cells (McKnight *et al.*, 1980) whereas the ovalbumin gene is not expressed in these cells despite the presence of functional oestrogen receptors (Deeley *et al.*, 1977). The function of steroid hormones in the regulation of the expression of this gene in oviduct tubular gland cells appears to be the relief of cell-specific repression of transcription (Chambon *et al.*, 1984; Gaub *et al.*, 1987). The apo Very Low Density

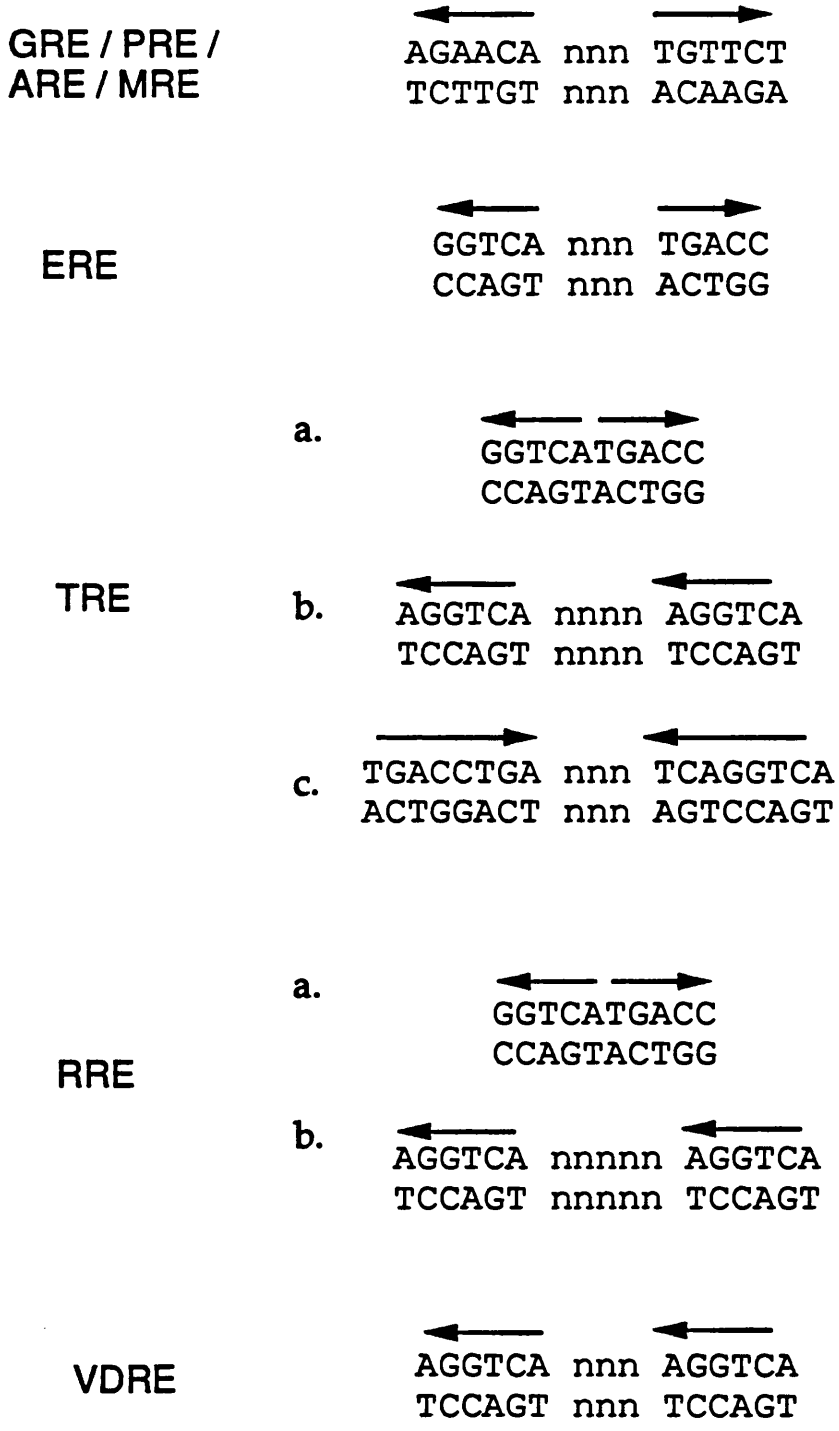
Lipoprotein gene (apoVLDL II) has also been shown to be oestrogen regulated in the liver (Van het Schip *et al.*, 1983 and 1986).

A sequence common to the promoters of these liver oestrogen responsive genes was identified by Walker and co-workers (1984). Vitellogenins are generally expressed from small gene families which are believed to have been generated by gene duplication. Comparison of the sequences of the promoters of four highly homologous Xenopus genes (A1, A2, B1 and B2) with that of the major chicken vitellogenin gene (Jost *et al.*, 1984) identified four blocks of high sequence homology (Walker *et al.*, 1984). Each of these blocks contained either one or two copies of a 13 base pair palindromic sequence, GGTCAnnnTGACC (Figure 1.2) which was also found in the oestrogen responsive chicken apo-VLDL II promoter

A number of oestrogen regulated genes have also been cloned from oestrogen-dependent breast tumour cell lines. The human pS2 gene was identified by differential screening of the MCF-7 cell line grown in the presence of oestrogen (Masiakowski *et al.*, 1982). The function of this protein is unknown although there is homology with a porcine pancreatic inhibitory protein (Thim, 1988). Other oestrogen regulated mRNAs were isolated from MCF-7 cells (May and Westley, 1986). One of these was identified as the lysosomal protease cathepsin D (Westley and May, 1987). This proved to be the 52K protein secreted by MCF-7 cells after treatment with oestradiol (Westley and Rochefort, 1980). The oestrogen receptor is also believed to be involved in the regulation of the expression of a number of other genes. This includes autoregulation of the oestrogen receptor gene expression (Mester and Baulieu, 1972; Westley and May, 1988), regulation of levels of progesterone receptor expression (Horwitz and McGuire, 1978) and creatine kinase B (Pentecost *et al.*, 1990).

Oestrogen response elements (EREs) were first identified in the 5' flanking region of the Xenopus vitellogenin genes (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Martinez *et al.*, 1987). MCF7 cells stably transformed with a vitellogenin A2 genomic clone (λXlv128, Wahli and Dawid, 1980) showed an increase of 2-3 fold in the number of RNA transcripts produced after oestrogen treatment (Klein-Hitpass *et al.*, 1986). In this first thorough analysis of an oestrogen responsive promoter, 5' sequences were fused to chloramphenicol acetyl transferase (CAT) coding

**Figure 1.2 - DNA binding sites for nuclear hormone receptors**




This figure shows consensus DNA sequences for the response elements of nuclear hormone receptors. GRE/PRE/ARE/MRE is a palindromic sequence through which glucocorticoid, progesterone, androgen and mineralocorticoid receptors regulate gene expression. ERE - the palindromic oestrogen response element . TRE- thyroid response elements, a. palindromic TRE, b. direct repeat TRE, c. inverse palindromic TRE. RRE - retinoic acid response elements a. palindrome, b. direct repeat. VDRE the vitamin D response element.

sequences. Transient transfections with these constructs mapped the sequences responsible for hormone responsiveness to between -482 and +14. Deletion mapping of these sequences indicated that the 5' boundary of the ERE in this gene was located between -331 and -324 and that sequences between -482 and -331 inhibited oestrogen inducibility. Deletion from the distal end of these sequences identified an element important in basal activity, and mapped the 3' end of the ERE to between -297 and -314. This indicated that the ERE was located between -331 and -297, a region which contains a single copy of the conserved 13bp palindrome identified by Walker *et al.*, (1984). Thus this study confirmed the functional significance of the GGTCAnnnTGACC motif. The perfect 13bp palindromic ERE has some similarity to the GRE consensus and substitution of one (TGACCT to TGTCCT) or two (TGACCT to TGTTCT) bases within the palindrome was sufficient to convert an ERE into a GRE (Klock *et al.*, 1987; Martinez *et al.*, 1987).

EREs have since been found in the promoter regions of chicken ovalbumin gene (Tora *et al.*, 1988), the rat prolactin gene (Waterman *et al.*, 1988) and the human pS2 gene (Berry *et al.*, 1989), but these do not conform exactly to the proposed consensus. The promoter of pS2 was mapped and sequences between -482 and -332 found to confer oestrogen inducibility on a heterologous gene (β-globin) in a heterologous cell line (HeLa) (Nunez *et al.*, 1989). The pS2 ERE differs from the consensus sequence by a single base pair and confers an oestrogen inducibility three-four times less than that of a perfect palindrome such as the A2 ERE (Berry *et al* 1989). The affinity of the oestrogen receptor is also five-fold more for the perfect palindrome compared with the pS2 ERE (Kumar and Chambon, 1988). An ERE in the chicken ovalbumin gene situated just upstream of the TATA box is unusual in that it contains only one half of the palindromic sequence (Tora *et al.*, 1988). This element is responsive to oestrogens in chicken embryo fibroblast cells but not in HeLa cells (Guab *et al.*, 1987), suggesting that cell-specific factors may be stabilising the binding of the oestrogen receptor to an element for which it has a relatively low affinity. Recently it has been demonstrated that interactions with Fos/Jun which binds to an overlapping site allow the receptor to regulate transcription even in the absence of a functional DNA binding domain (Gaub *et al.*, 1990).

**1.3 -3 Thyroid hormone and retinoic acid responsive genes**

Thyroid hormones affect most cells, influencing general metabolism, growth and development by regulating the expression of specific genes (Oppenheimer, 1983; Oppenheimer *et al.*, 1987). Gene expression known to be regulated by thyroid hormone includes the rat growth hormone (Glass *et al.*, 1987), TSHα (Burnside *et al.*, 1989) and TSHβ genes (Carr *et al.*, 1989; Darling *et al.*, 1989). Retinoids are also involved in many essential processes including development and differentiation (Brockes, 1989), with the effects on limb morphogenesis being particularly dramatic. Both thyroid hormone response elements (TREs) (Glass *et al.*, 1987) and retinoic acid response elements (RREs) have been identified and a consensus sequence AGGT(C/A)A, related to the ERE, has been derived. One sequence which acts as a TRE is a 12 base pair palindrome based on the ERE consensus but lacking the 3 base pair spacer present between the two halves of the palindrome (Figure 1.2). This palindromic sequence, the natural rat growth hormone TRE and the myosin heavy chain TRE was found to be capable of mediating both thyroid hormone and retinoic acid regulation of gene expression (Umesono *et al.*,1988; Bedo *et al.*, 1989; Graupner *et al.*, 1989). The thyroid hormone receptor is also able to bind with high affinity to an ERE but is unable to stimulate transcriptional activation from this element (Glass *et al.*, 1988). Recently Umesono and co-workers (1991) have demonstrated that direct repeats of the consensus motif separated by 3, 4 and 5 bases gave responses to vitamin D, thyroid hormone and retinoic acid respectively (Figure 1.2). This is further complicated by the observation that when separated by three base pairs, direct repeat, palindromic and inverse palindromic arrangements of the motif (Figure 1.2) gave transcriptional responses to retinoic acid, oestrogen and thyroid hormone respectively (Naar *et al.*, 1991). The thyroid and retinoic acid receptors appear to be able to bind to a number of different response elements and retain transcriptional activity.

The RXR family of retinoid binding proteins, which are structurally distinct from the retinoic acid receptor family, have been identified (Giguere *et al.*, 1987; Petkovich *et al.*, 1987). Although both families of receptor can regulate transcription via a single hormone response element (Mangelsdorf *et al.*, 1990), elements which are regulated by RXR and not retinoic acid receptor have been identified in the CRBPII promoter (Mangelsdorf *et al.*, 1991). The RXR responsive

sequence consists of five direct repeats of the AGGTCA spaced by a single nucleotide.

There are considerable similarities between the response elements of the different members of the steroid receptor superfamily. Glucocorticoid, progesterone, androgen and mineralocorticoid receptors stimulate transcription following binding to a palindromic TGTTCT motif. Oestrogen, thyroid, retinoic acid and vitamin D receptors act through various arrangements of the TGACC motif. Recently it has been proposed that all vertebrate hormone response elements are derived from an ancestral motif 5'-GGATCA-3' which is also found in DNA sequences bound by the invertebrate ecdysone receptor (Martinez *et al.*, 1991). The majority of steroid response elements identified fit simple palindromic consensus sequences, in contrast to the structure of response elements of other members of the nuclear hormone receptor superfamily which appear more complex.

**1.3 - 4 Synergy and cooperative binding**

Steroid response elements occur as part of the regulatory sequences which control hormone responsive gene expression. They are often found as pairs or groups of elements or in association with other transcription factor binding sites. Synergy can occur both between steroid receptors and also between steroid receptors and other transcription factors. There appear to be several different mechanisms by which synergy occurs; cooperative binding, cooperative interactions with other parts of the transcriptional machinery and indirect effects involving changes in chromatin structure.

Analysis of the MMTV LTR identified four glucocorticoid response elements, progressive deletion of which resulted in a gradual loss of inducibility (Hynes *et al.*, 1983; Kuhnel *et al.*, 1986). Other glucocorticoid responsive genes such as the chicken lysosome gene (Renkawitz *et al.*, 1984), rabbit uteroglobin gene (Cato *et al.*, 1984) and rat trytophan oxygenase gene (Danesch *et al.*, 1987) also appeared to consist of multiple response elements acting in synergy. Two glucocorticoid response elements located 2.5 kb upstream of the rat amino tyrosine aminotransferase gene were shown to induce transcription synergistically (Jantzen *et al.*, 1987). Similarly oestrogen regulation of the Xenopus vitellogenein B1 gene required at least two copies of the

imperfect (non-palindromic) oestrogen response elements (Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988). Cooperativity in this vitellogenin gene depended upon the EREs being closely adjacent (Martinez and Wahli, 1989; Strahle *et al.*, 1988). Synergy between different steroid receptors has also been reported. The chicken vitellogenin II gene, which contains progesterone and oestrogen response elements, a synergistic induction of transcription is observed in the presence of the two hormones (Cato *et al.*, 1988; Ankenbauer *et al* .,1988).

Co-operative binding between proteins on two adjacent binding sites was proposed as a model for the functional synergy between receptors. The first evidence for this was obtained using the gel retardation assay. Initially it was found that the binding affinity of partially purified progesterone receptor was 100 fold greater for a double GRE/PRE than for a single site (Tsai *et al.*, 1989), and purified glucocorticoid receptor also bound co-operatively to a double site (Schmid *et al.*, 1989). The alignment of binding sites on the DNA was found to be important, with correctly spaced response elements giving a more stable protein-DNA complex.

Oestrogen receptors have also been shown to bind co-operatively to DNA. Martinez and Wahli (1989) demonstrated that at higher oestrogen receptor concentrations occupancy of both imperfect EREs of the vitellogenin B1 gene is favoured over occupancy of a single site. However, similar experiments performed by others have failed to show co-operative binding to tandem perfect or imperfect EREs (Ponglikitmongol *et al.*, 1990). These groups also studied the effects of changing the alignment of binding sites along the DNA helix. Altering the spacing between the two imperfect EREs of the vitellogenin B1 gene, located just upstream of the tk promoter, by 2, 2.5, or 3 turns of DNA had no effect on the oestrogen inducibility of the gene (Klein-Hitpass *et al.*, 1988; Martinez and Wahli, 1989). These results again differed from those of Ponglikitmongkol and co-workers (1990) who found that the stereo-alignment of perfect EREs was only important when they were distant from the promoter (175 bp), whereas the stereo-alignment of imperfect EREs was important even when adjacent to the promoter. There appear to be two different observations being made, in the first oestrogen receptors bind co-operatively and their activity does not depend upon alignments of response elements, and in the second binding is not cooperative but alignment of elements is important. Comparison of the

systems used suggests that alignment of elements may only be important in the absence of other transcription factors as one group used a simple TATA region (Ponglikitmongkol *et al.*, 1990) and the others used a more complex promotor (Klein-Hitpass *et al.*, 1988; Martinez and Wahli, 1989).

Since not all synergistic activity could be accounted for by co-operative binding, an alternative mechanism for functional synergism which occurred at the level of transcription was proposed. This model involved 'bridging' proteins which did not themselves bind DNA but interacted with DNA binding proteins and other components of the transcriptional machinery (Ptashne, 1988). More than one protein interacting with this 'bridging' protein might then increase the transcriptional efficiency. The observation that overexpression of one steroid receptor can inhibit the effects of a second receptor (Adler *et al.*, 1988; Meyer *et al.*, 1989), presumably by titrating out a limiting transcription factor, supports this model.

Synergistic interactions between transcription factors play an important role in the control of gene expression especially in determining cell specificity of expression. Synergy between steroid receptors and other transcription factors has been observed in a variety of systems. The rat tryptophan oxygenase gene was only induced by glucocorticoids when a CACCC binding site sequence adjacent to the GRE remained intact (Danesch *et al.*, 1987). Similarly, deletion or mutation of an NF-1 binding site in the vicinity of a GRE in the MMTV promoter almost completely abolished glucocorticoid inducibility (Buetti and Kuhnel, 1986; Miksicek *et al.*, 1987; Cato *et al.*, 1988). Exonuclease III protection studies (Cordingley *et al.*, 1987) indicated that the NF-1 site is inaccessible in uninduced cells *in vivo* and it has been proposed that the functional co-operativity between these sites involves the repositioning of nucleosomes by the glucocorticoid receptor (Richard-Foy and Hager, 1987). Synergistic interactions between the glucocorticoid receptor and OTF-1 have also been observed on MMTV (Bruggemeier *et al.*, 1991)

The construction of synthetic regulatory units consisting of several receptor binding sites allowed the study of synergistic interactions in a more defined environment. To study the interactions of receptors with a single transcription factor, synthetic genes containing a MMTV PRE in combination with one of a number of different sites upstream of a thymidine kinase (tk) promoter were constructed. NF-1, CBP, Sp1 and OTF-1 were all able to synergise with the progesterone receptor, but not to

as great an extent as a second progesterone receptor (Schule *et al.*, 1988). Addition of a further GRE/PRE did not lead to a further increase in transcription (Strahle *et al.*, 1988; Tsai *et al.*, 1989). The effects of the second DNA binding site depended upon the cell line used, probably reflecting the relative levels of the transcription factors in the different lines (Strahle *et al.*, 1988). This might account for the observation that the activity of hormone responsive genes does not always correlate with the amount of receptor present (Tora *et al.*,1989a; Bocquel *et al.*, 1989).

The alignment of binding sites along the DNA helix appears to affect the degree to which they can synergise with each other. When the distance between a CACCC box and GRE were varied, a strong distance dependence was observed which showed a cyclic pattern of 10 bp or one turn of the DNA helix (Schule *et al*, 1988). However when the effects of spacing between a GRE and TATA box of the tk promoter was examined there was optimal spacing and distance dependence but no periodicity (Ham *et al.*, 1988). This study indicated that like other transcription factors (Chodosh *et al.*, 1987; Wirth *et al.*, 1987; Ruden *et al.*, 1988), steroid receptors do not require a specific stereo-alignment to interact with the TATA box. It also implied a more flexible interaction between these transcription factors compared to that between steroid receptors.

The MMTV promoter was induced through a synergistic interaction between OTF-1 and the glucocorticoid receptor which involved co-operative binding (Bruggemeier *et al.*, 1991). However, co-operative binding does not appear to be the only mechanism for synergy between steroid receptors and other transcription factors. Another group found no evidence for an increase in binding affinity when DNA fragments containing a GRE and NF-1 or OTF-1 site are incubated with purified or *in vitro* translated proteins (Muller *et al.*, 1991). This suggests that synergism between receptors and other transcription factors is more likely to be at the level of transcription.

### 1.3 - 5 Hormonal control of mRNA stability

The majority of studies on steroid hormone control of mRNA levels have concentrated on the regulation of gene transcription. However, the regulation of mRNA stability has also emerged as an important control point in a number of systems. Oestrogens regulate vitellogenin expression 10 fold at the level of transcription but also increases the half-life of vitellogenin mRNA from 15 hours to 500 hours

(Brock and Shapiro 1983). A 529 nucleotide minivitellogenin mRNA which contained 13 nucleotides of 5' untranslated sequence and 165 nucleotides of 3' untranslated sequence, but which lacked most of the internal coding region, was sufficient for oestrogen mediated mRNA stability in a Xenopus liver cell line (Nielsen and Shapiro, 1990). The effects of oestrogens are not simply to stabilise mRNA since hormone induces a system required for the rapid degradation of the avian apoVLDLII and vitellogenin mRNAs which occurs on hormone withdrawal (Gordon *et al.*, 1988).

Glucocorticoids can also regulate genes through control of mRNA stability. Transfection of expression vectors which contained the 3' noncoding region of phosphoenolpyruvate carboxykinase (PEPCK) fused to chloramphenicol acetyl transferase into rat hepatoma cells demonstrated that this 3' region gave increased mRNA stability in response to dexamethasone (Petersen *et al.*, 1989). It was proposed that this was the result of interactions of the mRNA with a glucocorticoid induced stabilising factor.

The mechanism by which hormones stabilise mRNAs is unclear but both oestrogen stabilisation of apoVLDLII mRNA (Cochrane and Deely, 1988) and glucocorticoid stabilisation of hGH mRNA (Paek and Axel, 1987) cause an increase in the length of their poly(A) tails. The oestrogen receptor is nuclear and yet influences the stability of the mRNA in the cytoplasm. One possible pathway for the stabilisation of mRNA by oestrogen could involve the oestrogen induction of a protein required for stability. However the protein induced would have to be very stable as effects on mRNA stability continue for several days after inhibition of synthesis with cyclohexamide (Brock and Shapiro, 1983; Blume and Shapiro, 1989). An alternative mechanism has been proposed, based on the observation that the oestrogen receptor can act as a protein kinase (Baldi *et al.*, 1986; Thampan, 1988). There is support for this hypothesis in the observation that oestradiol induces the reversible phosphorylation of two proteins on the 40S ribosomal subunit (Cochrane and Deely, 1984). Although ribosomes are required for vitellogenin mRNA stability the regulation appears to be through 3' untranslated sequences, and may be similar to histone mRNA degradation in which ribosomes must directly contact sequences in the 3' untranslated region (Graves *et al.*, 1987).

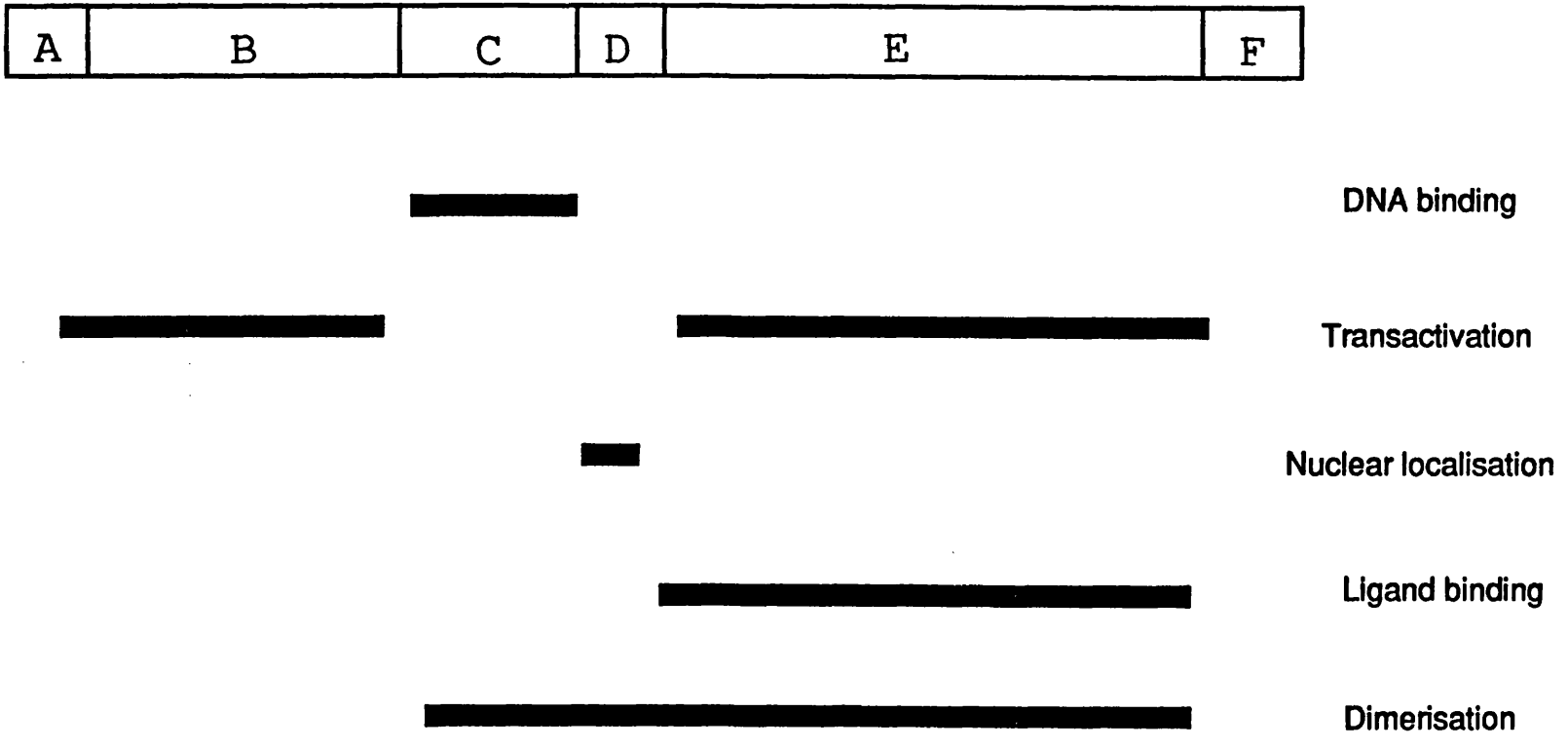
## 1.4 Cloning and sequence analysis of steroid hormone receptors

In 1985 the first complete sequence of a steroid hormone receptor was provided by the cloning of the human glucocorticoid receptor (hGR) cDNAs (Hollenburg *et al.* ). When the hGR was compared with sequences in the protein data base there was significant homology between the C-terminal 387 amino acids and the v-erb A oncoprotein of avian erythroblastosis virus, which suggested that these proteins shared a common ancestral gene (Weinburger *et al.*, 1985). The region of highest homology (45%) was within a 20 amino acid cysteine/lysine/arginine rich region and the authors proposed that this sequence formed part of the receptors DNA binding domain.

The human oestrogen receptor was cloned from cDNA libraries prepared from the MCF-7 breast cancer cell line (Walter *et al.*, 1985). The MCF-7 oestrogen receptor mRNA is 6322 nucleotides in length and encodes a 595 amino acid protein of 66kD (Green *et al.*, 1986). A number of cDNAs encoding oestrogen receptor have also been isolated from other species including chicken (Krust *et al.*, 1986), rat (Koike *et al.*, 1987), Xenopus (Weiler *et al.*, 1987) and rainbow trout (Pakdel 1989). The work described in this thesis was performed using the mouse oestrogen receptor cDNA isolated by White *et al.* (1987).

Comparison of the sequence of human and chicken oestrogen receptors indicated six regions, A-F, of different homology where the DNA binding domain (region C) and the ligand binding domain (region E) are the most highly conserved (Krust *et al.*, 1986) (Figure 1.3). Sequence alignment of oestrogen and glucocorticoid receptors revealed that the difference in size between the two receptors is almost entirely due to variations in the length of the N-terminal regions A and B. The N-terminus of the glucocorticoid receptor contains over 400 amino acids whereas the oestrogen receptor has only 185 amino acids in regions A and B. Over the receptor superfamily there is even greater variation in length of the N-terminus which is over 600 amino acids in the mineralocorticoid receptor but only 24 amino acids in the vitamin D receptor. Although the N-terminus is not well conserved between different steroid receptors, region A is highly conserved between human and chicken oestrogen receptors suggesting that it may play an important role in this receptor. The cysteine/lysine/arginine rich motif noted by Weinburger *et al.*, (1985) is within region C and it was proposed that eight

**Figure 1.3 - Functional domains in the oestrogen receptor**



of the nine invariant cysteines might form a zinc finger motif similar to those originally identified in Xenopus TFIIIA by Miller *et al* (1985b). Region E consists of approximately 250 amino acids and is hydrophobic in character. A number of groups have proposed that this region folds to form a ligand binding hydrophobic pocket. The region D between the highly conserved regions C and E is not conserved and was proposed to act as a hinge between these two functional domains (Kumar *et al.*, 1986). The length of region D may be increased or decreased without loss of receptor function (Kumar *et al.*, 1987) suggesting that the structure of this region is unlikely to be important in receptor function. Regions B and F show very low levels of homology and no function has been ascribed to them.

This work led to the suggestion that other steroid receptors could be divided into regions corresponding to region A-F of the oestrogen receptor. Progesterone (Jeltsch *et al.*, 1986; Loosfelt *et al.*, 1986; Gronemeyer *et al.*, 1987, Mishrahi *et al* 1987), androgen (Chang *et al.*, 1988, Lubahn *et al.*, 1988) and mineralocorticoid (Arriza *et al.*, 1987) receptors were cloned and the levels of sequence homology agreed with the six regions A-F proposed by Krust *et al.*, (1986). Each contained a highly homologous region C and a hydrophobic C-terminal domain which corresponded to the region E of oestrogen and glucocorticoid receptors. The cellular homologue of v-erb Aβ has been isolated and identified as a thyroid hormone receptor (Weinburger *et al.*, 1986; Sap *et al.*, 1986).

A number of cDNAs encoding other ligand-binding transcription factors with homology to the steroid hormone receptors have been isolated. These include the vitamin D receptor (Baker *et al.*, 1988) and several forms of retinoic acid receptor (Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988). There are also proteins, termed 'orphan' receptors which share homology with the receptors but for which no ligands have been identified such as COUP (Wang *et al.*, 1989), the oestrogen receptor related proteins ERR1 and ERR2 (Giguere *et al.*, 1988), the liver protein PPAR (Isseman and Green, 1990) and the Drosophila genes tailless (Pignoni, 1990) and seven-up (reviewed- Evans 1988). The steroid receptors and related proteins are referred to collectively as the nuclear receptor superfamily.

The cloning and mapping of the genomic sequences of the chicken progesterone (Huckaby *et al.*, 1987) and human oestrogen

(Ponglikitmongkol *et al.*, 1988) receptors has revealed that the genomic structure of the genes encoding the receptors is also well conserved despite varying in size from 15 to 200 kb. All receptors characterised to date contain at least eight coding exons but some also contain additional non-coding exons such as the extra 5' exon in the mouse (White *et al.* ,1987) and human (Keaveney *et al.*, 1991) oestrogen receptors, and the extra 5' and 3' exons in the human thyroid receptor (Zahraoui and Cuny 1987). The N-terminus is largely encoded by a single exon, whilst the DNA-binding domain is encoded by two exons (exons 2 and 3) each containing one zinc binding motif. The remaining five coding exons (exons 4-8) give rise to the hormone-binding domain. The intron/exon arrangement of the receptor family genes is of interest as it is possible that a single structural motif may be encoded in a single exon. The structure of the N-terminus and DNA binding domain agrees with the hypothesis (Gilbert, 1978) that a single functional structure may be encoded in a single exon whereas the C-terminus has a more complex structure.

The isolation and mapping of the genomic sequences of steroid receptor genes followed the identification of cDNA sequences. This has allowed comparison of the organisation of the promoters of these genes . It appears that multiple promoters may be a feature of the nuclear receptor family. In both chicken and human the progesterone receptor has two promoters (Kastner 1990a) which generate transcripts that encode receptors with different N-terminal sequences (Conneeley *et al* .,1989; Jeltsch *et al.*, 1990). Multiple transcripts are also generated by the human oestrogen receptor but these encode identical proteins (Keaveney *et al.*, 1991). The expression of thyroid and retinoic acid receptors has proved to be even more complex with multiple genes, alternative promoters and splicing patterns generating a number of different receptors (Lazer *et al.*, 1989; Miyajima *et al.*, 1989; Kastner *et al.*, 1990b).

## 1.5 Structure and function of steroid receptors

### 1.5 - 1 DNA binding domain

The DNA binding domain was originally identified in the glucocorticoid receptor by limited proteolysis. Carlstedt-Duke and co-workers (1982) performed a limited chymotrypsin digestion of the purified 94-98kD glucocorticoid receptor which yielded two fragments. The 50kD species, which was later found to correspond to the N-

terminus, contained sites for most of the anti-glucocorticoid receptor antibodies and was termed the immunological domain. The smaller 40 kD species contained both the DNA and hormone binding functions. Digestion of the 40kD species with trypsin yielded a 16kD fragment which contained the DNA binding domain and species of 27 and 31 kD both capable of binding ligand (Eisen *et al.*, 1985). Similarly, limited digestion of the oestrogen receptor with trypsin separated the DNA binding domain without affecting ligand binding activity (Sala-Trepat and Vallet-Strouve, 1974; Greene *et al.*, 1984). Subsequently sequence comparison between cloned steroid receptors suggested that the most highly conserved region, Region C, might contain this DNA binding domain.

The cloning of members of the steroid receptor family allowed functional domains to be studied in more detail by analysing the effects of natural and artificially generated mutants on gene transcription. A series of deletions throughout the human oestrogen receptor, each encompassing 66 amino acids, were generated by site directed and deletion mutagenesis (Kumar *et al.*, 1986). Nuclear association was used as an indirect assay for DNA binding. It appeared that DNA binding was not affected by removal of the N-terminal amino acids of the receptor but was abolished by deletion of region C or the proposed hinge region D. The DNA binding domain had sharply defined boundaries with sequences between 440 and 508 proving sufficient to mediate DNA binding (Kumar *et al.*, 1986). The mapping of the DNA binding domain to region C was supported by a mutant glucocorticoid receptor isolated from a glucocorticoid resistant lymphoma line (Danielsen *et al.*, 1986). The mutation of an arginine to histidine within region C resulted in a tenfold lower transcriptional activity of the glucocorticoid receptor in this cell line, due to a reduction in ability to bind DNA. The presence of conserved cysteine residues originally led to the hypothesis that the DNA binding domain of the steroid receptors was a cysteine (CC-CC) variant of the cysteine and histidine (CC-HH) 'zinc finger' motif found in a number of transcription factors. This was supported by the finding that zinc is important for DNA binding by the oestrogen receptor (Sabbah *et al.*, 1987).

Mutagenesis was also used to determine the sequences in the steroid receptors which determined DNA binding specificity. In 1987 Green and Chambon demonstrated that the region encoding the two 'zinc fingers' of the oestrogen receptor was involved in the recognition

of specific DNA sequences. By substituting the glucocorticoid receptor DNA binding domain for the oestrogen receptor DNA binding domain they generated a chimeric receptor which activated transcription through a GRE rather than an ERE in response to oestrogens. Although both 'zinc fingers' are required to bind DNA with high affinity it is the N-terminal finger which is of greater importance in determining specificity (Green *et al.*, 1988). Point mutagenesis has identified some of the amino acids in this N-terminal finger which determine the specificity of DNA binding. Conversion of the glycine-serine sequence between the the distal pair of cysteines in the first 'zinc finger' of the glucocorticoid receptor to the glutamine-glycine sequence found in the oestrogen receptor generates a receptor which binds to an ERE instead of a GRE (Danielsen *et al.*, 1989; Umesono and Evans, 1989). Conversely, changing the glutamine-glycine in an oestrogen receptor to the glycine-serine of a glucocorticoid receptor generates a receptor which no longer acts through an ERE and partially activates transcription through a GRE (Mader *et al.*, 1989). Hybrid receptors in which parts of the second finger or interfinger region of the glucocorticoid receptor are replaced by the corresponding oestrogen receptor sequence retain their ability to bind a GRE but some can also activate transcription through an ERE (Danielsen *et al.*, 1989). It has been proposed that these mutations disrupt the structure of the protein in a such a way that its ability to distinguish between a GRE and an ERE is reduced. These results were consistent with the recently solved structure of the glucocorticoid receptor on DNA (Luisi *et al.*, 1991), which confirmed that the N-terminal finger contacted DNA and the C-terminal finger made protein-protein contacts.

The initial mutagenesis experiments were followed by more detailed studies which began to investigate the possible structure of this domain. The 'zinc finger' DNA binding motif, first identified in the Xenopus transcription factor TFIIIA (reviewed - Klug and Rhodes, 1987), was one possible model. The DNA binding activity of TFIIIA is mediated by nine tandem repeats of a predominantly basic motif corresponding to the consensus $Y\,X\,C\,X_2\,C\,X_3\,F\,X_5\,L\,X_2\,H\,X_3\,H$ , in which the three hydrophobic residues and the cysteine and histidine pairs are invariant. It was shown that the DNA binding activity of TFIIIA was dependent upon zinc, present at a ratio of approximately one atom per tandem repeat (Miller *et al.*, 1985b). This led to the proposal that the zinc was tetrahedrally coordinated by the invariant cysteine and histidine pairs of

the repeats. These were proposed to form nine peptide loops, termed CC-HH 'zinc fingers', which interacted specifically with DNA. Subsequently this hypothesis was confirmed by extended X-ray absorption fine structure (Diakun *et al.*, 1986) and visible light (Frankel *et al.*, 1987) analysis. Similar zinc finger motifs have been identified in many other transcription factors, and confirmed to mediate DNA binding in a zinc dependent manner (Kadonaga *et al.*, 1987). In 1988 Berg proposed a model for the structure of the zinc finger consisting of an antiparallel β sheet followed by an α helix. The determination of the three dimensional structure of the 31st zinc finger of the Xenopus Xfin protein, which conforms to the consensus sequence, by NMR spectroscopy confirmed this model (Lee *et al.*, 1989). The polypeptide backbone forms a helix which packs against two antiparallel β sheets linked together by hydrogen bonds between the conserved tyrosine and phenanaline. The exposed face of the helix contains basic and polar residues which are proposed to form contacts with both the sugar-phosphate backbone and bases of the recognition sequence.

Another possible model for the nuclear receptor DNA binding domain was the zinc binding domain of GAL4. The yeast transcription factor GAL4 contains sequences which resemble the zinc finger motif described above but with cysteine residues in the place of the invariant histidine pair and acidic residues in the place of the conserved hydrophobics (Hartshorne *et al.*, 1986). The N-terminal region of GAL4 which contains the cysteine motif was shown to mediate DNA binding (Keegan *et al.*, 1986) in a zinc dependent manner (Pan and Coleman, 1989). Initially it was thought that the GAL4 DNA binding domain might form a structure similar to the Xfin zinc finger. In fact the actual structure of the domain, as determined by NMR proved to be a novel 'binuclear cluster' rather than a zinc finger (Pan and Coleman, 1990). This binuclear cluster, consisting of the two zinc ions coordinated by six cysteines was also found in PPR1 and LAC9, suggesting that the binuclear cluster may be a conserved DNA binding motif.

Since cysteines were involved in coordination of zinc in both zinc fingers and binuclear clusters it was predicted that this would also be the case in steroid receptors. In the second finger five conserved cysteines were present, and thus several alternative structures were possible. Point mutagenesis of cysteines within the proposed finger region of the glucocorticoid receptor identified which residues were involved in the

co-ordination of the zinc molecules (Severne *et al.*, 1988). It was found that all conserved cysteines in the region proposed to form the first finger were required for receptor function. However, within the second finger mutation of $Cys_{500}$ did not abolish transcriptional activity of the receptor. This was unexpected since $Cys_{500}$ is also highly conserved.

Physical studies of the steroid receptor DNA binding domain have revealed that it is structurally distinct from both the TFIIIA 'zinc finger' and GAL4 binuclear cluster. The three-dimensional structure of the DNA-binding domain of the glucocorticoid receptor was solved by two-dimensional nuclear magnetic resonance (2D NMR) of a fragment over-expressed in *E. coli* (Hard *et al.*, 1990). This revealed that the domain consisted of two globular structures formed by tetrahedral co-ordination of two zinc atoms by four pairs of cysteines (Freedman *et al.*, 1988). Immediately adjacent to the N-terminal side of each receptor finger were α-helices orientated perpendicular to one another and two turns near the beginning of the second finger. There was also a small region of antiparallel β-sheet in the first finger. The structure of the oestrogen receptor DNA binding domain has also been solved (Schwabe *et al.*, 1991) and it appears that the structures of the oestrogen and glucocorticoid DNA binding domains are similar. In contrast to the independent structures of the TFIIIA 'zinc fingers', the steroid receptor zinc fingers folded together to form a single structure. There are also differences in the positioning of the α-helical region.

Recently the structure of the glucocorticoid receptor bound to DNA has been solved (Luisi *et al.*, 1991). This confirmed earlier proposals, based on the results of mutagenesis, that the amino-terminal finger contains the residues which make phosphate contacts with DNA and the second pair of zinc coordinating cysteines begin an α-helix that provides all the base contacts in the major groove of the GRE. The carboxy-terminal motif provides additional phosphate contacts and the sequences involved in contacts between the two fingers also form contacts between receptors within a dimer.

### 1.5 - 2 Hormone binding

The earliest evidence for a discrete hormone binding domain came from proteolysis of receptors, which revealed that the DNA binding domain of the oestrogen (Sala-Trepat and Vallet-Strouve, 1974; Greene *et al.*, 1984) and glucocorticoid receptors (Carlstedt-Duke *et al.*, 1982; Eisen *et*

*al.*, 1985) could be removed without affecting hormone binding activity. Comparison of the sequences of the human and chicken oestrogen receptors identified a highly conserved hydrophobic region (Region E) in the C-terminus which was proposed to contain the hormone binding domain (Krust *et al.*, 1986). Region E is approximately 250 amino acids in length and corresponds in size to the smallest proteolytic fragment (27KD) which bound hormone (Eisen *et al.*, 1985). Deletions N-terminal of residue 301 or C-terminal of 552 did not affect the ligand binding affinity of the human oestrogen receptor expressed *in vitro* or in a transient transfection assay suggesting that region E is sufficient for ligand binding (Kumar *et al.*, 1986). Further deletions into the N-terminus resulted in a gradual loss of ligand binding activity but it was not possible to accurately map the N-terminal boundary of the hormone binding domain. In the mouse oestrogen receptor deletion of the C-terminus to residue 552 generated receptor with affinity for oestradiol similar to that of the full length receptor, deletion to 538 reduced affinity for ligand and deletion to 507 completely abolished ligand binding (Fawell *et al.*, 1990a). This suggested that sequences in this region were involved in ligand binding. Additional evidence which supported this came from point mutations in this region which also affected ligand binding.

The hormone binding domain of other members of the steroid receptor family have also been localised to the C-terminus. Mutagenesis of the glucocorticoid receptor identified region E as the hormone binding domain (Giguere *et al.*, 1986; Rusconi and Yamamoto, 1987). A point mutant in region E of the glucocorticoid receptor abolishes hormone binding (Danielsen *et al.*, 1986) and decreases transcriptional activity more than tenfold. Deletion of as few as 6 amino acids from region E reduced glucocorticoid receptor affinity for dexamethazone from 7nM to > 10μM (Weinberger *et al.*, 1985). The inability of v-erb-A, a viral homologue of the thyroid receptor, to bind hormone appears to be due to point mutations within region E and the loss of 9 amino acids from near the C-terminus (Munoz *et al.*, 1988).

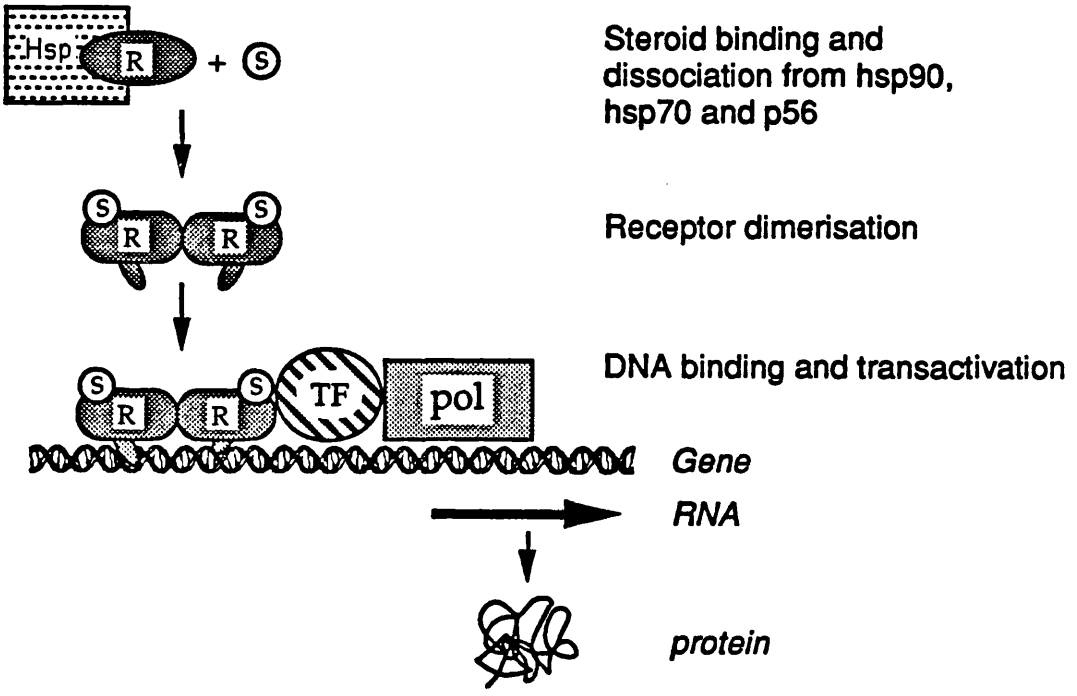
The size of the hormone binding domain precludes the use of NMR and as a result there is little structural information aside from predictions based on protein sequence.

### 1.5-3 Dimerisation

Many transcription factors have been shown to bind DNA as dimers (reviewed Pabo and Sauer, 1984; Jones, 1990). The palindromic nature of steroid response elements and their ability to bidirectionally activate transcription led to speculation that receptors also bound to DNA as dimers (Kumar and Chambon, 1988). A modification of the Jensen model of steroid hormone action that incorporates both dimerisation and heat shock protein complex is now widely accepted (Figure 1.4). In the absence of hormone the inactive receptor is associated with a heat shock protein complex. On addition of hormone the receptor dissociates from the other proteins and forms functional dimers which are capable of binding with high affinity to specific response elements and subsequently inducing transcription. Experiments using anti-oestrogen receptor antibodies (Linstedt *et al.*, 1986) and computer modelling studies (Gordon and Notides, 1986) were consistent with dimers being the transcriptionally active form of receptor.

The most extensively studied dimerisation motif is the leucine zipper (Landschultz *et al.*, 1988). Many dimerisation motifs appear to be closely associated, both functionally and spatially, with the DNA binding domain of the protein in question. This is true for both the bZIP family of proteins where the basic DNA binding sequences are directly N-terminal of the leucine zipper dimerisation motif and the helix-turn-helix proteins where the DNA binding motif precedes the first amphipathic helix (Davis *et al.*, 1990). The basic 'leucine zipper' DNA binding and dimerisation motif was first identified in C/EBP (Landschulz *et al.*, 1988). Deletion mutagenesis had shown that the C-terminal 60 amino acids of this protein bound DNA with wild-type affinity (Hope and Strul, 1986) and these sequences were also sufficient for dimerisation (Hope and Strul, 1987). The C/EBP DNA binding and dimerisation domain had significant homology with the C-terminal sequences of the v-Jun and v-Fos oncoproteins and the GCN4 protein. Landschulz *et al.* (1988) suggested that a heptad repeat of leucine residues, conserved across the three proteins, might form the dimerisation domain. This domain was proposed to form a long, ion pair stabilised α-helix with the leucine residues protruding along one side. Interdigitation with the leucines of a second polypeptide in an antiparallel configuration would then generate

Figure 1.4 - Revised model of steroid hormone action



a stable dimer interface thus positioning the basic DNA binding domains close together and allowing high affinity DNA binding.

The leucine zipper model was investigated by introducing point mutations in the conserved leucines. It was found that these residues were required for both dimerisation and DNA binding (Kouzarides and Ziff, 1988; Ransone *et al.*, 1989; Landschulz *et al.*, 1989). A peptide containing the C-terminal 33 amino acids adopted an α helical structure in solution and formed stable dimers (O'Shea *et al.*, 1989). These results confirmed that the structure of the region was α helical but revealed that the interaction was actually parallel, resembling the coiled-coil dimerisation interface of fibrous proteins such as tropomyosin (review-Cohen and Parry, 1986). In these proteins two parallel right-handed helices coil around each other with a slight left-handed twist which reduces the helical repeat to 3.5, placing the 4-3 repeat of hydrophobics on one face of the helix to form a hydrophobic dimer interface. In view of the parallel interaction between the leucine zippers a 'scissor-grip' model for the interaction of the dimer with DNA has been proposed (Vinson *et al.*, 1989). In this model the dimer is a 'Y' shape with the leucine zipper forming the base of the structure and the two basic regions orientated so that they fork away from each other to form a single DNA binding surface. Exchanging leucine zipper motifs between proteins has shown that both specificity and affinity of homo- and heterodimers are mediated by sequences within the zipper (Kouzarides and Ziff, 1989; Sellers and Strul, 1989; Neuberg *et al.*, 1989).

More recently another motif, the basic helix-loop-helix (bHLH) which comprises both DNA binding and dimerisation functions has been identified in the developmental control proteins Myo D, E12 and E47 (Murre et al., 1989). The helix-loop-helix consists of two short amphipathic α helices with conserved 4-3 hydrophobic repeat separated by a loop of variable length containing one or more helix breaking residues. Immediately N-terminal of the first helix lies a basic region shown by mutagenesis in MyoD to be involved in DNA binding (Davis *et al.*, 1990). The bHLH proteins, like the leucine zipper proteins, require formation of stable dimers for high affinity DNA binding.

There is direct evidence that both the human oestrogen receptor (Kumar and Chambon, 1988) and rat glucocorticoid receptor (Tsai *et al.*, 1988) bind to their respective response elements as dimers. This was obtained using techniques similar to those used by Hope and Struhl

(1986) on the leucine zipper proteins. Comparison of the ability of full-length and C-terminally deleted human oestrogen receptor to bind DNA in a gel retardation assay indicated that a major dimerisation activity is contained within the hormone binding domain (Kumar and Chambon, 1988). Studies of the nuclear uptake of the progesterone receptor (Guiochon-Mantel *et al.*, 1989) confirmed that the hormone binding domain contained sequences required for interactions between monomers. The C-terminus of the sequences required for dimerisation and subsequent DNA binding in the mouse oestrogen receptor was mapped by more detailed mutagenesis (Fawell *et al.*, 1990a). A receptor lacking residues C-terminal of 538 bound DNA with high affinity, whereas a further deletion to 507 reduced affinity to less than a tenth that shown by wild-type receptor. A potential leucine zipper was present in this region of the oestrogen receptor but was not conserved in other receptors and could be mutated with no effect upon DNA binding. Several sets of hydrophobic repeats were present in the ligand binding domain of the thyroid receptor (Forman *et al.*, 1989). Deletion of the thyroid receptor C-terminal hydrophobic repeat led to a loss of transcriptional activity. Since receptors which contained this region, but not the DNA binding domain, acted in a dominant negative fashion it appeared that these sequences were required for dimer formation. The hydrophobic repeats identified by Forman *et al* were conserved within the thyroid and retinoic acid receptors but not across the rest of the receptor superfamily.

Within the same region of the oestrogen receptor as the putative leucine zipper was another heptad hydrophobic repeat (Fawell *et al.*, 1990). In contrast this was conserved in other steroid receptors and through the nuclear receptor superfamily (Figure 1.5). It also overlapped the sequences which were required for thyroid receptor dimerisation but was not the C-terminal hydrophobic repeat identified by Forman *et al.*. The contribution of the conserved hydrophobic amino acids to dimerisation in the mouse oestrogen receptor was studied by introducing point mutations. Mutation of the N-terminal conserved hydrophobic residues (L-511 and I-518) abolished dimerisation and high affinity DNA binding whereas mutation of the two C-terminal residues (G-525 and M-532) of the repeat had no effect upon the formation of dimers. This mutagenesis suggested that only the N-terminal part of the repeat was

**Figure 1.5 - Steroid receptor superfamily sequence alignment**

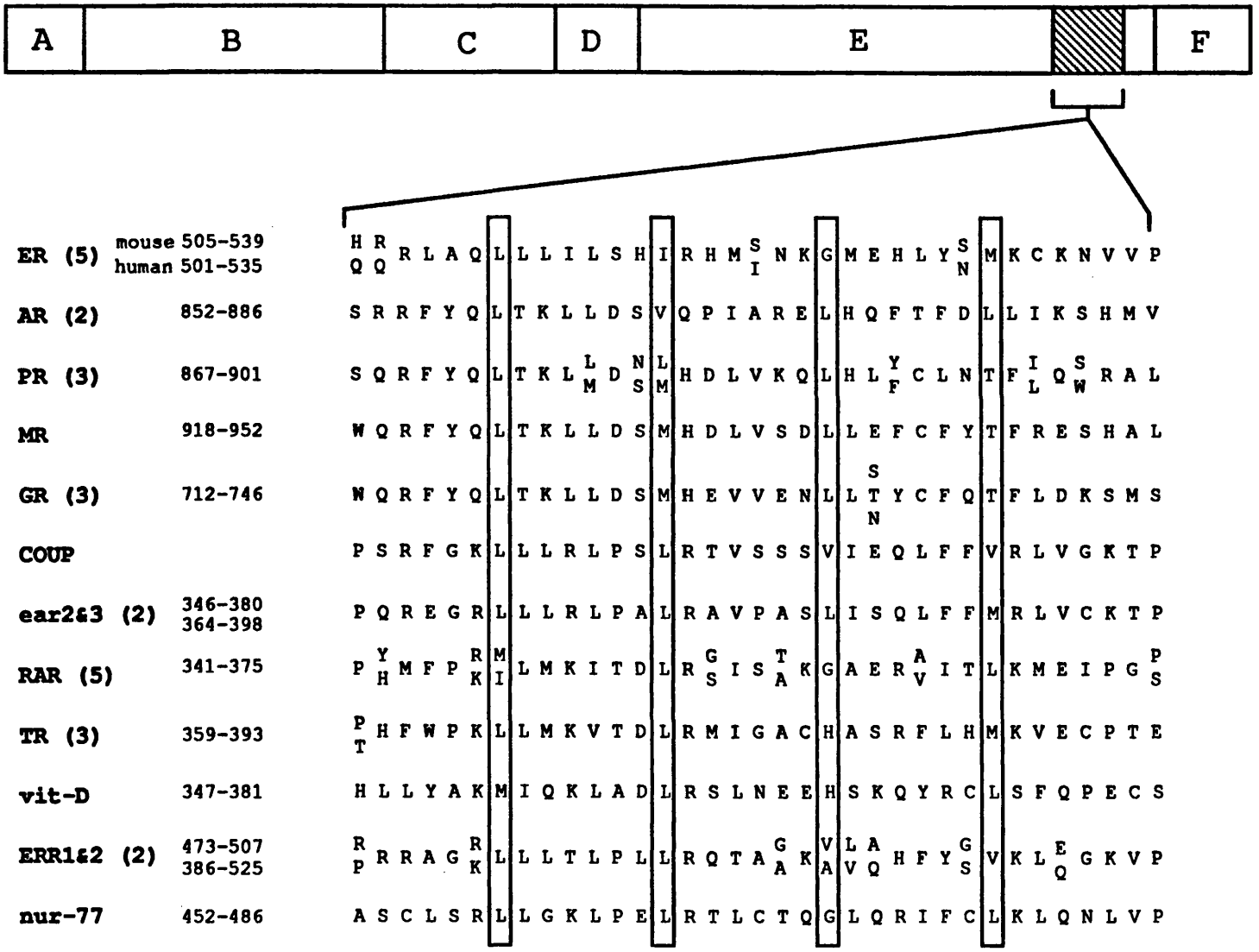


Figure 1.5

The protein sequence of members of the nuclear receptor family were aligned by computer (Genalign) or manually and a conserved region within the ligand bindng domain shown. Amino acid numbers are indicated and represent the human protein where appropriate. The figures in parenthesis for each class of receptor represent the number of different species or receptor subclasses used in the alignment and divergent residues are shown. A repeat of hydrophobic residues is highlighted in boxes. ER, estrogen receptor: mouse (White *et al.* 1987), human (Green *et al.* 1986), chicken (Krust *et al.* 1986), rat (Koike *et al.* 1987), Xenopus (Weiler *et al.* 1987). AR, androgen receptor: human and rat (Chang et al. 1988). PR, progesterone receptor: human (Mishrahi *et al.* 1987), rabbit (Loosfelt *et al.* 1986), chicken (Gronemeyer *et al.* 1987). MR, mineralocorticoid receptor: human (Arriza *et al.* 1987). GR, glucocorticoid receptor: human (Hollenberg *et al.* 1985), mouse (Danielson et al. 1986), rat (Miesfield *et al.* 1986). COUP: chicken (Wang *et al.* 1986). ear2&3: human (Miyajima *et al.* 1989). RAR, retinoic acid receptor: human (Petkovich *et al.* 1987; Giguere *et al.* 1987; Brand *et al.* 1988; Berbrook *et al.* 1988), mouse a,b.c forms (Zelent *et al.*1989 ). TR, thyroid hormone receptor: human (Weinberger *et al.* 1986), chicken (Sap *et al.* 1986), rat (Thomson *et al.* 1987). VIT D, vitamin D receptor: human (Baker *et al.* 1988). nurr-77: mouse (Hazel *et al.* 1988). ERR1&2: human (Giguere *et al.* 1988).

involved in dimerisation. The conserved sequence was within the region shown to be required for formation of thyroid hormone and retinoic acid receptor heterodimers (Glass *et al.*, 1989). A 22 amino acid peptide extending from amino acid 501-522 was capable of restoring DNA binding activity to a mutant receptor lacking most of the hormone binding domain (Lees *et al.*, 1990). This suggested that sequences between 501 and 522 were sufficient to induce DNA binding activity and appeared to encode a major portion of the dimer interface of the mouse oestrogen receptor. However, there is evidence that other sequences contribute to the formation of stable dimers *in vivo*. It has been demonstrated that removal of the entire hormone binding domain and N-terminus results in oestrogen receptor which is capable of binding to DNA, albeit weakly (Kumar and Chambon, 1988). This suggests that the DNA binding domain contains a minor dimerisation function . The ability of glucocorticoid receptor DNA binding domains to bind to DNA in a co-operative manner (Tsai et al., 1988; Dahlman-Wright et al., 1990) also provides evidence that there is a minor dimerisation function within the DNA binding domain itself.

**1.5-4 Transcriptional activation**

In the majority of systems studied, steroid receptors act by increasing levels of transcription from hormone responsive genes. The precursors of eukaryotic messenger RNAs (mRNAs) are transcribed by RNA polymerase II. The formation of a stable preinitiation complex is necessary for transcription to occur. It has been demonstrated in cell-free systems that both the progesterone receptor (Klein-Hitpass *et al.*, 1990) and oestrogen receptor (Elliston *et al.*, 1990) facilitate the formation of a stable preinitiation complex at the target gene promoter and thus augment the initiation of transcription by RNA polymerase II. Whilst other transcription factors, such as ATF (Horikoshi *et al.*, 1988a; 1988b), have been shown to interact with TFIID (the TATA box binding protein) steroid receptors have been shown to regulate expression from natural (Jeltsch *et al.*, 1990) and synthetic promoters (Thomson *et al.*, 1990) in the absence of a functional TATA box element. In the synthetic promoter the steroid response element was the only transcription factor response element present. Thus, in addition to interactions with classical transcription factors, receptors may be interacting directly with another component of the transcriptional machinery. Candidates for this protein

include RNA polymerase II, 'bridging' proteins (Ptashne, 1988) and an 'adaptor' protein proposed to mediate the transcriptional activity of the adenovirus E1a protein (Martin *et al.*, 1990).

Transcription factors appear to consist of separate transcriptional activation domains which retain their activity when fused to a heterologous DNA binding domain (Brent and Ptashne, 1985). The first transactivation domain to be identified was within the yeast regulatory proteins GAL4 and GCN4 (Hope and Struhl, 1986). Deletion mutagenesis identified two discrete regions which were able to independently activate the GAL1 promoter when fused to the GAL4 DNA binding domain (Ma and Ptashne, 1987a). Although there was little homology between the two regions both were highly acidic and the activity of the first region could be increased or reduced by changing the proportion of acidic residues (Gill and Ptashne, 1987). Acidic peptides generated from random E. coli DNA sequences (Ma and Ptashne, 1987b) or a synthetic ampipathic α-helix (Giniger and Ptashne, 1987) were able to functionally replace the transactivation domains of GAL4. This suggested that transcriptional activation can be mediated by acidic domains ('acid blobs') with no rigidly defined structure. GCN4 was also shown to have a predominantly acidic transactivation domain (Hope and Struhl, 1986) with no defined boundaries and transcriptional activity which correlated with acidity (Hope *et al.*, 1988). GAL4 is able to induce transcription from a responsive promoter in a range of species including plant (Ma *et al.*, 1988), Drosophila (Fischer *et al.*, 1988) and mammalian cells. Since one transcription factor is active in so many species it appears that the basic transcriptional machinery is conserved between yeast and higher eukaryotes. This also suggested that the 'acid blob' might be a structure functional in other eukaryotic transcription factors. Acidic transactivation domains have subsequently been identified in the VP16 protein of herpes simplex virus (Sadowski *et al.*, 1988; Treizenburg *et al.*, 1988) and the mammalian factor Jun (Struhl, 1988).

The mammalian transcription factors SP1 was found to contain a transactivation motif distinct from the 'acid blob'. Deletion analysis (Courney and Tjian, 1988) identified regions of little sequence homology but containing a high proportion (25%) of glutamine residues. Similar glutamine rich regions were found in other transcription factors and the glutamine rich region of the Drosophila Antennapedia homeobox protein was capable of activating transcription when fused to the SP1

DNA binding domain (Mitchell and Tjian, 1989). This suggested that the glutamine rich region may be a second common transcriptional activation domain motif. Another mammalian transcription factor CTF/NF-1 appears to contain a third class of transcriptional activation motif (Mermod *et al.*, 1989). The C-terminal 100 amino acids contain approximately 25% proline residues. Similar proline-rich regions have also been found in other transcription factors such as AP-2 (Williams *et al.*, 1988).

Two independent transcriptional activation functions (TAFs) have been identified in the oestrogen receptor. The N-terminal transactivation function (TAF-1) has been referred to as 'hormone independent' since in the absence of the hormone binding domain it is fully active without hormone in certain cell types and promoters. In the full length receptor, however, hormone is required for the activity of this N-terminal transactivation function, presumably for an earlier step in receptor activation such as dissociation from heat shock protein. The C-terminal transcriptional activation function (TAF-2) always requires the binding of ligand to induce gene expression and this function is referred to as 'hormone dependent'.

Deletion mutagenesis of the human oestrogen receptor located a transcriptional activation function (TAF-1) in the N-terminus which was active on the promoter of the oestrogen regulated pS2 gene but inactive on a vitellogenin ERE HSV-tk promoter (Kumar *et al.*, 1987). Lees *et al.* (1989) found removal of 89 amino acids at the N-terminus of the mouse oestrogen receptor abolished hormone-independent activity but had no effect upon the total receptor activity in the presence of oestradiol. Similar studies with human receptor identified no hormone independent activity in HeLa or CV-1 cells although C-terminal deletion mutants did retain a low constitutive activity which was highly cell and promoter-specific (Bocquel *et al.*, 1989). TAF-1 appears to be limited in the transcription factors with which it can interact and is therefore only active in particular cells and on some promoters. The difference in the hormone independent activities of the human and mouse receptors was proposed to be due to a point mutation found in the human MCF7 cell line clone as this Gly to Val mutation at amino acid 400 decreases the affinity of the receptor for oestradiol at 25°C (Tora *et al.*, 1989a). Since the 'hormone-independent' activity is proposed to be due to low levels of

residual steroids in the medium cells are grown in, a reduction in affinity might prevent this low level activity.

The N-termini of members of the steroid receptor superfamily are not well conserved (Evans, 1988), the vitamin D receptor has only 24 amino acids in this domain whereas the mouse glucocorticoid receptor has 427. The N-terminal domain of the glucocorticoid receptor also has transcriptional activity (Danielsen *et al.*, 1987; Hollenberg *et al.*, 1987; Misefeld *et al.*, 1987). Two transcriptional activation functions have been mapped to this domain: Enh2 (Godowski *et al.*, 1988) was located between amino acids 237 and 318, whereas τ1 was found between amino acids 77 and 262 (Hollenberg and Evans, 1988). Duplication of τ1 results in a 2-4 fold increase in levels of transcription indicating that the position of this element is not important but the overall structure of the N-terminus must be maintained as deletion or insertions of 3-4 amino acids inhibit transcriptional activity. Enh2 and τ1 overlap a highly acidic region of the receptor and it has been proposed that this may act as a transcriptional activator in a similar manner to the 'acid blob' of GCN4 and GAL4. The effects of insertions and deletions would however suggest that a more defined structural motif is involved.

Members of the steroid receptor superfamily also contain a second, hormone dependent transcriptional activation function in Region E towards the C-terminus. Oestrogen, glucocorticoid and progesterone receptors retain some activity when the N-terminus, but not the DNA binding domain is deleted (Kumar et al., 1987; Hollenberg *et al.*, 1987; Gronemeyer *et al.*, 1987). A fusion protein consisting of the entire oestrogen receptor hormone binding domain fused to the GAL4 DNA binding domain is able to stimulate transcription from promoters which contain a GAL4 binding site (Webster *et al.*, 1988). However, no fusion protein containing a peptide encoded by a single exon from this region of the oestrogen receptor is transcriptionally active (Webster *et al.*, 1989). This suggests that sequences from several exons come together to form TAF-2 in the oestrogen receptor. In contrast, other transactivation functions such as 'acid blobs' and ampipathic helices are encoded in a single exon. A series of deletions in the mouse oestrogen receptor have mapped the C-terminus of TAF-2. Whilst deletion to amino acid 552 does not affect transcriptional activity the removal of a further 14 amino acids to 538 reduces transcription to 1/20 wild-type activity (Lees *et al.* 1989). Studies on the synergistic interactions between TAF-1, TAF-2 and GAL4-

VP16 indicate that the two TAFs have properties distinct from one another and from those of 'acid blobs' (Tora *et al.*, 1989a; Tasset *et al.*, 1990).

An acidic C-terminal transactivation function, τ2, has been mapped to between residues 515 and 550 in the glucocorticoid receptor (Hollenberg and Evans, 1988). Godowski and co-workers (1988) also identified 'enh-1' a positively charged region between amino acids 440 and 525 in the C-terminus of the glucocorticoid receptor. The synergystic and and transcriptional interference properties of the two TAFs of the glucocorticoid receptor indicate that both are composed of acidic and nonacidic activation factors (Tasset *et al.*, 1990).

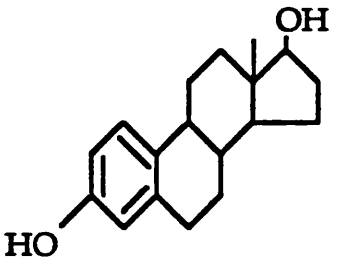
## 1.6 Antioestrogens

Antioestrogens are competitive inhibitors of oestrogen receptor function. These compounds fall into two broad classes, partial agonists such as tamoxifen which have oestrogenic effects in certain systems and the 'pure' antagonists such as ICI 164,384. Originally developed in the search for effective chemical contraception, antioestrogens have since been widely used in the treatment of breast cancer. The structure of oestradiol and these antioestrogens are shown in Figure 1.6.

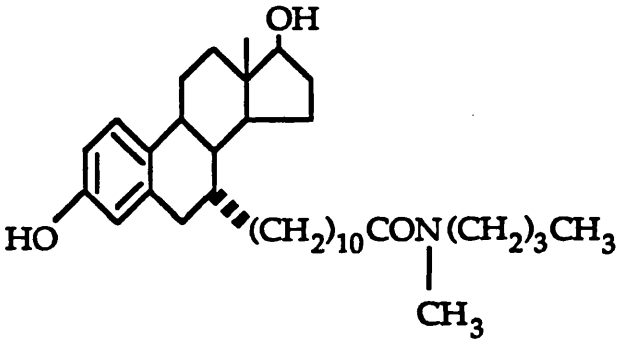
The first evidence for a relationship between oestrogens and the growth of breast tumours was obtained almost a century ago. In 1896 Beatson reported remissions in pre-menopausal breast cancer patients following bilateral ovariectomy. In 1932 the administration of ovarian extract to castrated male mice was found to induce mammary tumour formation (Lacassagne *et al.*, 1932). In women, breast cancer occurs almost exclusively after puberty (Akhtar *et al.*, 1983) and primary ovarian failure reduces the incidence of breast cancer to that observed in men. These observations led to the use of both endocrine therapy and surgery (ovariectomy/adrenalectomy) to control the disease. The oestrogen receptor can be detected in over half of human breast tumours and approximately two-thirds of these respond to endocrine therapy. In contrast only 5% of receptor negative tumours respond (Jordan, 1984). It was proposed that oestrogens were regulating cell growth and acting as mitogens through oestrogen receptor .

Tamoxifen is one of a number of clinically important nonsteroidal antioestrogens many of which are triphenylethylene derivatives

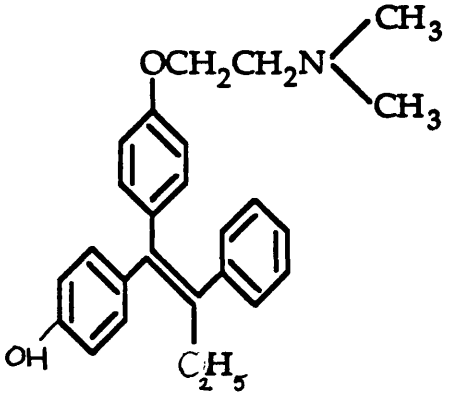
Figure 1.6 - Structure of oestradiol, ICI 164,384, tamoxifen and 4-hydroxytamoxifen



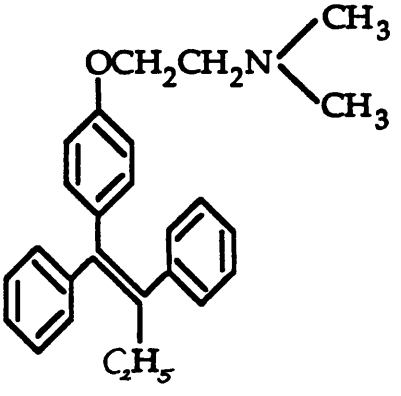

oestradiol




ICI 164,384




4-hydroxytamoxifen




tamoxifen

(reviewed - Furr and Jordan, 1984). Tamoxifen (ICI 46,474) has been used in treatment of receptor positive breast cancer with considerable success. Although it is tamoxifen which is administered clinically, *in vivo* this is converted to 4-hydroxytamoxifen which has a higher affinity for the oestrogen receptor (Wakeling and Slater, 1980). Since 4-hydroxytamoxifen appears to be the active compound *in vivo* (Bogna and Rochefort, 1981), all cell-free *in vitro* studies in this thesis were carried out using 4-hydroxytamoxifen.

The action of tamoxifen varies in different species. It acts as a pure antagonist in the chicken oviduct (Sutherland *et al.*, 1977) but as a partial agonist in the rat uterus (Callantine *et al.*, 1966; Lerner *et al.*, 1966; Katzenellenbogen and Ferguson, 1975). In contrast it is a strong agonist in the mouse uterus (Terenius, 1971). Tamoxifen stimulated growth of endometrial cancer cells but inhibited the growth of breast cancer cells when these were inoculated into the flanks of the same nude mouse (Gottardis *et al.*, 1988). This indicates that the difference in response to tamoxifen in different species is not due to metabolism of the compound *in vivo*.

The effects of this antioestrogen on the function and structure of the oestrogen receptor have been investigated using a variety of techniques. The gel retardation assay has been used to demonstrate that the oestrogen receptor recognises an ERE when either oestrogen or 4-hydroxytamoxifen is bound (Kumar and Chambon, 1988; Metzger *et al.*, 1988; Lees *et al.*, 1989). However the structure of the receptor when bound to tamoxifen and oestradiol does not appear to be identical. In a gel retardation assay the oestrogen receptor - oestradiol- DNA complex migrates with greater mobility than a receptor-DNA complex in the absence of ligand whereas the binding of tamoxifen does not affect the migration of receptor (Metzger *et al.*, 1988; Kumar and Chambon, 1988; Lees *et al.*, 1989). Further evidence that these ligands have different effects upon the oestrogen receptor comes from *in vivo* experiments in which wild-type and chimaeric receptors (ER-GAL4) stimulate transcription in HeLa cells in the presence of oestradiol but not hydroxytamoxifen (Webster *et al.*, 1988). These results suggest that oestrogens but not antioestrogens promote a structural change in the receptor which is necessary for the complete activation of a functional receptor.

The activity of tamoxifen as an agonist/antagonist depends upon the tissue and promoter examined (Jordan, 1984; May and Westley,1987). The partial agonist effect of this ligand appears to result from its ability to promote DNA binding by the oestrogen receptor *in vivo* (Webster *et al.*, 1988), allowing TAF-1 in the N-terminus to be active, but failing to induce the hormone dependent TAF-2 in the C-terminus. In chick embryo fibroblast (CEF) cells hydroxytamoxifen is a potent agonist, strongly activating several different oestrogen responsive promoters, whereas only the pS2 promoter was stimulated in HeLa cells (Berry *et al.*, 1990). This transcriptional activity correlated well with the activity of a mutant receptor lacking TAF-2 but retaining TAF-1. Thus the differences observed in the activity of tamoxifen in different tissues and via different promoters appears to be a result of interactions between TAF-1 and various tissue and promoter specific transcription factors. The variations in action of tamoxifen in different species are also likely to be due to the activities of TAF-1.

A series of steroidal 7α-alkylamide compounds, including ICI 164,384, has also been developed (Wakeling and Bowler, 1987). These appear to act as pure antagonists in all *in vivo* assays so far tested (Weatherill *et al.*, 1988; Bowler *et al.*,1989; Wilson et al., 1990). It has also been shown to be a pure antioestrogen for the regulation of expression of a range of natural and synthetic oestrogen responsive genes (Wiseman *et al.*, 1989; Berry *et al.*, 1990) . Thus it was hoped that they would provide a more effective treatment for receptor-positive breast tumours. Difficulties in the *in vivo* delivery of these highly lipophilic compounds prevents the clinical use of many of these antioestrogens. Recently however a related compound, ICI 182,780, in which the 7α side chain was modified by fluorination of the terminal alkyl function has been synthesised (Wakeling *et al.*, 1991). This compound is more potent in its antioestrogenic effects and is highly active *in vivo*. It will be interesting to observe the clinical effects of this antioestrogen.

In contrast to the effects of tamoxifen and other partial agonists, our group has shown that the receptor fails to bind DNA when bound to ICI 164,384 (Fawell *et al.*, 1990b) and proposed that this ligand acts by inhibition of dimerisation. There is however some controversy as different results have been obtained with receptor from other sources (Lees et al., 1989; Martinez and Wahli, 1989; Sabbah *et al.*, 1991). In addition a fusion protein containing oestrogen receptor sequences fused

to transactivation sequences from VP16 induced transcription on addition of ICI 164,384 (Pham *et al.*, 1991). ICI 164,384 is also capable of increasing the levels of progesterone receptor in endometrial cells, although the induction is not as great as that observed with tamoxifen (Jamil *et al.*, 1991). Despite the limited agonist effect of ICI 164,384 in some *in vitro* systems the pure antagonist effect of the 7a-alkylamide steroids and their derivatives *in vivo* suggest that these compounds may provide a more effective treatment for breast cancer than tamoxifen with its partial agonist effect *in vivo*.

# CHAPTER 2

## Materials and Methods

## 2.1 MATERIALS

### 2.1-1 CHEMICALS

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

| | |
|---|---|
| Absolute alcohol | James Burrough(FAD)Ltd, Witham, England. |
| Acrylamide | Boehringer Mannheim, Lewes, East Sussex, |
| Agarose | FMC Bioproducts, Rockland, USA. |
| Ammonium persulphate | BDH |
| Ampicillin | BeechamsResearchLaboratories, Brentford, England. |
| Amplify | Amersham International PLC, Amersham, England. |
| Bromophenol blue | BDH. |
| Bovine serum albumin (BSA) Fraction V | Sigma |
| Caesium chloride (ultra-pure) | Mallinckrodt, Paris, Kentucky, USA. |
| Dextran T-70 | Pharmacia P-L Biochemicals, Milton Keynes, England. |
| DTT | BioRad. |
| Glycogen | Boehringer Mannheim, Lewes, |
| East (molecular biology grade) | Sussex, England. |
| Nonidet P-40 | BDH. |
| Nucleotide triphosphates | Pharmacia. |
| Picofluor scintillant | Packard Instruments, Groningen, Netherlands. |
| RNA cap structure analogue [$m^7$G(5')ppp(5')G] | New England Biolabs Inc. Ma., USA. |
| SDS | Serva Feinbiochemica GMBH & Co. (Supplied by Cambridge Bioscience, England.) |
| TEMED | BioRad. |
| Urea (ultrapure) | BioRad. |
| Xylene cyanol | BDH. |

**2.1-1a) Radiochemicals.**

Amersham International PLC supplied radiochemicals with the following specific activities-

| | |
|---|---|
| [$^{35}$S]dATP | ~4000 Ci/mmol |
| [$\gamma$-$^{32}$P]ATP | ~3000 Ci/mmol. |
| L-[$^{35}$S]methionine | >1000 Ci/mmol. |
| [$\alpha$-$^{32}$P]dCTP | ~3000 Ci/mmol. |
| [1-$^{14}$C]acetyl-coenzyme A | 50-60 mCi/mmol. |

NEN supplied the following radiolabelled oestrogen-

| | |
|---|---|
| 16$\alpha$-[$^{125}$I]Iodoestradiol | ~2000 Ci/mmol. |

**2.1-1b) Miscellaneous.**

| | |
|---|---|
| NA-45 DEAE membrane | Schleicher & Schuell (Supplied by Andermann & Co. Ltd., Kingston-upon-Thames, UK.). |
| Oligonucleotides | Synthesised by I.Goldsmith ICRF. |
| Rabbit reticulocyte lysate | Promega, Southhampton, England |
| Sequenase sequencing kit | U.S. Biochemical Corporation, Cleveland, USA. |
| Abbott ER-EIA Monoclonal Kit | Abbott Laboratories, Chicago, USA |

**2.1-1c) Enzymes.**

Calf intestinal alkaline phosphatase, DNA polymerase I, DNase I (RNase-free), human placental ribonuclease inhibitor, Klenow fragment, RNase (DNase-free) and T4 polynucleotide kinase were all supplied by Boehringer. Restriction enzymes were routinely purchased from Biolabs and T4 DNA ligase from BRL. SP6 polymerase was supplied by Stratagene.

**2.1-1d) Cell culture media.**

| | |
|---|---|
| DMEM | ICRF media supplies. |
| Foetal calf serum (FCS) | Gibco (Gibco Ltd., Paisley, Scotland.) |
| PBSA | ICRF media supplies. |
| Trypsin (Stored at -20$^0$C) | ICRF media supplies [0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.01% (w/v) disodium 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]. |

**2.1-2 BUFFERS.**

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

| | |
|---|---|
| CAP buffer (5x) | 2.5mM ATP, UTP and CTP, 250mM GTP and 2.5mM $m^7G(5')ppp(5')G$ (stored at -20°C). |
| CIP buffer (10x) | 500mM Tris-HCl, pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$ 10mM spermidine (stored at -20°C). |
| DCC suspension | 0.025% (w/v) dextran - 0.25% (w/v) charcoal suspended in TE, pH7.4 and stored at 4°C. |
| Deoxy/dideoxy mixes (Made up in TE, pH8.4 and stored at -20°C)- | |
| d/ddGTP | 250 mM dTTP and dCTP, 12.5 mM dGTP, 250 mM ddGTP. |
| d/ddATP | 500 mM dTTP, dCTP, dGTP, 40 mM ddATP. |
| d/ddTTP | 250 mM dCTP, and dGTP, 12.5 mM dTTP, 500 mM ddTTP. |
| d/ddCTP | 250 mM dTTP and dGTP, 12.5 mM dCTP, 125 mM ddCTP. |
| DNA loading buffer (5x) | 0.25% bromophenol blue, 0.25% xylene cyanol, 25% (v/v) glycerol. |
| GTE | 50mM glucose, 25mM Tris-HCl, pH 8, 10mM EDTA, pH 8 (freshly made). |
| HBS (2x) | 40mM Hepes, 275 mM NaCl, pH 7.1. |
| Kinase buffer (10x) | 500mM Tris-HCl, pH 7.4, 100mM $MgCl_2$, 1mM EDTA, pH 8. |
| Luciferase Reaction Buffer | 25mM Glycylglycine pH 7.8, 5mM ATP pH 8.0, 15mM MgSO4 |
| Lysis Buffer | 0.6% NP40, 10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0, 150mM NaCl. |
| NTE (1x) | 0.1M NaCl in TE. |

| | |
|---|---|
| Phenol/chloroform | Redistilled phenol, equilibrated with TE pH 8.0, in a 50/50 mix (v/v) with chloroform. |
| Protein loading buffer (4x) | 4% SDS, 250mM Tris-HCl, pH 6.7, 20% glycine, 0.05% bromophenol blue, 2% b-mecaptoethanol. |
| Redox buffer (10x) | 10mM Glutathione (reduced), 10mM Glutathione (oxidised), 1M KCl, 0.5M Tris-Cl pH 8.0, 10mM EDTA,$10^{-7}$M oestradiol. |
| Repair buffer (10x) | 500mM Tris-HCl, pH 7.4, 70mM $MgCl_2$, 10 mM DTT (stored at -20°C). |
| Restriction Enzyme Buffers (stored at -20°C)- | |
| Low salt (10x) | 100mM Tris-HCl, pH 7.4, 100mM $MgCl_2$, 10mM DTT |
| Medium salt (10x) | As low salt with 500mM NaCl added. |
| High salt (10x) | As low salt with 1M NaCl added. |
| Very High salt (10x) | As low salt with 1.5M NaCl added. |
| Retardation Buffer (2x) | 20mM HEPES (pH 7.4), 50mM KCl, 1mM DTT, 10% glycerol. |
| Sequencing loading buffer | 80% (v/v) deionized formamide, 10mM NaOH, 1mM EDTA, pH 8.0, 0.1% (v/v) xylene cyanol, 0.1% (v/v) bromophenol blue. |
| SDS-PAGE buffer | 25mM Tris base, 190mM glycine, 0.1% (w/v)SDS. |
| STET buffer | 8% sucrose, 0.1% Triton X-100, 50mM Tris-HCl, pH 8, 50mM EDTA, pH 8. |
| T buffer (5x) | 200mM Tris-HCl, pH 7.4, 30mM $MgCl_2$, 10mM spermidine (stored at -20°C). |
| TBE (10x) | 89mM Tris-borate, 89mM boric acid, 2mM |
| EDTA, | pH 8.0. |
| TE (1x) | 10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0. |
| Tfb 1 | 30mM Potassium acetate, 100mM $RbCl_2$, 10mM$CaCl_2$, 50mM $MnCl_2$,15% (v/v) glycerol. Add 0.2M acetic acid to pH 5.8, filter sterilise and store at 4°C. |
| Tfb 2 | 10mM MOPES, 75mM $CaCl_2$, 10mM $RbCl_2$ and 15% glycerol. pH to 6.5 with KOH, filter and store at 4°C. |

| | |
|---|---|
| Whole Cell Extract Buffer | 0.4M KCl, 20mM HEPES pH 7.4, 1mM DTT, 20% glycerol, 0.5mg/ml bacitracin, 40ug/ml PMSF, 5ug/ml pepstatin, 5ug/ml leupeptin. |

### 2.1-3 BACTERIAL MEDIA AND PLATES.

All the organic components listed below were obtained from Difco.

| | |
|---|---|
| 2 X TY | 1.6% (w/v) bactotryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl. |
| 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. |
| Ya 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. |
| Yb | Ya plates without agar, filter sterilised prior to autoclaving |

## 2.2 METHODS

### 2.2-1 BACTERIAL TRANSFORMATION.

**1a) Storage of bacterial strains.**

Experiments described in this thesis use the DH5 *E. Coli* strain for all plasmid propagation and large scale purification. The cells 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 and therefore bacterial transformants were grown in the presence of 50 - 100 μg/ml ampicillin.

**1b) Preparation of competent cells.**

Bacteria were streaked out on a Ya plates and incubated at 37°C until colonies reached approximately 2mm in diameter. Single colonies were then innoculated into 5mls of Yb (in glassware prewashed with Yb) and grown at 37°C until the $OD_{550}$ reached 0.3 (approximately 3 hours). This was then subcultured (1/20) into 100mls of prewarmed Yb and grow until the $OD_{550}$ = 0.48 (approximately 2 hours). The cells were then chilled on ice and pelleted by spinning for 10 minutes at 3000 rpm and 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 500 μl aliquots on cardice. Competent cells were stored at -70°C.

**1c) Transformation.**

Competent cells were thawed on ice. A minimum of 25 μl of cells was added to each 10 μl prechilled DNA sample (maximum 1ng DNA/μl cells) and incubated for 30 minutes on ice. The cells were heat shocked for 90 seconds at 42°C and incubated at 37°C for 40 minutes after addition of 4 volumes of Y broth. Cells were then spread on L plates containing 50-100 μg/ml ampicillin which were inverted and incubated overnight at 37°C. Competent DH5 cells typically gave between $10^6$ and $10^8$ bacterial colonies per μg of supercoiled DNA.

## 2.2-2 PREPARATION OF PLASMID DNA.

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

### 2a) Plasmid mini-preparation (mini-preps).

Mini-preps frequently yield between 5 and 20 µg of DNA depending on the plasmid vector. Experiments have involved use of both the 'boiling' miniprep method and, more recently, the "CTAB" method of Del Sal, 1988.

i) Boiling method

A single bacterial colony was used to innoculate 5ml Lbroth(50µg/ml ampicillin) and grown shaking overnight at 37°C. Approximately 1ml of culture was pelleted for 10s in a microfuge and resuspended in 0.35ml of STET buffer. After addition of 25µl of 10 mg/ml lysosyme in 0.01M Tris-HCl, pH 8 and vortexing the tube was boiled for 40 seconds. The preparation was then spun for 10 minutes at room temperature in a microfuge. The pellet was removed with a toothpick and the supernatant precipitated by addition of 40µl of 3M NaoAc pH 7.0 and 420µl of isopropanol, vortexing and incubation on cardice for 10 minutes. The DNA was pelleted by centrifugation for 10 minutes at room temperature and resuspended in 50µl of distilled water. If the DNA was not to be used for sequencing, RNA was destroyed by addition of heat treated RNase A to a final concentation of 1mg/ml

ii) "CTAB" method. (Del Sal *et al.*, 1988)

The CTAB method gives a larger yield of DNA which contains fewer contaminants and is hence more suitable for direct plasmid sequencing. The Boiling method was followed up to removal of the boiled pellet. The DNA was then precipitated by addition of 8 µl of 5% CTAB and vortexing. The precipitate was recovered by centrifugation for 10 minutes at room temperature and resuspended in 300 µl of 1.2M NaCl by vigorous vortexing. The DNA was reprecipitated using ethanol, washed with 70% ethanol and resuspended in 50 µl of distilled water.

**2b) Large Scale Plasmid preparation.** (Unpublished method of D. Ish-Horowicz, ICRF).

A 5 ml overnight culture was innoculated into 400 ml of L broth containing 100 µg/ml ampicillin and grown at 37°C until the $OD_{550} = 1$ (approximately 3 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 necessary and 1ml of a 5ml overnight culture was innoculated directly into 400 ml ampicillin containing L broth and grown overnight at 37°C. The cell pellet was harvested in 400 ml Sorvall bottles by centrifugation at 7,000g for 10 minutes at 4°C. The bacterial pellet was resuspended in 20 ml GTE and after addition of 100 mg of lysozyme, incubated for 10 minutes at room temperature . Freshly prepared 0.2M NaOH, 1% SDS (40mls) was added and the suspension incubated for 5 minutes on ice. A further 15 minute incubation on ice followed the addition of 20 ml of 5M potassium acetate, pH 4.8. The debris was removed by centrifugation at 7,000g for 10 minutes at 4°C and filtration through medical gauze. The plasmid DNA was precipitated by addition of 48 ml of propan-2-ol and harvested by respinning at 7,000g for 10 minutes at 4°C. The dried precipitate was resuspended in 10 ml TE plus 0.75 ml 0.5M EDTA, pH 8 and neutralised by the addition of 200 µl of 1M Tris base. After addition of 10 gm of caesium chloride and 50 µl of 10 mg/ml ethidium bromide the volume was adjusted to fill a Beckman 5/8 x 3" Quick-seal centrifuge tube. The DNA was banded at 65,000 rpm for 20 hours in a Beckman L3-50 ultracentrifuge using a 70 Ti rotor. The supercoiled fraction was visualized using a low wavelength UV lamp and harvested with a 19g gauge needle and syringe. The DNA was mixed with 3 ml of distilled water and then shaken with an equal volume of isobutanol. The solvent phase, containing the ethidium bromide, was then discarded and the extraction procedure was repeated until the aqueous DNA solution was free from ethidium bromide (as judged by visualisation under UV). DNA was then precipitated by addition of 2 volumes of ethanol and centrifugation at 10,000g for 10 minutes at 4°C. The pellet was washed with ice-cold 70% ethanol until all traces of salt were removed and dissolved in 500 µl of distilled water. DNA concentration and purity were determined by measurement of the $OD_{260}$ and $OD_{280}$ as described by Maniatis *et al.* (1982).

## 2.2-3 DNA MANIPULATION AND SUBCLONING.

### 3a) Restriction endonuclease digestion.

Restriction enzyme digests were performed in low, medium or high salt buffers according to the suppliers instructions (except for Sma I digestions which were performed in 20mM KCl, 6mM $MgCl_2$, 6mM Tris-HCl, pH 8). Routinely 1-2 µg of DNA was digested at 37°C (Sma I 25°C) for 1 hour with a 3 fold excess of enzyme. The enzyme concentration did not exceed 1/10 of the final volume. Digestions were stopped by extraction with an equal volume of phenol/chloroform and, after spinning in a microfuge for 2 minutes, the aqueous phase transferred to a fresh Eppendorf tube. DNA was then precipitated by addition of 1/10 volume of 3M sodium acetate, pH 5.2 and 2.5 volumes of absolute ethanol at -20°C. Molecular biology grade glycogen was frequently added (1 µl of 20 mg/ml) to aid the precipitation of small amounts (<1µg) of DNA. The DNA was then pelleted for 15 minutes in an Eppendorf centrifuge. The pellet was washed with 180 µl of 70% ethanol, respun for 5 minutes and the pellet dried and resuspended in distilled water.

### 3b) Agarose gel electrophoresis.

Agarose [0.8 - 2.0% (w/v)] was dissolved in 1 x TBE by boiling in a microwave oven. The solution was cooled to 55°C and ethidium bromide added to 1 µg/ml before the gel was poured. DNA loading buffer was added to the sample to 20% of the final volume and the samples loaded onto the gel submerged in 1 x TBE. The agarose gel was run at 7.5 V/cm until the the DNA fragments were well separated. The fragments were visualised by illumination with a long wave UV light source and photographed using a Polaroid camera. A better image was obtained using a shorter wavelength UV source but since this can result in mutations this was only used when DNA was not to be used in subsequent cloning. The size of fragments was detemined by comparing their mobility relative to restriction fragments of known size (typically Lambda cut with Hind III + Eco RI ).

### 3c) Preparation of vectors.

Plasmid DNA (1 to 2 µg) was digested with the appropriate restriction endonuclease and then extracted with phenol/chloroform, ethanol precipitated and washed with 70% ethanol. The DNA pellet was

resuspended in 17 µl of water and 2 µl of 10 x CIP buffer and then phosphatased at 37°C for 30 minutes with 1 µl of calf intestinal alkaline phosphatase (20 units). This removed the 5' terminal phosphates so that self-ligation of the vector could not occur. The DNA was then re-extracted with phenol/chloroform, ethanol precipitated and 70% ethanol washed. Vectors were routinely resuspended to give a final concentration of 10 ng/ml.

**3d) Purification of restriction fragments**

DNA was run on an agarose gel to separate out restriction fragments prior to purification by one of the following methods.

i) NA-45 DEAE membrane

NA-45 DEAE membrane was presoaked in TE (pH8) and then inserted into a slot in the gel immediately in front of the band of interest. 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 minutes allowing the DNA to bind to the paper. This was then transferred to an Eppendorf tube containing 200 µl 1M NaCl and the DNA eluted by heating to 70°C for 15 - 30 minutes. DNA was recovered by ethanol precipitation, washed in 70% ethanol and then resuspended in distilled water.

ii)Electroelution

The electroelution apparatus was filled with TBE, ensuring that no air remained in the capillary tubes connecting the two buffer tanks. The band of interest was excised from an agarose gel and placed in one of the rear chambers of the tank. 50µl of 3M NaoAc with 1µl DNA loading buffer was introduced to the elbow of each capillary tube with a .3mM Gilson tip. The two buffer tanks were isolated and 100 volts applied across the tank for 30-40 minutes depending on the size of the fragment. The tanks were then reconnected and the buffer levels reduced so that the capillary was isolated and 200µl of buffer containing the DNA fragment removed from this tube. The DNA was precipitated by addition of 1µl moleclar biology grade glycogen and 400µl ethanol. After centrifugation the pellet was washed with 70% ethanol and resuspended in distilled water. This method was particularly useful in purifying larger fragments or oligonucleotides from acrylamide gels.

**3e) Conversion of 5' overhanging ends to blunt ends.**

5' overhanging ends were converted to blunt ends by filling in using the Klenow fragment of DNA polymerase I. DNA restriction fragments (1-2 µg) were incubated for 30 minutes at room temperature in a final volume of 20 µl containing 2 ml of 10 x repair buffer, 2 µl of each 2mM dNTP stock and 1 µl of Klenow enzyme (5U/µl). The DNA was then extracted with phenol/chloroform, ethanol precipitated and washed with 70% ethanol.

**3f) Oligonucleotide kinasing and annealing.**

A number of experiments involved the cloning of annealed oligonucleotides to introduce either short coding sequences into mutants or binding sites into reporter constructs. 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. Equal amounts (10 ng) of each oligonucleotide were mixed in 10 µl containing 1 µl 10 x kinase buffer, 1 µl 10mM dATP and 1 µl of T4 polynucleotide kinase (approximately 5 units) and incubated at 37°C for 60 minutes. After the addition of 10 µl of 10 x TNE buffer and 80 µl of distilled water the oligonucleotides were annealed by heating to 80°C for 3 minutes followed by slow cooling to room temperature.

**3g) Ligations.**

Ligations were routinely carried out with 20 ng of vector and an equimolar ratio of insert. This was generally either a DNA fragment isolated from agarose gels, or an annealed pair of oligonucleotides. Ligation was in a final volume of 10 µl containing 10mM $MgCl_2$, 50mM Tris-HCl, pH 7.5, 1mM dATP, 5mM DTT and 1-10 units of T4 DNA ligase. Ligations (both blunt and sticky ends) were incubated for 2 hours at room temperature or overnight at 14°C.

**2.2-4 DNA SEQUENCING**

**4a) Preparation of DNA and Sequencing reactions**

2µg of miniprep or caesium chloride banded DNA was routinely used for sequencing, although miniprep DNA could also be used. The DNA was denatured by addition of NaOH to give a final concentration of

0.2M in 20µl and incubation at 70°C for 5 minutes. The single strands were then precipitated on cardice by the addition of 8 µl of 5M ammonium acetate, pH 5.4 and 100 µl ethanol. The pellet was recovered by spinning in a microfuge for 10 minutes at room temperature, washed in 70% ethanol, and resuspended in 7 µl of distilled water. Double stranded sequencing was carried out exactly according to the protocol of Sequenase (USB).

**4b) Electrophoresis of Sequencing reactions**

The reactions were boiled for 5 minutes to separate the freshly synthesised strands from the template and these were analysed on a 6% denaturing polyacrylamide gel. The gel solution contained:-

42 gms electrophoretic grade urea
15 mls acrylamide stock (38% acrylamide, 2% Bis-acrylamide)
10 mls 10 x TBE

This was made up to 100ml with distilled water and degassed prior to the addition of 80µl of TEMED and 800µl of 10% ammonium persulphate. The gel was then poured between plates separated by 0.25mm spacers and the blunt side of a '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 individual sample wells. The gel was pre-run for 30 minutes at 2500 V in 1 x TBE before the samples were loaded and the gel run for a further 1 - 2 hours to separate bands. The gel was fixed for 15 minutes in 10% methanol and 10% acetic acid. The fixed gel was transferred to Whatmann 3MM and dried at 80°C for 20 minutes. The labelled bands were visualised by autoradiography using Kodak XAR film.

## 2.2-5 *IN VITRO* PROTEIN SYNTHESIS

**5a) Complementary RNA synthesis.**

Complementary RNA (cRNA) was prepared to allow *in vitro* synthesis of the receptor deletion mutants in a rabbit reticulocyte lysate system. Receptor mutants were subcloned into the vector pSP65 which contains the bacteriophage SP6 promoter. The resultant plasmids were then linearised for cRNA synthesis using a unique restriction site 3' of the coding sequence, generally HinDIII. The linear DNA template was

then phenol/chloroform extracted, ethanol precipitated and pelleted in a microfuge. The pellet was washed with 70% ethanol before being resupended in 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 5 x T buffer
20 µl 5 x CAP buffer
1 µl 1M DTT
48-43 µl DEPC treated water
4 µl human placental ribonuclease inhibitor
2 units SP6 polymerase

The reaction was made up to 100µl and incubated for 1 hour at 37°C followed by the addition of a further 1unit of SP6 polymerase and another 30 minutes at 37°C. The DNA template was then digested by incubation for 15 minutes at 37°C with 5 µl DNase-free RNase (23 units/µl) and an additional 3µl human placental ribonuclease inhibitor. The RNA was then extracted with phenol/chloroform and precipitated by the addition of 100 µl 5M ammonium acetate, pH 5.4 and 400 µl ethanol and 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.

**5b) *In vitro* translations.**

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 rabbit reticulocyte lysate (with $ZnCl_2$ added to 20mM), 3 µl cRNA (~500 ng) and was made up to 30 µl with DEPC treated water. An aliquot (9 µl) of this reaction mix was then removed to a second Eppendorf tube containing 1 µl [$^{35}$S]methionine. To the remaining 20µl 1µl of unlabelled 1mM methionine 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%. Translated receptors labelled with [$^{35}$S]methionine were used to assess both the size, purity and yield of receptor mutants. Unlabelled translations were used for ligand binding and electrophoretic mobility shift (gel retardation) assays.

5c) SDS polyacrylamide gel electrophoresis of proteins

Proteins were analysed on discontinous polyacrylamide gels using the Atto corporation AE-6220 dual slab chamber. The gel plates (14cm x 16 cm) were separated by 0.75mm spacers and sealed with silicon gaskets. Gels were prepared from two solutions forming the resolving and stacking gels respectively. The running gel routinely contained between 10-15% acrylamide (30% acrylamide, 0.8% bis-acrylamide stock) depending on the size of the proteins, 375mM Tris-HCl, pH 8.8 and 1% SDS. The solution was degassed prior to the addition of AMPS to 0.1% (w/v) and TEMED 0.1% (v/v). The solution was poured between the plates to within 3 cm of the top and then overlayed with water saturated isobutanol. Once the gel had set (10-30 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, 125mM Tris-HCl, pH 6.8, 1% SDS, 0.1% (w/v) AMPS and 0.1% (v/v) TEMED] before the stacking gel was poured and a blunt-toothed comb inserted. The gel was always run within 30 minutes of the stacking gel setting. Prior to loading on the gel, the proteins were reduced by the addition of 25% volume of 4 x Protein loading buffer and heating for 2 minutes at 95°C. 10-50 µg of total protein was routinely loaded per 6 cm well using 0.3mm flat capillary loading tips. The gel was run in 1 x SDS-PAGE buffer at 250V until the bromophenol blue marker reached the bottom of the gel. Protein gels were fixed by shaking for 30 minutes in 10% glacial acetic acid, 30% methanol and then incubated in Amplify for 15 minutes before drying. The bands were then visualised by fluorography using Kodak XAR film.

## 2.2-6 *IN VITRO* PROTEIN ASSAYS

6a) Ligand binding. (Modified from Coffer *et al.*, 1980).

Receptor expressed in reticulocyte lysate or insect cells was incubated overnight at 4°C in 50 µl containing 10mM Tris-HCl, pH 7.4, 1mM EDTA, pH 8, 1mM DTT, 1 mg/ml BSA and 16α-[$^{125}$I]oestradiol at a final concentration of $10^{-8}$ to $10^{-10}$M. Non-specific binding was determined by the inclusion of 100 fold excess cold competitor ($10^{-6}$M diethylstilbestrol) in parallel reactions. Free steroid was removed by 10 minute incubation at 4°C with DCC suspension followed by 5 minute centrifugation. The activity of the supernatant was quantitated in a γ

counter. The affinity of receptor for ligand was then determined by Scatchard analysis.

**6b) Gel retardation assay.**

DNA binding activity of oestrogen receptor was determined in an electrophoretic mobility or gel retardation assay. The DNA probes were prepared by annealing oligonucleotides corresponding to either the vitellogenin A2 ERE (5'-CTAGAAAGTCAGGTCACAGTGACCTGATC AAT-3'), a palindromic TRE (5'-CTAGAAAGTCAGGTCATGACCTGAT CAAT-3') or creatine kinase ERE (Figure 6.1). These were then labelled by filling in the 5' overhanging ends as described in section 2.2-5c but with a two molar excess of [$\alpha$-$^{32}$P] dCTP in place of the 2mM dCTP. Aliquots of receptor translated in reticulocyte lysate (1-5 µl) or whole cell extract from baculovirus-infected insect cells (0.1-1 µl) were pre-incubated for 15 min at room temperature in 15 µl containing 1 µg poly-dI.dC: poly-dI.dC, 0.1 mg BSA, 10µl 2x Retardation Buffer with or without added hormone or antihormone. $^{32}$P labelled double-stranded oligonucleotide probe (0.05 - 20 ng in 5 µl) was added and the samples incubated for a further 30 min at room temperature. Samples were then applied directly to a 6% polyacryamide (30% acrylamide, 0.8% bis -acrylamide stock), 0.5xTBE gel which had been pre-run for 30 minutes at 100V in an Atto Corporation AE-6220 dual slab chamber. The samples were then analysed by electrophoresis in 0.5xTBE at 250V for 60 minutes. Gels were fixed for 15 minutes in 10% acetic acid, 30% methanol, dried and subjected to autoradiography.

**6c) Proteolytic gel retardation**

The gel retardation described above was also modified by including a proteolysis step. Whole cell extracts containing receptor were preincubated in binding buffer for 10 minutes before addition of $^{32}$P labelled double-stranded oligonucleotide probe. After 10 minutes protease was added at between $5x10^{-5}$ and $5x10^{-3}$ units/ul and incubated for a further 10 minutes at room temperature before analysis by electrophoresis as described above.

**6d) Enzyme immunoassay**

This was carried out using the Abbott ER-EIA Monoclonal kit for oestrogen receptor quantitation according to the suppliers protocol.

**6e) Methylation Interference Footprinting**

In a method similar to 2.2-3f, 100ng of oligonucleotide containing an oestrogen response element was labelled by kinasing with $\gamma^{32}P$ ATP. The labelled oligonucleotide was annealed with 100ng complementary cold oligonucleotide, and precipitated twice to remove unincorporated nucleotide. The annealed oligonucleotide was resuspended in 200 µl 50mM Sodium cacodylate, 1mM EDTA with 1 µg poly dI.dC and cooled on ice. Methylation by addition of 1 µl of DMS was stopped after 5 minutes at room temperature by addition of 50 µl 1.5M NaoAC, 1M mercaptoetanol and precipitation with 750 µl ethanol. All waste was immersed in 5M NaOH before disposal. After centrifugation and 70% ethanol wash the pellet was resuspended in 20 µl distilled water. A bandshift gel was run with two tracks for each probe, one containing 20ng probe alone, the other probe and 5 µl of oestrogen receptor expressed in insect cells. The gel was run for 45 minutes then wrapped in saranwrap and wet exposed to Kodak XAR film for 1 hour with markers so that the retarded band and free band could be located and excised. DNA was eluted from the gel by electroelution and precipitated with 1 µg dI.dC. The pellet was redissolved in 100 µl 1M piperidene, capped tightly and heated to 90°C for 30 minutes. The piperidene cleavage products were precipitated with 1 µg dIdC, 0.3M NaoAc, 10mM $MgCl_2$ and two volumes ethanol. After centrifugation the pellets were dried in a speedvac, redissolved in 100 µl distilled water and transferred to a fresh tube. Two further precipitations and two 70% ethanol washes removed the last traces of piperidene. The final pellet was dissolved in 10 µl distilled water and equal cpm of each sample denatured at 100°C in DNA formamide loading buffer. The samples were then analysed by electrophoresis on a 10% denaturing polyacrilamide gel and run at 50mA for 1 hour.

**6f) Disulphide Linkage of receptors**

To reduce crosslinking between free surface cysteine groups a pretreatment with iodoactamide was carried out. An aliquot (4µl) of receptor expressed in the reticulocyte lysate system and labelled by incorporation of [$^{35}S$] methionine was incubated in 10µl $10^{-7}$ M oestradiol for 10 minutes. After incubation with 1µl 0.5M iodoacetamide for 1 minute, cysteine blocking was terminated with 0.07M TRIS base. After 10 minutes 10µl 10x Redox Buffer was added, the reaction made up to 100µl

with distilled water and incubated for 90 minutes at room temperature. This reaction was split equally and 20µl exposed to reducing conditions (0.01M DTT, under nitrogen) whilst the remaining 20µl was left in oxidising conditions under oxygen for 2 hours at room temperature. Each of these reactions was then divided and half added to a protein loading buffer containing the reducing agent mercaptoethanol and the remainder to a buffer lacking mercaptoethanol. The samples were boiled for 2 minutes and run out on a 10% SDS gel.

## 2.2-7 CELL CULTURE METHODS.

### 7a) Maintenance of cell stocks.

Cells were routinely maintained as monolayer cultures on T-175 tissue culture flasks at 37°C in a humidifying atmosphere of 10% $CO_2$ in air. All the cell lines used in this thesis [COS-1, NIH 3T3 D4, ZR-75 and HeLa cells (kindly provided by M. Freid, G. Stark or ICRF central cell culture services) ] were grown in DMEM supplemented with 10% (v/v) foetal calf serum (FCS). The ZR-75 cell line was also supplemented with $10^{-8}$ oestradiol. Cell stocks were subcultured twice weekly. Prior to passage the growth media was removed and the cell monolayer washed with 20 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. When the cells had detached from the flask trypsin was inactivated by the addition of 10 ml of serum containing culture media. The cells were then subcultured into fresh media at a dilution of between 1 in 5 and 1 in 20. Frozen stocks of each cell line were prepared after the first passage. Cells were trypsinised as described and pelleted by centrifugation at 1500 rpm for 5 minutes. The pellet was resuspended in 9 ml of DMEM containing 10% FCS and 1 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 polystyrene and frozen at -70°C overnight before being transferred to liquid nitrogen for long term storage.

### 7b) Charcoal treatment of serum. (Modified from Page and Parker, 1983).

Foetal calf serum (FCS) 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. 200 ml of dextran-coated charcoal suspension 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 repelleted. The serum was decanted into bottles containing a fresh dextran-coated charcoal pellet, incubated and pelleted as above. The serum was then filter sterilised using a 0.22 mm Nalgene nitrocellulose filter unit.

**7c) Transient transfection**

i) Calcium phosphate precipitation

Cells were seeded for transient transfection at a density between 0.5-5 x $10^5$ per 5 $cm^2$ dish in 4 mls of phenol red free DMEM containing 10% charcoal treated foetal calf serum. After 24 hours the cells were transfected by calcium phosphate precipitation with a total of 10 µg of DNA per 5 $cm^2$ dish. The precipitate was prepared by mixing two freshly prepared solutions:-

| | |
|---|---|
| Solution A | 500 µl 2 x HBS, pH 7.1 |
| | 10 µl of 70mM sodium phosphate, pH 7.1 |
| Solution B | 500 µl distilled water |
| | 60 µl 2 M $CaCl_2$ |
| | 20 µg of supercoiled plasmid DNA |

Solution B was added to A at a rate of 1 drop every 2 seconds while air was continuously bubbled through to aid mixing. The mixture was left standing for 20 minutes to allow the precipitate to form and then 0.5 ml added dropwise to each of two duplicate dishes.

The transfection solution/precipitate was incubated with the cells for 6 hours at 37°C. The media was then removed and the cells were washed three times with phenol-red free DMEM and fed with phenol-red free DMEM containing 10% dextran-charcoal treated FCS in the presence or absence of $10^{-8}$M oestradiol.

ii)Electroporation

This transfection method produces a larger proportion of transfected cells but appears unsuitable for NIH 3T3 cells. It was used primarily in transfections into COS cells to show that mutant receptor constructs used in transfections were being expressed at similar levels in a mammalian system. COS cells grown to confluence in T75 flasks were trypsinised as described in general cell culture methods, pelleted by centrifugation and resuspended in 3ml PBSA on ice. An 800µl aliquot of suspension was transferred to a 0.4cm Biorad Genepulsar cuvette and plasmid DNA added. The cells were electroporated by a pulse of current at 450Volts and capacitance 250µF with a time constant of 4.3-5.2 allowing uptake of DNA. Transfected cells were then plated down on a 9cm dish containing 10mls DMEM with 10%FCS and grown to confluence for harvesting by the whole cell extract method described below.

**7d) Harvesting cell monolayers.**

The transfected cells were harvested when the monolayer was 80% confluent - routinely 24 to 48 hours after addition of hormone. The cell monolayer was washed three times with PBSA after removal of the media and 100 µl of lysis buffer added to each plate. When only nuclei were visible under a microscope the lysate was transferred to a microfuge tube 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 luciferase enzyme in cellular extracts (24 hours) this assay was performed as soon as possible. In contrast chloramphenicol acetyl transferase (CAT) activity remains stable in these extracts and after the luciferase assay they can be stored at -70°C for several months.

**7e) Luciferase assay**

This assay was routinely used as a control for cell number and transfection efficiency. Aliquots of cell extract (20 µl) were added to 350 µl of luciferase reaction buffer in luminometer cuvettes. These were loaded into an LKB 1251 luminometer which injected 33 µl of 3mM Luciferin (Na salt from Sigma) and recorded peak activities. Control assays with extracts from untransfected cells gave zero rate and had peak activities of

less than 0.6 units. Transfected cell extracts routinely gave peak activities of 30-200 units.

**7f) Assay of cytoplasmic CAT activity.** (Sleigh, 1986).

The remaining 60 µl of cell extract was then heated to 65°C for 10 minutes, to denature the endogenous acetyl coenzyme A degrading activity of the cells, and stored at -70°C until required. CAT activity was determined by analysis of the transfer of the [$^{14}$C] acetyl group from [$^{14}$C]Acetyl Co-A to chloramphenicol. Chloramphenicol and its acylated derivatives are soluble in organic solvents whereas acetyl Co-A is not. The acetylated chloramphenicol products can therefore be separated from the labelled substrate by extraction with ethyl acetate allowing direct quantitation of CAT activity without using chromatographic separation techniques. The assay was set up with 20 µl 8mM chloramphenicol, 20 µl acetyl CoA mix (0.1 mCi of [$^{14}$C]acetyl Co-A in 45mM unlabelled acetyl Co-A), 10 µl lysis buffer and cell extract up to 20 µl. Reaction volume was adjusted to 100 µl with 0.25M Tris pH 7.8 and then incubated at 37°C for 1 hour. The reaction was quenched by the addition of 100 µl of ice cold ethyl acetate and vortexing. This was then spun for 2 minutes in an microfuge and 80 µl of the upper phase transfered directly to a scintillation vial. The extraction was repeated with a further 100 µl of ethyl acetate and a 100 µl of the upper phase was removed. Organic extracts were pooled and counted by liquid scintillation. This assay is only linear up to 55,000 DPM (P. Webb, 1987) and therefore extracts with higher activities were routinely diluted and reassayed.

**7g) Stable transfections**

NIH 3T3 cells were transfected by calcium phosphate precipitation as described above, using 10 µg of receptor expression plasmid and 1 µg of the hygromycin resistance plasmid PY3 for 2 x $10^5$ cells in a 6cm dish. The cells were allowed to grow for 24 hours then split 1:10 and grown for a further 24 hours before addition of hygromycin to 350 µg/ml to select transfected cells. The media was changed every 4 days, continuing selection conditions, until colonies had reached 3-4mm diameter when ring-cloning was carried out. Colonies were trypsinised into 5mm sterile cloning rings and plated in 3cm well dishes. At confluence the cells were split into 9cm plates and grown until several vials could be frozen down for storage in liquid nitrogen.

**7 h)Whole Cell Extract**

Cells grown to 80% confluence in 15cm plates were washed twice with cold PBSA and scraped off the dishes into 5ml of PBSA. After centrifugation at 1500rpm for 5 minutes the cell pellet was frozen at -70°C. Cell pellets were thawed into approximately 10 volumes of high salt buffer containing protease inhibitors (0.4M KCl, 20mM HEPES pH 7.4, 1mM DTT, 20% glycerol, 0.5mg/ml bacitracin, 40µg/ml PMSF, 5µg/ml pepstatin, 5µg/ml leupeptin), passed 5 times through a 25 gauge needle and insoluble material removed by centrifugation (50,000g for 15 minutes). The protein concentration was determined in a Biorad Protein Assay exactly according to the protocol. The resulting whole cell extracts were stored in aliquots at -70°C and were stable for several months.

# CHAPTER 3

## Mutagenesis and functional analysis of a region within the hormone binding domain involved in ligand binding and dimerisation

## 3.1 Introduction

This chapter describes the mutagenesis of conserved sequences within the hormone binding domain of the mouse oestrogen receptor to investigate which amino acids are involved in dimerisation and hormone binding. Amino acids between Q-503 and R-519 have been mutated since earlier studies had indicated that this region is involved in both functions (Kumar and Chambon, 1988; Fawell *et al.*, 1990a). In addition this chapter includes experiments directed towards determining the structure of the dimerisation interface.

## 3.2 Strategy for mutagenesis of the mouse oestrogen receptor

The methods available for the introduction of point mutations into the mouse oestrogen receptor cDNA included 'site-directed' mutagenesis, Polymerase Chain Reaction (PCR) and the use of double-stranded oligonucleotides. The simplest method to introduce mutations is to clone a double-stranded oligonucleotide bearing the mutation directly into the cDNA at suitable restriction enzyme sites. This method is however limited to the use of oligonucleotides of up to around 100 bases. Since the plasmids pMORK and pMORHK (Fawell *et al.*, 1990a) provide cDNAs which contain suitable restriction sites to introduce mutations by double-stranded oligonucleotide cloning, this method was chosen. The cloned DNA was finally sequenced to ensure that no errors had been inadvertently introduced into either strand during the synthesis of the oligonucleotide or the cloning steps.

Modified forms of the mouse oestrogen receptor containing a unique Kpn I restriction enzyme site at 1739 (pMORK) or the Kpn site and a Hpa I site at 1618 (pMORHK) were used as vectors to generate mutations between residues 501 and 530. To mutate amino acids between positions 501-509 inclusive, complementary oligonucleotides were synthesised extending between a Hpa I site and a unique Cel II site. Amino acids between positions 511 and 532 inclusive were changed by use of oligonucleotides extending between the Cel II site and the Kpn I site. The amino acids A-509 and Q-510 were mutated with an oligonucleotide extending from a Hpa I site to the Kpn I site The sequences of the oligonucleotides used are shown in Figure 3.1 and the cloning steps for each type of oligonucleotide in Figures 3.2-4. Where possible, oligonucleotides were designed to introduce a Nde I site at 1683, while maintaining correct amino acid sequence. This facilitated the

Figure 3.1 - Oligonucleotides used to generate mutant receptors

**A. Coding strand sequence of oligonucleotides cloned into Cel II - Kpn I vector to generate mutants between 511 and 530.**

L-511R/K

```
          Lys
T CAG ARG CTT CTC ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC
   Gln Arg Leu Leu Ile Leu Ser His Ile Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
 Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val
```

L-511E/D

```
          Glu
T CAG GAW CTT CTC ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC
   Gln Asp Leu Leu Ile Leu Ser His Ile Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
 Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val
```

L-511A

```
T CAG GCA CTT CTC ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC
   Gln Ala Leu Leu Ile Leu Ser His Ile Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
 Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val
```

L-512X

```
                Glu
                Gly
T CAG CTC GXG CTC ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC
   Gln Leu  Val Leu Ile Leu Ser His Ile Arg His Met Ser Asn
                Ala
AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
 Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val
```

L513K

```
T CAG CTC CTT AAG ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC
   Gln Leu Leu Lys Ile Leu Ser His Ile Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
 Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val
```

L-513X

Glu
T CAG CTC CTT GNG ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC
Gln Leu Leu Gly Ile Leu Ser His Ile Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

I514X

Cys
Arg
T CAG CTC CTT CTC NGT CTT TCC CAT ATC CGG CAT ATG AGT AAC
Gln Leu Leu Leu Gly Leu Ser His Ile Arg His Met Ser Asn
Ser
AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

L515X

Val
Ala
T CAG CTC CTT CTC ATT GNG TCC CAT ATC CGG CAT ATG AGT AAC
Gln Leu Leu Leu Ile Gly Ser His Ile Arg His Met Ser Asn
Glu
AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

S516A

T CAG CTC CTT CTC ATT CTT GCG CAT ATC CGG CAT ATG AGT AAC
Gln Leu Leu Leu Ile Leu Ala His Ile Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

H517A

T CAG CTC CTT CTC ATT CTT TCC GCG ATC CGG CAT ATG AGT AAC
Gln Leu Leu Leu Ile Leu Ser Ala Ile Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

I518A

T CAG CTC CTT CTC ATT CTT TCC CAT GCA CGG CAT ATG AGT AAC
Gln Leu Leu Leu Ile Leu Ser His Ala Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

I518R/K

Lys

T CAG CTC CTT CTC ATT CTT TCC CAT ARG CGG CAT ATG AGT AAC

Gln Leu Leu Leu Ile Leu Ser His Arg Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C

Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

I-518E/D

Glu

T CAG CTC CTT CTC ATT CTT TCC CAT GAW CGG CAT ATG AGT AAC

Gln Leu Leu Leu Ile Leu Ser His Asp Arg His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C

Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

R-519X

Gly
Val

T CAG CTC CTT CTC ATT CTT TCC CAT ATC GNG CAT ATG AGT AAC

Gln Leu Leu Leu Ile Leu Ser His Ile Ala His Met Ser Asn

AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C

Lys Gly Met Asp His Leu Tyr Asn Met Leu Cys Lys Asn Val Val

Y530TF

T CAG CTC CTT CTC ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC

Gln Leu Leu Leu Ile Leu Ser His Ile Arg His Met Ser Asn

Phe

AAA GGC ATG GAG CAT CTC TYC AAC ATG AAA TGC AAG AAC GTG GTA C

Lys Gly Met Asp His Leu Thr Asn Met Leu Cys Lys Asn Val Val

**B. Coding strand sequence of oligonucleotides cloned into Hpa I - Cel II vector to generate mutants between 503 and 508**

Q503X, Q504A

```
                    Ala
                    Val
A ACT CTG CAG GNT GCA CAT CGA CGC CTA GC
  Thr Leu Gln Gly Ala His Arg Arg Leu
                  Asp
```

H505X,R506A

```
                              Pro
A ACT CTG CAG CAA CAG NCT GCA CGC CTA GC
  Thr Leu Gln Gln Gln Ala Ala Arg Leu
                         Thr
```

L-508X

```
                                          Asp
                                          Gly
A ACT CTG CAG CAA CAG CAT CGA CGC GNT GC
  Thr Leu Gln Gln Gln His Arg Arg Ala
                                          Val
```

**C. Coding strand sequence of oligonucleotides cloned into Hpa I - Kpn I vector to generate mutants between 509 and 510**

Q-510X

```
                                                  Asp
                                                  Gly
A ACT CTG CAG CAA CAG CAT CGA CGC CTA GCT GNG CTC CTT CTC
  Thr Leu Gln Gln Gln His Arg Arg Leu Ala Val Leu Leu Leu
                                                  Ala

ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC AAA GGC ATG GAG CAT
 Ile Leu Ser His Ile Arg His Met Ser Asn Lys Gly Met Asp His

CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
 Leu Tyr Asn Met Leu Cys Lys Asn Val Val
```

A-509X

```
                                          Arg
A ACT CTG CAG CAA CAG CAT CGA CGC CTA ANG CAG CTC CTT CTC
  Thr Leu Gln Gln Gln His Arg Arg Leu Met Gln Leu Leu Leu

ATT CTT TCC CAT ATC CGG CAT ATG AGT AAC AAA GGC ATG GAG CAT
 Ile Leu Ser His Ile Arg His Met Ser Asn Lys Gly Met Asp His

CTC TAC AAC ATG AAA TGC AAG AAC GTG GTA C
 Leu Tyr Asn Met Leu Cys Lys Asn Val Val
```

Figure 3.2 - Generation of MORL-508A, L-508G, L-508D and L-508V

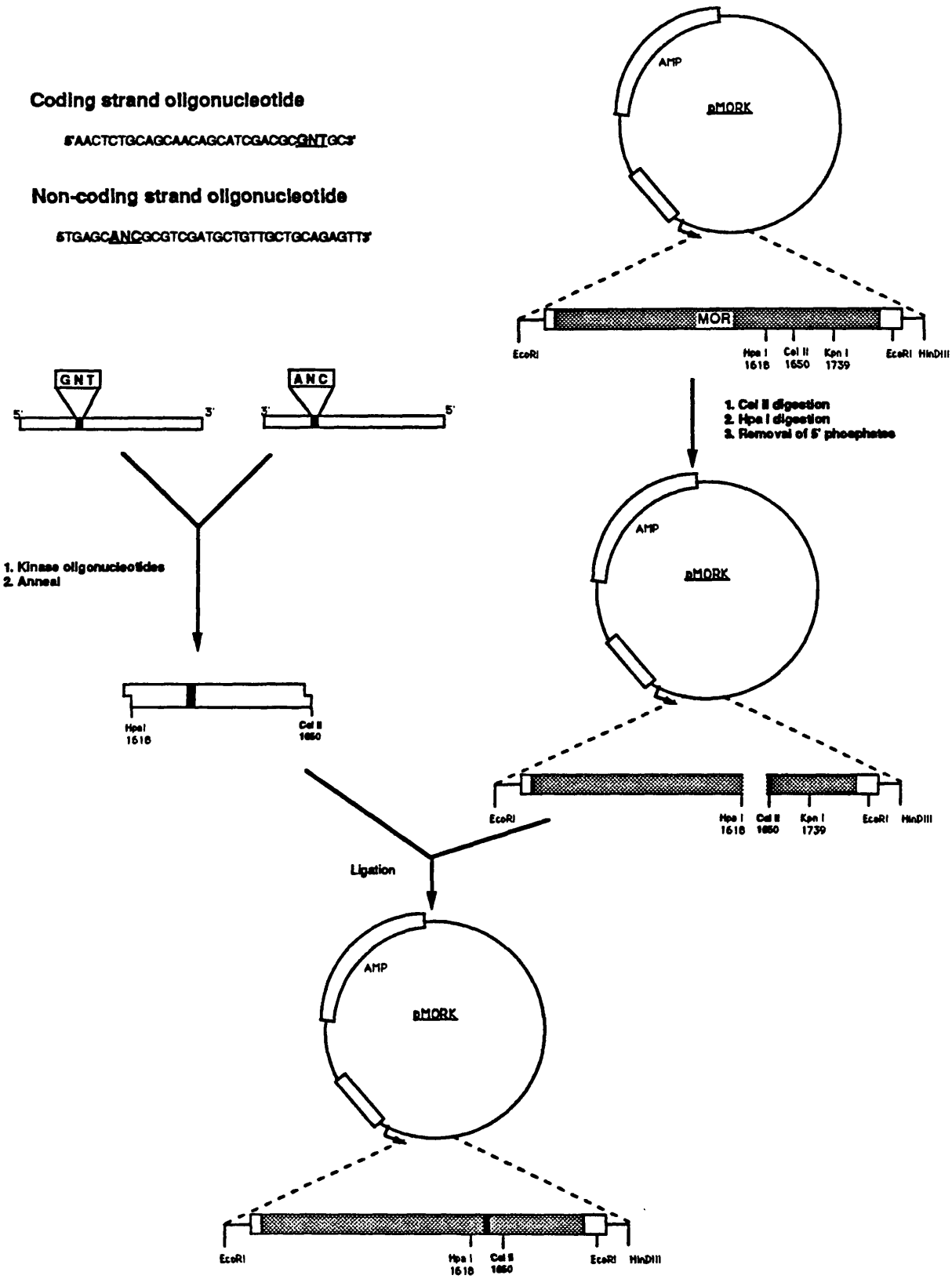


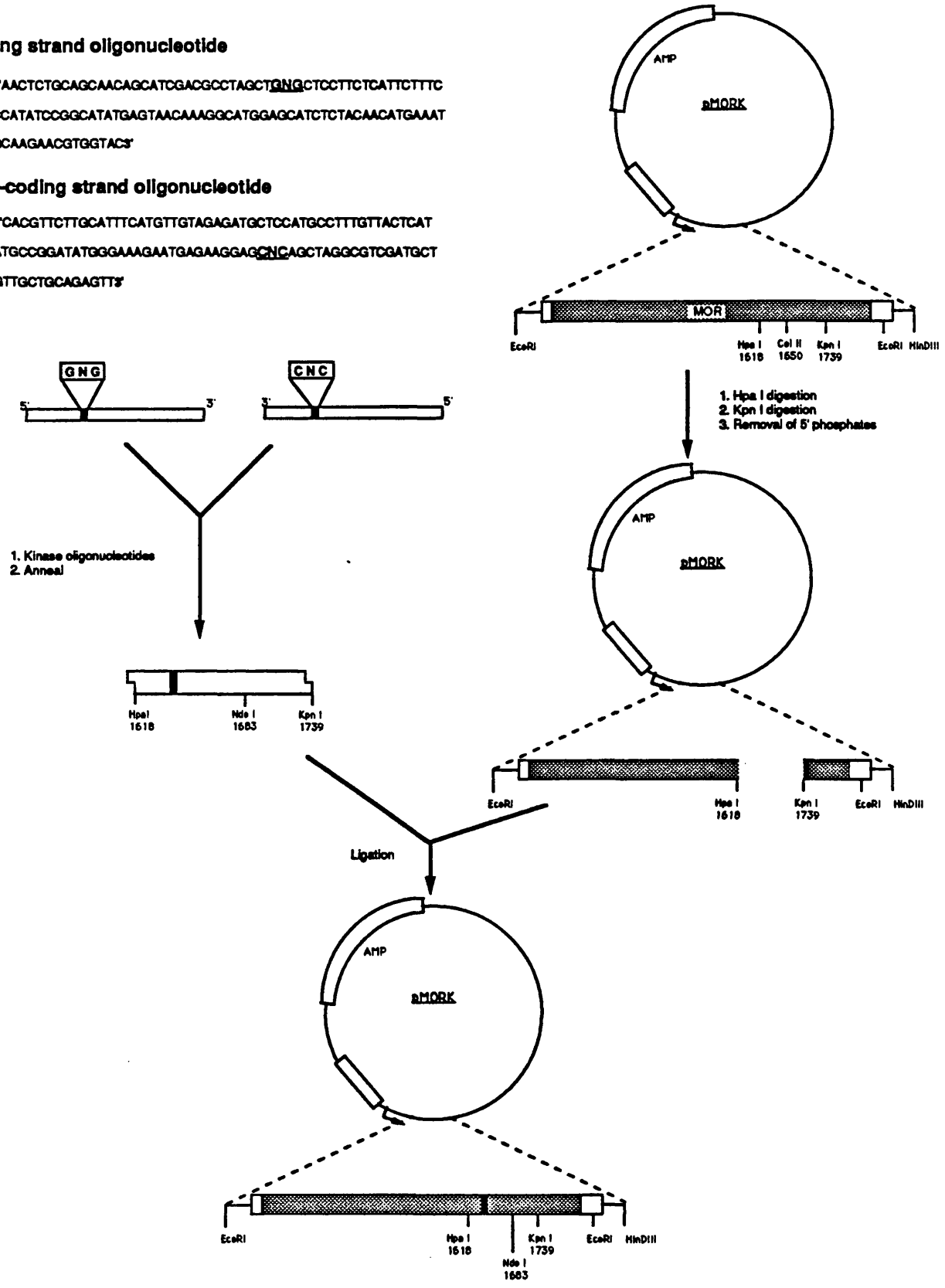
Figure 3.3 - Generation of MORQ-510A, Q-510G, Q-510E and Q-510V

**Coding strand oligonucleotide**

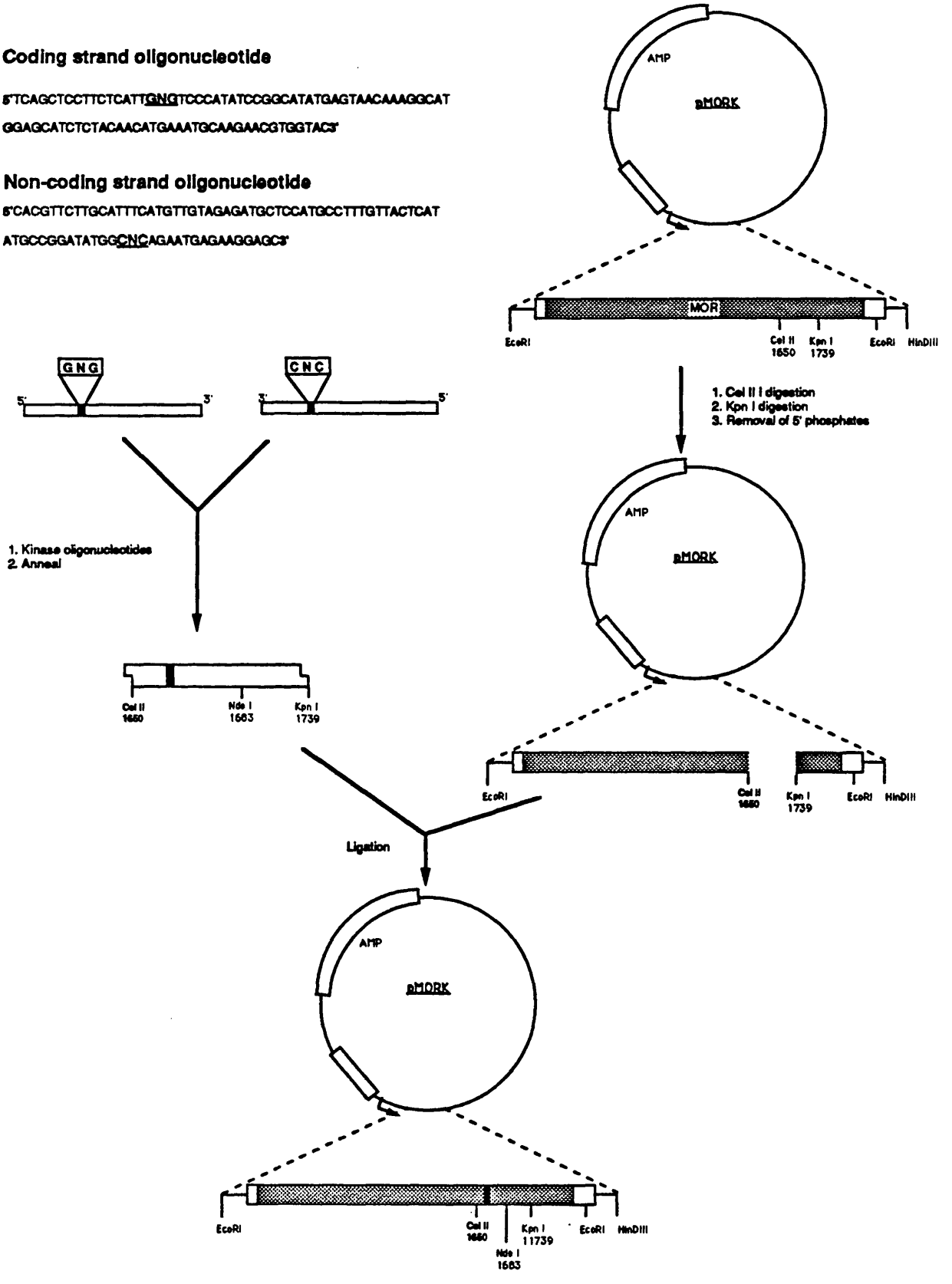
5'AACTCTGCAGCAACAGCATCGACGCCTAGCTGNGCTCCTTCTCATTCTTTC
CCATATCCGGCATATGAGTAACAAAGGCATGGAGCATCTCTACAACATGAAAT
GCAAGAACGTGGTAC3'

**Non-coding strand oligonucleotide**

5'CACGTTCTTGCATTTCATGTTGTAGAGATGCTCCATGCCTTTGTTACTCAT
ATGCCGGATATGGGAAAGAATGAGAAGGAGCNCAGCTAGGCGTCGATGCT
GTTGCTGCAGAGTT3'



## Figure 3.4 - Generation of MORL-515A, L-515G, L-515E and L-515V



identification of recombinants during cloning. The mouse oestrogen receptor contains a single Nde I site at base 1410 which linearised the plasmid MORK. When this restriction enzyme was used with mutant receptors constructed using an Nde I site containing oligonucleotide, a 300bp fragment was released.

To generate more information about the region of the oestrogen receptor being investigated a number of different residues were introduced into each position. This was achieved by synthesising oligonucleotides degenerate in one position within the codon for the amino acid to be mutated. The oligonucleotide was then annealed and cloned into an mouse oestrogen receptor cDNA plasmid. For example by specifying the codon for amino acid 515, a leucine in the wild-type receptor, as GNG four different clones could be isolated containing valine (L-515V), alanine (L-515A), glycine (L-515G) or glutamic acid (L-515E) at that position. This enabled us to study the effects of introducing a hydrophobic amino acid, a small relatively neutral amino acid, a small helix-breaking amino acid and a charged amino acid into a conserved hydrophobic position. The length of many of the oligonucleotides used (up to 120 nt) created some problems with up to 25% of clones with the second Nde I site containing a mistake in the sequence. These random changes were occasionally useful in generating novel mutations or double substitutions. All mouse oestrogen receptor mutants created were named to describe the mutated residue, the position of the residue, and the residue introduced ie. the construct in which the leucine at position 515 had been mutated to an alanine was L-515A.

## 3.3 Choice of methods for testing dimerisation and ligand binding

One of the main aims of this mutagenesis was to investigate the effect of particular changes in the amino acid sequence on the ability of the mouse oestrogen receptor to form stable dimers. It is possible to demonstrate the formation of dimers by immunoprecipitation. The mutation is introduced to both full-length and N-terminally truncated receptor which are then cotranslated. The receptors are precipitated with an antiserum which recognises an epitope present only in the full-length receptor. When visualised on an SDS acrylamide gel the truncated receptor is only present if a stable dimer has formed between the two

forms of receptor. Since dimerisation is essential for high affinity DNA binding activity (Kumar and Chambon, 1988) the ability of receptor to dimerise can be tested in a DNA binding assay. Subsequently it has been found that DNA binding activity and dimerisation of mutants in this region correlate completely (Fawell *et al.*, 1990a). In view of this correlation we have used the ability of oestrogen receptor mutants to bind to an ERE in a gel retardation assay to determine the effects of mutations on dimerisation.

The second major aim of this study was to investigate the effects of mutagenesis on the ability of the receptor to bind ligand with high affinity. Earlier studies used covalently labelling ligands to investigate which regions of the receptor were involved in hormone binding (Ratajczak *et al.*, 1989; Harlow *et al.*, 1989). Point mutagenesis and quantitation of radiolabelled ligand binding has advantages over affinity labelling in characterisation of ligand binding activity. It is possible to study the effects of particular mutations on the affinity of receptor for oestrogen and detect whether any amino acid is making important contacts with the ligand.

## 3.4 Identification of specific amino acids required for oestrogen binding.

Receptor mutants were linearised with the restriction enzyme HinD III and cRNA transcribed by SP6 RNA Polymerase. Translations were carried out in the Rabbit Reticulocyte Lysate system. Proteins labelled with [$^{35}$S]-methionine were separated from free methionine on an SDS acrylamide gel The dried gel was subjected to autoradiography to compare the relative amounts of each receptor mutant translated. The results of this were used to control receptor input into both gel retardation and ligand binding assays.

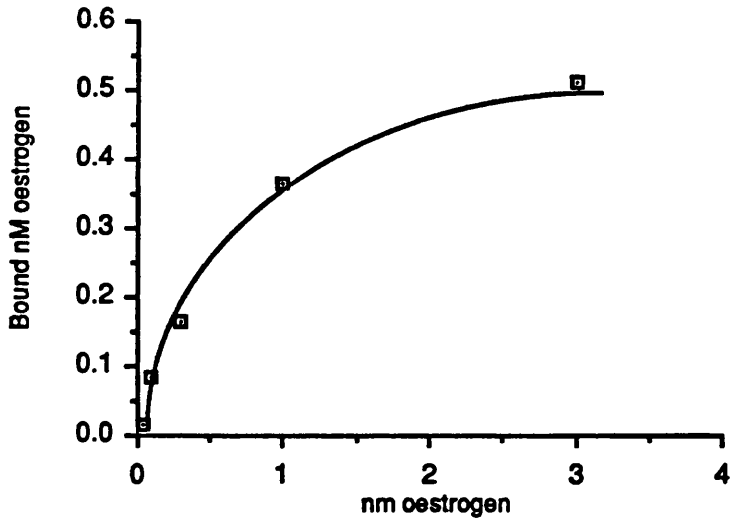
Mutant oestrogen receptors translated in reticulocyte lysate from their corresponding cRNAs in the absence of labelled amino acids were tested for their ability to bind oestrogen. Aliquots of receptor were incubated with [16$\alpha$-$^{125}$I]-oestradiol and unbound ligand removed with dextran-coated charcoal. Non-specific binding was determined in the presence of excess diethylstilbestrol. The binding affinity of the mutant was then determined by further binding assays with a range of concentrations of ligand and the Kd determined by Scatchard analysis (Figure 3.5). However, it became evident that the single point assay at

Figure 3.5

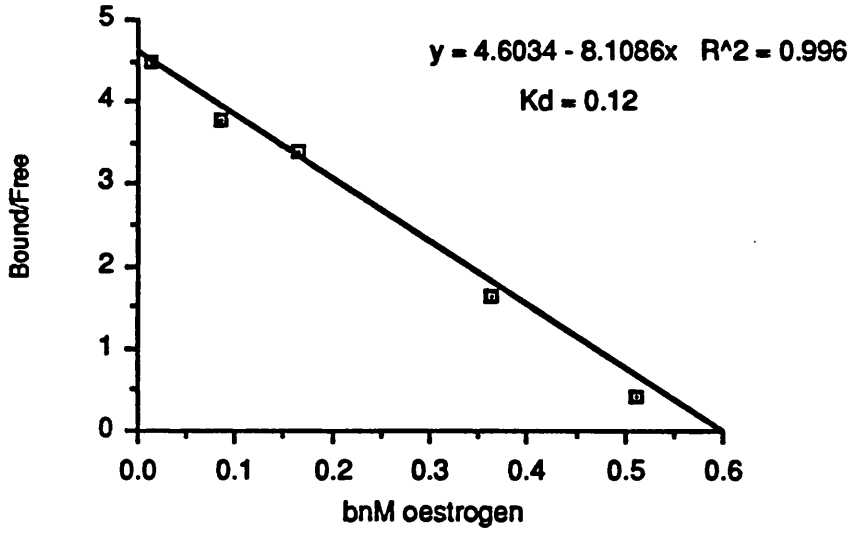
This figure shows the oestrogen binding affinity determined for a range of mutant oestrogen receptors. A representative binding curve (A.) and Scatchard analysis (B.) obtained for the mutant L-511E is shown. Table C presents the oestrogen binding affinity of other mutant oestrogen receptors .

Figure 3.5 - Binding affinities of mutant receptors

A.



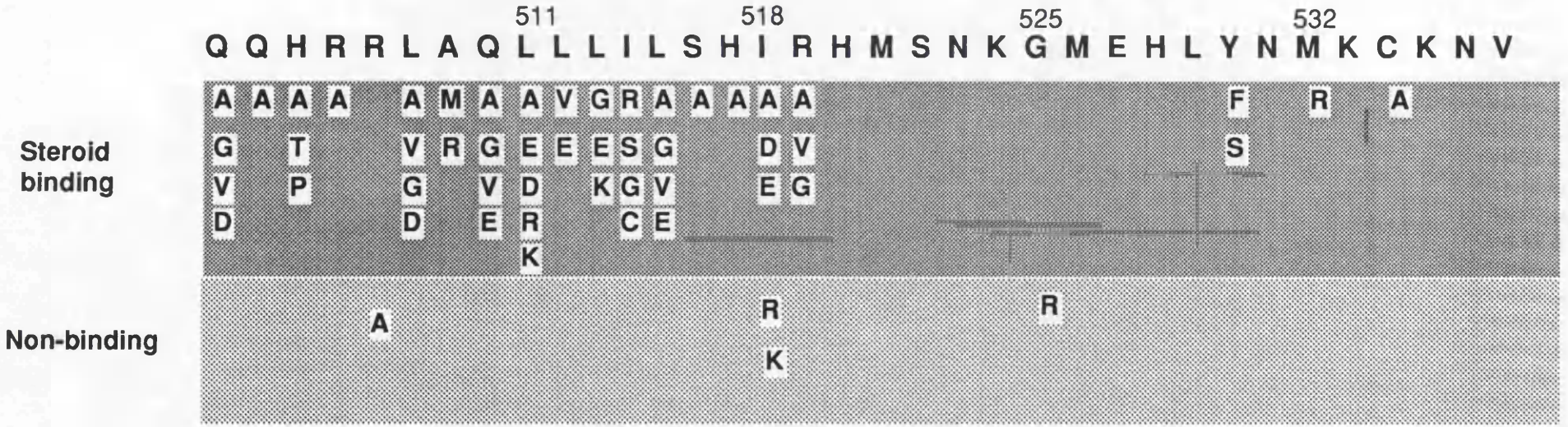

B.




C.

| | KD nM | | KD nM |
|---|---|---|---|
| R-507A | 2.9 | | |
| Q-510A | 0.11 | | |
| L-511E | 0.12 | L-511R | 1.1 |
| L-513K | 0.19 | | |
| L-515K | 0.11 | | |
| I-518E | 0.42 | I-518R | - |
| R-519A | 0.10 | | |
| G-525R | - | | |
| Y-530F | 0.12 | Y-530S | 0.15 |
| M-532R | 0.56 | | |

Figure 3.6 - Summary of ligand binding activity of mouse oestrogen receptor point mutants



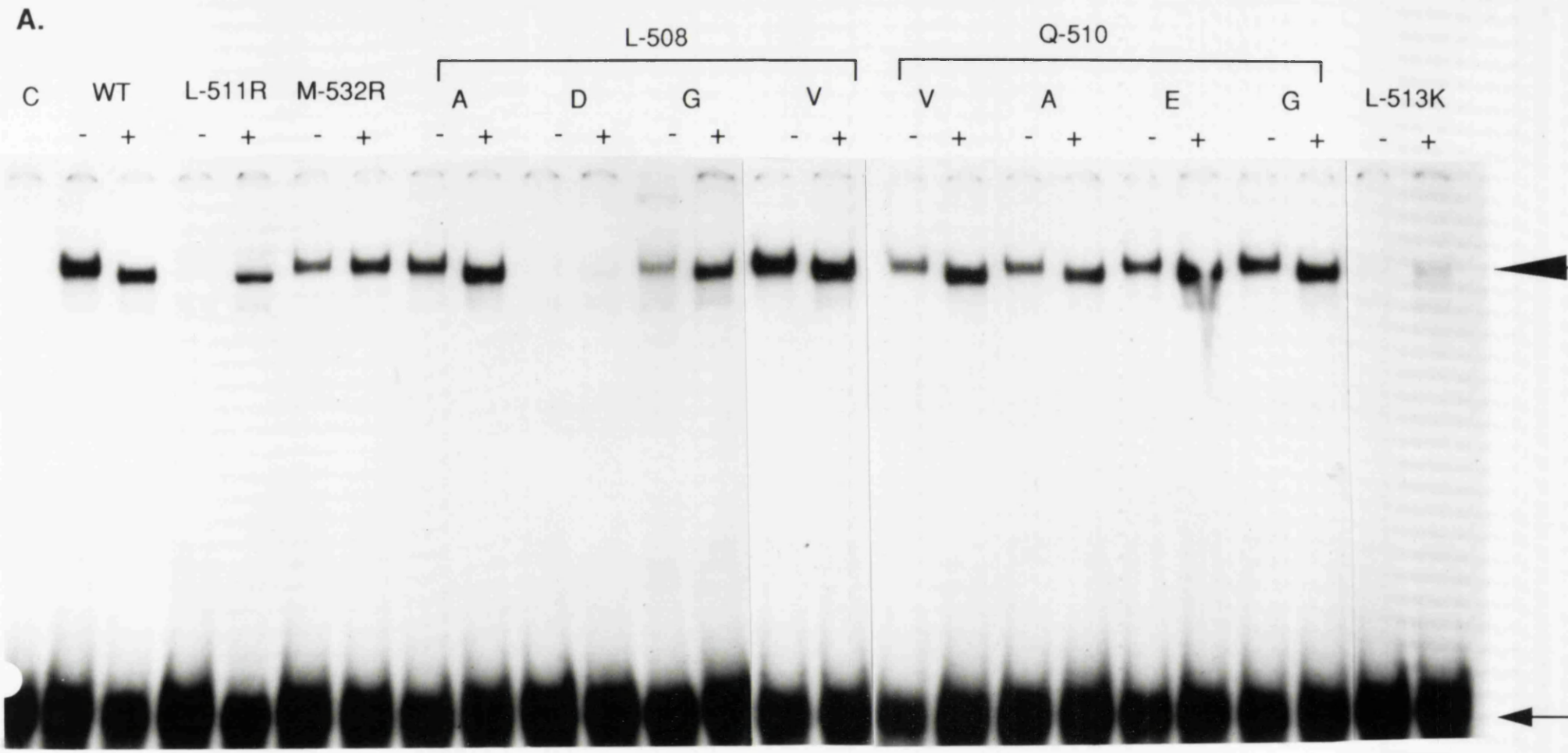
1nM iodoestradiol was sufficient to reveal whether the affinities of mutant receptors were similar to that of wild-type receptor. Subsequently, actual affinities were determined only for mutants which showed significantly lower binding than the wild-type receptor at 1nM. The results of these ligand-binding assays are summarised in Figure 3.6.

Mutagenesis revealed that the isoleucine at position 518 and the glycine at position 525 were particularly important for the binding of oestrogen. The introduction of mutations to positions 507 and 511 could also reduce affinity for oestrogen, but ligand binding was not abolished. However it was noted that the reduction in the affinity for oestrogen depended on the amino acid substitution. I-518A had wild-type affinity for oestradiol whereas substitution of an acidic residue reduced affinity four-fold and a basic residue abolished ligand-binding entirely. This established that only a relatively conservative change in this position could be tolerated. A number of different hydrophobic residues in this region were changed to charged residues with no effect on ligand binding. This demonstrated that it was not the overall hydrophobicity of the region which was important for ligand binding but the contribution of particular residues. The amino acids R-507, L-511, I-518 and G-525 appeared to be most important but were adjacent to residues such as L-508, L-512 and R-519 which could be mutated without any effect upon ligand binding.

## 3.5 Point mutations in the hormone binding domain disrupt DNA binding

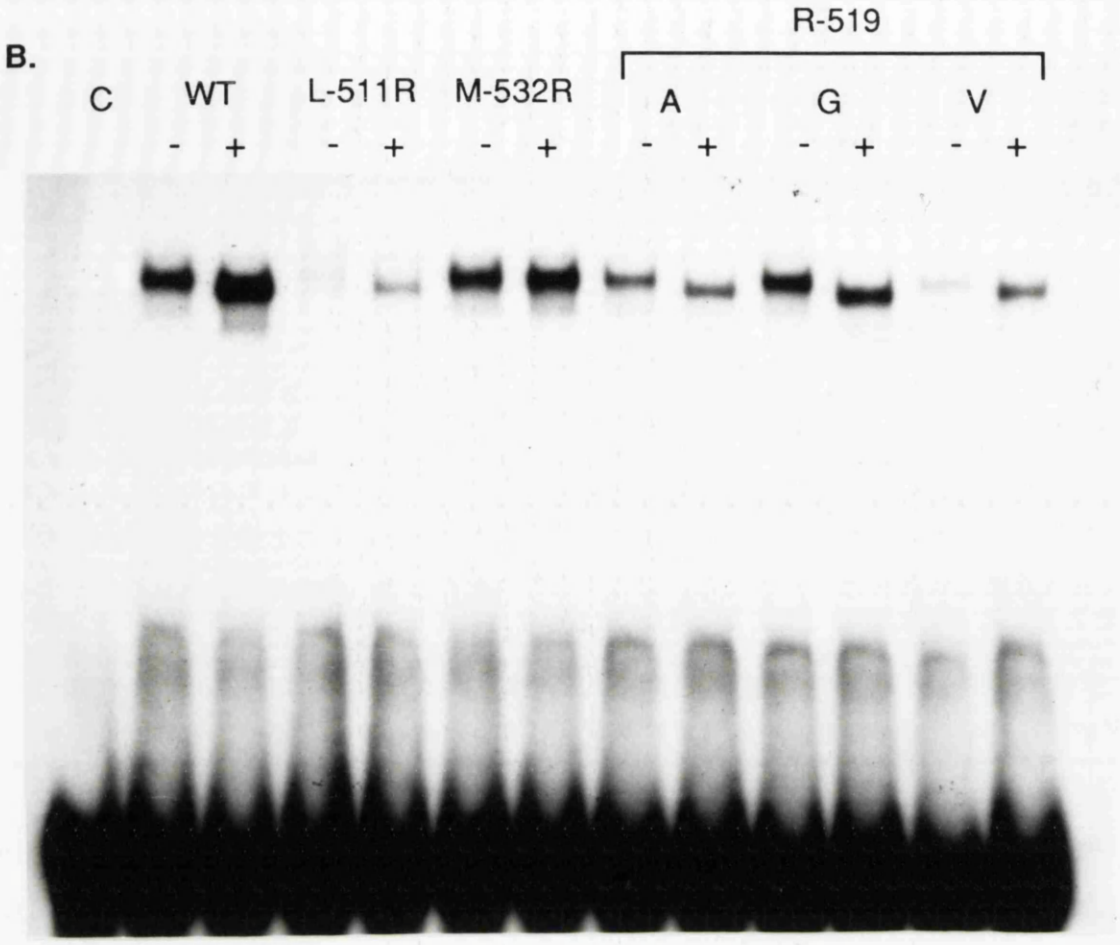
In this study the ability of oestrogen receptor mutants to bind to a vitellogenin A2 ERE in a gel retardation assay has been used to assess the effect on dimerisation of substitutions of individual amino acids in the hormone binding domain. Gel retardation assays were carried out as described in the Methods section, using receptor translated with non-radioactive methionine. This was incubated with $^{32}P$ labelled double-stranded oligonucleotide probe containing the palindromic A2 ERE in the absence or presence of oestradiol. The receptor/DNA complex was separated from free probe on a polyacrylamide gel. Typical gels are shown in Figure 3.7 and a summary of the ability of the mutants to bind DNA is shown in Figure 3.8.

**Figure 3.7 - Ability of oestrogen receptor point mutants to bind DNA**




Wild-type (WT) and mutant oestrogen receptors were translated in vitro and preincubated in binding buffer containing 10nM oestradiol ( + ) or no added hormone ( - ). The proteins were then tested for their ability to bind to A2 ERE in a gel retardation assay. The lane marked 'C' contains a control translation. Free ERE probe is marked with an arrow and the receptor/DNA complex with an arrowhead.

Figure 3.7 - Ability of oestrogen receptor point mutants to bind DNA



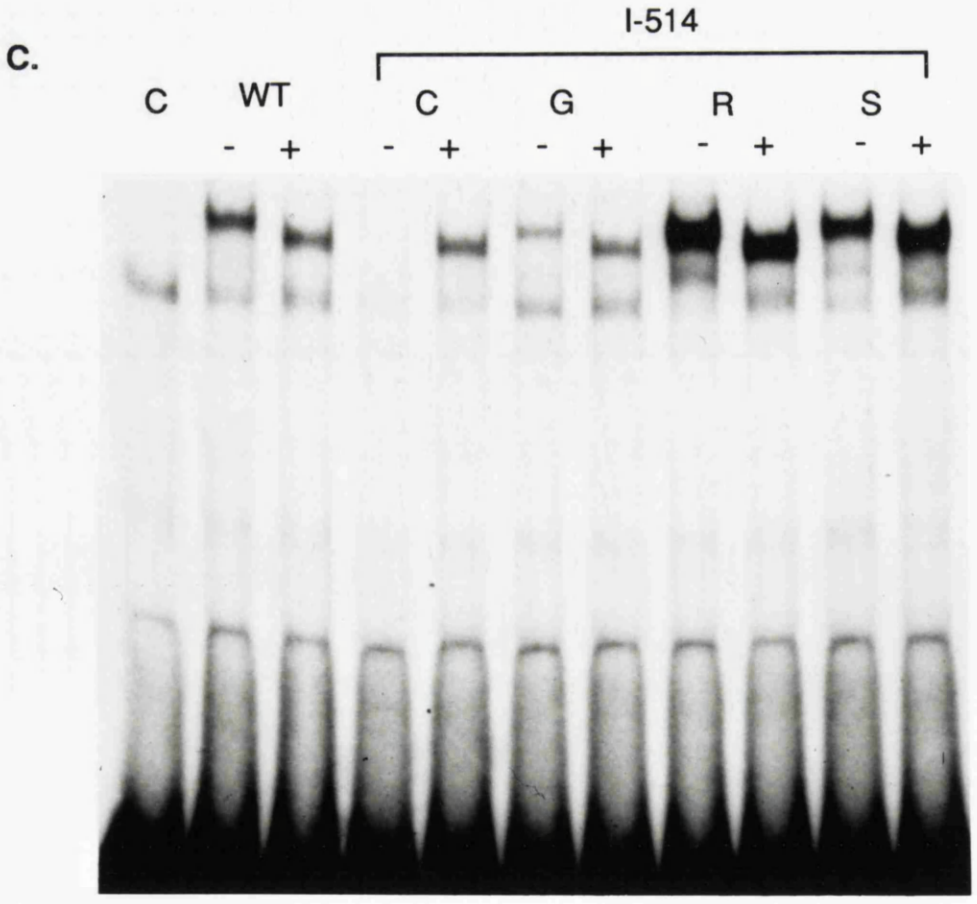
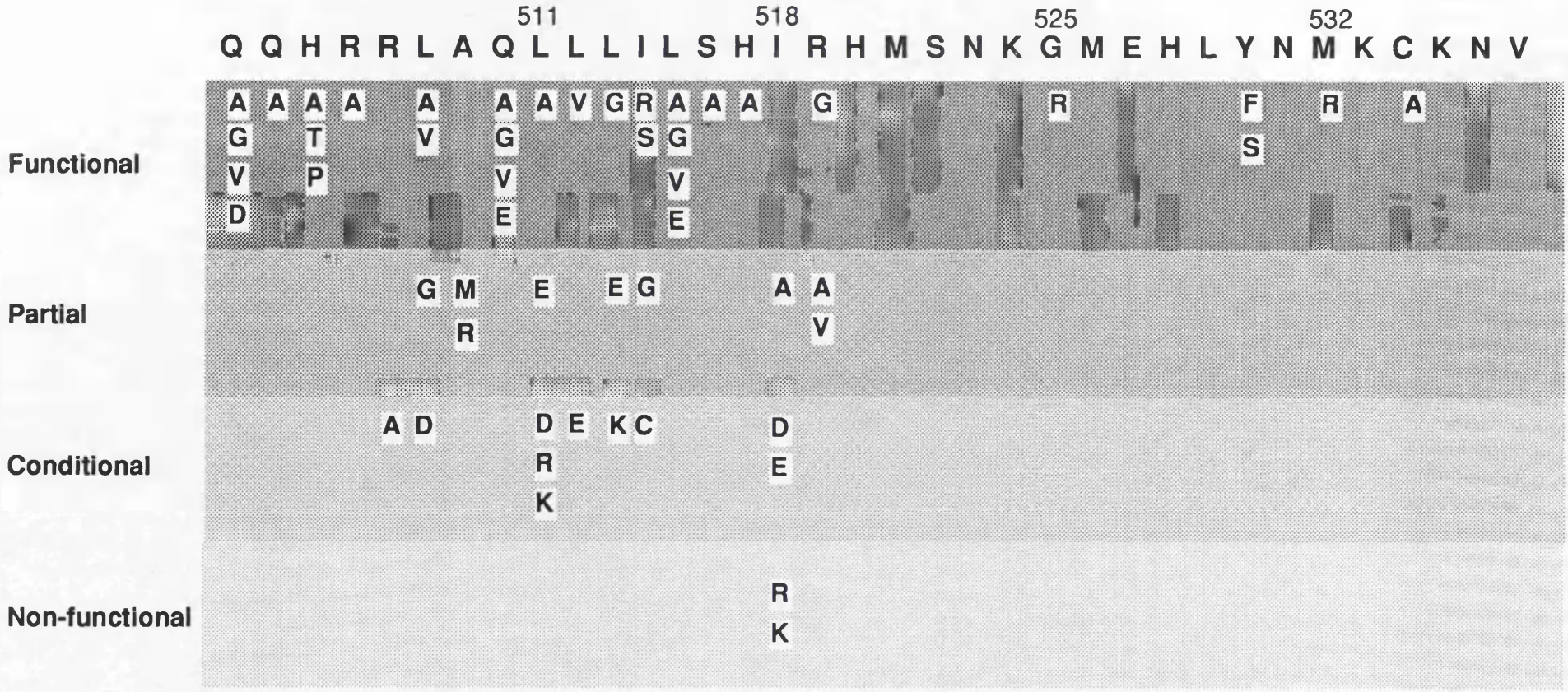



Figure 3.8 - Summary of DNA binding activity of mouse oestrogen receptor point mutants



The DNA binding activities of the mutant receptors were divided into 4 broad groups;

(+++) **Functional** - where binding was indistinguishable from wildtype MOR, (90-100% eg L-508V )

(++) **Partial**- where binding was less than with wildtype but still occurred even in the absence of ligand.(10-90% eg L-508G )

(+) **Conditional** - where even low levels of DNA binding occurred only in the presence of ligand.(0.5-10% eg L-511R )

(-) **Non-functional** - no DNA binding even in the presence of ligand. (<0.5% eg I-518R - see fig. 3.11)

A number of different residues within the region tested were important for DNA binding. Mutations in R-507, L-508, L-511, L-512, L-513, I-514 and I-518 could abolish DNA binding in the absence of oestrogen. Additionally mutations in A-509 and R-519 could reduce DNA binding in the absence of ligand. These results also demonstrated that the effect of each mutation depends on two factors, firstly the importance of the wildtype residue in the dimerisation interface, and secondly the nature of the amino acid introduced. L-515A, L-515V, L515-G and L-515E are all capable of wild-type DNA binding which suggested that this position was not involved in the dimerisation interface. In contrast mutation of I-518 to amino acids other than the small relatively neutral residue alanine greatly reduced or completely abolished DNA binding. This suggested that I-518 was an important part of the dimerisation interface.

These results also show that amino acids directly N-terminal of R-507 are unlikely to contribute to the dimerisation interface as mutation of residues 503-506 did not reduce DNA binding. It appears that the sequences involved in this major dimerisation interface are contained within residues 507-519. An $\alpha$ helical structure had been predicted for these sequences using the Chou-Fasman algorithm. It was therefore surprising that three different amino acids could be mutated to glycine, a residue which tends to disrupt helices, and generate functional receptor (eg L-508, I-514 and R-519).This suggested that maintaining a helical structure over this region was not essential for dimerisation.

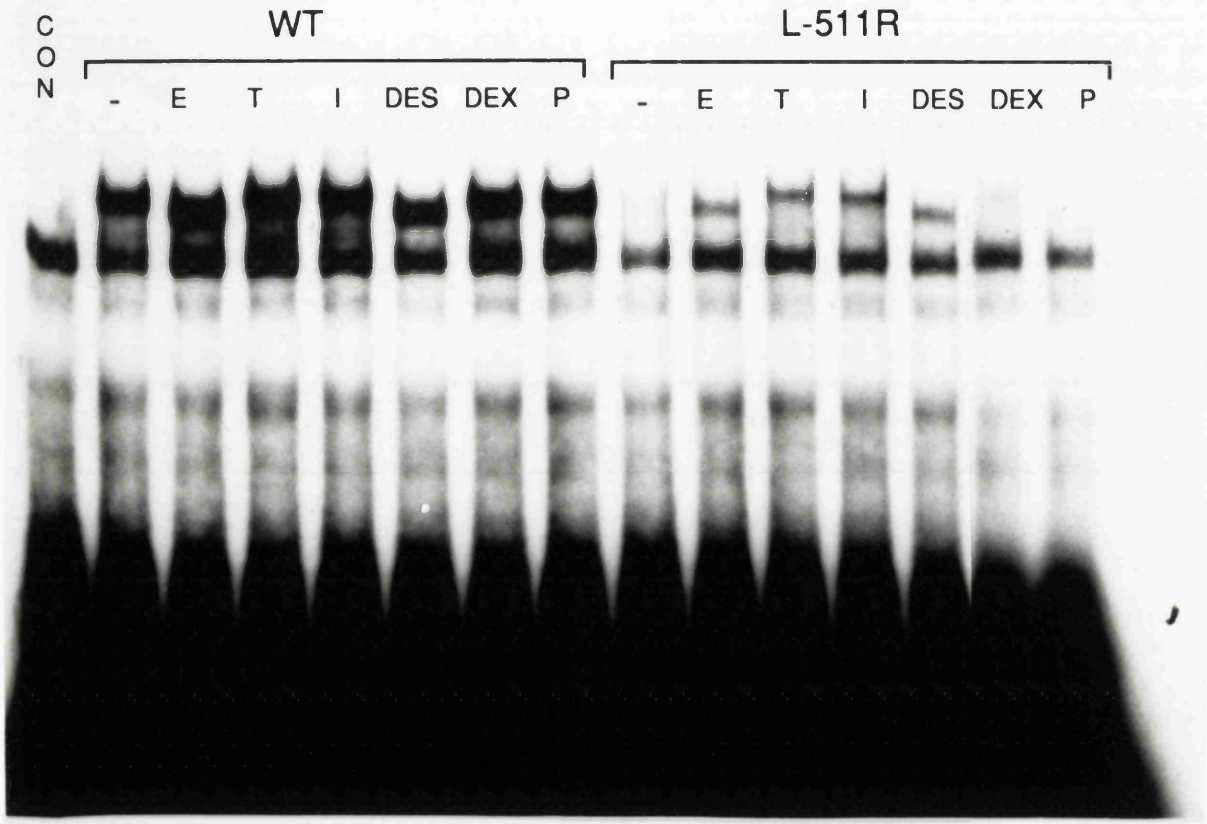
The effects of a number of mutations were unexpected. The androgen, glucocorticoid, progesterone and mineralocorticoid receptors

contain a lycine at the position which corresponds to L-513 in the oestrogen receptor. It was therefore predicted that the mutation L-513K would, if it had any effect at all, alter the ligand-binding affinity of the receptor. Surprisingly the substitution had only a very small effect on ligand-binding but completely abolished DNA binding in the absence of ligand. Even in the presence of ligand, the ability of this mutant to bind DNA was very limited. Also unexpected was the observation that some highly conserved amino acids could be mutated with no deleterious effect. The leucine at position 515 is an example of a residue which is conserved across the steroid receptor superfamily and yet mutations in this residue appeared to have no effect upon DNA binding. Similarly the conserved Q-510 can be mutated to a range of amino acids (alanine, valine, glycine or glutamic acid) without affecting the ability of the receptor to dimerise. These mutations also failed to interfere with oestrogen binding. Since these residues were highly conserved it was possible that they were involved in another function such as transcriptional activation (see Chapter 4).

## 3.6 Effect of ligand on DNA binding activity of oestrogen receptor.

The DNA binding activity of wild-type and mutant receptors was determined in the absence and presence of hormonal ligands in a gel retardation assay (Figure 3.9). It was found that oestrogen increased the mobility of the wild-type receptor but had no effect upon the levels of DNA binding. Since the conditions of the gel retardation assay have been optimised for DNA binding by the oestrogen receptor, cation conditions were adjusted to look for any ligand dependence in DNA binding under sub-optimal conditions. The receptor was incubated under a range of conditions from 0-5mM $MgCl_2$ and run on an acrylamide gel under the same conditions. Increases in the concentration of magnesium produced an overall decrease in the level of DNA binding but failed to show any ligand dependence in DNA binding. An effect of oestradiol which was apparent under all salt conditions was an increase in mobility of receptor which had been preincubated with this ligand. Preincubation with diethylstilbestrol also increased the mobility of receptor in a gel retardation assay. In contrast mobility was not altered by preincubation with the antioestrogens 4-hydroxytamoxifen and ICI 164,384 or a glucocorticoid or progestin.

Figure 3.9 - Effect of ligand on DNA binding activity of mutant



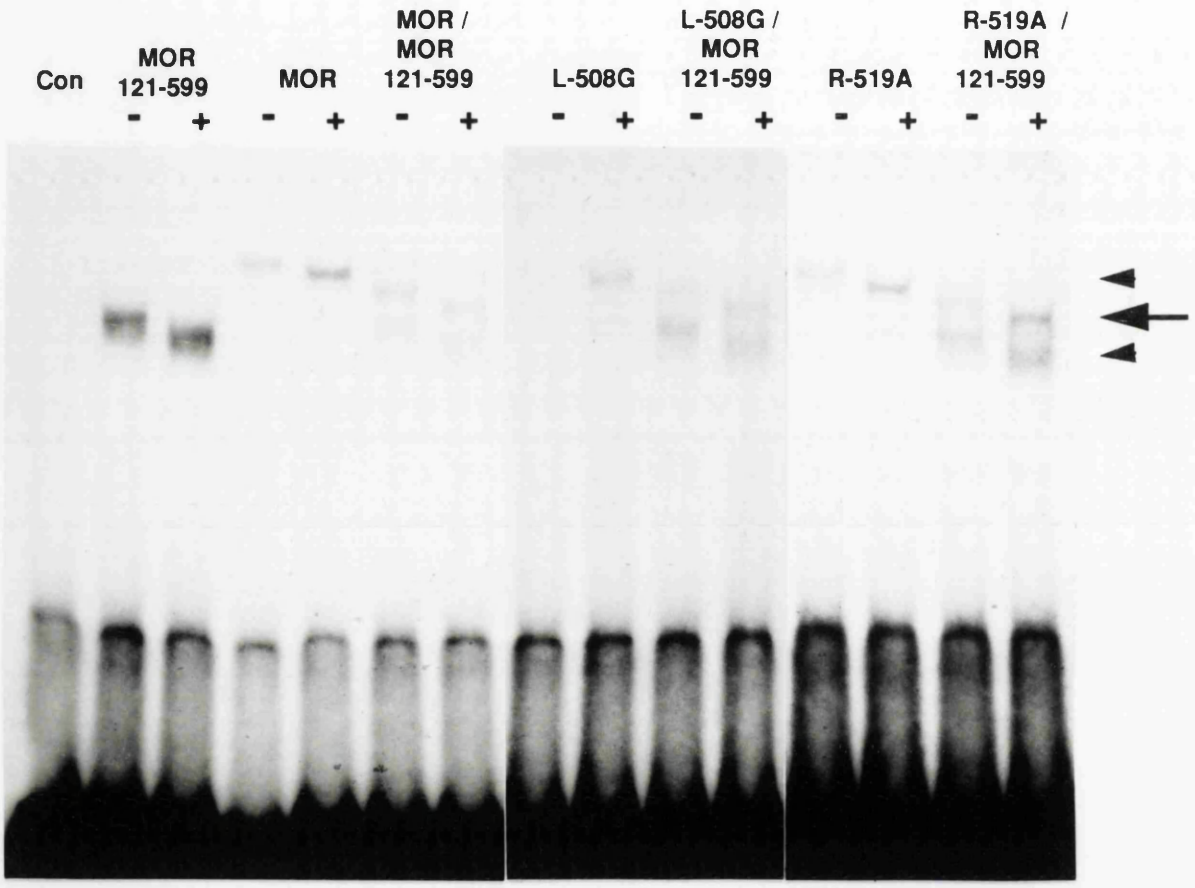

Wild-type (WT) receptor and the point mutant L-511R were translated *in vitro* and tested in a gel retardation assay for their ability to bind DNA after preincubation with a number of ligands, 10nM oestradiol (E), 100nM 4-hydroxytamoxifen (T), 1μM ICI 164,384 ( I ), 100nM diethylstilbestrol (DES), 1μM dexamethazone (DEX), 1μM R5020 (P) or no added hormone ( - ). 'Con' indicates that a control translation was used.

Ligand has however been shown to increase the level of DNA binding in a number of dimerisation deficient mutants. Many mutants which were unable to bind to DNA in the absence of ligand showed low levels of DNA binding after incubation with oestradiol. The antioestrogen tamoxifen restored binding to a similar extent as did, somewhat unexpectedly, the 'pure' antioestrogen ICI 164,384 . Neither the glucocorticoid dexamethazone nor the progestin R5020 restored binding which confirmed that this was not a non-specific effect of steroids. Mutants with low DNA binding levels in the absence of ligand generally showed an increase in binding on the addition of hormone.

## 3.7 Ability of point mutants to bind DNA as a heterodimer with wild-type receptor.

When full-length and N-terminally truncated receptors are cotranslated *in vitro* a third band, intermediate in size between the bands corresponding to homodimers of each species, is visible in a gel retardation assay (Figure 3.10). This third band is a heterodimer consisting of one full-length receptor and one N-terminally truncated receptor. In the DNA binding/dimerisation assays described above, two point mutations were present in each dimer, or rather a single mutation was present in each monomer of the pair. It was possible that if only one of the monomers interacting carried the mutation which interfered with dimerisation then the effect might not be so severe. The mutants L-508G and R-519A which showed low levels of dimerisation were cotranslated with the N-terminal deletion mutant of the mouse oestrogen receptor, 121-599 MOR, to investigate whether the point mutation had to be present in both monomers for disruption of dimerisation to occur. The cotranslated receptors were tested for DNA binding in a gel retardation assay as described above, and levels of binding compared with wild-type and mutant receptors alone. It was found that the L-508G receptor mutant, which only bound DNA in the presence of ligand as a homodimer, also required ligand to bind DNA as a heterodimer with wild-type receptor. Similarly the increase in binding of a R-519A homodimer which occurred upon addition of ligand was similar to that seen on addition of ligand to the wild-type/mutant heterodimer. With both mutant receptors the levels of heterodimer binding in the presence or absence of ligand were similar to those of the mutant homodimer. The introduction of a deleterious residue within the dimerisation

**Figure 3.10 - Ability of point mutants to heterodimerise with MOR121-599**




Full-length wild-type (MOR), point mutant and N-terminally truncated (MOR121-599) receptors were translated individually or cotranslated *in vitro* as indicated. 'Con' indicates a control translation. The receptors were tested for their ability to bind as homo- and heterodimers to an ERE in a gel retardation assay in the presence of 10nM oestradiol ( + ) or absence of added hormone ( - ). Homodimers are indicated with arrowheads and the heterodimer with an arrow.

interface interfered with dimerisation even when the mutation was only present in one half of the dimer.

## 3.8 Uteroglobin as a model for oestrogen receptor dimerisation.

A model for the dimerisation of the oestrogen receptor has been proposed based on the structure of the major dimerisation interface of the uteroglobin protein. The mutagenesis described in this chapter was also directed towards testing this model. Uteroglobin is a dimeric protein which binds progesterone with high specificity (Beato and Baier, 1975). Interestingly it shows limited sequence homology with the progesterone receptor over the region corresponding to sequences involved in oestrogen receptor dimerisation (Figure 3.11-I). Uteroglobin is the only steroid binding protein for which a three-dimensional structure has been determined by X-ray diffraction analysis (Mornon *et al.*, 1980; Morize *et al.*, 1987; Bally and Delettre,1989). The dimer consists of a globular protein with two-fold symmetry which surrounds an internal hydrophobic cavity in which a single molecule of progesterone is believed to bind (Beato *et al.*, 1977; Temussi *et al.*, 1980). Each monomer is divided into four regions of irregular α helix and four β turns and a major dimer interface is formed by the interaction of residues 39-49, which are part of helix 3 and β turn 2, with the corresponding residues in the other monomer in an antiparallel manner. Several amino acids within this region, including Gln-40, Lys-43, Asp-46 and Ser-47, are believed to contribute key intermolecular hydrogen bonds and these are well-conserved in the progesterone receptor, though less well in the oestrogen receptor.

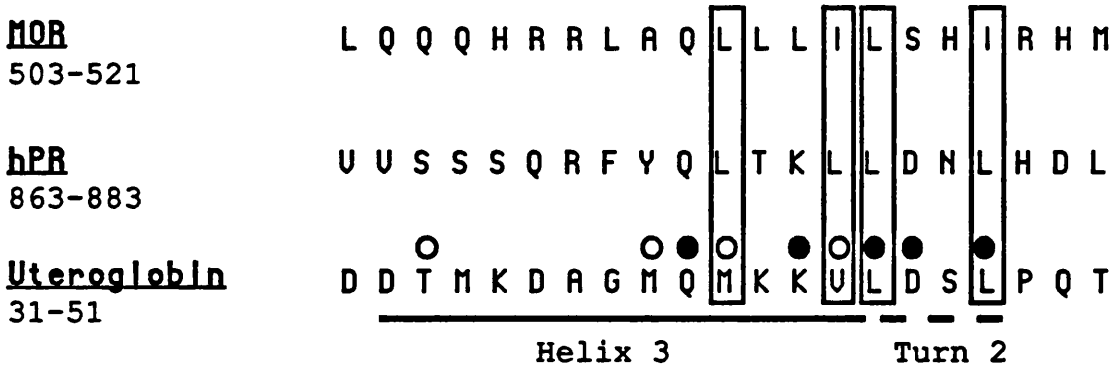
If the oestrogen receptor dimerisation interface resembles that of uteroglobin it should be possible to substitute the oestrogen receptor residues for those in the uteroglobin model without disrupting the structure. To test this idea the uteroglobin co-ordinates were entered into a computer modelling program (QUANTA) and a 3D structure generated. The basic uteroglobin structure was displayed (Figure 3.11-IIa-b) and then the corresponding oestrogen receptor residues (see alignment 3.11-I) introduced over the region amino acids 39-49 with a protein mutagenesis program written by P.Bates (Figure 3.11-IIc). In the figure, one monomer of uteroglobin is shown in blue and the other in red. In

**Figure 3.11 - Uteroglobin as a model for receptor dimerisation**

**3.11-I** The region of the mouse oestrogen receptor (MOR) shown to be required for dimerisation was aligned with the equivalent region of the human progesterone receptor (hPR). A region of the rabbit uteroglobin sequence has also been aligned. Residues conserved between hPR and uteroglobin (filled circles) or having a conservative change (open circles) are indicated. Conserved hydrophobic residues are boxed.

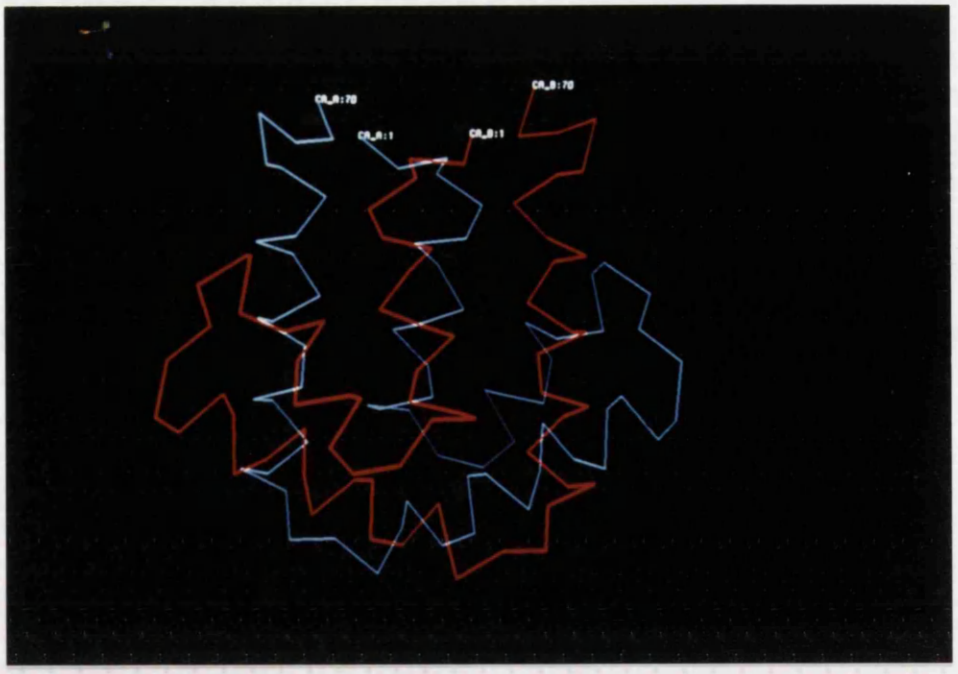
**3.11-II** This figure was generated using the QUANTA suite of programs. It shows a dimer of uteroglobin in which the main chain of one monomer is in red and the other monomer in blue. A. Uteroglobin dimer - axis of symmetry is vertical and parallel with the page. B. Dimer rotated through 90 degrees relative to A. The major dimerisation interface is highlighted in green. C. Dimer in same orientation as B. Side groups of oestrogen receptor residues have been introduced over dimerisation interface. Hydrophobic and neutral residues are shown in green, charged and polar residues in yellow.

**3.11-I**

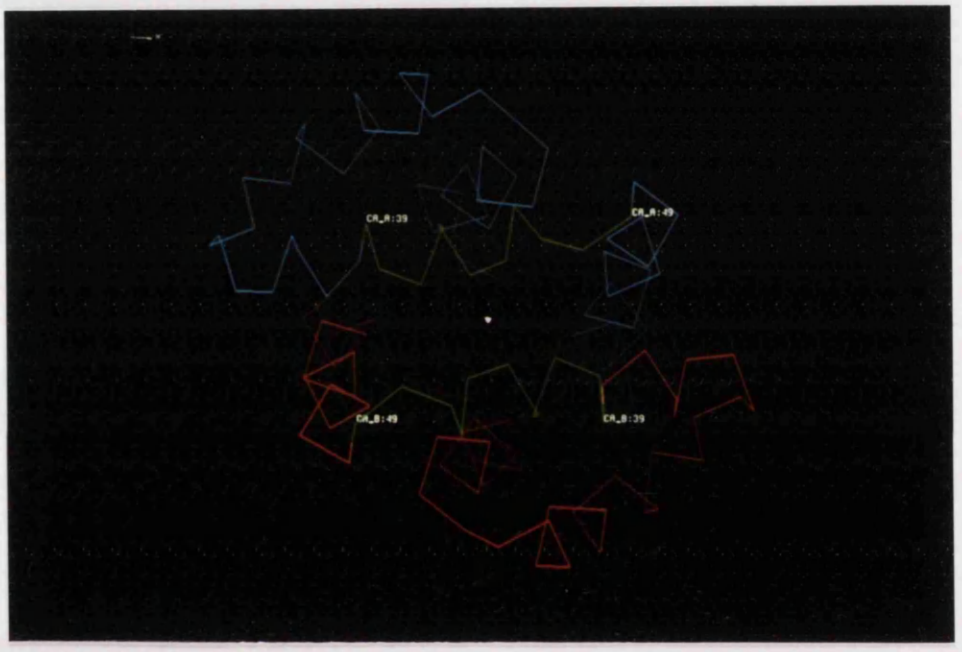


3.II–II

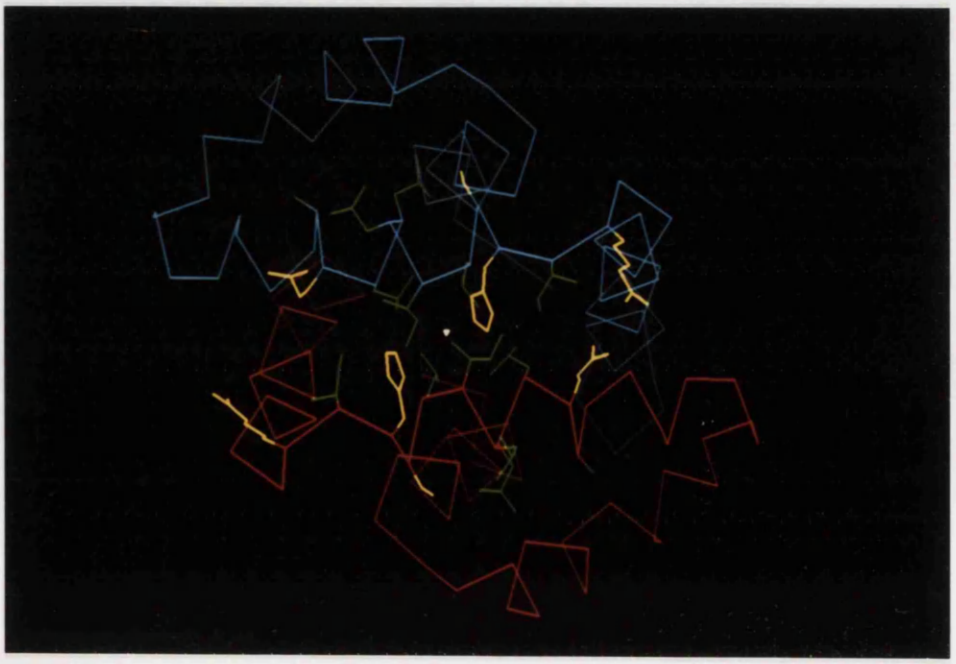
A.




B.




C.



panel B the residues 39-49, the major dimerisation interface, are highlighted in green. Panel C has the hydrophobic and neutral amino acids of the oestrogen receptor in green and charged or polar groups in yellow.

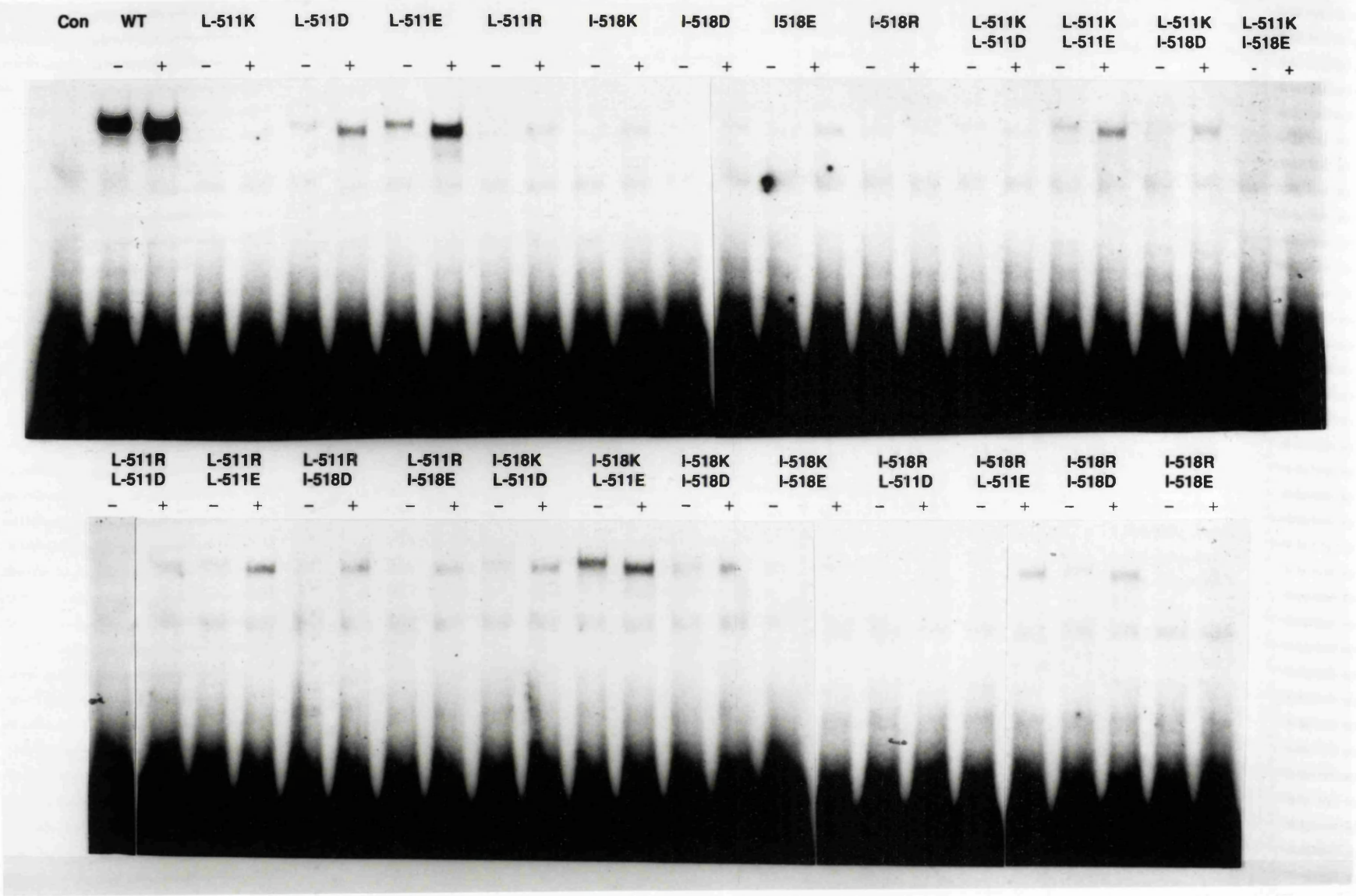
The oestrogen receptor residues appeared to fit into the uteroglobin structure well. Charged or polar residues (eg Q-510, S-516, R-519) tended to project away from the dimerisation interface and residues which projected into the dimerisation interface ( eg L-511, I-514, I-518) tended to be hydrophobic or neutral in nature. The results of mutagenesis in this region of the oestrogen receptor also fitted this model. Mutation of residues predicted to project away from the dimerisation interface generated functional receptor ( eg R-519A) whereas mutation of residues predicted to be buried within the dimerisation interface ( eg L-511R ) to charged residues disrupted dimerisation. Since the point mutagenesis supported the uteroglobin model the following experiments to test this model were performed.

The model for oestrogen receptor dimerisation based on that of uteroglobin predicts that the corresponding sequences in each monomer interact in an antiparallel manner. Thus the residues L-511 and I-518 would be predicted to project into the dimerisation interface in a way which would place the L-511 of one monomer opposite the I-518 of the other monomer with the distal ends of the residues very close to one another (<6Å). To test this prediction we investigated whether the disruption of dimerisation caused by mutation to an acidic residue in one molecule of oestrogen receptor could be rescued by dimerising with a second molecule which contained a basic residue. It was proposed that an interaction of basic and acidic residues to form a salt bridge, removing the charge from the dimerisation interface might be sufficient to restore DNA binding. Since the exact distance between the residues was unknown both basic and acidic residues of different side group length were introduced at 511 and 518. This allowed for a range of different length covalent bonds to form. The individual cRNAs corresponding to each of these mutant oestrogen receptors were translated with one another. If rescue by complementation of charge was possible, might have allowed us to determine whether the sequences of the dimerisation interface interact in a parallel or antiparallel manner.

The results of these complementation experiments are shown in Figure 3.12. Although the individual mutants varied in their DNA

**Figure 3.12 - Ability of heterodimers of dimerisation deficient mutants to bind DNA**

Mutant oestrogen receptors were translated in reticulocyte lysate either individually or cotranslated with a mutant of opposite charge. Con indicates a control translation . Equal amounts of total receptor were tested for their ability to bind to 1ng of radiolabelled A2 ERE in a gel retardation assay.

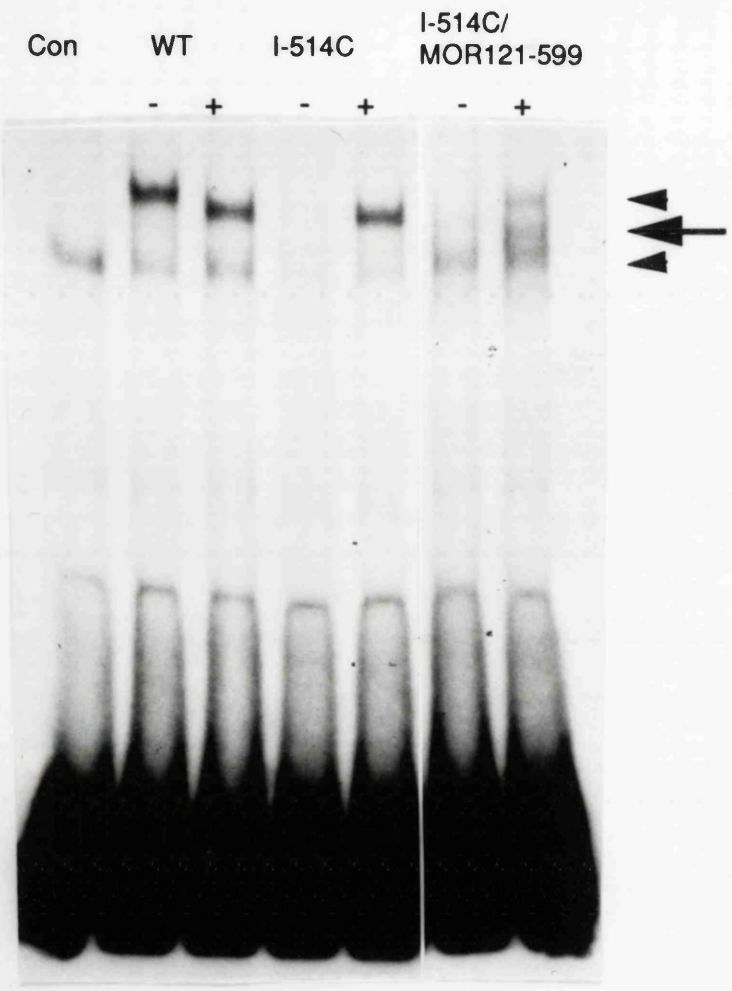

Con
WT
L-511K
L-511D
L-511E
L-511R
I-518K
I-518D
I518E
I-518R
L-511K L-511D
L-511K L-511E
L-511K I-518D
L-511K I-518E
- + - + - + - + - + - + - + - + - + - + - + - + - +
L-511R L-511D
L-511R L-511E
L-511R I-518D
L-511R I-518E
I-518K L-511D
I-518K L-511E
I-518K I-518D
I-518K I-518E
I-518R L-511D
I-518R L-511E
I-518R I-518D
I-518R I-518E
- + - + - + - + - + - + - + - + - + - + - + - +

binding activities (compare L-511K and L-511E), no combination of mutations tested appeared to be able to restore dimerisation to levels higher than those of the individual mutants. In fact in many cases the level of binding actually seemed to decrease (compare I-518R and L-511E translated individually and together). This suggests that the mutant receptors were unable to form a stable heterodimer. We also tested the effect of oestrogen on dimerisation between these receptor mutants. In the presence of this ligand the amount of DNA binding tended to be greater overall but there was still no increase in the formation of heterodimers over homodimers.

A second experiment was also carried out to investigate whether the sequences studied do in fact approach each other very closely within the dimer. Introduction of cysteine residues, which can be linked by a disulphide bridge, may allow directed crosslinking of proteins at specific amino acid positions. This technique had previously been used to determine that the leucine zipper dimerisation was a parallel and not an antiparallel interaction (O'Shea *et al.* 1988). Whilst the latter work was carried out with peptides we have used a modified form of this technique to attempt to link the dimerisation domains of two monomers through a disulphide bridge to aid in the elucidation of the structure of the dimer interface. Whether the sequences involved in dimerisation were in an antiparallel or parallel configuration, the isoleucine at position 514 was predicted to approach the corresponding residue in the other receptor very closely.

The isoleucine at 514 was mutated to a cysteine with a double-stranded oligonucleotide as described previously. Since this experiment depends upon the ability of the cysteine mutant to form stable dimers, I-514C was tested for its ability to dimerise and bind DNA with high affinity (Figure 3.13). I-514C proved to be a conditional DNA binding mutant which suggests that this mutation inhibits but does not block dimerisation. As a consequence all subsequent experiments were carried out in the presence of 10nM oestradiol. Both wildtype and and I-514C receptor cRNAs were translated individually and cotranslated to produce [$^{35}$S]-methionine labelled protein. Receptors were incubated under oxidising conditions to investigate whether or not they could be cross-linked via the cysteine residue then run out on a 10% SDS acrylamide gel. Initially we found that under these conditions much of the label was retained at the stacking gel/resolving gel interface. This was probably due

**Figure 3.13 - Ability of I-514C to dimerise**




Wild-type (WT) and mutant (I-514C) receptors were translated *in vitro* and preincubated in binding buffer containing 10nM oestradiol ( + ) or no added hormone ( - ). The proteins were then tested for their ability to bind to a doublestranded oligonucleotide ERE probe in a gel retardation assay. 'Con' indicates that a control translation was used. Homodimers are marked with arrowheads and heterodimer with an arrow.

to large complexes forming by linkage of surface cysteine groups or linkage of unfolded receptor.

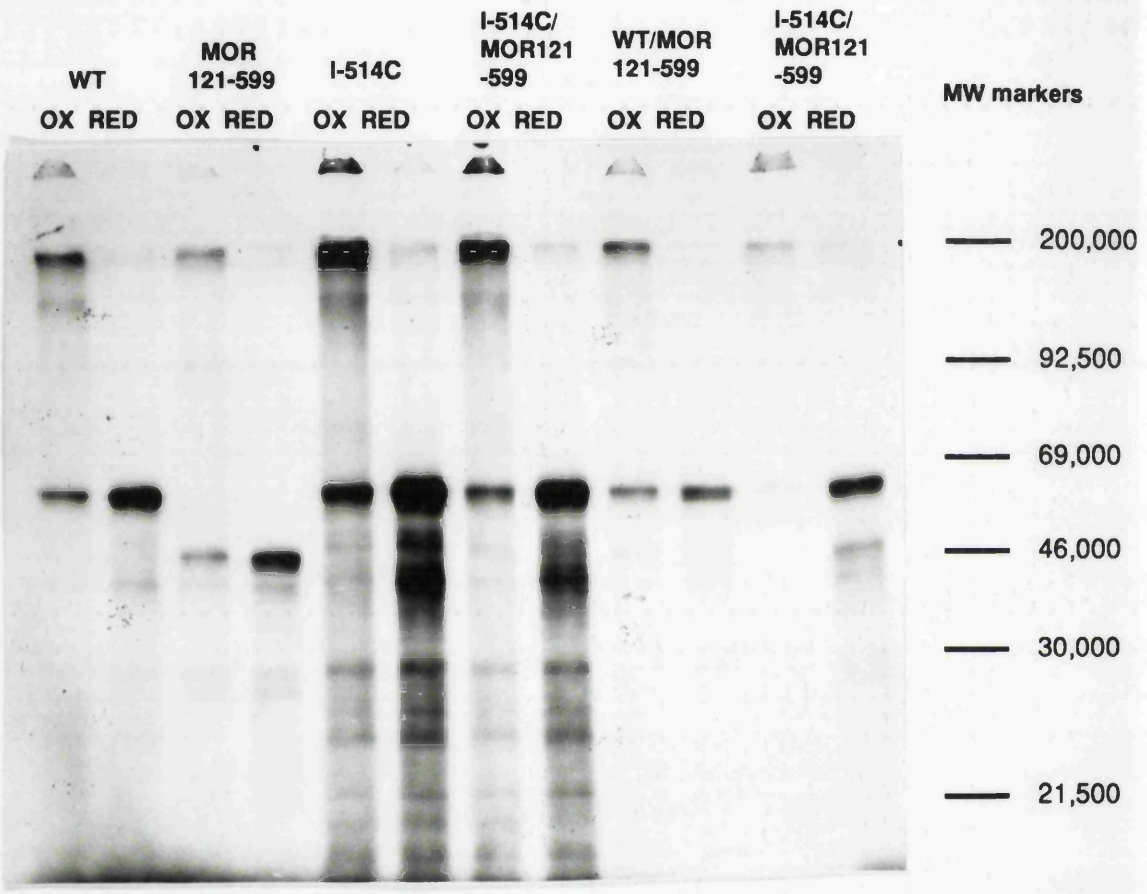
The experiment was then repeated but with an additional iodoacetamide step prior to oxidation. Pretreatment with iodoacetamide blocked free cysteine groups on the surface of the receptor and reduced but did not entirely eliminate the high molecular weight band at the stacking gel/resolving gel interface . A band of approximately 66 KD was visible in all the tracks containing wild-type or I-514C receptor (Figure 3.14). The deletion mutant also showed a mobility consistent with its predicted size. A high molecular weight band which might correspond to two linked molecules of receptor was also visible in the gel. However since it was also present with wildtype receptor it did not correspond to a dimer linked at the introduced cysteine. It may however represent two unfolded receptor molecules linked at native cysteines.

The simplest explanation for the lack of success in linking the oestrogen receptor dimers either through a salt bridge or a disulphide bond was that the model itself was wrong. There are however limitations with the methods used which could account for the failure to link the molecules. Although amino acids with different length side chains were used in the heterodimerisation experiment the exact positioning of the residues involved may have been wrong for formation of covalent bonds. The presence of disruptive residues within the dimerisation interface may have interfered with the formation of dimers to such an extent that the amino acids were never in a position to form a salt bridge or cross-link via a disulphide bond. In view of this whilst the heterodimerisation and cysteine cross-linking experiments do not support the proposed model they do not disprove it.

## 3.9 Conclusions

The residues in this region involved in dimerisation appear to be between R-507 and R-519. Hydrophobic interactions are important in the dimerisation interface. Point mutation of hydrophobic residues tended to generate functional receptor whereas the introduction of charged residues tends to disrupt dimerisation. This was particularly true of residues predicted to project into the dimerisation interface in the model of dimerisation based on the structure of the uteroglobin major dimer interface. The mutagenesis and structural studies described in this

## Figure 3.14- Cross-linking I-514C




Radiolabelled MOR, MOR121-599 and MORI-514C were translated *in vitro* and treated with iodoacetamide. The proteins were then incubated under reducing (RED) or oxidising (OX) conditions in the presence of 1mM oestradiol and run on a 10% SDS acrylamide gel, with size markers, under oxidising conditions.

chapter tend to support this model indirectly but there is no direct evidence for the structure of the interface.

Several amino acids in this region appear to be involved in ligand binding. Mutation of the highly conserved R-507 and L-511 gave receptor with lowered affinity for ligand. Mutation of I-518 generated receptors which did not bind ligand or bound with lowered affinity. Several ligands, both agonist and antagonists of oestrogen action, were capable of restoring low level DNA binding to a number of dimerisation deficient mutants.

# CHAPTER 4

## Transcriptional activity of mutant mouse oestrogen receptors

## 4.1 Introduction

This chapter describes the testing of a series of point mutant oestrogen receptors for their ability to induce hormone responsive gene expression *in vivo*. The mutants chosen for analysis fall into two groups. Firstly there are mutations in amino acids which are highly conserved across the nuclear receptor superfamily, but which have little or no effect upon ligand binding or dimerisation, for example Q-510, L-515 and R-519. A high level of conservation suggested that these positions were functionally important. Since the hormone dependent transcriptional activation function, TAF-2, has been mapped to this region of the receptor (Lees *et al.*, 1989) it was possible that these residues were involved in transcriptional activation. The second group of mutant receptors tested were impaired in their ability to dimerise and bind DNA *in vitro*. Since DNA binding is required for transcriptional activity, expressing these mutants in cells also allowed their ability to dimerise *in vitro* and *in vivo* to be compared. Mutations in both conserved (L-511E and I-511E) and non-conserved residues (L-513K) were tested.

## 4.2 Choice of cell line and receptor for transient transfection

Oestrogen receptor cDNAs encoding full-length and truncated receptor had been introduced into the eukaryotic expression vector pJ3Ω (Lees *et al.*, 1989) This vector contains the early promoter and enhancer, small-t intervening sequence and large T polyadenylation signal of the simian virus (SV 40) (Morgenstern and Hand, 1990). The full-length plasmid pJ3MOR has been tested in a number of cell lines containing negligible oestrogen receptors. Since on transfection with a receptor expression vector and reporter the NIH 3T3 cell line showed a large induction of oestrogen responsive genes in response to oestradiol, these cells were chosen for studying hormone dependent transcription activity.

There are two transactivation functions (TAF) in the oestrogen receptor, one hormone independent (TAF-1) in the N-terminus and a second hormone dependent function (TAF-2) towards the C-terminus. It has been demonstrated that TAF-1 and TAF-2 are able to synergise (Webster *et al.*, 1989). Therefore to test the effects of mutations on TAF-2 activity alone, mutants were expressed in pJ3MOR121-599 as truncated receptors which lacked TAF-1.

## 4.3 Transfer of point mutants into pJ3MOR121-599

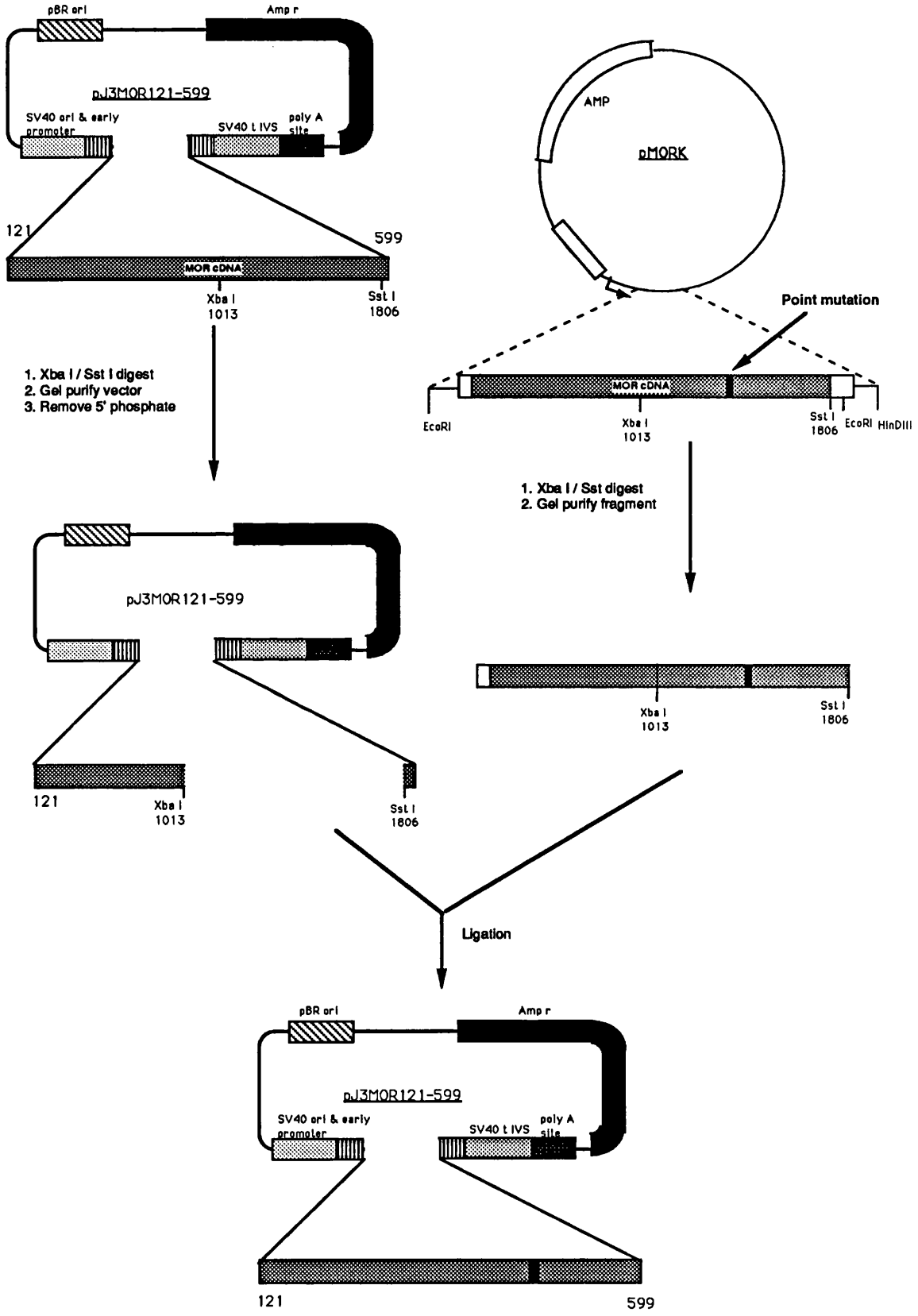
All point mutants had previously been introduced into the SP65 based vectors pMORK or pMORHK to generate a series of constructs for *in vitro* expression of the receptors in the reticulocyte lysate system. The point mutant pMOR plasmids were digested with the enzymes Xba I and Sst I to generate 700 base pair fragments which contained the point mutations. These fragments were then purified on an agarose gel. The vector pJ3 MOR121-599 was also cut with Xba I and Sst I and the linearised vector separated from the 700 base pair fragment and treated with phosphatase. Vector and fragment containing point mutation were ligated and the resultant plasmid used to transform DH5 E.coli. Minipreps were checked by digestion with appropriate restriction enzymes and sequenced through the mutant codon. Large-scale plasmid preps were checked on an agarose gel to ensure that there was at least 80% supercoiled DNA. The transfer of the point mutants to pJ3MOR121-599 is summarised in Figure 4.1.

## 4.4 Quantitation of expression levels of mutant receptors in COS cells.

Prior to comparison of levels of transcriptional activity it was necessary to establish that the mutant receptors were being expressed *in vivo* at similar levels. A luciferase control (see 4.5) is adequate to correct for transfection efficiency, ie differences in the quality of precipitates and number of cells transfected, but obviously cannot reflect differences in translation and stability of the final oestrogen receptor protein. For example a subtle change in the structure caused by a point mutation might have caused a major change in the steady state levels of receptor by altering the rate of degradation of the protein. The NIH 3T3 cell line, whilst suitable for the investigation of oestrogen dependent transactivation, poses some problems for the quantitation of receptors expressed. Immunofloresence assays had demonstrated that less than 1% of NIH 3T3 cells transfected by calcium phosphate precipitation actually express receptor (P.Danielian-unpublished data). As a result of this it has not been possible to detect the protein in a whole cell extract prepared from transfected 3T3 cells. In view of the high levels of receptor expressed in COS-1 cells in transient transfection the relative levels of expression of the various receptor mutants were analysed in these cells.

COS-1 cells were transfected by electroporation with each of the receptor plasmids then plated down in DMEM with 10% FCS. At confluence the cells

**Figure 4.1 - Construction of mutant pJ3MOR121-599 vectors**



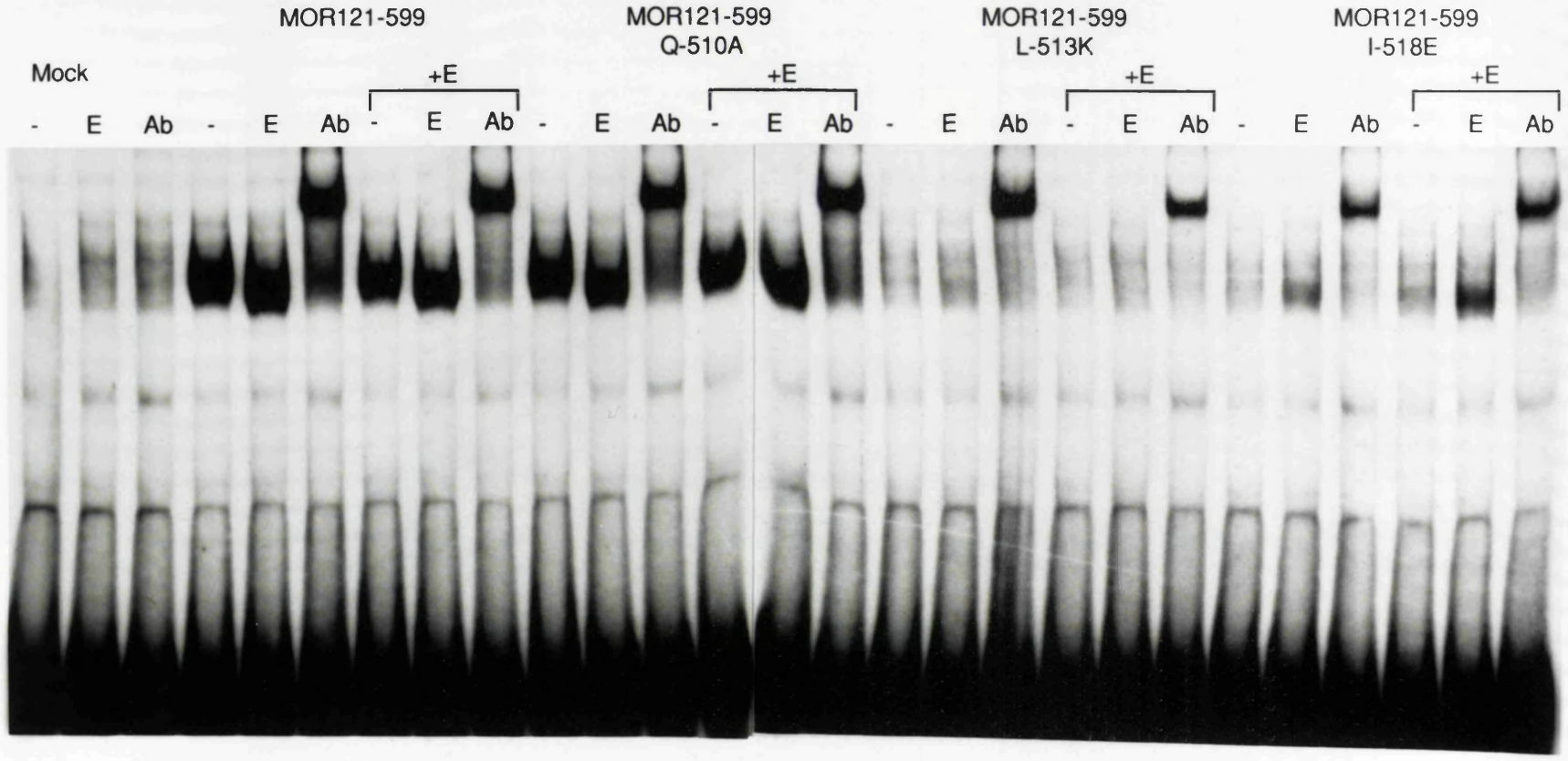
were harvested and receptor prepared by the whole cell extract method. The protein levels present in the extracts were quantified in a Biorad protein assay. Equal amounts of protein were tested in a gel retardation assay to assess the relative levels of expression of the different mutants. Since these mutants vary in their ability to dimerise and bind DNA *in vitro* direct comparison of DNA binding levels would not give an accurate idea of the relative levels of receptor expressed. It has been found that the antiserum MP16, raised against the mouse oestrogen receptor, is capable of inducing dimerisation deficient receptor to bind to DNA with high affinity (Fawell *et al.*, 1990b). The relative levels of expression of each receptor mutant were therefore determined by comparison of the intensity of the band in the presence of MP16, since this overcame the variation in binding due to differences in dimerisation.

It was found that four mutant forms of receptor (L-511E, Q-510A, L-515E and R-519A - data not shown) were expressed at similar levels in COS-1 cells. However the expression of L-513K was reduced when prepared from cells grown in the presence of oestradiol (Figure 4.2). There is no clear explanation for this observation since there is no evidence that oestrogen down-regulates receptor in this system. I-518E receptor levels were also lower than the other receptor constructs. The levels of expression of I-518E were similar whether the COS-1 cells were grown in the presence of 10nM oestradiol or absence of hormone (Figure 4.2).

## 4.5 Transient transfection of point mutants into NIH 3T3 cell line

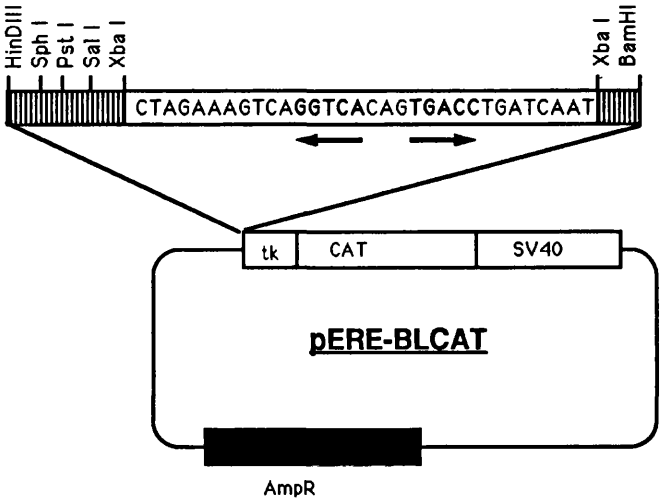
Since the presence of endogenous steroids would mask the response to added hormone these were removed from the medium in which cells were grown during transient transfections. The foetal calf serum (FCS) used was incubated twice with dextran-coated charcoal to remove endogenous steroids. In addition cells were grown in medium lacking the pH indicator phenol red as this has been shown to act as a weak oestrogen (Bertholis, 1986). The NIH 3T3 cell line is particularly sensitive to the concentration of FCS and does not grow well in dextran-coated charcoal treated foetal calf serum (DCFCS). Therefore the cells were routinely grown in DMEM containing 10% FCS and then plated down for transfection in phenol-red free DMEM containing 10% DCFCS at a density of $2 \times 10^5$ cells per 6cm dish. Cells were transfected with the mutant receptor expression vector, pERE-BLCAT oestrogen responsive reporter plasmid, pJ3LUC control plasmid, and

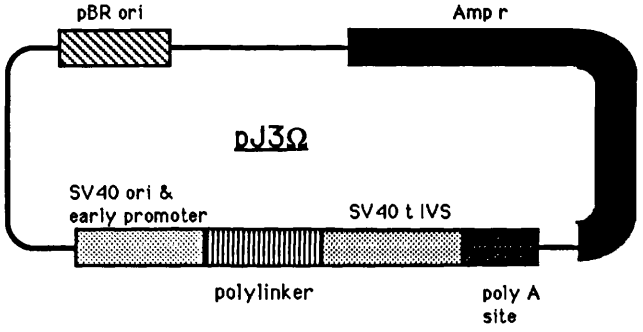
**Figure 4.2 - Levels of mutant oestrogen receptors expressed in COS-1 cells**

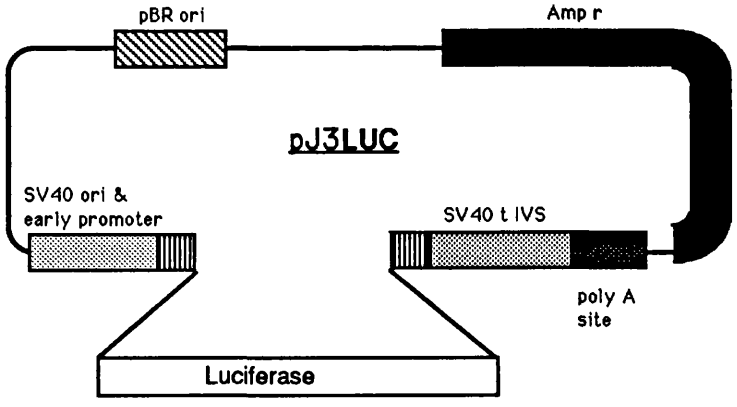



COS-1 cells were transiently transfected with wild-type or mutant pJ3MOR121-599 by electroporation. The cells were grown in the presence or absence of 10nM oestradiol for 48 hours prior to harvesting. Extracts were prepared by the whole cell extract method and tested for receptor content in a gel retardation assay. Extracts were preincubated under standard conditions (-) or with 10nM oestradiol (E) or wih MP16 antiserum (Ab).

**Figure 4.3 - Reporter and control plasmids used in transient transfections**



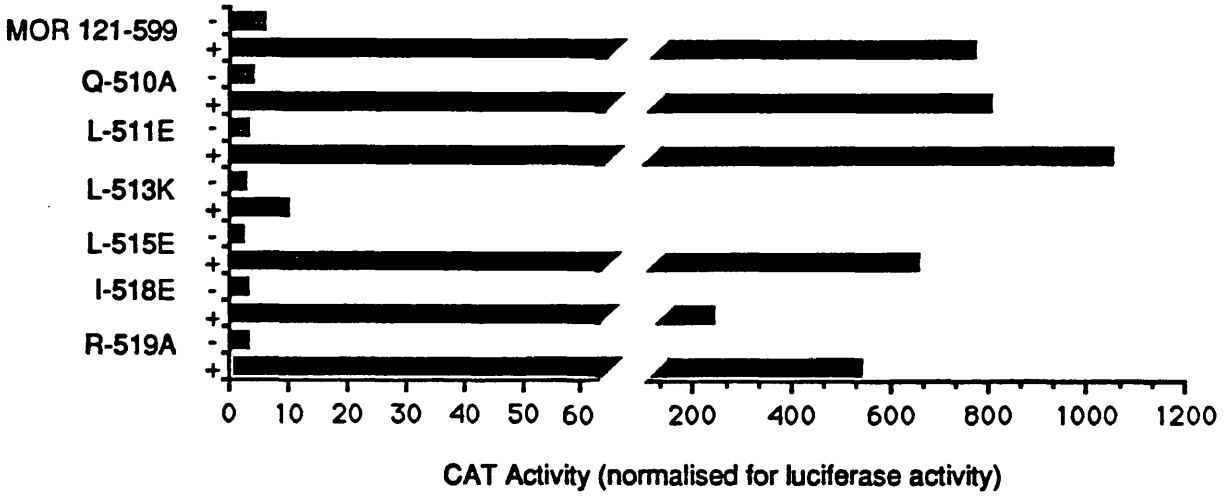
pJ3Ω ( Figure 4.3 ) by the calcium phosphate method. The pJ3LUC plasmid expresses the enzyme luciferase which can be quantitated in a luminesence assay. The expression of the luciferase gene is completely hormone insensitive and the levels of expression were used to correct for efficiency of transfection and cell number. The pJ3Ω plasmid, which expresses no protein in a mammalian system, was added to maintain DNA concentration in each transfection. After 6 hours the cells were washed three times and cultured for 24-48 hours in the presence or absence of $10^{-8}$ M oestradiol prior to harvesting. CAT activity was determined by the method of Sleigh (1986). In a typical experiment 6cm dishes of 3T3 cells were transfected in duplicate with 5μg of the reporter pERE-BLCAT, 1μg of control plasmid pJ3LUC, 0 or 0.5μg of pJ3MOR121-599 or point mutant, and pJ3Ω to give a total of 10μg of DNA.

## 4.6 Transcriptional activity of MOR point mutants

There was no difference in the levels of CAT activity in the presence and absence of oestradiol in NIH 3T3 cells transfected with pERE-BLCAT alone. All transfections including receptor expression plasmids gave a greater CAT activity than pERE-BLCAT alone, and the latter value was used to calculate fold inductions. The results of the transient transfection of point mutants of the mouse oestrogen receptor into NIH 3T3 cells are shown in Figure 4.4. The wildtype receptor MOR121-599 induced transcription over 400 fold in the presence of oestrogen. Hormone independent activity was a little over threefold. The mutant receptors, particularly L-513K, displayed a slightly lower transcriptional activity in the absence of added oestrogen than that shown by wildtype. However the total transcriptional activity of the mutants Q-510A, L-511E, L-515E and R-519A were similar to those of wildtype MOR121-599. The mutant I-518E had a reduced ligand dependent activity which is reflected in its total induction factor of only 127 fold, approximately a third that of wildtype receptor. However the most striking reduction in transactivation was observed in L-513K which displayed a ligand dependent transcriptional activity of only 4 fold. Although it was possible that this might be a direct effect of the point mutation on the transcriptional activity of TAF-2, the effect of this mutation on dimerisation *in vitro* made this appear unlikely. L-513K is a 'conditional' DNA binding mutant when expressed *in vitro*, and fails to bind DNA in the absence of the dimerising antibody MP16 when expressed in COS-1 cells. It is therefore more likely that a failure to dimerise explains the lack of transcriptional activity.

**Figure 4.4 - Transcriptional activity of mouse oestrogen receptor point mutants**

**4.4 a)**




**4.4 b)**

| | Induction Factor | | |
|---|---|---|---|
| | - Oestrogen | + Oestrogen | Total |
| MOR 121-599 | 3.1 | 131 | 407 |
| Q-510A | 2.1 | 201 | 423 |
| L-511E | 1.6 | 346 | 553 |
| L-513K | 1.2 | 4 | 5.3 |
| L-515E | 1.6 | 296 | 347 |
| I-518E | 1.7 | 80 | 127 |
| R-519A | 1.6 | 173 | 285 |

5µg of pERE-BLCAT was cotransfected with 1µg of pJ3LUC and 0.5µg of either wild-type or mutant pJ3MOR121-599 and 4.5µg of pJ3Ω into NIH 3T3 D4 cells in quadruplicate. The cells were then cultured in duplicate in absence of hormone or in the presence of 10nM oestradiol and harvested after 36 hours when the cells were 80% confluent. Luciferase activity was used to normalise the CAT data (4.3 a) and the induction factors were calculated by dividing the average normalised CAT activity for each receptor construct by the values of pERE-BLCAT alone (4.3 b).

## 4.7 Stable transfections

The work described above identified a mutant oestrogen receptor (L-511E) with greatly reduced DNA binding activity, but which was nevertheless capable of transcriptional activity to similar levels as wild-type receptor in a transient transfection assay. Another mutant (L-511R) which disrupted dimerisation to an even greater extent than L-511E was also transcriptionally active in transient transfection. The earlier *in vitro* studies suggested that when these amino acid substitutions were made there was a reduction in the amount of receptor present as a dimer (Chapter three). If the amount of dimeric protein were also reduced *in vivo* it had no effect upon the level of transcription induced by oestradiol from a pERE-BLCAT construct.

When NIH 3T3 cells are transiently transfected an immunofloresence assay detected that only a small proportion of the cells, (<1%), were expressing receptor (P.Danielian-unpublished data). However those cells which express receptor appear to do so at very high levels. If levels of receptor in the nucleus were elevated then this might have favoured dimerisation even between mutant receptors unable to dimerise at physiological levels. The total amount of receptor in individual cells may have been so great that, even if only a low proportion of receptor was present as a dimer, this was sufficient to generate a large oestrogen dependent transcriptional activity. To investigate this hypothesis a number of stably transfected lines expressing oestrogen receptor at low levels were generated. Since the level of expression of receptor can be accurately determined in a clone of cells this allowed us to determine if any relationship between levels of receptor and ability of dimerisation deficient mutants to bind DNA *in vivo* existed.

When DNA is introduced into cells by transient transfection the majority of it is not integrated into the host genome but is slowly lost from the cells. A small proportion of the cells transfected will incorporate the transfected DNA into their genome. To select this subset of cells a gene which confers resistance to a cytotoxic drug is introduced along with the plasmid containing the gene of interest. The cells are then grown in medium containing the drug, only the stably transfected cells survive and give rise to clones. To ensure that any cells which have integrated the drug resistance plasmid also contain the gene of interest ten-fold more of the latter is included in the initial transfection.

Preliminary experiments were performed to determine suitable conditions for the production of stable cell lines. The plasmid PY3 (Blochlinger and Diggelmann, 1984), which carries the gene for hygromycin resistance, was chosen for cotransfection into the 3T3 cell line to enable clones of cells which had stably integrated both receptor and PY3 DNA to be isolated. An assay was set up to determine the concentration of hygromycin which would kill any 3T3 cells that did not contain the hygromycin resistance plasmid within 4-5 days. A range of concentrations of hygromycin from 100 to 800μg/ml/ml was added to NIH 3T3 cells at 20% confluence. After 5 days the plates were scored for cell survival.

Table 4.1

| [Hygromycin] μg/ml | Approximate % cell survival |
|---|---|
| 10 | 100 |
| 100 | 75 |
| 200 | 20 |
| 300 | 5 |
| 400 | <1 |
| 500 | 0 |

Although some cells survived at 400 μg/ml hygromycin for 5 days their morphology indicated they were unlikely to survive much longer. Based on these results the concentration of hygromycin chosen for all subsequent work was 350 μg/ml.

Approximately $2x10^5$ NIH 3T3 cells were transfected by calcium phosphate precipitation with 1μg of hygromycin resistance plasmid PY3 and 10μg of either pJ3MOR or pJ3MOR L-511R. The transfected cells were grown for 24 hrs before being split 1:10 into DMEM with 10%FCS and 350μg/ml hygromycin to select for transformed cells. The media was changed, maintaining the selective pressure, every 4 days and the surviving cells allowed to grow until they had formed colonies of 3-4mm diameter. There were no colonies on the plates mock-transfected with pJ3Ω alone, 16 colonies on the plates transfected with the wild-type expression vector pJ3MOR and 42 colonies on the plates transfected with the mutant receptor expression vector pJ3MORL-511R. At this point 12

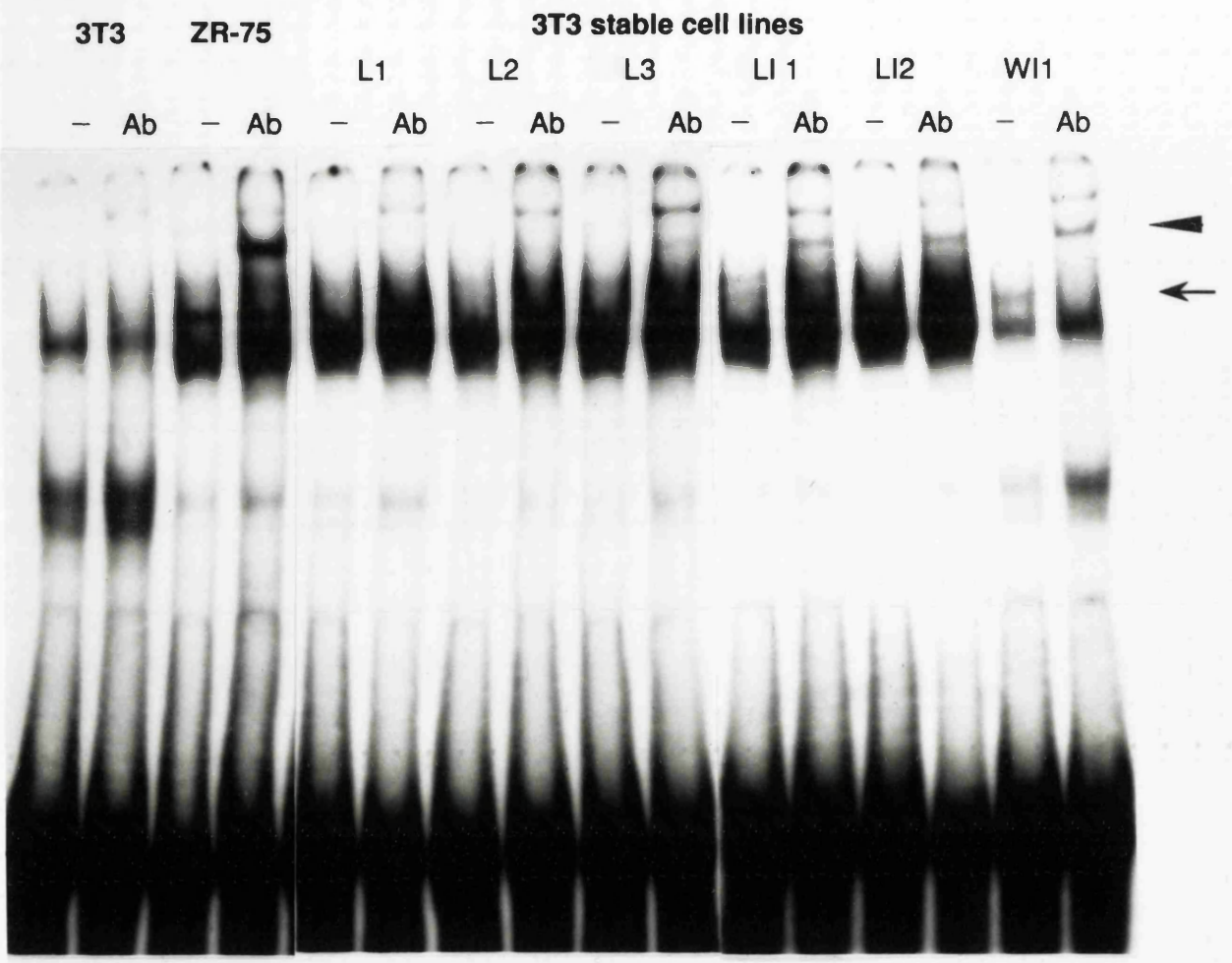
colonies from each transfection were ring-cloned and plated out in 3cm well dishes under the same selection conditions. Many of the cell lines failed to grow well after ring-cloning and only three viable cell lines with wild-type receptor and six with mutant receptor were established.

## 4.8 Oestrogen receptor content of stable cell line extracts

The initial ratio of 10:1 receptor plasmid : hygromycin resistance plasmid were expected to produce stable lines where all cells resistant to hygromycin also contained stably integrated receptor DNA. Stable cell lines were grown under routine conditions (DMEM+10%FCS) until 24hr before harvesting when media were removed and new media containing 10nM oestradiol added. Cells were then harvested and whole cell extracts prepared and tested for oestrogen receptor content using two assays. Firstly a commercially available enzyme linked immunoassay (Abbott ER-EIA Monoclonal kit) was used to determine receptor content. In the second,whole cell extracts were tested for DNA binding activity using an ERE as the DNA probe in a gel retardation assay.

No receptor could be detected in the whole cell extracts from the three stable cell lines established after transfection with the wild-type receptor. Three of the cell lines established with the mutant L-511R receptor (L1, L2 and L3) appeared to contain receptor in the immunoassay (Table 4.3). Since the L-511R mutant dimerises and binds DNA weakly, even in the presence of oestrogen, the antiserum MP16 which restores DNA binding to dimerisation deficient mutants was added to the gel retardation assay. When incubated with MP16, L2 and L3 produced a protein-DNA complex of a similar size to that generated by a ZR-75 cell line whole cell extract (Figure 4.5). However L1 was virtually undetectable in this assay. Comparison of the intensity of the bands indicated that the amount of mutant receptors in these stable lines is 5% (L2) and 25% (L3) of the levels in ZR-75 cells. The two assays are not entirely consistent since we fail to detect receptor in the L1 cells in a gel retardation. In addition whilst the immunoassay indicates that the receptor content of L2 and L3 is similar, the gel retardation indicates that there is fivefold more receptor in L3. This difference may reflect a lower sensitivity of the gel retardation assay or interference from other proteins in the extract.

**Figure 4.5 - Oestrogen receptor content in stable 3T3 Lines**




Stable cell lines were cultured in E4 medium containing 10% foetal calf serum and 10nM oestradiol for 24 hours and whole cell extract prepared as previously described. Each extract was incubated in gel retardation binding buffer in the presence or absence of the MP16 antiserum prior to addition of labelled ERE oligonucleotide probe. The binding activity of the stable cell extracts were compared to that of untransfected 3T3 extracts and also to that of extracts from ZR-75 cells grown in the absence of oestradiol. The arrow marks the position of the receptor/DNA complex and the arrowhead the position of the supershifted complex.

## 4.9 Stable transfections in the absence of oestrogen and presence of antioestrogens.

The experiment described above generated stable cell lines which expressed the mutant L-511R, but failed to generate NIH 3T3 cell lines which contained wildtype oestrogen receptor. It was possible that the expression of oestrogen receptor was somehow interfering with the normal growth of this cell line. The transfection was repeated under different conditions to minimise the activity of expressed oestrogen receptor, which in this cell line is mainly hormone dependent. One approach was to grow the cells in phenol-red free medium which contained 10% DCFCS in which oestrogens are undetectable by normal techniques. An alternative approach to reducing the activity of the oestrogen receptor was to grow the cells in the presence of an antioestrogen. The antioestrogen 4-hydroxytamoxifen was unsuitable as it is a partial agonist in many cell lines. ICI 164,384, unlike 4-hydroxytamoxifen, is a 'pure' antioestrogen with only antagonist activity reported in the majority of cell lines tested.

A further calcium phosphate transfection was performed as described for the previous experiment, but with one third the plates grown in phenol red-free DMEM with 10% DCFCS and 350μg/ml hygromycin, one third DMEM with 10% FCS and 350μg/ml hygromycin alone and the remainder in DMEM with 10% FCS and 350μg/ml hygromycin supplemented with $10^{-6}$M ICI 164,384. Again colonies were ring-cloned and expanded until enough cells were available for testing as described above.

Table 4.2.

| Transfected Construct | No. of Colonies | | |
|---|---|---|---|
| | FCS | | DCFC |
| | -ICI | +ICI | |
| Mock(pJ3Ω) | 0 | 0 | 0 |
| pJ3MOR | 4 | 3 | 0 |
| pJ3MORL-511R | 40 | 15 | 0 |

As shown by Table 4.2 there were no colonies in the absence of full serum. Some cells survived beyond a week and showed signs of forming a colony but their growth was very slow and the cells were dead by the end of the third week. The sensitivity of the 3T3 cell line to the serum conditions under which it is grown prevented comparison of levels of oestrogen receptor expression in clones grown in the presence and absence of steroids

Where cells were grown in full serum this second stable transfection generated a number of colonies which displayed hygromycin resistance. However only eight of these survived to form stable cell lines. Out of these eight only two, LI1 and LI2, expressed the MORL-511R protein as detected in a gel retardation assay (Figure 4.6). Out of the three lines transfected with wildtype receptor, only WI1, expressed receptor as detected in a gel retardation assay. All three stably transfected lines which contained receptor were derived from cells grown in the presence of the antioestrogen ICI 164,384.

The oestrogen receptor content of the different cell lines generated was also determined in the Abbott ER-EIA Monoclonal immunoassay (Table 4.3).

Table 4.3

| Cell Line | Oestrogen receptor fmol/mg |
|---|---|
| ZR-75 | 69.3 |
| L1 | 9.4 |
| L2 | 17.6 |
| L3 | 21.2 |
| LI 1 | 13.5 |
| LI 2 | 42.0 |
| WI 1 | 222.5 |

The relative receptor content of the cell lines generated by this second transfection is also not in agreement with the levels detected in the gel retardation assay. The levels of LI1, LI2 and WI1 are similar as determined in the gel retardation assay but their concentrations vary greatly in the immunoassay. The amount of wild-type receptor detected by immunoassay in the 3T3-WI1 cell line is far higher than the levels of receptor in the breast cancer cell line, but lower as detected in the gel retardation. In contrast all the mutant MORL-511R lines express receptor at lower levels than the ZR-75 cell line. It is possible that other proteins in the cell extract are interfering with the binding in the gel retardation assay.

## 4.10 Transcriptional activity of oestrogen receptor expressed stably in 3T3 cells

To test whether the dimerisation deficient oestrogen receptor L-511R was capable of transactivation when expressed at physiological levels, transient transfections were performed with the stable cell lines which contained receptor .

The control plasmid pJ3LUC and the reporter pERE-BLCAT were cotransfected into each stable line. A control 3T3 cell line was transfected both with these plasmids and also with pJ3 MOR. Cells were grown for 36 hours in the presence or absence of 10nM oestradiol prior to harvesting. CAT assays were performed and the results normalised for luciferase activity. The results of this experiment are shown in Figure 4.6.

Basal levels of CAT activity were measured in the parental 3T3 cell line transfected with pERE-BLCAT alone. The stable line which expressed wildtype oestrogen receptor showed levels of transcriptional activity similar to that obtained for the ZR-75 cell line in the presence and absence of oestradiol. Transcriptional activity was not observed in the absence of oestrogen in cell lines expressing MOR L-511R. The addition of 10nM oestradiol elevated transcriptional activity in all cell lines. Fold inductions were difficult to determine for the wildtype receptor line (WI1) since it had hormone independent activity. Comparison with the CAT activity of the parental 3T3 cells in the absence of oestrogen receptor was used to estimate the hormone independent induction of transcription. Cell lines containing mutant receptor ( L1, L2, L3, LI1 and LI2) had oestrogen dependent transcriptional activities of between 33 and 70 fold. The level of expression of the receptors as judged from the gel shift assay or the immunoassay did not appear to correlate with the size of the ligand induced transactivation. Since the total activity for L-511R in each of the cell lines was similar to that of the wildtype receptor it appears that L-511R is active when expressed at physiological levels.

## 4.11 Conclusions

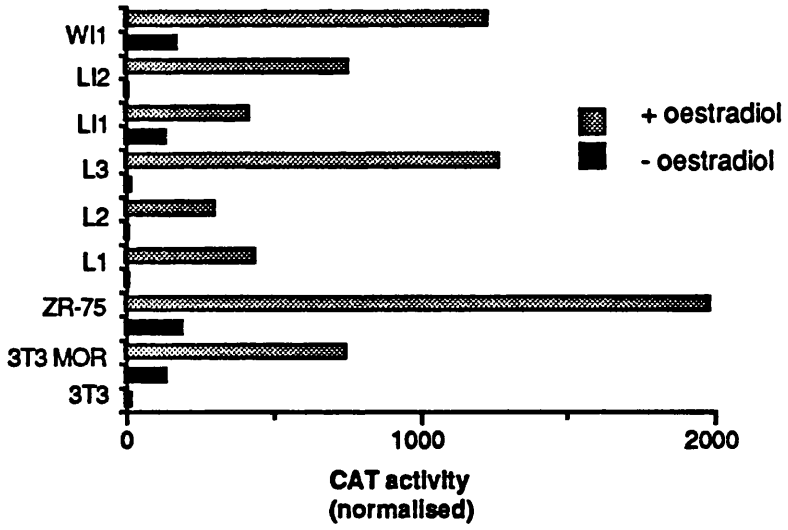
The transient transfection experiments have shown that a number of highly conserved residues (eg Q-510, L-515, R-519), which are not important in ligand binding or dimerisation also appear to have no function in transcriptional activity of the oestrogen receptor. Mutation of I-518 to glutamic acid reduces transcriptional activity whereas the mutation L-513K virtually abolishes transcription. Initially it was unclear whether the

## Figure 4.6 - Transcriptional activity of stable 3T3 lines

**4.6 a)**

| Cell line | - Oestradiol | | + Oestradiol | | Total |
|---|---|---|---|---|---|
| | CAT Activity | Fold Induction | CAT Activity | Fold Induction | Induction |
| **ZR-75** | 195 | - | 1985 | 10 | 10 |
| **3T3;** | | | | | |
| 3T3 | 21 | | 20 | | |
| 3T3 (+MOR) | 140 | 7 | 749 | 5 | 38 |
| WI1 | 173 | 8 | 1231 | 7 | 56 |
| LI 2 | 12 | - | 762 | 66 | 66 |
| LI 1 | 6 | - | 424 | 74 | 74 |
| L3 | 21 | - | 1270 | 60 | 60 |
| L2 | 9 | - | 301 | 33 | 33 |
| L1 | 6 | - | 435 | 68 | 68 |

**4.6 b)**




Stable cell lines were plated out at $2x10^5$ cells per 6cm plate and transfected by the calcium phosphate method with 5µg pERE-BLCAT and 1µg pJ3LUC in quadruplicate. The original 3T3 line was also transfected with the same precipitate to assess basal levels, and with 0.5 µg pJ3MOR, 5µg pERE-BLCAT and 1µg pJ3LUC as a positive control. Cells were grown in the absence of added hormone or in the presence of 10nM oestradiol and harvested after 36 hours. CAT activity was determined and normalised for luciferase activity. 4.4 a) is a table of the fold inductions in CAT activity, 4.4 b) represents the CAT activity in the form of a graph.

mutation L-513K was a transactivation mutant or whether its low levels of transcriptional activity in the presence of oestradiol reflected an inability of ligand to restore dimerisation effectively *in vivo*. It is however more likely that a reduction in DNA binding accounts for the low transcriptional activity of L-513K since ligand is also incapable of inducing DNA binding when this mutant is expressed in COS-1 cells.

The difficulties in obtaining a stable NIH 3T3 line expressing the wild-type receptor appeared to be due to a toxic effect of the receptor on this line, as cloning in the presence of a potent antioestrogen to some extent helped overcome this problem. The results obtained after stable transfection of the oestrogen receptor into NIH 3T3 cells are consistent with those obtained by transient transfection. The dimerisation deficient receptor mutant L-511R is capable of wild-type transcriptional activity in the presence of oestradiol in both transient and stable transfections. Thus it is unlikely that overexpression of receptor in the transient transfection assay accounts for the transcriptional activity of this mutant. It appears that oestrogen is able to stabilise dimerisation of receptors both *in vitro* and *in vivo*.

# CHAPTER 5

## Effect of ligand binding and DNA binding on the structure of the mouse oestrogen receptor

## 5.1 Introduction

This chapter describes investigations into the effects of different hormonal ligands and DNA response elements on the structure of the oestrogen receptor. The behaviour of the oestrogen receptor varies greatly depending on the hormonal ligand or DNA binding sites it is associated with. Since it is likely the differences in receptor activities are due to changes in the structure of the receptor we have investigated this using proteolysis.

## 5.2 Proteolytic techniques for structural studies

Proteolytic cleavage has previously been used to investigate the structure of oestrogen receptors. In 1984 immunological analyses by Greene and coworkers showed that the MCF-7 human oestrogen receptor had DNA binding and steroid binding regions separable by enzymatic cleavage. Proteolysis of the oestrogen receptor (Katzenellenbogen *et al.*, 1987; Elliston and Katzenellenbogen, 1988) and other steroid receptors (Reichman *et al.*, 1984; Birnbaumer *et al.*, 1983; Puri and Toft 1986) labelled covalently with radiolabelled ligands gave further information on the structure of the hormone binding domain.

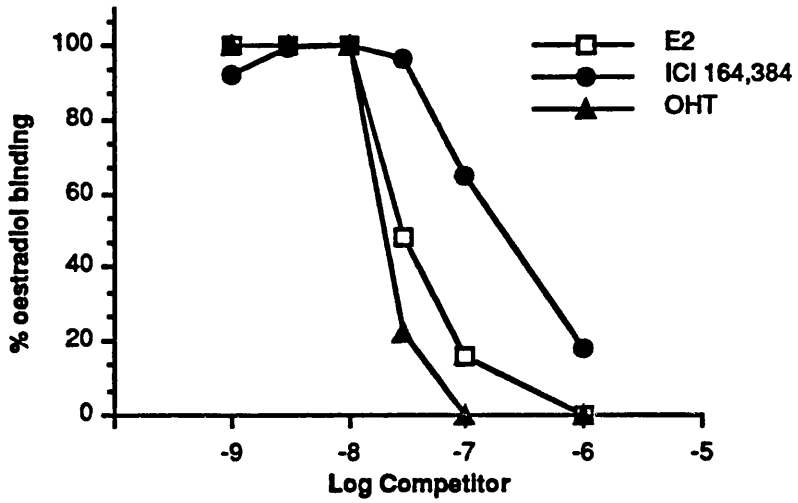
We have used a refinement of proteolytic cleavage which allows changes in the proteolysis of DNA binding proteins to be examined. Termed the proteolytic clipping bandshift assay (PCBA) this technique consists of subjecting the DNA binding protein to limited proteolysis prior to analysing the cleavage products in a gel retardation assay. PCBA was originally described in a paper reporting the identification of an octomer binding protein (Schreiber *et al.*, 1988). Subsequently PBCA was used successfully to distinguish between the structure or conformation of the yeast transcriptional activator PRTF on two different DNA binding sites (Tan and Richmond, 1990). Earlier studies relied on the retention of either specific antibody epitopes or an intact ligand binding domain for detection of oestrogen receptor fragments (Katzenellenbogen *et al.*, 1987; Elliston and Katzenellenbogen, 1988). The proteolytic gel shift assay detects fragments which retain the ability to bind DNA, so loss of ligand binding activity does not prevent detection of fragments. The gel shift assay also has the advantage of allowing the use of non-covalently labelling ligands and visualisation of differences in mobility or fragment patterns in a gel system.

The gel shift assay is very sensitive, able to detect very small amounts of DNA binding proteins. Proteolysis was expected to produce a number of different truncated oestrogen receptors capable of binding DNA. In consequence there would be a reduction in signal due to division into a number of bands. It was also possible that proteolytic fragments which bound DNA with reduced affinity would be generated. Therefore to maximise the probability of detecting all proteolytic fragments capable of binding DNA it was necessary to use a protein source which contained a large amount of oestrogen receptor. The oestrogen receptor expressed in *S.frugiperda* cells under the control of the baculovirus polyhedron promoter was chosen. Whole cell extracts from these insect cells contained 150-250 fmol receptor/mg protein, 50 fold more than present in an *in vitro* translation of the oestrogen receptor .

## 5.3 Oestrogen receptor expressed in insect cells binds ligands and DNA with high affinity

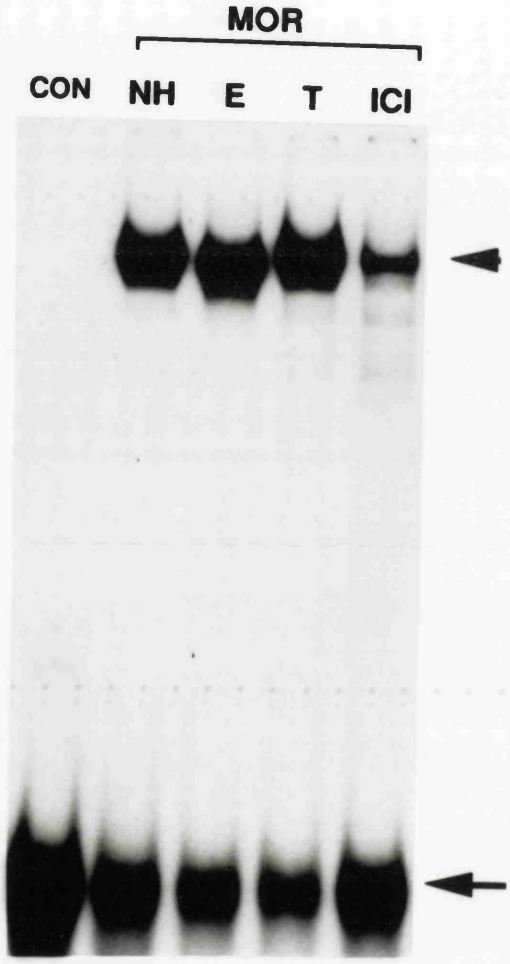
Before proceeding with the analysis of the structure of the receptor it was first necessary to determine that receptor expressed in *S.frugiperda* cells bound the ligands to be tested with a similar affinity to native receptor. Aliquots of infected insect cell extract were incubated with [16α-$^{125}$I]-oestradiol and unbound ligand removed with dextran-coated charcoal. Non-specific binding was determined in the presence of excess diethylstilbestrol. Scatchard analysis indicated that the receptor bound oestradiol with high affinity, Kd=0.3nM, a similar dissociation constant to that reported for endogenous receptor. The Relative Binding Affinities (RBA) of the antioestrogens 4-hydroxytamoxifen and ICI 164,384 were determined by incubating receptor with [16α-$^{125}$I]-oestradiol in the presence of increasing concentrations of competing ligand. The competition curves are shown in Figure 5.1. RBAs were calculated from competition curves by determining the molar concentrations of unlabelled ligands that reduced radiolabelled ligand binding by 50%. This assay indicated that the RBA of 4-hydroxytamoxifen was 100% whereas ICI 164,384 had a RBA of 10% compared to iodoestradiol. These RBAs were in approximate agreement with previous reports for the mouse receptor. Thus the mouse oestrogen receptor expressed in *S.frugiperda* cells appears to bind to the ligands being studied with a similar affinity to endogenous receptor.

**Figure 5.1 - Relative binding affinities of antiestrogens for MOR expressed in insect cells.**




Whole cell extract containing mouse oestrogen receptor was prepared by infecting *S.frugiperda* cells with recombinant MOR baculovirus vector. The relative binding affinities were determined by incubating the extract with 3nM [16α-$^{125}$I]-oestradiol in the presence of increasing concentrations of oestradiol, 4-hydroxytamoxifen and ICI 164,384.

**Figure 5.2 - Effect of ligands on DNA binding activity of oestrogen receptor**




Whole cell extract was prepared from *S.frugiperda* cells infected with MOR recombinant baculovirus (MOR) or uninfected cells (CON). The receptor was preincubated with 1nM oestradiol ( E ), 100nM 4-hyroxytamoxifen ( T ), 1μM ICI 164,384 ( I ) or no hormone ( NH ) and DNA binding activity determined in a gel shift assay with a double-stranded ERE oligonucleotide as probe. The positions of retarded complexes and free probe are indicated with an arrowhead and arrow respectively.

The effects of oestrogen, 4-hydroxytamoxifen and ICI 164,384 on the DNA binding of the oestrogen receptor expressed in insect cells were also tested. In a gel retardation assay the effect of the ligands on the mobility of oestrogen receptor expressed in insect cells was similar to their effects on receptor expressed in breast cancer cells (S.Dauvois-unpublished data). The receptor bound DNA with high affinity both in the presence and absence of oestradiol (Figure 5.2). Upon oestrogen binding a slight increase in the mobility of the receptor-DNA complex was observed. This was consistent with the possibility that ligand induced a change in receptor conformation. In contrast neither of the antioestrogens caused a change in the mobility of the receptor in the gel retardation assay. Whereas oestrogen and 4-hydroxytamoxifen had no apparent effect on the amount of receptor binding in this assay, ICI 164,384 strongly inhibited DNA binding by the receptor expressed in *S.frugiperda* cells. It seemed likely that the alteration in mobility of the receptor-DNA complex which occur on oestrogen binding resulted from differences in the conformation or post-translational modification of the receptor. In view of this it seemed possible that changes in the availability of proteolytic sites might occur on ligand binding. Therefore the following proteolytic gel retardation assays were performed to investigate whether the binding of oestradiol, 4-hydroxytamoxifen and ICI 164,384 induced a structural change which exposed new proteolytic cleavage sites or concealed pre-existing sites.

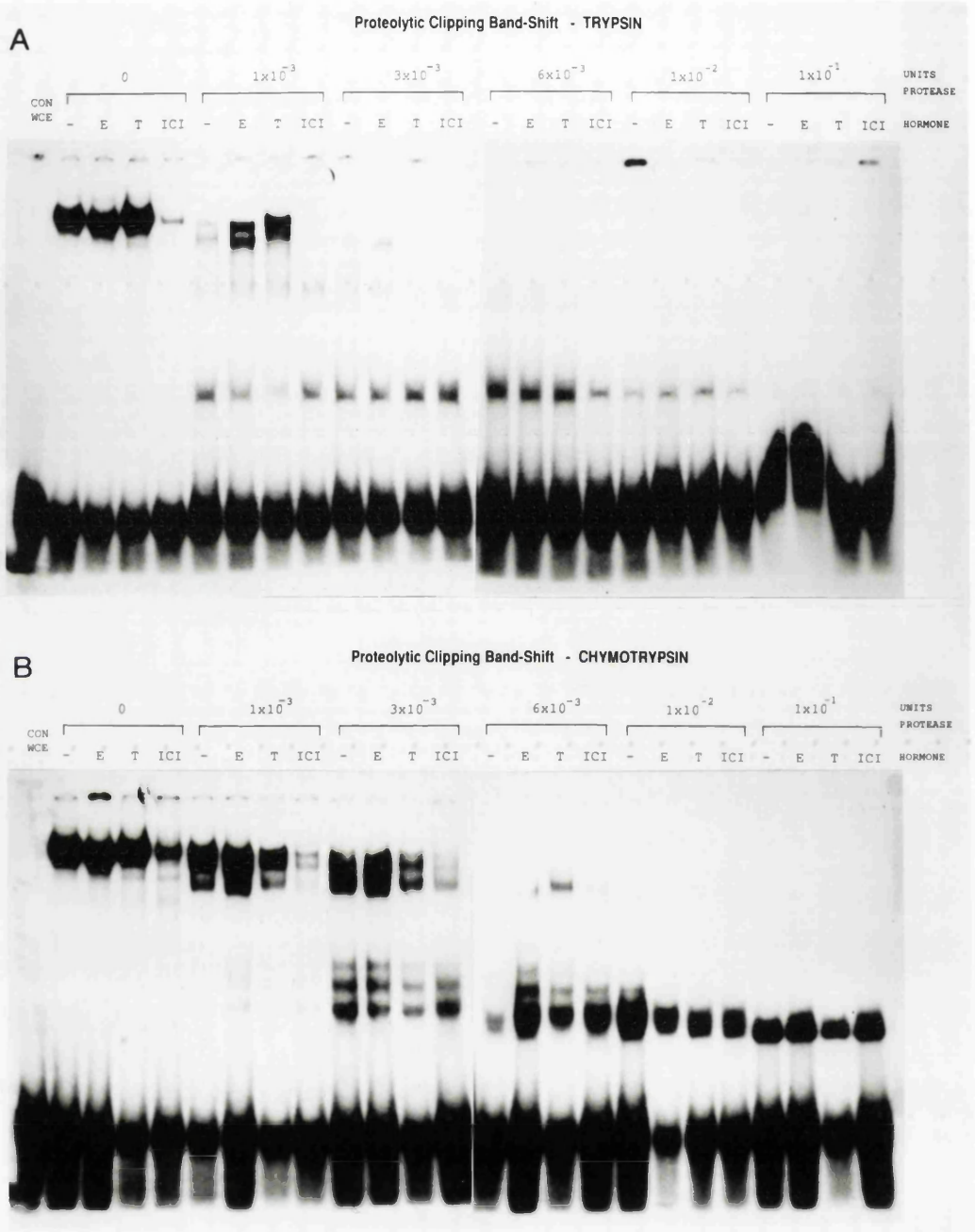
## 5.4 Influence of hormonal ligands on proteolytic cleavage of oestrogen receptor

Aliquots of *S.frugiperda* whole cell extract were preincubated in binding buffer containing 10nM oestradiol, 100nM 4-hydroxytamoxifen, 1mM ICI 164,384 or no added hormone for 10 minutes before addition of radiolabelled probe. After 10 minutes between $5x10^{-5}$ and $5x10^{-3}$ units/µl of the proteases chymotrypsin, trypsin, *Staphylococcus aureus* V8, elastase or papain were incubated with receptor for 10 minutes and the products analysed on a nondenaturing polyacrylamide gel as described for the gel retardation assay.

The receptor/DNA complexes obtained with the proteases were similar irrespective of which ligand was bound (Figure 5.3). As the amount of protease increased the size of fragments observed decreased. The enzyme chymotrypsin generated a number of high molecular weight

Figure 5.3 - Effect of ligands on DNA binding activity of proteolytic fragments of oestrogen receptor.

Whole cell extracts containing oestrogen receptor were preincubated with no added hormone ( - ), 1nM oestradiol ( E ), 100nM 4-hydroxytamoxifen ( T ), 1$\mu$M ICI 164,384 ( I ) and [$^{32}$P]-labelled ERE probe. The extracts were then incubated with a range of concentrations of trypsin (panel **A**), chymotrypsin (panel **B**), Staphylococcus aureus V8 (panel **C**), elastase (panel **D**) and papain (panel **E**) and the DNA-receptor complexes analysed by gel retardation analysis.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Proteolytic Clipping Band-Shift - CHYMOTRYPSIN
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6x10⁻³
1x10⁻²
1x10⁻¹
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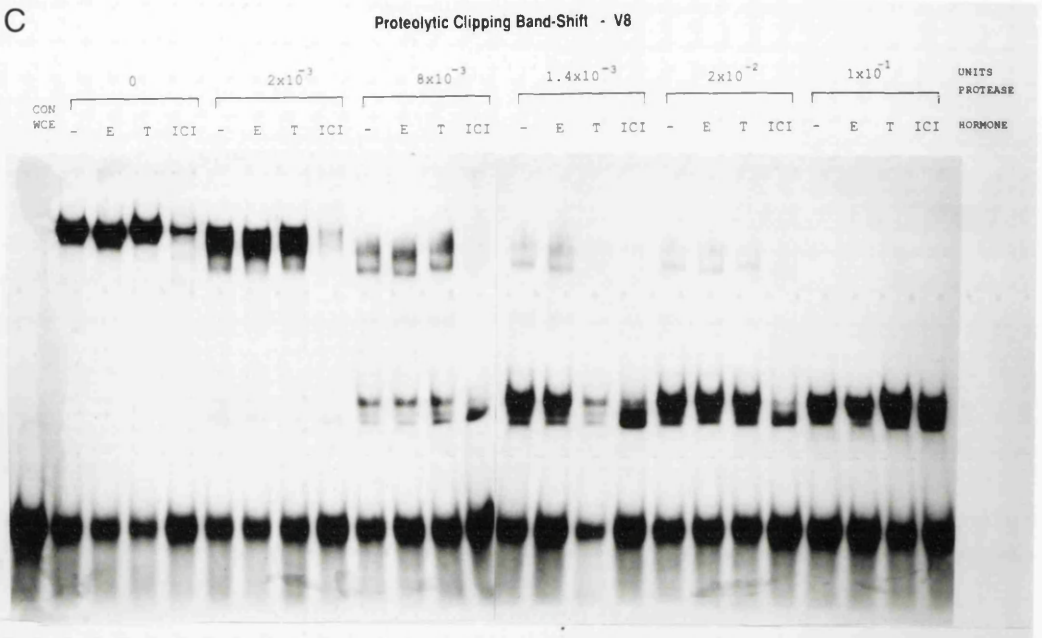
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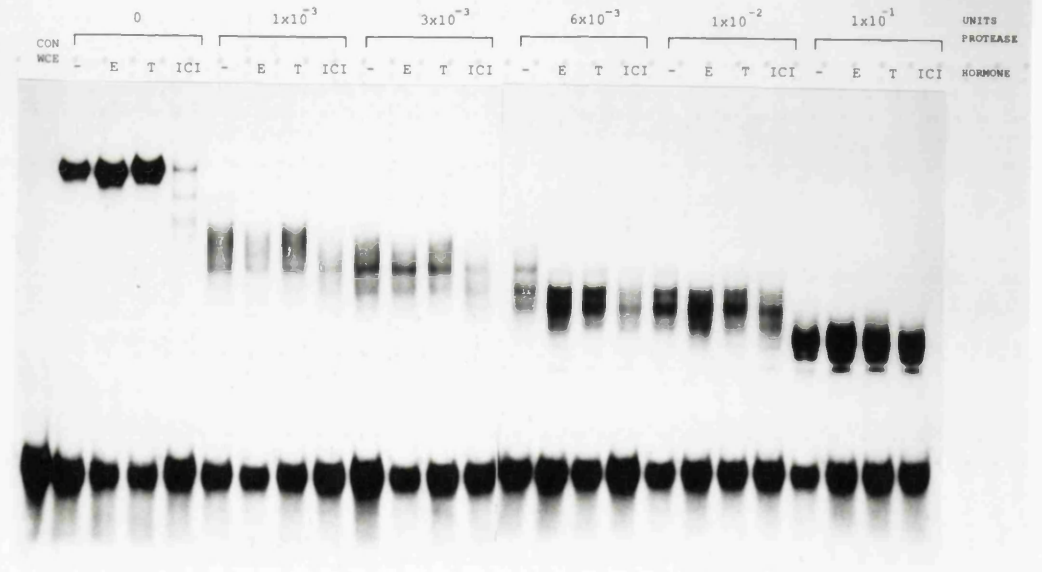
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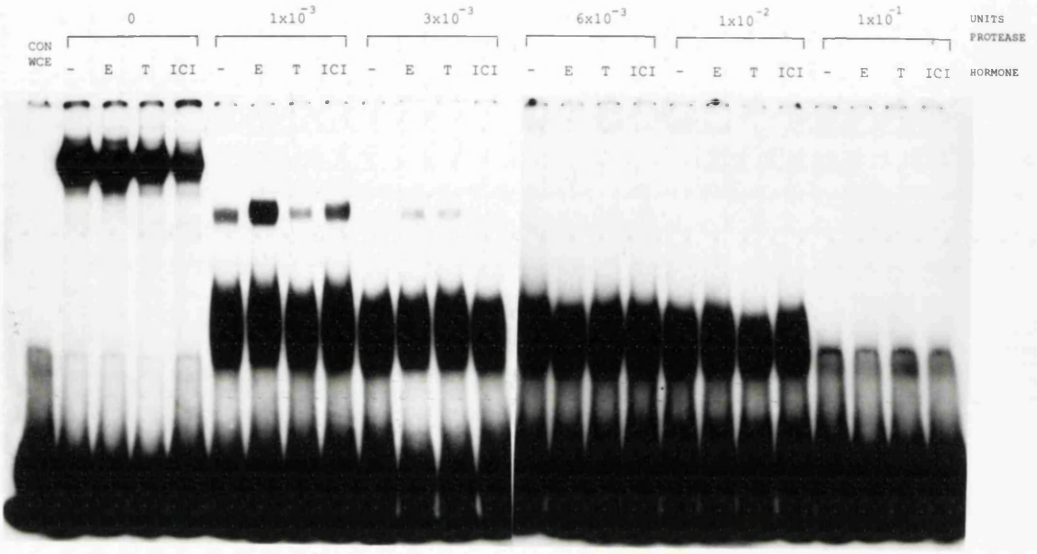


complexes which retained the ligand induced differences in relative mobilities, However as the size of these products decreased so the difference in mobility was lost (Figure 5.3 B). Comparison of the position of these complexes suggested that the smaller fragments migrated with similar mobility to a truncated receptor which lacks both N- and C-terminal sequences. It therefore seemed likely that these fragments lacked the hormone binding domain and thus they no longer bound ligand. There were no significant differences between the chymotrypsin cleavage patterns obtained with the receptor bound to oestradiol, 4-hydroxytamoxifen, ICI 164,384 or no hormone which might have suggested a change in the availability of cleavage sites.

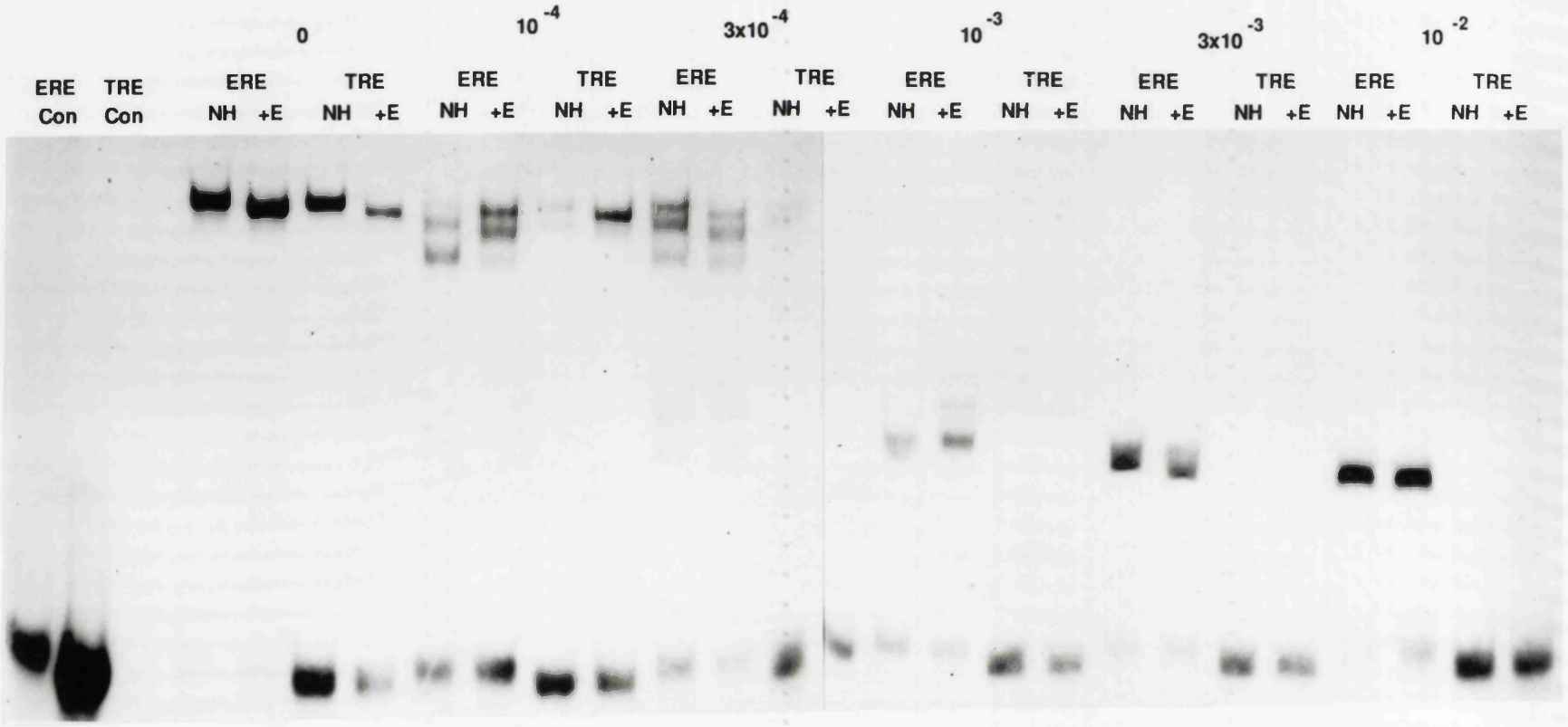
Proteolysis with other enzymes produced similar results although the cleavage patterns themselves varied. Trypsin and *Staphylococcus aureus* V8 (Figure 5.3- A and C) also gave cleavage patterns in which the large fragments retained ligand induced differences in mobility and small fragments did not. Proteolysis with trypsin, unlike the other enzymes used, did not produce a DNA binding fragment resistant to cleavage. This is probably due to cleavage of a trypsin site within the DNA binding domain itself. Elastase (Figure 5.3 D) produced a large series of progressively smaller bands, none of which retained the ligand effects on mobility, whereas papain reduced the receptor to a small DNA binding fragment at relatively low protease concentrations (Figure 5.3 - E).

Proteolysis of the receptor bound to the pure antioestrogen ICI 164,384 showed a qualitatively similar pattern to that obtained with other ligands. There was however a strong inhibition of DNA binding of the high molecular weight fragments with this antioestrogen. ICI 164,384 did not inhibit the DNA binding activity of the fragments whose size indicated that they lacked the hormone binding domain. Thus it appeared that it was not possible to detect significant differences in the cleavage pattern of the oestrogen receptor that could be attributed to a ligand induced change in receptor structure in this assay.

## 5.5 Effect of DNA binding site on the DNA binding activity of the oestrogen receptor.

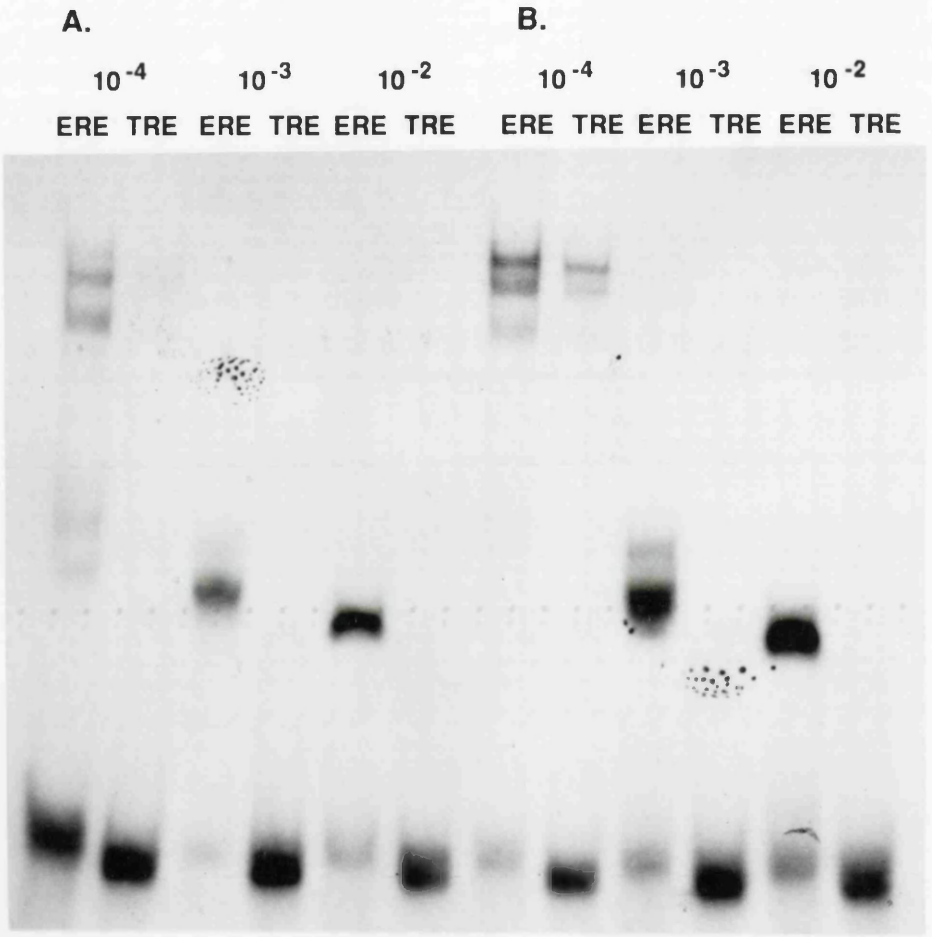
The oestrogen receptor is able to bind to both an ERE and a palindromic TRE in the absence of hormone and generates complexes with increased mobility upon oestrogen binding when bound to either element (Figure 5.4). If this reflects a ligand induced change in

Figure 5.4 - Effect of protease treatment on DNA binding activity of oestrogen receptor




Whole cell extract was prepared from S.frugiperda cells infected with MOR recombinant baculovirus and preincubated with no added hormone (NH) or 10nM oestradiol (E) and incubated with either an ERE or TRE. After limited proteolysis with $10^{-4}$to $10^{-2}$units chymotrypsin receptor complexes were analysed in a gel shift assay. Con indicates that whole cell extract from uninfected cells was used.

Figure 5.5 - Effect of order of addition of protease on proteolysis of oestrogen receptor




1µl whole cell extract prepared from S.fugiperda cells infected with recombinant MOR baculovirus was incubated in binding buffer as described in the methods . **A**. chymotrypsin was added and proteolysis terminated with TPCK after 10 min before addition of 1ng ERE probe and incubation for a further 10 min. **B** 1ng ERE probe was incubated with receptor before addition of chymotrypsin and incubation for a further 10 min. In both A and B the proteolytic products were analysed in a gel shift assay.

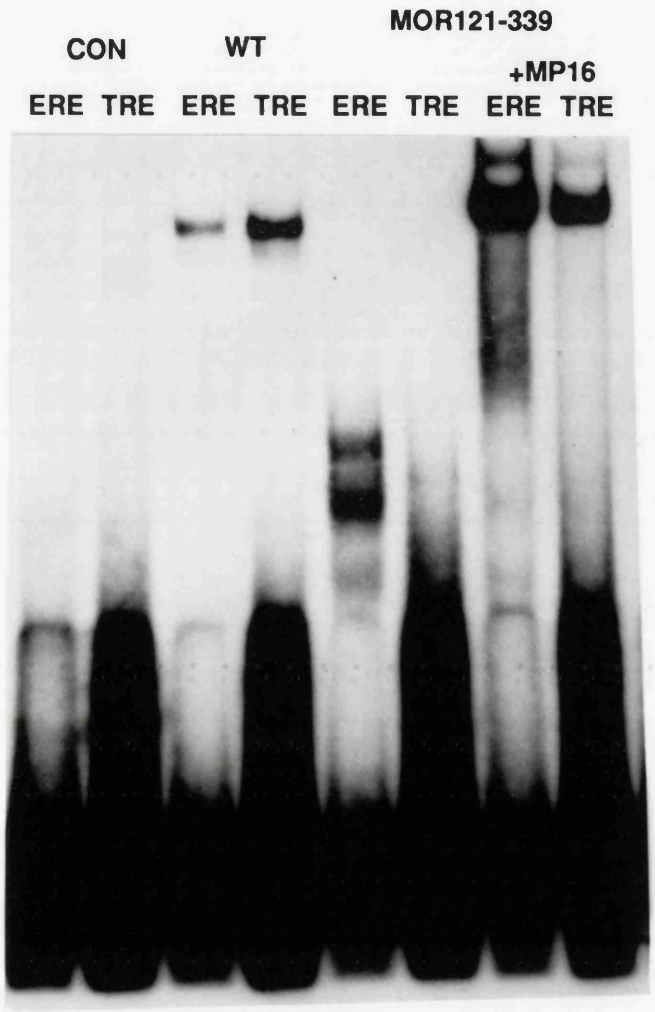
conformation it appears to occur irrespective of the site to which the receptor is bound. Analysis of the cleavage pattern generated with chymotrypsin by gel retardation assay revealed a difference in the patterns generated on the ERE and TRE. Although binding to the TRE was weaker, the larger fragments generated on these elements were similar. However the smaller proteolytic fragments which bound to the ERE were absent in the corresponding TRE track.

A second experiment was performed to determine whether this difference was due to a change in proteolysis of receptor induced by the binding sites or whether proteolysis itself was affecting binding. The receptor was preincubated for 10 minutes in binding buffer before addition of protease for 10 minutes. Proteolysis was terminated by addition of chymotrypsin inhibitor TPCK. A further 10 minute incubation with the ERE or TRE probes was carried out prior to analysis of products in the gel retardation assay. The proteolytic cleavage patterns obtained were similar whether the receptor was exposed to chymotrypsin before or after the addition of DNA, suggesting that the cleavage sites preexisted in the receptor and were not induced on DNA binding (Figure 5.5).

The smaller fragments, which fail to bind DNA, probably lack the hormone binding domain. Since this domain contains sequences important in dimerisation the inability of fragments to bind to a TRE might have resulted from their inability to dimerise efficiently on this element. Indirect evidence for this was obtained by analysing the DNA binding activity of the deletion mutant MOR121-339 lacking the hormone binding domain. This mutant was also expressed in insect cells. MOR121-339 binds to an ERE but, like the small proteolytic fragments described above, appeared incapable of binding to a TRE (Figure 5.6).

The antiserum MP16 has previously been demonstrated to be able to restore high affinity DNA binding activity to dimerisation defective oestrogen receptors in a gel retardation assay (Fawell *et al.*, 1990b). This restoration is believed to be achieved by the antibodies linking the two monomers in such a way that the DNA binding domains can interact with a response element in a similar manner to a natural dimer. Therefore, to determine if the loss of binding was due to a lack of dimerisation, the affinity of the truncated receptor for TRE and ERE was determined in the presence and absence of MP16. The concentration of $^{32}P$ labelled, double-stranded oligonucleotides was calculated from a gel

## Figure 5.6 - DNA binding activity of MOR121-339




Whole cell extract was prepared from *S.frugiperda* cells infected with either MOR or MOR121-339 recombinant baculovirus and incubated with an ERE or TRE DNA probe. The DNA binding activity of the full-length receptor (WT) and MOR121-339 were analysed in the presence and absence of an oestrogen receptor antiserum MP16 by gel shift analysis. CON indicates that an uninfected *S.frugiperda* extract was used.

Figure 5.7

Whole cell extracts prepared from *S.frugiperda* cells infected with baculovirus were incubated with the vitellogenin A2 ERE at concentrations ranging from 0.25nM to 75nM in the presence of MP16 antiserum. Concentrations of over 2.5nM were generated by dilution of the radiolabelled ERE with unlabelled ERE oligonucleotide. The receptor-DNA complexes were then analysed in a gel shift assay (A.). The gels were fixed and dried and the levels of radioactivity present in both the receptor-DNA complex and the free probe were quantitated by direct scanning with an Ambis Systems β-scanner. The equivalent position for control lysate with a range of probe concentrations was subtracted as backgroundand the probe values adjusted to account for any reduction in specific activity resulting from dilution of the probe. These corrected values were then used to plot the binding curves (B.) and Scatchard analyses (C.).

Figure 5.7 - Affinity of MOR121-339 for TRE in presence of MP16

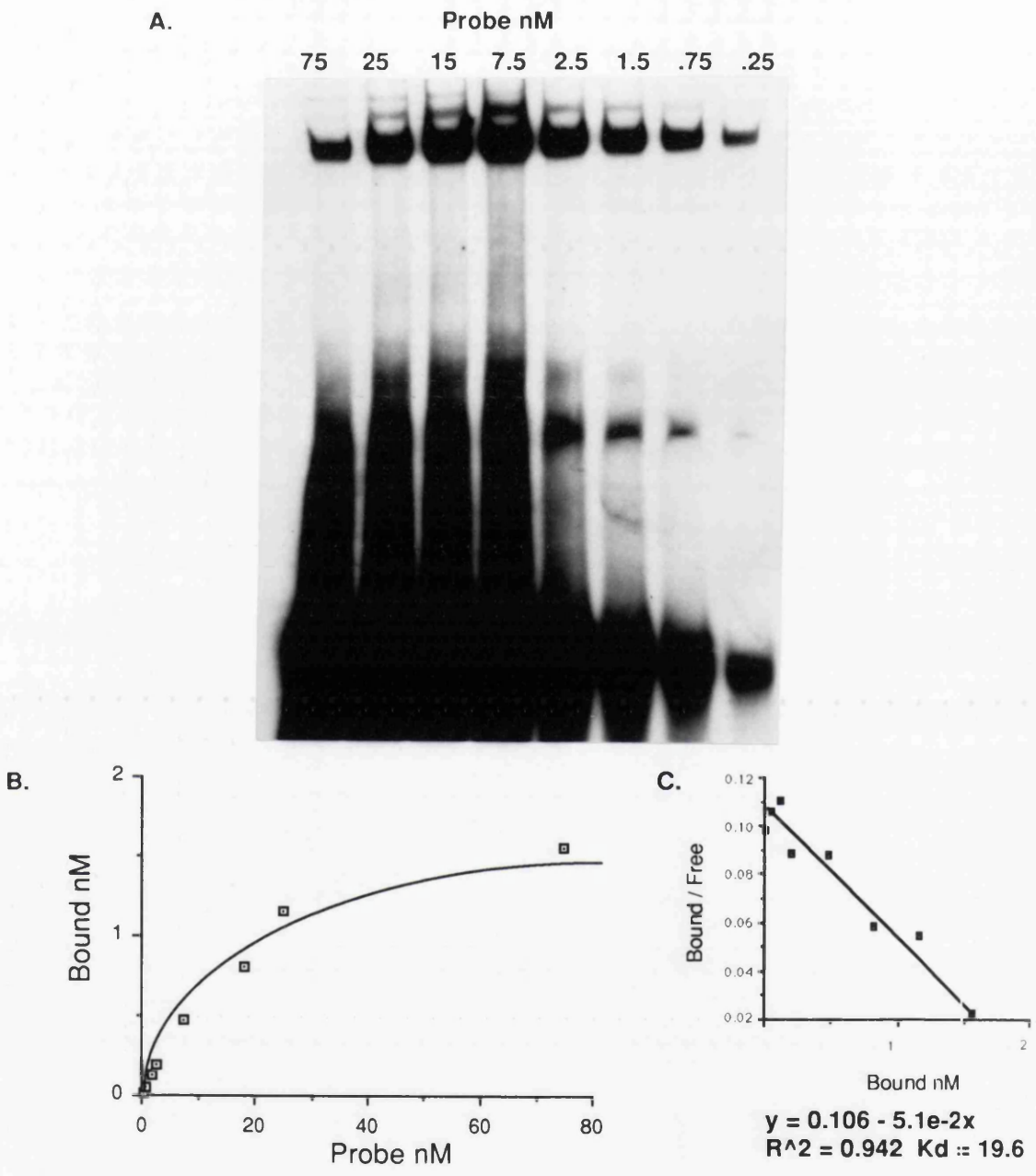
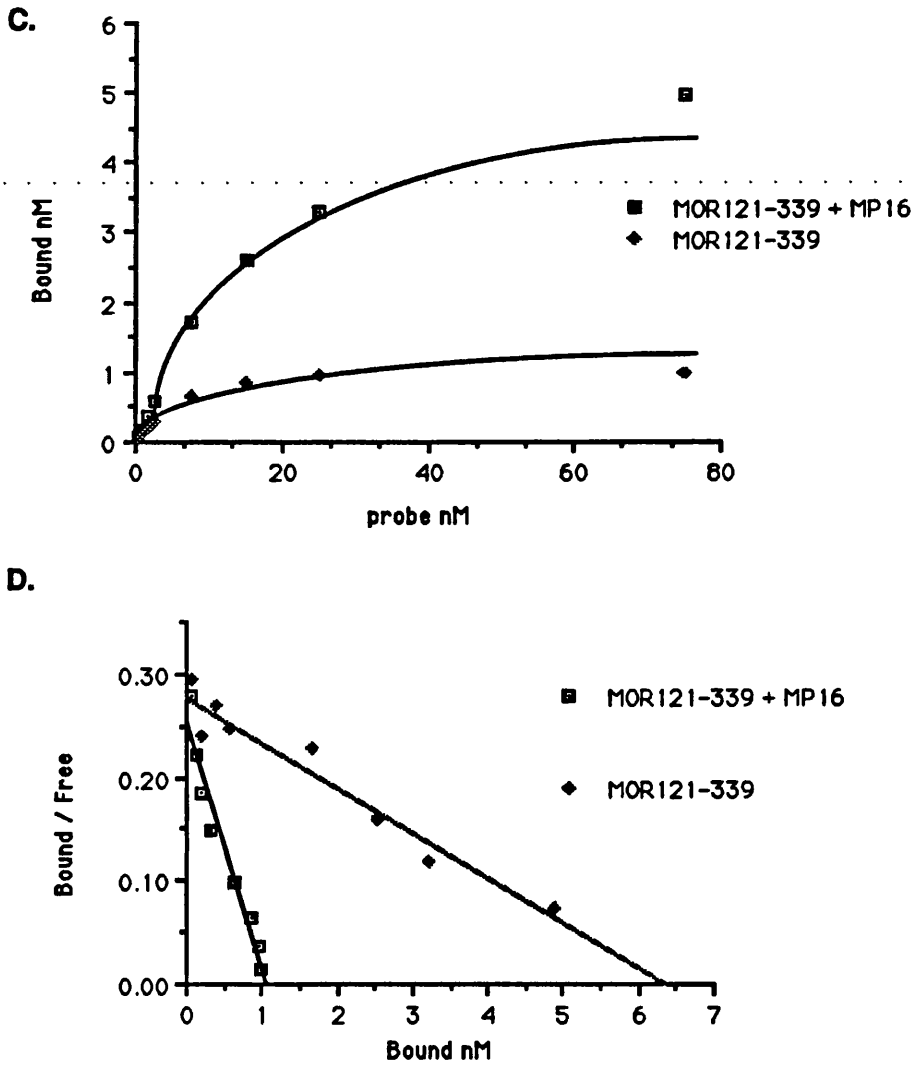


Figure 5.8 - Affinity of MOR121-339 for A2 ERE in the presence and absence of MP16

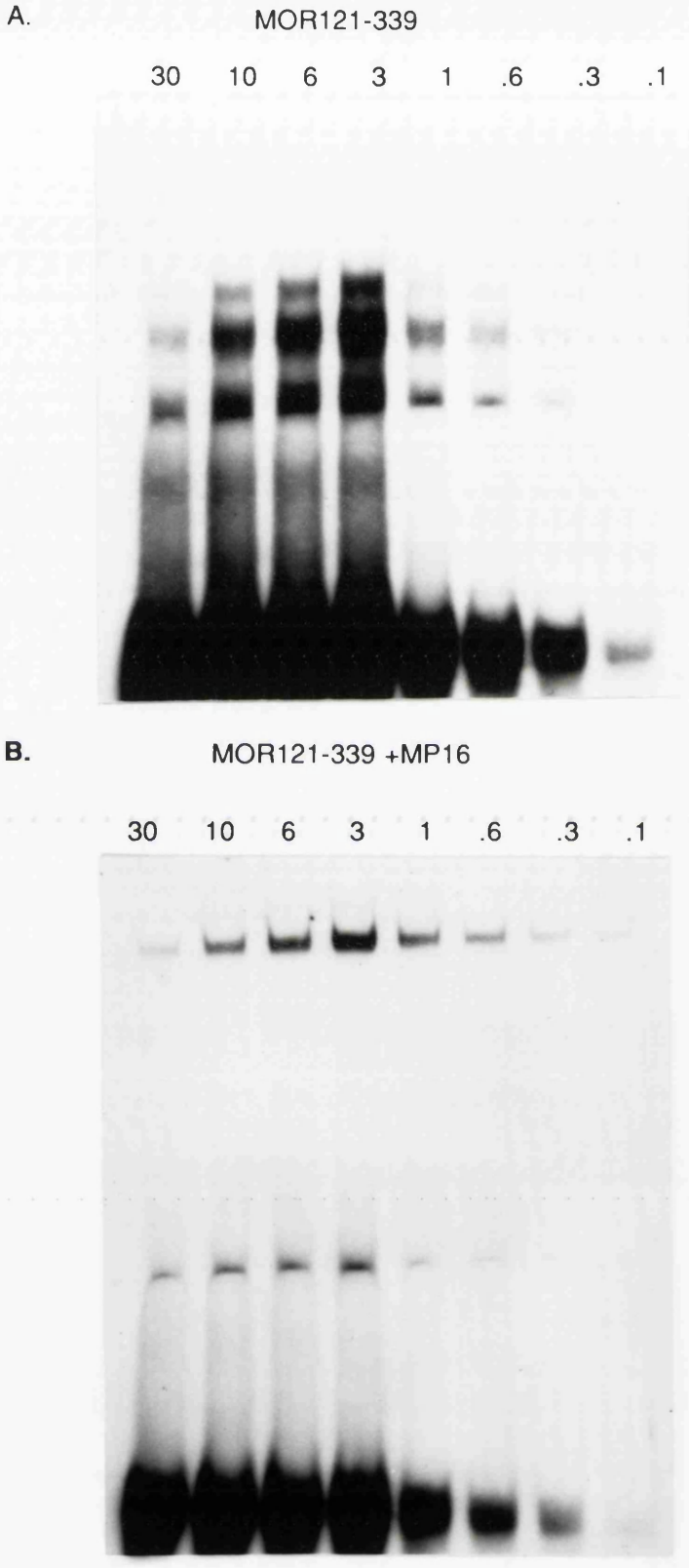



MOR121-339 y = 0.25369 - 0.23697x Kd = 4.2 nM

MOR121-339 + MP16 y = 0.27779 - 4.3383e-2x Kd = 23 nM

Whole cell extracts prepared from *S.frugiperda* cells infected with baculovirus were incubated with the vitellogenin A2 ERE at concentrations ranging from 0.25nM to 75nM in the presence or absence of MP16 antiserum. Concentrations of over 2.5nM were generated by dilution of the radiolabelled ERE with unlabelled ERE oligonucleotide. The receptor-DNA complexes were then analysed in a gel shift assay (A. and B.). The gels were fixed and dried and the levels of radioactivity present in both the receptor-DNA complex and the free probe were quantitated by direct scanning with an Ambis Systems β-scanner. The equivalent position for control lysate with a range of probe concentrations was subtracted as backgroundand the probe values adjusted to account for any reduction in specific activity resulting from dilution of the probe. These corrected values were then used to plot the binding curves (C.) and Scatchard analyses (D).

Figure 5.8 - Affinity of MOR121-339 for A2 ERE in the presence and absence of MP16



retardation assay with increasing proportions of unlabelled oligonucleotide of known concentration. Gel retardation assays were then carried out in DNA excess with a constant amount of protein and increasing amounts of DNA. The dried gels were quantitated on an Ambis β system and the results used to determine the various Kds.

No Kd could be determined for the MOR121-339 alone on the TRE as binding was virtually undetectable. However in the presence of MP16 MOR121-339 was able to bind the TRE with an affinity of 22nM, similar to this receptor's affinity for an ERE (Figure 5.7). Preincubation with the antiserum MP16 increased the affinity of MOR121-339 for an ERE from 23 nM to close to wildtype affinity (3nM) (Figure 5.8). Thus it appears that the inability of MOR121-339 to bind to a TRE was due to a failure to dimerise on this element. This is also likely to provide an explanation for the failure of the small proteolytic fragments to bind to a TRE.

## 5.6 Conclusions

Mouse oestrogen receptors prepared from *S.frugiperda* cells infected with recombinant MOR specifically bound oestradiol, 4-hydroxytamoxifen and ICI 164,384 with dissociation constant and relative binding affinities similar to those reported for endogenous receptor. Oestradiol, 4-hydroxytamoxifen and ICI 164,384 differ markedly in their effects on the activity of the oestrogen receptor but we were unable to detect any alterations in the structure of the receptor expressed in insect cells by limited proteolysis and gel retardation analysis. Although this study has not demonstrated any changes in the availability of proteolytic cleavage sites when the MOR is bound to an ERE or TRE, differences in the ability of proteolytic fragments of the receptor to bind these two elements have been detected. This appears to be due to differences in the ability of receptor lacking the hormone binding domain to dimerise on these elements.

# CHAPTER 6

## Analysis of the putative creatine kinase ERE

## 6.1 Introduction

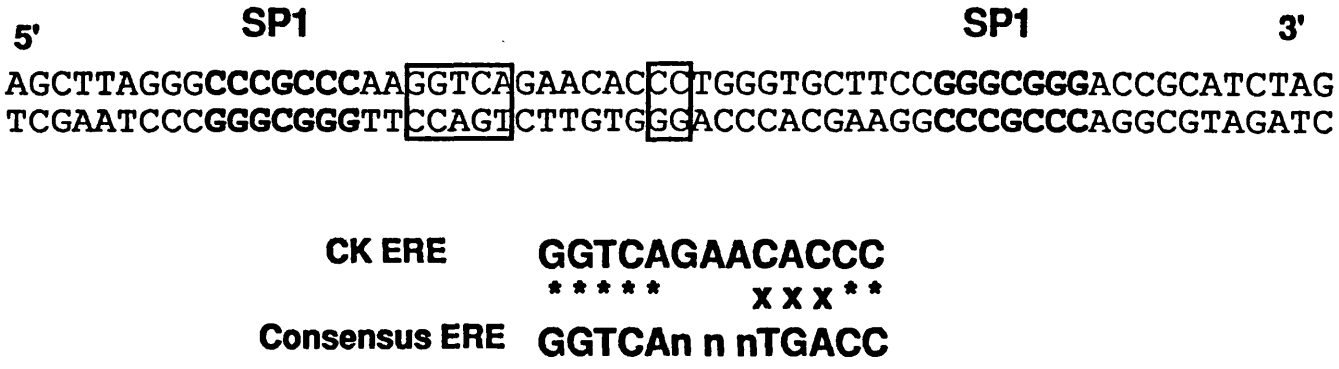
Creatine kinase-B (CK) is induced by oestrogens in the uterus of the immature rat (Notides and Gorski, 1966). This is at least in part due to an 7-10 fold increase in mRNA levels (Pentecost *et al.*, 1990). This activity has been localised to a 1.7 kb DNA fragment upstream of the start of transcription. An ERE half site, flanked by sequences with 90% homology to the decamer SP1 consensus binding site, is located approximately -550bp upstream of the cap site (Figure 6.1). The bases which correspond to the second half of the palindrome contain only two nucleotides which agree with the ERE consensus sequence. Response elements are not always palindromic since a half-site ERE, 40 nucleotides upstream of the TATA box (Tora *et al.*, 1988) has been shown to increase the expression of the ovalbumin gene (Gaub *et al.*, 1990). However imperfect palindromic EREs, such as that found in the promoter of the pS2 gene (Berry *et al.*, 1989) and perfect palindromes (Klein-hitpass *et al.*, 1986) have been more widely studied.

Surprisingly the 'pure' antioestrogen ICI 164,384 acts to increase expression of the creatine kinase gene (Pentecost- personal communication). In view of the suggestion that ICI 164,384 in inhibits dimerisation (Fawell *et al.*, 1990) it was possible that the receptor was binding to the GGTCA half-site as a monomer. Another possibility was that the receptor, whether monomer or dimer, was stabilised in its interaction with DNA by factors binding to the flanking SP1 sites. This chapter describes experiments performed to determine the mechanism by which the responses to ICI 164,384 mediated by the creatine kinase-ERE and A2-ERE differ.

## 6.2 Oestrogen receptor binds to the creatine kinase ERE as a dimer

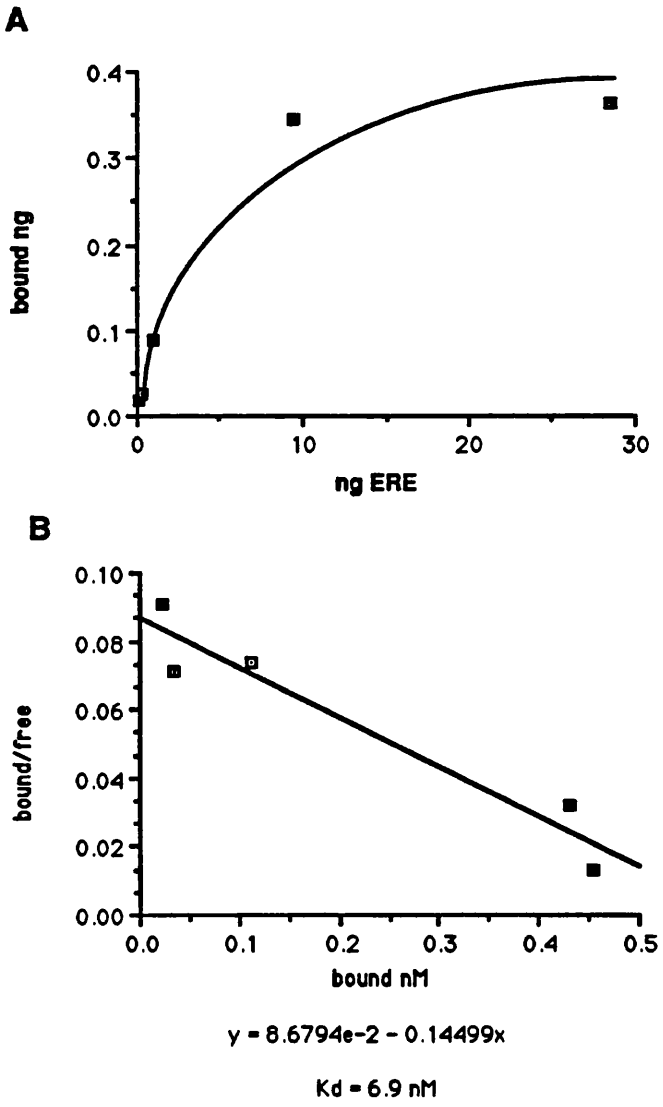
Before investigating the effects of ICI 164,384 on the putative creatine kinase ERE element it was necessary to check that the oestrogen receptor bound this sequence with high affinity. The affinity of receptor for the creatine kinase element was determined so that binding could be optimised. Gel retardation assays were carried out in DNA excess with a constant amount of protein and increasing amounts of creatine kinase ERE. The dried gels were then quantitated using an Ambis β system and the results used to determine the Kd of the receptor for this sequence (Figure 6.2). Scatchard analysis gave an affinity for the DNA binding site

**Figure 6.1 - Oligonucleotide containing Creatine Kinase ERE and flanking sequences**




The sequence of the creatine kinase oligonucleotide probe after labelling with klenow is shown. The the larger box contains the ERE half-site and the smaller box contains the bases which conform to the consensus. The flanking SP1 sites are highlighted in bold. Below is a comparison of creatine kinase ERE and the consensus ERE; bases which conform to the consensus are starred and those which differ marked with a cross.

Figure 6.2 - Affinity of MOR for Creatine Kinase ERE




Whole cell extracts prepared from *S.frugiperda* cells infected with recombinant MOR baculovirus were incubated with oligonucleotide containing the creatine kinase ERE (CKERE) at concentrations ranging from 0.1nM to 30nM. Concentrations of over 1nM were generated by dilution of the radiolabelled CKERE with unlabelled CKERE oligonucleotide. The receptor-DNA complexes were then analysed in a gel retardation assay. The gel was fixed and dried and the levels of radioactivity present in both the receptor-DNA complex and the free probe were quantitated by direct scanning with an Ambis Systems β-scanner. The equivalent position for control lysate with a range of probe concentrations was subtracted as backgroundand the probe values adjusted to account for any reduction in specific activity resulting from dilution of the probe. These corrected values were then used to plot the binding curves (A.) and Scatchard analyses (B).

of 6.9 nM. To ensure gel retardation assays were carried out in probe excess, subsequent studies were performed using 6 ng of the sixty base pair oligonucleotide.

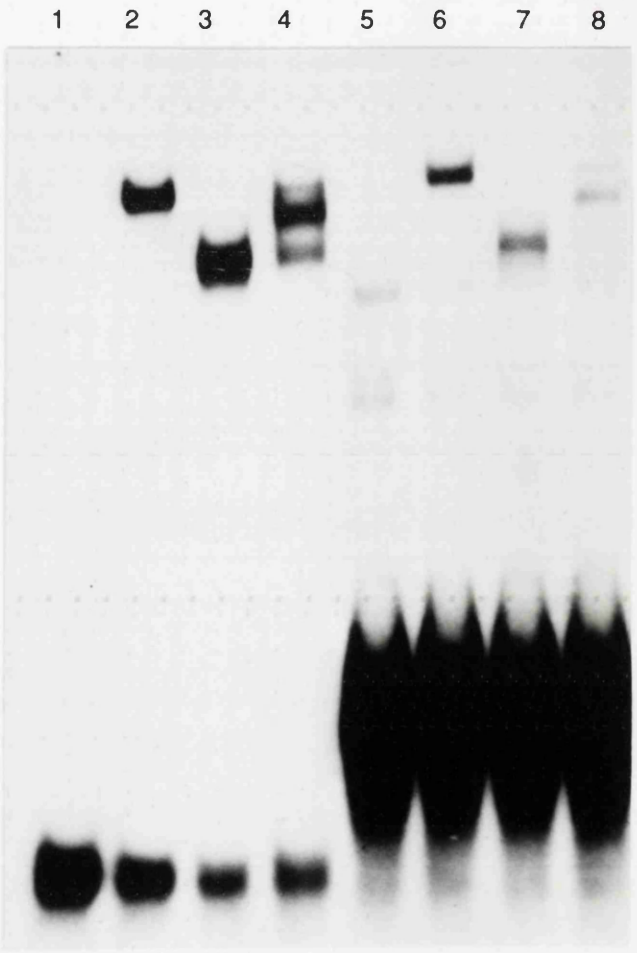
An ability of oestrogen receptor to bind the creatine kinase ERE as a monomer might account for the stimulatory effects of ICI 164,384 *in vivo*. In view of this the oestrogen receptor was tested in a gel retardation assay to determine whether it bound to the creatine kinase ERE as a monomer or dimer. Full length (MOR) and N-terminally deleted (MOR121-599) oestrogen receptors were translated individually or together in a rabbit reticulocyte system. The *in vitro* translated receptors were incubated in binding buffer for 10 min prior to incubation for 30 min at room temperature with either a creatine kinase ERE radiolabelled probe or vitellogenin A2 ERE as a control. The receptor/DNA complexes were analysed on a polyacrylamide gel. Figure 6.3 shows that a heterodimer formed between the full-length and N-terminally deleted receptor on both the A2 and creatine kinase EREs. Thus it appeared that the oestrogen receptor bound to both elements as a dimer.

## 6.3 Oestrogen receptor dimer contacts both consensus and non-consensus halves of creatine kinase ERE

The previous experiment demonstrated that the oestrogen receptor binds to the creatine kinase element as a dimer. It did not however distinguish between the binding of a preformed dimer to only one half of the site, and a dimer with both monomers making specific contacts with the response element. To determine whether the receptor was contacting the non-consensus half of the element as well as the consensus sequence, methylation interference footprinting was performed.

Oligonucleotide was labelled and methylated with 0.5% DMS for 5 min at room temperature. This gave on average one methylated guanidine per oligonucleotide. The radiolabelled probe was incubated in the presence or absence of oestrogen receptor expressed in insect cells and run on a nondenaturing polyacrylamide gel. The gel was subjected to autoradiography without drying to locate the position of the probe and receptor/probe complex. These bands were excised from the gel and DNA recovered by electroelution. The DNA was cleaved at methylated Gs with 1M piperidene and after multiple precipitations to remove piperidene

**Figure 6.3 - Ability of MOR to bind the Creatine Kinase ERE as a Dimer**




Full-length (MOR) and N-terminally deleted oestrogen receptors (MOR121-599)were translated individually or cotranslated *in vitro* and tested for their ability to bind either the vitellogenin A2 ERE or the CK ERE in a gel shift assay. Lanes 1-4 contain A2 ERE and lanes 5-8 contain CK ERE. Lanes 1 and 5 contain a control translation, lanes 2 and 6 contain MOR, lanes 3 and 7 contain MOR121-599 and lanes 4 and 8 contain cotranslated MOR and MOR121-599.

the products analysed on a 20% denaturing acrylamide gel. The results are shown in Figure 6.4.

Since DNA binding proteins cannot contact methylated residues the cleavage pattern of the probe from the receptor/DNA complex lack the bands corresponding to the Gs contacted by the protein. Comparison of the intensity of bands in the absence or presence of receptor allows identification of the G nucleotides which are contacted by receptor. On the palindromic vitellogenin A2 ERE the receptor contacts Gs in both halves of the site as seen in previous studies. On the creatine kinase sequence the oestrogen receptor appears to be contacting the consensus half of the element at the two Gs on the upper or coding strand, and also the G on the upper or non-coding strand. Although the tracks representing the lower or non-coding strand of the creatine kinase sequence are not equal in intensity it appears that the receptor contacts at least the outer of the two Gs which correspond to the consensus. This contact is weaker than that on the other half of the element. The footprinting data suggests that despite the non-consensus sequence of this creatine kinase ERE the receptor is binding to both halves of the element.

## 6.4 ICI 164,384 inhibits oestrogen receptor binding to the creatine kinase ERE

The oestrogen receptor had been shown to bind the creatine kinase ERE as a dimer but the effects of ICI 164,384 on the ability of the receptor to bind were unknown. Oestrogen receptor expressed in *S.frugiperda* cells was incubated in binding buffer containing no additions, MP16 antiserum, ICI 164,384, or ICI 164,384 and MP16 antiserum for 10 minutes. The A2 or creatine kinase ERE probes were then added and and the complexes formed after 30 minutes analysed in a gel retardation assay. As previously observed in the presence of ICI 164,384 the binding of oestrogen receptor to the A2 ERE was strongly inhibited. Surprisingly the binding of receptor to the creatine kinase ERE was inhibited to a similar extent (Figure 6.5). MP16 antiserum restored DNA binding in the presence of ICI 164,384 to receptor on both response elements. This suggested that it was an inability to dimerise that prevented DNA binding in both cases. No receptor/DNA complex which might correspond to the binding of a monomer could be seen migrating lower

Figure 6.4 - Methylation interference assay

[$^{32}$P]-labelled oligonucleotides corresponding to the upper (U) and lower (L) strands of the vitellogenin A2 (A2 ERE) and creatine kinase (CK ERE) response elements were methylated on approximately one base per molecule. 20ng of of probe was used in a gel retardation assay in the presence or absence of 5ul oestrogen receptor expressed in insect cells. The bound (+) and unbound (-) band were excised and the eluted probe cleaved with piperidene. Fragments were analysed on a denaturing acrylamide gel (A.). The protected residues are indicated with arrowheads in A. and on the creatine kinase probe sequence (B.) below.

B.

Figure 6.4 - Methylation interference assay

A.

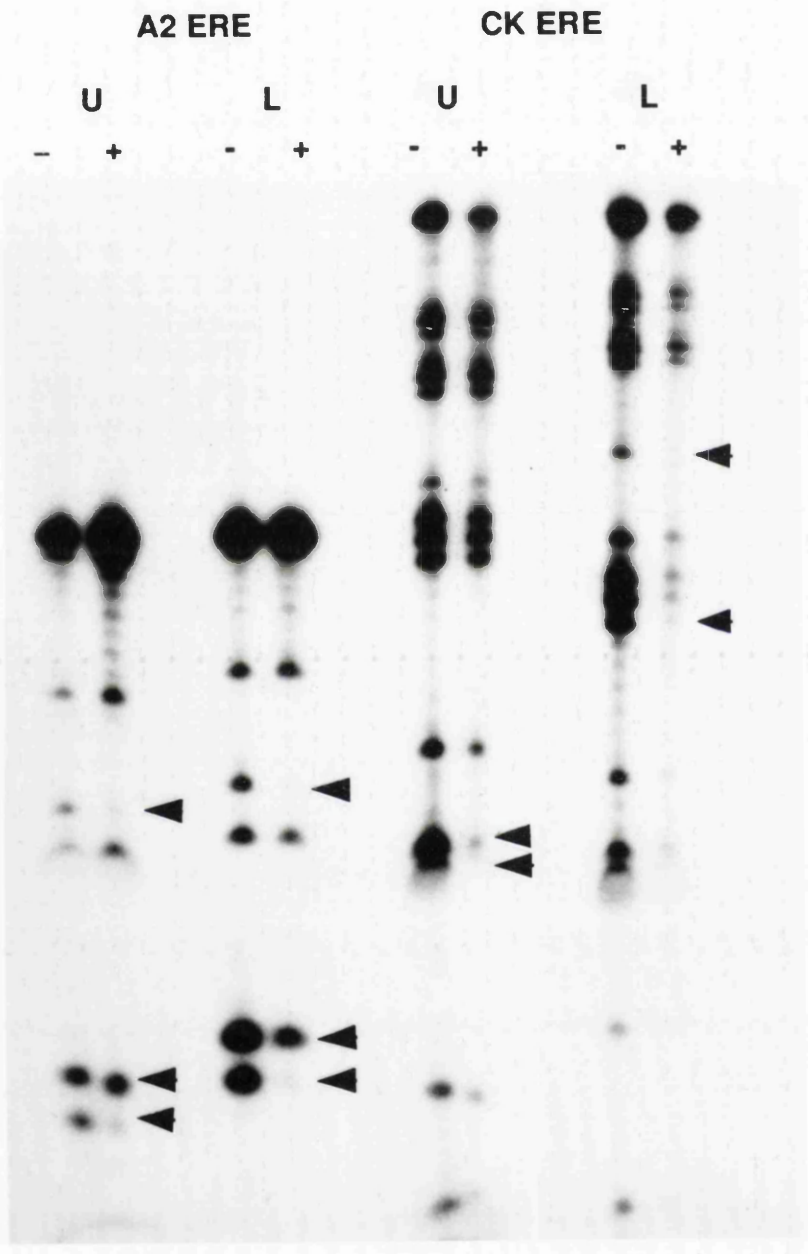
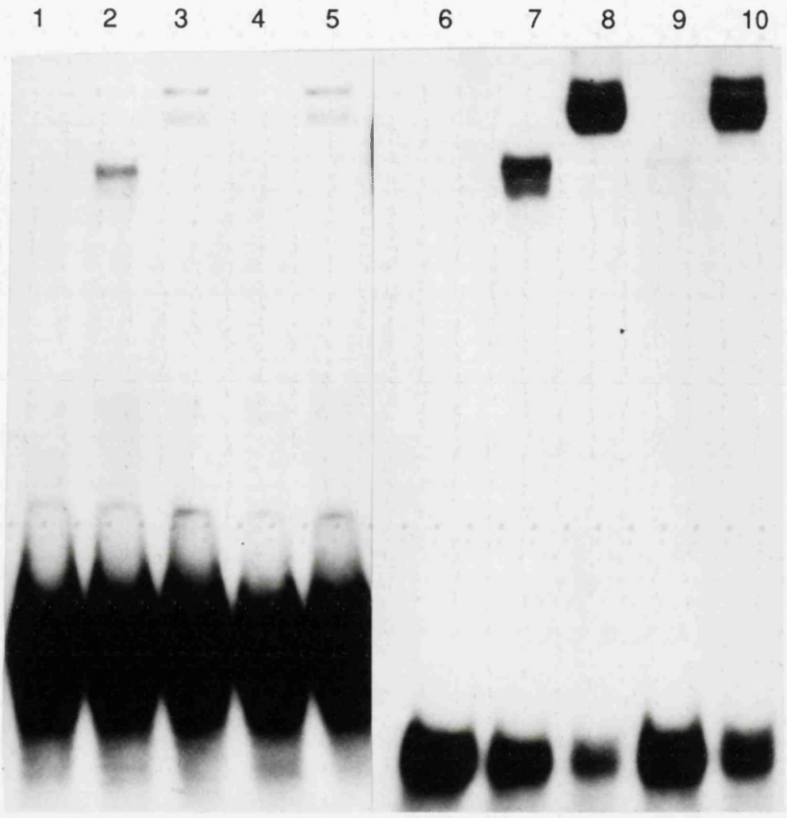


**Figure 6.5 - Ability of ICI 164,384 to interfere with MOR binding to Creatine Kinase ERE**




Whole cell extracts were prepared from *S.frugiperda* cells infected with recombinant MOR baculovirus and tested for their ability to bind to either a creatine kinase ERE (Lanes 1-5) or vitellogenin A2 ERE (Lanes 6-10). Lanes 1 and 6 contain a control insect cell extract, lanes 2 and 7 contain MOR, lanes 3 and 8 contain MOR and MP16, lanes 4 and 9 contain MOR and 1 $\mu$M ICI 164,384 and lanes 5 and 10 contain MOR, 1mM ICI164,384 and MP16.

in the gel with the creatine kinase ERE in the presence of ICI 164,384. Thus in this system no difference in the effects of ICI 164,384 on oestrogen receptor binding to vitellogenin A2 and creatine kinase EREs could be detected.

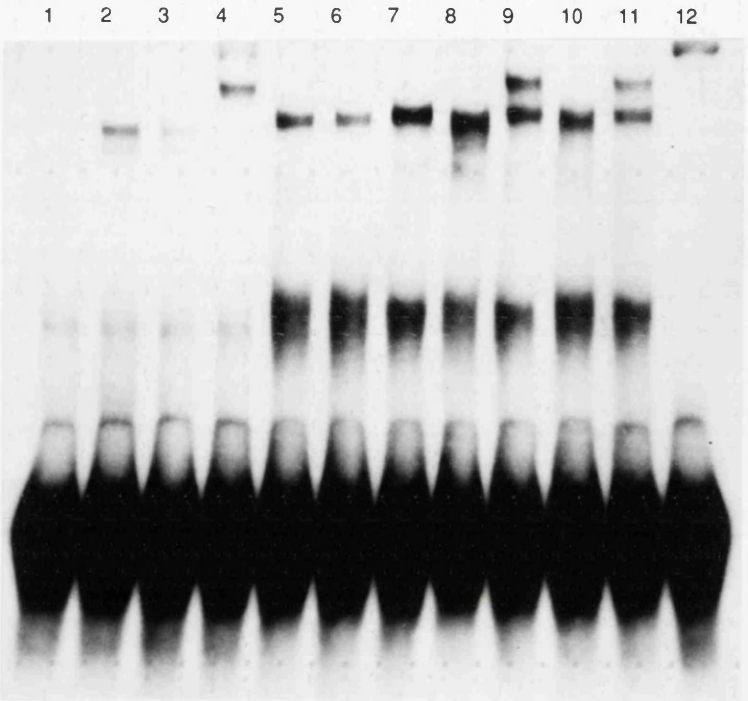
## 6.5 HeLa extract fails to restore ICI 164,384 inhibited DNA binding.

The original data demonstrating the agonist effect of ICI 164,384 was obtained in HeLa cells whereas the gel retardation data was generated using insect whole cell extract. The creatine kinase ERE is flanked by two consensus SP1 sites in the natural promoter. It was therefore possible that, *in vivo*, binding of SP1 protein stabilised the binding of the oestrogen receptor, possibly as a monomer. Since the insect cells do not contain SP1 any stabilising effects of this protein would not have been observed in the previous assay. To ensure that SP1, or other potential transcription factors that might be involved in the agonist effect of ICI 164,384 were present in the reaction, whole cell extract prepared from HeLa cells was tested in the gel retardation assay. Since SP1 binds to DNA in the presence of magnesium ions and the oestrogen receptor in inhibited in DNA binding by the presence of magnesium experiments were performed to optimise cation conditions. It was found that both a HeLa specific protein and the receptor were able to bind the oligonucleotide at 1mM magnesium.

The HeLa cell extract generated a specific band when incubated with the creatine kinase ERE oligonucleotide but not with the A2 ERE. This protein/DNA complex migrated with a slightly lower mobility than the oestrogen receptor/DNA complex. The protein was likely to be SP1 as the complex migrated with a similar mobility to the same extract incubated with an oligonucleotide containing a consensus SP1 site. When oestrogen receptor and HeLa extract were incubated together in the presence of creatine kinase ERE complexes of higher molecular weight were not observed (Figure 6.6 ). Each individual complex could be discerned, although slightly smeared possibly due to the two bands being so similar in size. However no band corresponding to both proteins on the same oligonucleotide was observed. ICI 164,384 prevented receptor binding to the oligonucleotide even in the presence of the HeLa extract.

This experiment was initially carried out in DNA excess but if binding of receptor and HeLa protein were not co-operative it would be

Figure 6.6 - Effect of Hela proteins on ability of MOR to bind the creatine kinase ERE




The following whole cell extracts were tested for their ability to bind to 1ng radiolabelled creatine kinase ERE in a band shift assay as described in the methods. BVMOR ; oestrogen receptor expressed in *S.frugiperda* cells under control of the baculovirus polyhedrin promoter, 1µl of 1:10 dilution of whole cell extract. Hela extract : 1µl of 1: 20 dilution of whole cell extract. MP16 : 1µl antiserum. ICI 164,384: $10^{-6}$M

Lane 1 : Uninfected insect cell whole cell extract
Lane 2 : BVMOR
Lane 3 : BVMOR and ICI 164,384
Lane 4 : BVMOR and MP16
Lane 5 : Hela extract
Lane 6 : Hela extract and ICI 164,384
Lane 7 : Hela extract and MP16
Lane 8 : BVMOR and Hela extract
Lane 9 : BVMOR, Hela extract and MP16
Lane 10 : BVMOR, Hela extract and ICI 164,384
Lane 11 : BVMOR, Hela extract, ICI 164,384 and MP16
Lane 12 : MP16

unlikely for both proteins to bind to the same oligonucleotide. To determine whether the two proteins could bind to the same oligonucleotide the experiment was repeated under conditions where DNA was limiting. Again, binding of both oestrogen receptor and HeLa protein to the same element was not observed (data not shown). Thus it would appear that the two proteins were incapable of interacting with the same oligonucleotide in the gel retardation system.

## 6.7 Conclusions

In the experiments described in this chapter only one significant difference between the interactions of the oestrogen receptor with vitellogenin A2 ERE and the creatine kinase ERE could be discerned. This was the lower affinity of the receptor for the creatine kinase ERE. Both the effects of the antioestrogen ICI 164384 and the antiserum MP16 on receptor binding to the two elements appeared to identical *in vitro*. Incubation with a HeLa whole cell extract containing proteins capable of binding to the creatine kinase oligonucleotide does not overcome the inhibition of receptor binding by the antioestrogen. It appears therefore that in this assay the creatine kinase sequence is acting as a weak but conventional ERE.

# CHAPTER 7

# Discussion

## 7.1 Introduction

The aim of this chapter is to review the data presented in this thesis in the light of other studies and discuss the implications for our understanding of the structure and function of steroid receptors.

The work described in this thesis concentrates on investigations of the functions which are associated with the hormone binding domain of the mouse oestrogen receptor. Sequences within this domain are involved directly or indirectly in all the major steps which transform an inactive oestrogen receptor into an active transcription factor. Biochemical studies using proteases demonstrated that the hormone binding function is contained within a discrete structural domain (Green *et al.*, 1984). This was confirmed by subsequent deletion mutagenesis (Kumar *et al.*, 1986). One of two transcriptional activation functions identified in the oestrogen receptor, TAF-2, has also been mapped to this domain (Webster *et al.*, 1988; Webster *et al.*, 1989; Tora *et al.*, 1989; Lees *et al.*, 1989). In addition sequences towards the C-terminus of the hormone binding domain are involved in the formation of dimers (Kumar and Chambon, 1988).

Since ligand binding is required for the dissociation of oestrogen receptor from a complex containing heat shock protein, it appears that all subsequent receptor activities depend on this initial step. Dimerisation and DNA binding are also linked as high affinity DNA binding requires the formation of stable dimers (Kumar and Chambon, 1988). Following high affinity DNA binding TAF-1 is able to function constitutively while TAF-2 requires oestrogen for its activity. Dimerisation and TAF-2 map to the hormone binding domain and their activity depends on earlier steps in receptor activation such as ligand binding. Therefore it is important to be able to distinguish between the effects of these different functions as analysis of any one function might be disrupted by effects on a distinct function. For example a mutation which caused a loss of transcriptional activity may not necessarily be a direct effect on TAF-2 since it might disrupt ligand binding or dimerisation.

The structure and function of the hormone binding domain of the oestrogen receptor has been investigated by using two major approaches. Firstly specific single amino acids were replaced and receptor function analysed *in vitro* and *in vivo*. Secondly the receptor was subjected to limited proteolytic cleavage and analysed in a gel retardation assay. It is possible to investigate the role of ligand in receptor function using

steroidal and non-steroidal ligands. For clinical purposes a large number of oestrogen antagonists have been developed to inhibit oestrogen receptor function. By investigating the mechanism by which these antioestrogens disrupt receptor action, it is also possible to gain insights into the normal function of the oestrogen receptor.

## 7.2 Sequences involved in hormone binding

The C-terminus of the oestrogen receptor is required for high affinity hormone binding. A series of deletions through the human oestrogen receptor demonstrated that region E contained all sequences required for ligand binding (Kumar *et al.*, 1986). Subsequent deletion mutagenesis of the mouse oestrogen receptor mapped the C-terminal boundary of the hormone binding domain to between residues 538 and 552 (Lees *et al.*, 1989). Complete loss of ligand binding occurred on deletion to residue 507 (Fawell *et al.*, 1990a). The investigation of which residues are involved in ligand binding has therefore focused on this region.

To study the importance of individual amino acids most positions were mutated to several different amino acids. Several residues which appeared to be involved in ligand binding were identified. It was found that the affinity of receptor for oestradiol was reduced when certain mutations were introduced at amino acids R-507, L-511 and I-518. Substitution of basic amino acids into position 518 and 525 completely abolished ligand binding. The extent to which any particular mutation interfered with hormone binding depended on two factors; firstly how close to the hormone binding site the mutated amino acid was located, and secondly on the charge and size of the residue introduced. Where the affinity for oestrogen is affected by mutation to the small, relatively neutral amino acid alanine (R-507), it appears likely that the amino acid in the wildtype receptor interacts directly with hormone. However, in cases where the introduction of charged residues, but not alanine, disrupted ligand binding (I-518,L-511) the amino acid in the wildtype receptor may be closely associated with the binding site but not necessarily contacting the hormone.

It has been proposed that the cysteine in this region was also involved in binding hormone. Experiments using covalently labelling ligands had suggested that cysteine 530 in the human receptor (corresponding to C-534 in the mouse) was closely associated with

sequences involved in ligand binding (Harlow *et al.*, 1989). However, we found that mutation of C-534 to alanine generated receptor which bound oestradiol with high affinity and this amino acid is therefore unlikely to be involved in directly binding ligand. It has recently been shown that a human oestrogen receptor in which the corresponding cysteine has been mutated to alanine can still be covalently labelled, but the site of labelling in this receptor is likely to be another cysteine within the hormone binding domain (Reese and Katzenellenbogen, 1991). As the alternative cysteines are found at positions 381, 417, and 447 this suggests that more than one region of the hormone binding domain may be involved in ligand binding. This is also in agreement with mutagenesis of the human oestrogen receptor which demonstrated that 66 amino acid deletions within the hormone binding domain abolished hormone binding (Kumar *et al.*, 1986)

Evidence that other sequences within the hormone binding domain are required for the high affinity binding of hormone has been obtained from a mutant human oestrogen receptor. The original human oestrogen receptor cloned from MCF-7 cells was later found to have a point mutation of glycine to valine at amino acid 400 (corresponding to 404 in the mouse) (Tora *et al.*, 1989). This mutation was associated with a reduction in the affinity of receptor for oestradiol at 25°C but not 4°C. The reduced hormone binding affinity of this mutant also suggested that amino acids N-terminal of the region we have studied are important in hormone binding.

Naturally occurring mutations are often useful in identifying sequences important in the wildtype protein but to date there are no reports of endogenous full length oestrogen receptors with point mutations which abolish ligand binding (Grahame *et al.*, 1990; Murphy, 1990). As they do not occur naturally, it is possible that such mutations are fatal during development.

There are however reports of truncated oestrogen reporter transcripts in breast tumours (Fuqua *et al.*, 1991), and mRNA species which lack the region encoding the hormone binding domain (Murphy and Dotzlaw, 1989). Since receptors lacking the entire hormone binding domain have constitutive transcriptional activity (Tora *et al.*, 1989a; Lees *et al.*, 1989), these truncated mRNAs might generate constitutively active oestrogen receptor. A number of abnormal oestrogen receptor cDNAs have been isolated from the antioestrogen resistant T-47D cell line

(Grahame *et al.*, 1990). These cDNAs have been sequenced and amongst the mutations are changes which would cause frame shift mutations resulting in receptors truncated after the second zinc finger or within exon 5 or result in a deletion of all of exon 4. The presence of abnormal receptors such as these might account for the lack of response to tamoxifen in hormone independent, receptor positive, breast tumours. A human oestrogen receptor variant, associated with increased levels of spontaneous abortion (Lehrer et al., 1990), has been identified. Although the variant is associated with slightly reduced oestrogen binding this is due to a point mutation in region B (Garcia *et al.*, 1988) and not a direct effect on ligand binding and the basis of this curious observation is not known.

The importance of the region we have studied in hormone binding is supported by work on other steroid receptors. Mutations in the androgen receptor hormone binding domain have been identified in human subjects with complete androgen insensitivity (CAIS). Mutation of valine 866 to methionine (V-866M) reduced affinity for ligand four-fold (Brown *et al.*, 1990). Alignment of receptor sequences revealed that this amino acid corresponded to I-518 in the oestrogen receptor. Since mutation of I-518 in the oestrogen receptor also disrupts hormone binding this suggests that the position is important in hormone binding in more than one member of the receptor superfamily. In other CAIS subjects the mutation of the arginines at 774 and 831 to cysteine and glutamine respectively, gave rise to receptors completely incapable of binding androgens, indicating that amino acids from different parts of the hormone binding domain are involved in androgen binding. The molecular basis of androgen insensitivity in the testicular feminised (Tfm) rat was due to mutation of arginine 734 to glutamine which reduced androgen binding affinity to only 10% that of the wildtype receptor (Yarbrough *et al.*, 1990). Interestingly, alignment of oestrogen and androgen receptor sequences revealed that this mutation corresponded to a position only a few amino acids N-terminal of the glycine shown to be important in ligand binding in the human oestrogen receptor (Tora *et al.*, 1989a). These results suggest that this region of steroid receptors might also be important in hormone binding.

Affinity labelling has identified cysteines in the glucocorticoid receptor which are associated with hormone binding (Carlstadt-Duke *et al.*, 1988). Additionally mutation of one of these cysteines, which is

within the highly conserved region we have been studying, abolished ligand binding (Danielsen, 1991). Deletion mutagenesis of the glucocorticoid receptor demonstrated that the loss of only 5 amino acids from the C-terminus was sufficient to increase Kd for ligand 30 fold, and more extensive deletions reduced affinity further (Rusconi and Yamamoto, 1987). As found with the androgen receptor, a number of point mutations from different parts of the hormone binding domain (amino acids 546, 547,742 and 770) also affected the ability of glucocorticoid receptor to bind ligand (Danielsen *et al.*, 1986; Danielsen, 1991). These data indicate that amino acids from sequences in the hormone binding domain over 200 amino acids apart are involved in glucocorticoid binding.

In conclusion, we have identified a number of residues in a limited region towards the C-terminus of the hormone binding domain which are important for oestrogen binding, but there is evidence that other sequences in the domain are also involved in this function. These data suggest that a number of residues from different parts of the hormone binding domain contribute to the ability of oestrogen, androgen and glucocorticoid receptors to bind their respective ligands. It is likely that the structure involved in ligand binding in each receptor is formed from several regions of the hormone binding domain which are brought together in the tertiary structure. Confirmation of this hypothesis awaits the results of X-ray crystallographic studies of steroid receptor structure.

## 7.3 Sequences and structure of a dimerisation interface

The work of Kumar and Chambon (1988) provided direct evidence that the human oestrogen receptor bound to DNA as a dimer and suggested that a dimerisation activity was contained within the hormone binding domain. The ability of receptors to form stable dimers can be measured directly in an immunoprecipitation assay which determines whether heterodimers form between full-length and N-terminally truncated receptor in solution. However since dimerisation has been shown to correlate completely with DNA binding (Fawell *et al.*, 1990a), the gel retardation DNA binding assay was used in this study to determine if mutations were affecting the formation of stable dimers.

Work on the mouse oestrogen receptor demonstrated that deletion to residue 538 had no effect on DNA binding, whereas deletion

to 507 reduced DNA binding affinity to only 10% that of the wild-type receptor (Fawell *et al.*, 1990a). This analysis therefore indicated that the C-terminal boundary of sequences required for dimerisation mapped to this region. However, there appeared to be no discrete dimerisation domain within the hormone binding domain. This thesis describes a thorough mutagenesis of these sequences essential for dimerisation and discusses some possible models for the structure of this region.

During the course of this work two models had been proposed for dimerisation of transcription factors, the leucine zipper and helix-loop-helix. The leucine zipper motif has been well characterised in a number of transcription factors. It consists of a heptad repeat of leucine residues forming a coiled coil with the hydrophobic residues projecting along one side (Landschulz *et al.*, 1988). The hydrophobic leucines of each monomer interact with the leucines of the second monomer in a parallel alignment (O'Shea *et al.*, 1989) to form a stable dimerisation interface. An alternative model for dimerisation is the helix-loop-helix found in c-myc, MyoD (Davis *et al.*, 1990) and a number of Drosophila homeobox proteins. This motif consists of two ampipathic α helices connected by a nonconserved loop region of varying length (Murre *et al.*, 1989). It was possible that the sequences involved in oestrogen receptor dimerisation were related to one of these two motifs.

A putative leucine zipper was identified in the oestrogen receptor between residues 494 and 515 (Fawell *et al.*, 1990a). This was unlikely to be involved in dimerisation as it was not conserved in other receptors and could be mutated without affecting the ability of receptors to form stable dimers. Other so-called heptad repeats were identified in the thyroid and retinoic acid receptors (Forman *et al.*, 1989). These 'heptad repeats' were however generally shorter than a leucine zipper, consisting of just two or three hydrophobic repeats, and only one of the sequences identified contained four hydrophobic repeats. Additionally since these repeats are not conserved in other members of the nuclear receptor superfamily, they are unlikely to be functionally significant.

The study described in this thesis concentrated on a second hydrophobic repeat identified in the hormone binding domain of the mouse oestrogen receptor (Fawell *et al.*, 1990a). This repeat is conserved across the nuclear receptor superfamily and a consensus sequence identified receptors and a number of proteins with a coiled coil structure from a protein database. Although hydrophobic, this conserved repeat

did not contain the leucines characteristic of a leucine zipper and was unlikely to be a related structure. Changing the spacing between the N-terminal and C-terminal pairs of conserved residues so as to disrupt the heptad repeat and change the alignment of the residues did not interfere with dimerisation. Of the four residues of the heptad hydrophobic repeat the C-terminal conserved positions could be mutated with no effect upon dimerisation. This indicated that the structure of the region 511 to 532 was not a coiled coil and that it did not form a helix-loop-helix.

The mutagenesis described in this thesis further defined the extent of the sequences in this region of the receptor which were important in the formation of dimers *in vitro*. Site-directed mutagenesis indicated that sequences between R-507 and R-519 appear to be important in the formation of stable dimers. This work is in agreement with the ability of a 22 amino acid peptide (501-522) to restore DNA binding activity to dimerisation deficient mutant mouse oestrogen receptor (Lees *et al.*, 1990). The peptide fused to a truncated oestrogen receptor, MOR121-339, generated a receptor capable of binding DNA. However DNA binding activity was reduced compared with that of wild-type receptor. It seems likely that other sequences within the hormone binding domain also contribute to the formation of dimers. It has also been suggested that there is a weak dimerisation function present within the DNA binding domain of the oestrogen receptor (Kumar and Chambon, 1988).

If a discrete dimerisation domain were present in this region of the oestrogen receptor it might be possible to exchange it with the corresponding region of another member of the steroid receptor family. Thus indirect evidence that there is no discrete dimerisation domain within this region is provided by the failure to generate dimeric oestrogen receptors when the corresponding androgen receptor sequences are introduced (White *et al.*, 1991). In addition preliminary experiments with a synthesised peptide corresponding to the oestrogen receptor dimerisation sequences, failed to detect dimer formation between the peptides *in vitro* (S.Fawell-personal communication). This is in contrast to proteins which contain the leucine zipper in which there is a single dimerisation domain encompassing only 30 amino acids (Landschulz *et al.*, 1988) which forms stable dimers when expressed as a peptide (O'Shea *et al.*, 1989). The leucine zippers of Fos, Jun and other related proteins can also be swapped successfully, again demonstrating

that this is a discrete domain (Sellers and Struhl, 1989; Neuberg *et al.*, 1989; Agre *et al.*, 1989).

On the basis of their DNA binding activities it was possible to divide the oestrogen receptor point mutants into four classes. This allowed a number of conclusions to be drawn about the effects of different types of mutations within this region. The mutagenesis confirmed that L-511 and I-518 were important in dimerisation as these positions only tolerated very minor mutations. However, these were not the only residues which were involved in receptor dimerisation. Several other residues, particularly those which were hydrophobic, affected DNA binding when mutated (eg. I-514, L-512). The introduction of hydrophobic residues in this region tended to result in fully functional receptor, whereas the introduction of charged residues produced mutants with a reduced ability to bind DNA. These results suggested that the oestrogen receptor dimerisation interface consisted mainly of hydrophobic interactions.

As mentioned previously, a naturally occurring mutation in the human androgen receptor which replaces valine 866 with methionine reduces affinity for androgen only four-fold (Brown *et al.*, 1990). Data presented in this thesis indicates that similar reductions in hormone binding affinity do not prevent transcriptional activity of the oestrogen receptor *in vivo*. In view of this, the reduction in androgen binding is unlikely to account for the complete insensitivity to hormone in subjects carrying this mutation. Interestingly, when receptor sequences are aligned, the valine at 866 in the androgen receptor corresponds to I-518 in the mouse oestrogen receptor. Since mutation of I-518 affects both ligand binding and dimerisation in the oestrogen receptor, it is possible that the complete androgen insensitivity generated by mutation of the valine is in part due to impairment of dimerisation.

Protein structure programs predicted that the sequences in the region of the oestrogen receptor mutated would form an α-helical structure. Since changing the spacing of the two halves of the hydrophobic heptad repeat did not impair receptor dimerisation (Fawell *et al.*, 1990a) it had been proposed that the dimer interface was not an extended α helix. The mutagenesis described in chapter three suggested that even within the N-terminal part of this conserved region the structure was unlikely to be α helical. Three different residues in this region (Q-510, L-513 and L515) generated functional receptor when

mutated to glycine, a residue which tends to disrupt helices. This indicated that the structure of the dimerisation interface is also unlikely to be a helix-loop-helix in which the region mutated forms the C-terminal helix.

These results are in contrast to similar mutagenesis of the leucine zipper of GCN4 in which the leucines of the heptad leucine repeat and the hydrophobic (usually valine) residues at the intermediate position in the repeat (LxxxVxxL) were randomly mutated (Hu *et al.*, 1990). In the GCN4 study it was found that the majority of mutations of a single leucine or valine which resulted in functional protein were to an aliphatic or aromatic hydrophobic residue. The functional proteins contained no mutations which introduced helix-breaking residues and a low percentage (15%) of these were strongly polar. Mutations in the JunD leucine zipper, have generated transcription factors with either decreased or increased ability to form homodimers (Hirai and Yaniv, 1989). Mutation of residues other than the leucines in the leucine zipper domain of Fos transcription factor has also been performed. Substitution of certain amino acids in Fos with the corresponding Jun amino acids facilitates the association of two Fos leucine repeats (Schuermann *et al.*, 1991). Thus while the steroid receptor dimerisation interface and coiled coils such as leucine zipper are both hydrophobic, their structure appears to differ greatly. The structure of the receptor dimer interface is not $\alpha$ helical and there is a greater tolerance of conservative substitutions than is allowed in the leucine zipper.

The structure of the dimeric uteroglobin protein has been determined by X-ray diffraction crystallography (Mornon *et al.*, 1980; Morize *et al.*, 1987; Bally and Delettre, 1989). Since uteroglobin binds the steroid hormone progesterone (Beato and Baier, 1975) we considered the possibility that the structure of this protein might provide a model for dimerisation of steroid receptors. The uteroglobin dimer has a two-fold symmetry around an internal hydrophobic cavity. A major part of the uteroglobin dimer interface is formed by part of a helix and $\beta$ turn incorporating residues 39-49. There is a limited homology between uteroglobin and the hormone binding domain of the progesterone receptor in this region. Interestingly the region of homology corresponds to the sequences in the oestrogen receptor shown to be important in dimerisation. It was therefore proposed that the structure of uteroglobin in this region, in particular, might provide a good model for the

oestrogen receptor dimer interface. In view of this, mutagenesis was directed towards testing whether the structure of the dimerisation interface resembled that of uteroglobin. Other residues of the uteroglobin protein are also involved in dimerisation but the region corresponding to the conserved sequences in the oestrogen receptor does form a major part of the dimer interface.

The three dimensional structure of uteroglobin was displayed using the computer graphics QUANTA program. The residues over the major uteroglobin dimerisation interface were converted in the display to the corresponding oestrogen receptor side groups at positions 509-519. The positions of charged and hydrophobic oestrogen receptor residues were unlikely to disrupt the uteroglobin structure. The residues which projected into the dimerisation interface were hydrophobic or neutral in nature (eg. L-511, I-514, I-518), whereas charged or polar residues tended to project away from the dimerisation interface (eg Q-510, S-516,R-519). Additionally mutation of residues predicted by this model to be buried within the dimerisation interface did, in fact, disrupt dimerisation (eg. L-511R). Mutation of those residues predicted to project away from the interface tended to be less disruptive (eg. R-519A). Thus it appeared that the oestrogen receptor residues would be able to form a similar structure to the corresponding uteroglobin residues.

A series of mutations directed towards distinguishing between the anti-parallel arrangement of uteroglobin monomers and a parallel arrangement similar to the leucine zipper motif were tested for their ability to form heterodimers. It was hoped that one or more combinations of different mutants would generate a stable heterodimer. However, no combination of mutant receptors with charged residues in the dimer interface were able to dimerise and bind DNA more than the individual mutant receptors. An experiment designed to link cysteines, which had been introduced into the dimerisation interface, via a disulphide bond was also unsuccessful. It was therefore not possible to distinguish between a parallel and antiparallel alignment, or confirm the model of dimerisation based on uteroglobin experimentally. There are a number of possible explanations for this. The positioning of the residues may be incompatible with the formation of a salt bridge as the acidic and basic side groups may not be close enough for the formation of a covalent bond. In addition the residues introduced at these positions have been shown to disrupt the formation of dimers. It is possible that

even if dimers containing the charged residues formed, the structure of the interface might have been changed so that the residues were never in a position to form a covalent bond. It is also of course possible that the model is incorrect and the residues do not interact in the manner predicted. If the model is incorrect then the receptors must form a novel dimerisation structure distinct from that of uteroglobin.

Although there is homology between the hormone binding domain of receptors and uteroglobin, this only extends over a limited region. Recent point mutagenesis of the uteroglobin protein has identified residues essential for progesterone binding but these all lie outside the region of homology with the progesterone receptor (Peter *et al.*, 1991). The stoichiometry of ligand binding suggests that steroid receptors bind one molecule of ligand per receptor monomer. Uteroglobin, on the other hand, has been shown to bind a single molecule of progesterone per dimer (Beato *et al.*, 1977; Temussi *et al.*, 1980). The structure of uteroglobin is therefore not likely to be a good model for the structure of the receptor hormone binding domain outside the limited region tested.

Initially it was assumed that members of the nuclear hormone receptor superfamily formed homodimers exclusively. However, there is now evidence that the retinoic acid receptor and thyroid hormone receptor can interact cooperatively on a thyroid hormone response element, and this effect appears to be mediated through the formation of heterodimers (Glass *et al.*, 1989). The retinoic acid/thyroid hormone receptor heterodimer differs from the homodimers in its affinity for certain DNA binding sites. The heterodimer does not recognise a thyroid hormone response element in the growth hormone gene which is bound with high affinity by the thyroid hormone receptor (Glass *et al.*, 1987). It is therefore likely that the two receptors may have a novel regulatory effect on the patterns of gene expression in tissues in which they are coexpressed *in vivo*. This seems to be a feature of many other types of transcription factor. The exact combination of leucine zipper proteins in a homodimer or heterodimer determines to which site the dimer binds. Whereas the Jun/Fos heterodimer binds to the AP-1 site in preference to the related cyclic AMP response element (CRE), a Jun/CREB heterodimer binds preferentially to the CRE (Benbrook and Jones, 1990; Macgregor *et al.*, 1990).

The sequences through which the retinoic acid and thyroid receptors dimerise appear to correspond to those we have identified as important in the dimerisation of the mouse oestrogen receptor. The hydrophobic repeat identified in the steroid receptors is present in both thyroid hormone and retinoic acid receptors and deletion of the thyroid hormone receptor β to amino acid 422 (corresponding to L-515 in the mouse oestrogen receptor) abolished the formation of heterodimers (Glass *et al.*, 1989). Since the heptad hydrophobic repeat is highly conserved it is likely that other less conserved residues are responsible for specificity of dimerisation. We have identified one candidate for such a specificity function, L-513, in the mouse oestrogen receptor. The position corresponding to this residue in the receptors for glucocorticoid, progesterone, androgen and mineralocorticoids is conserved as a lycine. It was therefore somewhat surprising that the mutation L-513K in the oestrogen receptor resulted in a protein which dimerises only very weakly even in the presence of ligand. This indicated that there may be important differences between the dimerisation interfaces of steroid receptors. The failure to successfully swap sequences involved in dimerisation between oestrogen and androgen receptors also supported this idea (White *et al.*, 1991). These data suggest that, although the sequences involved are highly conserved, there are significant differences in the structures of steroid receptor dimerisation interfaces. This may account for the lack of heterodimerisation between different steroid receptors.

The dimerisation interface of the oestrogen receptor, like the leucine zipper and helix-loop-helix, appears to consist of hydrophobic interactions. Unlike these other structures however, the sequences involved in oestrogen receptor dimerisation are not α helical. Whilst the mutagenesis of individual amino acids tends to support the model of oestrogen receptor dimerisation based on the structure of uteroglobin, there is no direct evidence which confirms it. We are therefore unable to draw any firm conclusions about the actual structure of this oestrogen receptor dimerisation interface. Furthermore, there is evidence that residues in other regions of the receptor also contribute to the formation of dimers. It also appears likely that non-conserved amino acids (such as L-513) are involved in controlling specificity of dimerisation between nuclear hormone receptors. We appear to have reached the limit of the useful information which can be generated by mutagenesis of these

sequences. Whilst interesting data might be generated if a similar extensive mutagenesis were to be carried out in another part of the hormone binding domain, we must await the results of the physical NMR and crystallographic studies on purified receptors currently in progress to discover if our structural predictions are accurate.

## 7.4 Effect of different response elements on the ability of oestrogen receptor to bind DNA

Oestrogen receptors are able to bind to certain DNA sites with relatively high affinity and yet fail to induce gene expression. The site to which the receptor binds, particularly the spacing between the two halves of the palindrome, also determines whether the receptor is transcriptionally active. The oestrogen receptor was originally shown to bind with high affinity to elements (EREs) which normally consist of inverted repeats of the sequence TGACC separated by three base pairs. Subsequently it was found that the oestrogen receptor could also bind to a palindromic thyroid response element (TRE) (Holloway *et al.*, 1990). This TRE consists of an inverted repeat of the TGACC motif with no gap between the repeats. Although the oestrogen receptor is able to bind to the TRE it is unable to stimulate transcription from this response element. This is presumably due to the smaller size of the element requiring a different receptor conformation for binding which may perturb the hormone binding domain and thereby disrupt transcriptional activation.

The possibility that a difference in conformation of oestrogen receptor could be detected was investigated using a proteolytic clipping band shift assay (PCBA). This consists of subjecting the DNA binding protein to limited proteolysis and analysing cleavage products in a gel retardation assay (Schreiber *et al.*, 1988). PCBA has previously been used to detect changes in the structure of the PRTF factor when bound to different DNA sites (Tan and Richmond, 1990). This yeast transcription factor binds to both an a-specific and an α-specific response element, but only activates transcription from an a-specific response element. Comparison of the DNA/PRTF complexes in a gel shift assay after proteolysis with chymotrypsin revealed that additional complexes were present with an a-specific probe that were not seen with an α-specific probe. These additional complexes were not observed if the proteolysis was terminated prior to addition of probe. We have now used this

proteolytic clipping band shift technique to determine whether the observed differences in the ability of the oestrogen receptor to transactivate from the ERE and TRE were also reflected by changes in structure which could be detected by proteolysis.

When receptor was cleaved with chymotrypsin there was a clear difference between the the pattern of receptor/DNA complexes obtained with ERE and TRE. Whilst there was no significant difference between high molecular weight bands with the two response elements, there was a complete absence of low molecular weight bands with the TRE. Initially differences in the ability of smaller proteolytic fragments to bind to the ERE and palindromic TRE were thought to be due to changes in the availability of proteolytic sites. If this were correct then it would be expected that, as with PRTF (Tan and Richmond, 1990), pretreatment with protease before DNA binding would abolish differences in the protein/DNA complexes. However, the same pattern was generated whether the protease was added before or after DNA. Therefore the difference in the receptor/DNA complexes observed on the two elements were unlikely to be due to changes in the proteolytic cleavage sites available.

An alternative hypothesis was that the inability of the fragments to bind to the TRE was in part due to differences in their ability to dimerise on this element. It has previously been shown that high affinity DNA binding by the oestrogen receptor is dependent on protein dimerisation and that this is mediated by sequences within the hormone binding domain (Kumar and Chambon, 1988). The isolated DNA binding domain was capable of binding to an oestrogen response element but with an affinity of only $10^{-8}$M compared to $10^{-9}$M for the full length receptor. An antiserum against the mouse oestrogen receptor, MP16, is capable of restoring high affinity DNA binding to receptors which are unable to dimerise (Fawell *et al.*, 1990b). The epitope for MP16 is in the N-terminal domain and it is thought to link the N-termini of two receptor monomers so that they subsequently bind DNA as a dimer. It was found that this antiserum increases the affinity of an oestrogen receptor lacking the hormone binding domain for an ERE from 20nM to 4nM and induced this truncated receptor to bind to a TRE. It is therefore likely that the inability of the isolated DNA binding domain and smaller receptor fragments to dimerise on the TRE prevented binding.

Studies on oestrogen, glucocorticoid and thyroid hormone receptors have demonstrated that amino acids within the DNA binding domain not only determine to which half-site sequences the receptor can bind, but also the spacing (Mader *et al.*, 1989; Danielsen *et al.*, 1989; Umesono and Evans, 1989). DNA binding specificity was shown to depend on a region in the base of the first zinc finger and also the region between the two zinc fingers. Mutation of just three amino acids was sufficient to convert a glucocorticoid receptor into a receptor which activated transcription from an ERE. Changing a further five amino acids in the stem of the second finger of this mutated receptor to the corresponding residues in the thyroid hormone receptor generated a mutant which bound a thyroid hormone response element (Umesono and Evans, 1989). Further information on the effects of half-site spacing on DNA binding has been obtained from investigations into co-operative binding by the glucocorticoid receptor DNA binding domain. It was found that in this truncated receptor a region in the second finger is important for co-operative binding to a GRE. Co-operative binding is lost when the the spacing between the inverted repeats is altered (Dahlman-Wright *et al.*, 1990). Mutation of these sequences in the second finger also abolishes transcriptional activity in the full-length receptor *in vivo* (Dahlman-Wright *et al.*, 1991). Presumably the co-operative interactions between monomers depend upon their precise position on DNA.

Interactions between the second zinc fingers of monomers are also likely to be important for DNA binding by oestrogen receptors which lack the C-terminal dimerisation sequences. Altering the spacing of half-sites would change the relative positions of receptors bound to them. Thus the spacing of the TRE could prevent contacts between amino acids in the second finger of oestrogen receptors which might stabilise binding to an ERE. Alternatively, the truncated receptor might form stable dimers in solution and, with the relative positions of receptor subunits fixed, be unable to bind both halves of the element. This second explanation is supported by recent crystallographic data obtained on the interaction of the glucocorticoid receptor DNA binding domain with DNA (Luisi *et al.*, 1991). In this study it was found that contacts between the two subunits determined the sequences bound. There is however no evidence that the oestrogen receptor DNA binding domains form dimers in solution (Schwabe *et al.*, 1990).

The importance of protein-protein interaction between subunits of dimers in precisely positioning proteins on a DNA binding site has also been found in other transcription factors. Fos and Jun dimerise through a leucine zipper structure. Mutation of the leucine closest to the basic DNA binding sequences did not significantly affect the ability of the proteins to dimerise in solution (Gentz *et al.*, 1989). It did however interfere with the ability of the dimer to bind DNA (Ransone *et al.*, 1989) and induce transcription (Schuermann *et al.*, 1989). Unlike the leucine zipper family, steroid hormone receptors have a second, major dimerisation function in the C-terminus. This second function is not affected by interactions with DNA. The ability of the full length receptor to bind to a TRE as a dimer probably arises from stable interactions between these C-terminal dimerisation functions in the hormone binding domain. The failure of full length receptor to stimulate promoter activity upon binding to a TRE *in vivo* (Holloway *et al.*, 1990) may arise from an effect of aberrant DNA binding. It may be necessary for the receptor to adopt a different conformation for binding to the smaller element and this might inhibit transcriptional activity mediated by TAF-2 in the hormone binding domain. This is somewhat surprising as the hormone binding domain is a discrete structural domain which can be moved to other positions within oestrogen receptor or even fused to a heterologous transcription factor and retain transcriptional activity (Webster *et al.*, 1988; Lees *et al.*, 1989).

In contrast it has recently been demonstrated that thyroid hormone and retinoic acid receptors are not so limited in either the spacing or orientation of response elements through which they induce gene expression (Naar *et al.*, 1991; Umesono *et al.*, 1991). When separated by three base pairs, direct repeat, palindromic and inverted palindromic arrangements of the TGACC motif gave transcriptional responses to retinoic acid, oestrogen and thyroid receptors respectively (Naar *et al.*, 1991). Another group found that direct repeats separated by 3, 4 and 5 bases gave responses to vitamin D, thyroid hormone and retinoic acid respectively (Umesono *et al.*, 1991). Unlike the perfectly palindromic TRE used in this thesis and other studies response elements which conform to these consensus sequences are found in natural genes (Devergne *et al.*, 1991; Munoz-Canoves *et al.*, 1990; Demay *et al.*, 1990; Noda *et al.*, 1990). The ability of retinoic acid and thyroid receptors to transactivate successfully from both palindromic, inverse palindromic

and direct repeats is somewhat surprising. Protein-protein contacts which can occur when a dimer is bound to a palindromic repeat with no intervening bases are unlikely to occur when the two proteins are bound to a direct repeat separated by three bases. Thyroid and retinoic acid receptors can bind as heterodimers and it was initially thought that different combinations of receptor might bind to the various sequences. However, similar heteromeric complexes were identified on all elements (Naar *et al.*, 1991). It is therefore unlikely that the formation of different heterodimers on these elements explains how the receptors interact on different sites. These authors suggest receptor orientation changes with altered arrangement of DNA binding motifs. This implies either a flexible C-terminus which acts independent of DNA binding domain orientation, or the presence of multiple dimerisation interfaces. The requirement of the C-terminal 34 amino acids of the thyroid receptor for high affinity binding to the inverted palindrome but not other sites supports the idea that there are multiple dimerisation interfaces (Naar et al., 1991).

In conclusion, it appears that the inability of C-terminally deleted oestrogen receptor to dimerise on a palindromic TRE inhibits DNA binding. Important contacts between sequences in the second zinc finger cannot occur when the spacing of the palindrome is changed. The presence of additional dimerisation sequences in the C-terminus of the full-length receptor is sufficient to restore DNA binding but not transcriptional activity. Whilst the spacing of response element half-sites is important for interactions between DNA binding domains of steroid receptors, other members of the nuclear hormone receptor family are less restricted.

## 7.5 Effects of point mutations on transcriptional activity

Deletion mutagenesis of the mouse oestrogen receptor has mapped the C-terminus of the ligand dependent transcriptional activation function TAF-2 to between amino acids 538 and 552 (Lees *et al.*, 1989). We have therefore investigated whether the adjacent conserved sequences involved in ligand binding and dimerisation might also be involved in transcriptional activity. The receptors chosen for testing included those with mutations in highly conserved amino acids which had little or no effect upon dimerisation and ligand binding. Other mutants were also tested to determine whether impairment of

dimerisation activity *in vitro* would be reflected in a reduction in transcriptional activity in mammalian cells.

All the mutant receptors which were not impaired in dimerisation or ligand binding *in vitro* were transcriptionally active *in vivo*. The levels of transcription induced from an oestrogen responsive promoter by these receptors (eg. Q-510A, L-515E and R-519A) were similar to those induced by wild-type MOR121-599 receptor. In contrast two point mutants which were dimerisation deficient *in vitro* had impaired transcriptional activity. The first (I-518E) gave transcriptional activity of only 30% that detected from the wild-type receptor. In addition to an impairment in dimerisation this mutant receptor was also expressed at lower levels than wildtype receptor in mammalian cells. Thus the reduction in transcriptional activity was unlikely to be due to a direct effect on TAF-2. The effect of the second mutant (L-513K) was more dramatic, reducing transcriptional activity to less than 5% that of the wildtype receptor. The level of *in vitro* DNA binding by L-513K was very low even in the presence of oestradiol, placing it on the borders of the conditional and non-functional classes (Chapter 3). This mutation interfered strongly with the ability of the receptor to dimerise. Moreover, when this receptor was expressed in COS-1 cells, oestradiol was unable to induce detectable levels of dimerisation and DNA binding. These data suggest that L-513K is not a mutation which affects TAF-2 directly but that the reduced transcriptional activity reflects the inability of oestrogen to induce dimerisation *in vivo*. They also indicate that the formation of stable dimers is required for transcriptional activity *in vivo*.

Despite the conclusion that dimerisation is necessary for transcription *in vivo* we have identified mutants (L-511E and L-511R) which fail to dimerise and bind DNA *in vitro* and yet are transcriptionally active *in vivo*. We therefore conclude that these mutants are able to form sufficient dimers *in vivo* to induce transcription. Since this could have been a consequence of high levels of expression of receptor in transient transfection, a series of stable lines were established which expressed mutant receptors at levels similar to those found in breast tumour cell lines. The mutant L-511R was chosen as there was a striking contrast between its low DNA binding activity *in vitro* and full transcriptional activity *in vivo* .

Difficulties were encountered establishing stable lines, particularly those containing wildtype receptor. Initially only three stable lines

expressed the mutant receptor (L-511R) and none expressed wildtype receptor. Since a number of other lines expressing the selectable marker were generated it was possible that these cells had selectively lost the oestrogen receptor DNA or expressed receptor at levels beneath the sensitivity of our assays. Although problems of this nature were apparently not encountered when the mouse glucocorticoid receptor was expressed at high levels in the CHO cell line (Israel and Kaufman, 1989; Hirst *et al.*, 1990) the over-expression of human oestrogen receptor in CHO cells has been more difficult (Kushner *et al.*, 1990). These problems were ascribed to either a 'squelching' effect whereby the receptor was titrating out factors necessary for the transcription of essential genes or a promiscuous activation of CHO genes by the receptor which had a lethal effect. It was possible that the difficulties in expressing the wild-type receptor in the NIH 3T3 cell line were caused by similar effects. To block any toxic effects a second series of stable lines were generated in the presence of an antioestrogen to reduce the activity of the oestrogen receptor. ICI 164,384 was chosen in preference to tamoxifen as partial agonist effects have been reported for tamoxifen in many mammalian cell lines, whereas ICI 164,384 acts as an antagonist in the vast majority of cell lines tested (May *et al.*, 1989; Berry *et al.*, 1990) The only exception to date is the Ishikawa endometrial cell line (Jamil *et al.*, 1991). Recent work has demonstrated that ICI 164,384 also increases the rate of degradation of the oestrogen receptor *in vivo* (S.Dauvois-submitted; Gibson *et al.*, 1991). By reducing the amount of receptor protein present and inhibiting dimerisation of remaining receptors it was expected the antioestrogen would inhibit transcriptional activity and thereby reduce any deleterious effects of receptor expression. This was only partially successful as when cells were grown with ICI 164,384 one cell line expressing wildtype oestrogen receptor was obtained.

The transcriptional activity of both mutant and wild-type receptor in the stable cell lines was lower than their activities in transient transfection. However, the total activity of L-511R and wildtype receptor was similar in both transient and stable transfections. The varying levels of expression of L-511R in the different stable lines did not seem to correlate with the level of induction of the oestrogen responsive gene. This suggests that there is sufficient oestrogen receptor to induce transcriptional activity even at the lower levels. This is in contrast to studies in which the levels of glucocorticoid regulation in cell lines

correlated with receptor levels (Vanderbilt *et al.*, 1987; Hirst *et al.*, 1990). The range of levels of expression of glucocorticoid receptor were however greater than found in our 3T3 lines and this may account for the difference in correlation. It is clear that the ability of dimerisation deficient receptor mutants to transactivate *in vivo* is not due to overexpression of the protein. The low levels of dimeric receptor present may be sufficient for full transcriptional activity. Alternatively there may be differences in ability of L-511R to dimerise which reflect differences in conditions in an *in vitro* gel retardation compared to those in an intact cell nucleus. Other proteins present *in vivo* may also influence activity of receptor.

In conclusion, it appears that no amino acids in the conserved region important in dimerisation and hormone binding are directly involved in the transcriptional activity of TAF-2. There is now evidence that sequences C-terminal of this region comprise a major part of TAF-2 (Danielian *et al.* - submitted). Since no one exon from the C-terminus has transcriptional activity (Webster *et al.*, 1989) other sequences must also contribute to this function but these do not appear to overlap the region we have mutated. Whilst some mutations which disrupt dimerisation *in vitro* appear to inhibit transcriptional activity *in vivo*, others do not. Variations in the extent to which oestrogen can stabilise dimerisation by mutant receptors *in vivo* appear to account for this difference.

## 7.6 Influence of hormonal ligands on dimerisation and structure of the hormone binding domain.

### 7.6 - 1 Effects of oestrogen and 4-hydroxytamoxifen on dimerisation

Hormone binding is required for the transcriptional activity of steroid receptors *in vivo* (Yamamoto, 1985). It has also been shown using the glucocorticoid receptor that this is due to an inability to bind DNA *in vivo* in the absence of hormonal ligand (Becker *et al.*, 1986). In contrast mouse oestrogen receptor binds to an oestrogen response element *in vitro* in the absence of ligand and incubation with oestrogen does not increase the levels of DNA binding. However, oestrogen does induce an increase in the mobility of receptor/DNA complex in a gel retardation assay. When oestrogen receptor is treated with the antioestrogen 4-hydroxytamoxifen there is no inhibition of DNA binding, but the increase in mobility observed with oestrogen does not

occur. This suggests that changes in the structure of the receptor which may result from oestrogen binding is not induced by 4-hydroxytamoxifen.

Although oestrogen did not influence the level of DNA binding by wildtype receptor it did induce or increase the DNA binding activity of mutant receptors impaired in dimerisation. This implies a role of oestrogen in stabilising the dimerisation of receptors where the formation of dimers is disrupted. Since the wildtype oestrogen receptor dimerises efficiently under the conditions used, no effect of hormone on dimerisation is observed. Evidence that stabilisation of dimerisation by oestrogen also occurs *in vivo* is provided by the transcriptional activity of the dimerisation deficient mutant L-511E in transient and stable transfections. Interestingly, the antioestrogen 4-hydroxytamoxifen also appears to be able to stabilise dimerisation, since it too increased DNA binding of a mutant receptor. Many of the mutations tested replace a hydrophobic residue with a charged residue. Since the dimerisation interface is hydrophobic in nature, it is possible that these ligands are stabilising dimerisation by shielding the disruptive charged residue.

Oestrogen has also been shown by other groups to increase DNA binding by a mutant human oestrogen receptor. The original human oestrogen receptor cloned from the MCF-7 cell line (Walter et al., 1985) contained a point mutation in region E, which converted the glycine at position 400 to a valine. Since this receptor did not bind DNA in the absence of ligand (Tora *et al.*, 1989) it was possible that the mutation affected the ability of the oestrogen receptor to dimerise. The effect of ligand in increasing DNA binding appears to be more important under sub-optimal conditions, such as in buffers which contain magnesium (Brown and Sharp, 1990). In the absence of magnesium the human oestrogen receptor was able to bind DNA in the presence or absence of ligand. However, in the presence of magnesium ions DNA binding became ligand dependent. This stabilisation effect was more pronounced at higher temperatures. Brown and Sharp also confirmed that tamoxifen was capable of inducing DNA binding. Conditions in the cell and gel retardation assay are unlikely to be identical and this may account for the different effects of ligand *in vivo* and *in vitro*. It is possible that one function of ligand *in vivo* might be to stabilise dimerisation.

One explanation for the lack of requirement of hormone for DNA binding *in vitro*, in contrast to the absolute requirement *in vivo*,

involves the interaction between receptor and heat shock proteins. Sequences within the hormone binding domain of the oestrogen receptor are required for interactions with heat shock protein (Baulieu, 1987) but no single region of this domain was sufficient (Chambraud *et al.*, 1990). This suggested that the association between receptor and hsp90 involved multiple weak binding sites. The glucocorticoid receptor expressed in reticulocyte lysate associates with the hsp90 present in that system (Dahlman *et al.*, 1989; Denis and Gustafsson, 1987) and binds hormone with high affinity. It is therefore likely that the oestrogen receptor expressed in the same system is also associated with hsp90. Thus the lack of hormone dependence for DNA binding *in vitro* is unlikely to be due to lack of interaction with hsp90. There is also evidence for the involvement of other proteins, p56 and hsp70, in the receptor/heat shock protein complex (Tai *et al.*, 1986; Sanchez *et al.*, 1990), but this has not been demonstrated with receptors expressed in the reticulocyte lysate system. It is possible that interactions with one of these other components of the heat shock complex may account for differences between the behaviour of the protein *in vivo* and *in vitro*. Heat shock proteins may play more than a simple role as repressors of receptor activity. There is now evidence that the glucocorticoid receptor expressed in a wheat germ lysate, which lacks hsp90, is incapable of binding steroid (Dalman *et al.*, 1990). In addition Picard and coworkers (1990) have shown in a yeast system that oestrogen and glucocorticoid receptors are not functional in the absence of a homologue of hsp90. These data suggest that hsp90 is required to keep receptor in a conformation capable of binding hormone with high affinity. If the receptor expressed *in vitro* were already partly 'transformed' then ligand would not necessarily be required for DNA binding.

We have shown that both oestrogen and 4-hydroxytamoxifen induce DNA binding by a dimerisation deficient oestrogen receptor. These ligands may shield the charged residue introduced from the hydrophobic dimer interface and thus increase dimerisation. The difference in the requirement for oestrogen for high affinity DNA binding *in vitro* and *in vivo* is likely to be due to the different conditions found in a gel retardation assay and an intact nucleus. Investigations into which proteins the oestrogen receptor interacts with in different systems may reveal the source of the differences between *in vivo* and *in vitro* DNA binding.

**7.6 - 2 Action of ICI 164,384**

The work in this thesis has added to the growing body of conflicting data on the action of ICI 164,384. This ligand acts as a pure antioestrogen with only antagonist activity in intact animals (Weatherill *et al.*, 1988; Bowler *et al.*, 1989; Wilson *et al.*, 1990). Our group has previously shown that this antioestrogen inhibits DNA binding by oestrogen receptor expressed in insect cells and proposed that this is due to an inhibition of dimerisation (Fawell *et al.*, 1990b). A similar inhibition of DNA binding has been reported for oestrogen receptor prepared from human and pig uterus (Wilson *et al* 1990). In contrast we found that ICI 164,384 does not affect the DNA binding of receptor translated *in vitro* in a reticulocyte lysate system (Lees *et al.*, 1989). Failure of ICI 164,384 to inhibit DNA binding was also observed when oestrogen receptors were expressed in HeLa cells (Martinez and Wahli, 1989) or isolated from calf uterus (Sabbah *et al.*, 1991). In the latter case the results must be treated with caution since $10^{-8}$M ICI 164,384 was used whereas other groups found that $10^{-7}$M ICI 164,384 was required for inhibition of DNA binding activity.

Surprisingly we found that ICI 164,384 was actually able to restore low level DNA binding by the dimerisation deficient mutant L-511R, translated *in vitro,* in a gel retardation assay. This was not a non-specific effect of incubation with steroid as neither the synthetic glucocorticoid dexamethazone or progestin R5020 restored binding. This result was completely unexpected in view of the inhibitory effect of ICI 164,384 on dimerisation of receptor expressed in *S.frugiperda* cells (Fawell *et al* 1990b). One possible explanation is that, since the mutation L-511R affects both dimerisation and ligand binding activity the structure of the region is altered in such a way that the 7α aliphatic side chain of ICI 164,384 is no longer able to interfere with dimerisation. In the absence of inhibitory effects from the side chain, the ring system of the antioestrogen might increase dimerisation by shielding the charged residue from the dimer interface. It is also possible that the effects of ICI 164,384 on this mutant are not due to an altered dimer interface. This 'pure' antioestrogen may fail to disrupt the dimerisation of any in *vitro* translated oestrogen receptor, but only stabilise dimerisation as do oestradiol and tamoxifen. This is obviously inconsistent with the effect of ICI 164,384 in disrupting dimerisation of receptor expressed in insect cells (Fawell *et al.*, 1990b). An

additional mechanism by which ICI 164,384 acts as an antagonist has now been identified. Recently our group and others have found that this antioestrogen decreases the amount of oestrogen receptor by increasing its rate of turnover (S.Dauvois-submitted; Gibson *et al.*, 1991). It appears that ICI 164,384 targets the receptor to lysosomes for degradation. This effect on receptor levels, in conjunction with inhibition of dimerisation, is likely to account for the pure antagonist effect of this antioestrogen *in vivo*.

An exception to the pure antioestrogen behaviour of ICI 164,384 has been reported for the progesterone receptor gene. Although ICI 164,384 inhibits the expression of the progesterone receptor gene in a breast cancer cell line (May *et al.*, 1989), it induces expression of this gene in an endometrial cell line (Jamil *et al.*, 1991). ICI 164,384 also appears to induce expression of the creatine kinase gene. The expression of this gene is regulated by oestrogen at the level of transcription (Pentecost *et al.*, 1990). It has also been found that in transient transfection there is a significant induction of gene expression in the presence of ICI 164,384 (Pentecost-unpublished data). The work described in chapter six was directed towards finding an explanation of this agonist effect. However, in a series of gel retardation assays carried out *in vitro* the only difference between the binding of oestrogen receptor to a perfect ERE from the vitellogenin A2 gene and the proposed creatine kinase ERE was in the DNA binding affinities. The lower affinity of the oestrogen receptor for response elements which diverge from the consensus 13bp palindrome has previously been noted (Berry *et al.*, 1989), but no differences in the response to antioestrogens were reported. The antioestrogen ICI 164,384 inhibited the binding of the oestrogen receptor to a creatine kinase ERE as efficiently as it inhibited the binding to an A2 ERE. It was a possibility that in the presence of ICI 164,384 the receptor was binding to DNA as a monomer. However, no bands with higher mobility, corresponding in size to a monomeric form of receptor, were observed in the gel retardation assay.

Cooperative binding and synergy between transcription factors are important in the regulation of gene expression. Steroid receptors regulate transcription in synergy with a range of different transcription factors (Strahle *et al.*, 1988; Schule *et al.*, 1988). It was therefore possible that the ability of the oestrogen receptor to mediate transcriptional activity through the creatine kinase promoter in the presence of ICI

164,384 might be due to interactions with other transcription factors. These interactions might take place with the monomeric receptor. Since the creatine kinase ERE is flanked by sequences which fit the SP1 binding site consensus, cooperative binding between oestrogen receptor and SP1 proteins might have allowed the receptor to bind in the presence of ICI 164,384. To test this hypothesis it was necessary to add HeLa cell extract which contains SP1 as this is not present in insect cells. However, addition of HeLa extract to the gel retardation assay did not change the ability of the oestrogen receptor to bind to the oligonucleotide. This was not due to a lack of SP1 binding but cooperative binding was not observed.

There are a number of possible explanations for the inability to observe *in vitro* the agonist effect of ICI 164,384 reported *in vivo*. It is possible that conditions in our *in vitro* assay were not suitable for interactions with other transcription factors which might be required for stabilisation of DNA binding by oestrogen receptor in the presence of ICI 164,384. An alternative explanation might be that a transcription factor other than SP1 is required for oestrogen receptor to bind DNA in the presence of ICI 164,384. An ERE in the vitellogenin B1 gene acts in synergy with an NF-1 site over 200 bases downstream of the ERE (Corthesy *et al.*, 1989). It is therefore possible that a protein binding outside the 60 bp tested may be required for expression of the creatine kinase in response to ICI 164,384. There is also the possibility that the monomeric oestrogen receptor may stimulate transcriptional activity not by binding to an ERE but by interacting with a distinct protein and acting through its binding site. The regulation of a number of genes involves interactions between steroid receptors and members of the leucine zipper family (Schule *et al.*, 1990; Yang-Yen *et al.*, 1990; Jonat *et al.*, 1990; Diamond *et al.*, 1990). Oestrogen receptor and a Fos/Jun heterodimer coactivate the ovalbumin gene promoter through an element containing a half-palindromic ERE which also mediates induction by phorbal esters (Gaub *et al.*, 1990). Surprisingly, direct contact between oestrogen receptors and DNA are not required for this coactivation since a mutant lacking the DNA binding domain induces gene expression as efficiently as the wild-type receptor. In view of the evidence that the antioestrogen ICI 164,384 inhibits dimerisation and subsequently DNA binding (Fawell *et al.*, 1990b), it is possible that the activation of the creatine kinase gene is also independent of DNA binding.

Interactions between the oestrogen receptor and Fos/Jun and other leucine zipper proteins may in part account for narrow tissue specific regulation of hormone responsive genes particularly where no direct contact of receptor with DNA is required. The particular leucine zipper protein present governs whether glucocorticoid receptor induces or represses expression of the mouse proliferin gene (Diamond *et al.*, 1990). It has been found that the ability of Fos to repress glucocorticoid receptor activity is not shared by other highly related proteins (Lucibello *et al.*, 1990) and the Jun proteins which can inhibit oestrogen receptor activity are also limited (Doucas *et al.*, 1991). Variations in the Fos and Jun proteins expressed might explain the lack of ovalbumin expression in cells with functional oestrogen promoter (Deeley *et al.*, 1977; Jost *et al.*, 1978; Dierich *et al.*, 1987)

In conclusion, since the creatine kinase sequences appear to act as a weak but conventional ERE it is possible that the partial agonist effect of ICI 164,384 previously observed may be due to interactions of the oestrogen receptors with other transcription factors. In at least one other system the ability to bind DNA is not required for coactivation of a gene in conjunction with other transcription factors. ICI 164,384, although possibly inhibiting the formation of dimers *in vivo*, may act as a weak agonist by freeing receptor from the heat shock protein complex. This would make the monomer available for interactions with other transcription factors available in some cell systems. This hypothesis could be tested using the mutant L-513K which fails to dimerise *in vivo*.

### 7.6 - 3 Effects of ligands on structure of oestrogen receptor

A series of proteolytic gel retardation assays were performed in order to investigate whether the difference in activity of oestrogen receptors when they are bound either to an oestrogen or antioestrogen was reflected in alterations in receptor structure. The receptors used in this study were expressed in insect cells. The full length receptor specifically bound oestradiol with a dissociation constant similar to that reported for endogenous receptor (Sakai and Gorski, 1984). The relative binding affinities of 4-hydroxytamoxifen and ICI 164384 were also in agreement with previous reports for the mouse and human receptors (Pons *et al.*, 1984; Wilson *et al.*, 1990). On oestradiol binding the receptor expressed in insect cells showed the increase in mobility in a gel retardation assay previously noted with endogenous (Kumar and

Chambon, 1988) and *in vitro* expressed receptors (Lees *et al.*, 1989). More recently the increase in mobility with oestradiol and the lack of a similar down-shift with 4-hydroxytamoxifen has been confirmed in the human oestrogen receptor expressed in *S.frugiperda* cells (Brown and Sharp, 1990).

Oestradiol, 4-hydroxytamoxifen and ICI 164,384 differ markedly in their effects on the DNA binding activity and transcriptional activity of the oestrogen receptor. It might therefore have been expected that a change in the structure of the receptor would be reflected in a change in sensitivity to one or more of the proteases tested. However, no alterations in the structure of the oestrogen receptor were detected by the limited proteolysis and gel retardation described in this thesis. One limitation with the approach used is that not all fragments generated might have been detected. High affinity DNA binding depends upon sequences between residues 507 and 522 (Fawell *et al.*, 1990). Thus any proteolytic fragments which lacked these sequences might have been incapable of binding to DNA with high affinity in a gel retardation assay. Smaller fragments which lack the entire hormone binding domain were able to bind weakly to DNA. These fragments and similar deletion mutants were probably able to dimerise through a weak dimerisation function in the DNA binding domain (Kumar and Chambon, 1988).

Other studies have been made in which receptors from a number of sources were covalently labelled with the oestrogen [$^3$H]-ketononestrol aziridine or [$^3$H]-tamoxifen aziridine, cleaved with proteases and analysed in SDS gels. The proteolytic cleavage patterns obtained with receptor bound to the two ligands using this technique were also indistinguishable (Elliston and Katzenellenbogen, 1988). In contrast, work which analysed proteolytic fragments in a sucrose gradient found that oestrogen increased the rate of degradation relative to that observed with tamoxifen (Attardi and Happe, 1986). Both studies report the same patterns of degradation of receptor complexed with oestrogen and antioestrogen. It is unlikely that the differences in degradation rate observed by Attardi and Happe result from the type of ligand used since we have also used these non-covalently labelling ligand yet find no difference in degradation rate. It appears that any changes in the structure of the oestrogen receptor which occur on ligand binding do not affect the sites available for proteolytic cleavage.

Similar studies on the effects of ligand have been performed on other steroid receptors. The compound RU486 is an antagonist of glucocorticoid and progesterone receptor action in man (reviewed Baulieu, 1985). In the presence of the synthetic progestin R5020 the progesterone receptor migrated more slowly in a gel retardation assay than when treated with the antagonist RU486 (El-Ashry *et al.*, 1989; Meyer *et al.*, 1990). The antiprogestin RU486 is able to stimulate transcription from progesterone receptor N-terminal transactivation domain but not the C-terminal transactivation domain *in vivo* (Meyer *et al.*, 1990). Whilst this ligand appears able to induce DNA binding by the receptor it is incapable of inducing the formation of an active transcriptional activation function in the hormone binding domain. In this respect it appears to be similar in function to the antioestrogen tamoxifen. In a gel retardation assay both antisteroids cause a change in the mobility of their receptor relative to hormone bound receptor. The antioestrogen treated oestrogen receptor migrates with lower mobility than oestrogen treated receptor (Kumar and Chambon, 1988; Lees *et al.*, 1989; Brown and Sharp, 1990) whereas the antiprogestin RU486 causes an increase in progesterone receptor mobility (Meyer *et al.*, 1990). Limited proteolysis experiments performed on the progesterone receptor treated with the progestin ORG 2058 or the antiprogestin RU486 have failed to show any difference in the cleavage of receptor with these ligands (Geier *et al.*, 1990)

Despite the markedly different effects of ligands on the transcriptional activity of receptors *in vivo* and the mobility of the ligand-bound receptors in gel retardation assays, no changes in the proteolytic cleavage of receptors can be detected. Thus changes in structure which occur in the oestrogen receptor appear to be very small and fail to expose or conceal any novel sites for the proteolytic enzymes used. This is in contrast to results obtained with the ACE1 transcription factor where a change in conformation which occurred on copper binding was detected by limited proteolysis and SDS gel analysis (Furst *et al.*, 1988). The modification which may take place and change the mobility of oestrogen receptor in the gel retardation assay could be phosphorylation. It has been proposed that oestradiol binding stimulates the tyrosine phosphorylation of receptor (Auricchio *et al.*, 1987) and that tamoxifen inhibits this activity. The involvement of phosphorylation in activation of the oestrogen receptor has now been demonstrated *in vitro*

(Lahooti *et al.*, 1990a) and *in vivo* (Lahooti *et al.*, 1990b) in MCF-7 cells but there is no further evidence that this involves a tyrosine residue. Hormonal regulation of phosphorylation has also been reported for glucocorticoid and progesterone receptors (Orti *et al.*, 1989; Hoeck *et al.*, 1989; Denner *et al.*, 1990a). Recent work suggests that phosphorylation of progesterone receptor can modulate receptor mediated transcription *in vivo*. since treatment of CV-1 cells with cAMP, a stimulator of protein kinase A, was able to mimic progesterone dependent receptor mediated transcription in the absence of progesterone (Denner *et al.*, 1990b). Studies on other transcription factors demonstrate that phosphorylation can modulate DNA binding activity (Cherry *et al.*, 1989; Manak *et al.*, 1990; Bagchi *et al.*, 1989; Raychaudhuri *et al.*, 1989) and interactions with other elements of the transcription machinery (Gonzalez *et al.*, 1989).

The domain structure of the receptor offers another explanation for the inability to detect changes in structure upon ligand binding. The results described in Chapter 5 may merely be reflecting a very compact structure of the hormone binding domain which is resistant to cleavage. Less structured areas of the receptor such as Region D, the 'hinge' region directly C-terminal of the DNA binding domain would be more susceptible to proteolysis. Before proteolysis within the 250 amino acid hormone binding domain it might be cleaved as a unit. This would be consistent with earlier reports of 27KD 'meroreceptors', highly resistant to degradation which retain the ability to bind hormone (Sherman and Stevens, 1984). These meroreceptors are generated by degradation of a number of steroid receptors with a range of proteases (Birnbaumer *et al.*, 1983; Reichman *et al.*, 1984; Puri and Toft, 1986; Katzenellenbogen *et al.*, 1987). Other transcription factors are also composed of protease resistant domains and proteolysis was used to help define structural domains in a variety of multifunctional proteins. The DNA binding domains of a range of different transcription factors have all been mapped using partial proteolysis (Ptashne, 1986; Sauer *et al.*, 1988; Smith *et al.*, 1984). It appears that division of the protein into several tightly folded, protease resistant structural domains is common amongst both prokaryotic and eukaryotic transcription factors.

In conclusion, there are no significant differences between the proteolytic cleavage patterns of the oestrogen receptor bound to oestradiol, 4-hydroxytamoxifen or ICI 164,384. There appear to be two possible explanations for this. Firstly, the ligand-induced changes in

receptor structure may not affect the proteolytic sites available for cleavage. Secondly, the hormone binding domain may be cleaved as a unit before less accessible sites within the domain. Since receptors are composed of discrete domains this latter hypothesis is more likely to be correct. Alternative biophysical methods are therefore necessary for investigations into the effects of hormonal ligands on the structure of the oestrogen receptor.

As the hormone binding domain of the oestrogen receptor contains a number of different functions it might be expected that one function could influence the activity of another. In fact we have found that the binding of ligand affects either directly or indirectly, dimerisation, DNA binding, and the transcriptional activity of the receptor. The lack of hormone-dependence in DNA binding by wildtype receptor appears to reflect differences between our *in vitro* assay and conditions in intact cells. Since antioestrogens inhibit activity of the oestrogen receptor it is assumed that they must interfere with at least one of the effects of ligand listed. The antagonist effect of 4-hydroxytamoxifen does not result from inhibition of dimerisation or DNA binding. In fact we have found that this antioestrogen can induce DNA binding by a dimerisation deficient oestrogen receptor. The inhibition of gene expression appears to be due to failure to activate TAF-2. In contrast the mechanism by which ICI 164,384 exerts its antagonist activity is less clear. This antioestrogen also fails to induce the formation of TAF-2 but there is a further inhibitory effect on the formation of dimers in some systems. Since DNA binding is not required for oestrogen induction of gene expression in at least one system it is possible that ICI 164,384 allows receptor to interact with other transcription factors as a monomer. There is also growing evidence that an increase in the turnover of receptor accounts for the major inhibitory effects of this antioestrogen.

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