The role of ligand in oestrogen receptor dimerisation and DNA binding

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Thesis presented for the degree of Doctor of Philosophy at the University of London
May 1994
To Mum and Dad,

This thesis is a tribute to the ambition that you instilled in me and to your unfailing loving support which has been, as will always be, beyond measure.

This thesis is dedicated to my parents who have given me far more than can be acknowledged or ever returned, the work was theirs.
All experiments included in this thesis were performed by myself unless indicated otherwise in the text.
ABSTRACT

The oestrogen receptor is a member of the nuclear receptor superfamily which stimulates gene expression in response to oestradiol after recognising and binding to palindromic sequences known as oestrogen response elements. The receptor binds to DNA as a preformed dimer and dimerisation is required to stabilise the receptor-DNA complex. Treatment with the class II antioestrogens has been proposed to inhibit DNA binding activity as a consequence of disrupting the dimerisation of the receptor.

The ability of the class II antioestrogens to inhibit the DNA binding activity of the mouse oestrogen receptor was found to be dependent on the structure of the aliphatic side-chain which characterises this family of ligands. A direct correlation was observed between the antagonism mediated by the pure antioestrogens in vivo and their ability to inhibit the DNA binding activity of the oestrogen receptor in vitro. The antagonists were found to bind to a similar region of the hormone binding domain to oestradiol and the aliphatic side-chain influences both the ability of the ligands to bind to the receptor and subsequently the efficiency with which each compound can antagonise DNA binding.

To study the influence of ligand on the dimerisation of the oestrogen receptor, the hormone binding domain of the mouse protein was expressed as a glutathione S-transferase fusion protein in bacteria and purified to homogeneity. The fusion protein was found to bind agonist and antagonist with a similar affinity to the full length receptor and was subsequently analysed by fast protein liquid chromatography and electron microscopy. The hormone binding domain was observed to dimerise constitutively in vitro and is converted to a monomeric form following treatment with the pure antioestrogen ICI 182,780. This supports the hypothesis that the class II antagonists inhibit the DNA binding of the oestrogen receptor as a consequence of disrupting receptor dimerisation. Using a gel retardation assay, the ability of the antagonists to inhibit DNA binding was found to be sensitive to the inherent stability of oestrogen receptor dimers which appears to differ between different types of cells. As a result, although the pure antioestrogens may prevent the dimerisation of the oestrogen receptor during translation, the ligands may not necessarily disrupt the dimerisation of preformed receptor dimers depending on the strength of the dimerisation interaction.
Acknowledgements

This thesis would not have been possible or so enjoyable without the contributions made by a large number of people. I would like to begin by thanking Malcolm Parker for bravely supervising this fast spoken lass throughout the last three years and for all his guidance, support and inspiration which were invaluable. An enormous thankyou is due to everyone in both the Molecular Endocrinology and Viral Carcinogenesis laboratories for making my time at the ICRF so much fun and most importantly for all their help and friendship. In particular, I wish to express my gratitude to Roger White, Sophie Dauvois and Sue Hoare for their continual support and advice throughout the years. I would also like to acknowledge the help received from other colleagues at the ICRF including Richard Newman who performed the electron microscopy analysis presented in this thesis and to both Richard and Alice Vrielink for patiently introducing me to protein structural techniques during our collaborations. Thankyou to Arnold Coffer and Jane Fellows for all their help and for kindly providing the affinity purified oestrogen receptor, to Anna Florence for preparing large scale plasmid preparations at amazing short notice and to Ian Goldsmith for synthesising the oligonucleotides used in these studies. Thanks also to Steven McGary (Cardiac Medicine Department, National Heart and Lung Institute, London) for analysing the structures of the antioestrogen analogues by computer modelling. This thesis was made possible as a result of continued help from family and friends to whom I am particularly grateful, especially to Kirsten, Allison Butler and Anna Florence for keeping up my spirits in times of need and to Robin, Jane Mitchell, Lenna Cockburn and Samuel Waters for their kindness and laughter during my writing. I must thank especially my family in London, Jacqueline Taylor and Steven McGary for giving me a home with all the kindness and support which went far beyond the call of friendship. Finally and most dearly, I thank my fiancé for all the support and understanding which made an impossible dream become a reality.
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Antisera
Ligands

BUFFERS
Buffers
Bacterial media and plates

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Transformation of competent bacteria
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Abbreviations

Amp\(^R\)  ampicillin
ATP  adenosine 5' triphosphate
bp  base pair
BSA  bovine serum albumin
dATP  2' deoxyadenosine 5' triphosphate
DTP  2' deoxycytosine 5' triphosphate
dGTP  2' deoxyguanosine 5' triphosphate
dTTP  thymidine 5' triphosphate
DBD  DNA binding domain
DCC  dextran coated charcoal
CPM  counts per minute
DEAE  diethylaminoethylamine
DEPC  diethyl pyrocarbonate
DES  diethylstilbestrol
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
D.W.  distilled water
cDNA  complementary deoxyribonucleic acid
DTT  dithiothreitol
E2  oestradiol
EDTA  ethylenediaminetetraacetic acid
EtBr  ethidium bromide
ER  oestrogen receptor (h=human, m=mouse)
ERE  oestrogen response element
EtOH  ethanol
FPLC  Fast Protein Liquid Chromatography
GR  glucocorticoid receptor
GRE  glucocorticoid response element
GST  glutathione S-transferase
Hepes  N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HBD  hormone binding domain
HRCD  horseradish colour development solution
hsp  heat shock protein
ICI 164,394  N-n-butyl-11-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)-N-methylundecanamide
ICI 169,784  N-n-butyl-11-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7β-yl)-N-methylundecanamide
ICI 163,964  N-n-butyl-11-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)-undecanamide
ICI 165,801  N-n-decyl-5-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)-pentanamide
ICI 165,375  N-n-butyl-15-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)-pentadecanamide
ICI 165,889  N-n-methyl-8-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)-octanamide
ICI 182,780  7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]oestra-1,3,5,(10)-triene-3,17β-diol
ICRF  Imperial Cancer Research Fund
IPTG  isopropyl-β-D-thiogalactoside
kb  kilobase pair
kD  kilodalton
Kd  dissociation constant
LTR  long terminal repeat
MeOH  methanol
MMTV  mouse mammary tumour virus
NMR  nuclear magnetic resonance spectroscopy
NP40  nonidet P40
4-OH  4-hydroxy
O.D.x  optical density at wavelength x
4-OHT  4-hydroxytamoxifen
PAGE  polyacrylamide gel electrophoresis
PBSA  phosphate buffered saline A
PCR  Polymerase Chain Reaction
PMSF  phenyl methylsulfonyl fluoride
PR  progesterone receptor
PRE  progesterone response element
RAR  retinoic acid receptor
RARE  retinoic acid response element
RBA  relative binding affinity
RNA  ribonucleic acid
mRNA  messenger RNA
RNase  ribonuclease
RXR  retinoid X receptor
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<tr>
<td>S</td>
<td>sedimentation coefficient</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
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<td>TEMED</td>
<td>tetramethylethylenediamine</td>
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<td>TR</td>
<td>thyroid hormone receptor</td>
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<td>TRE</td>
<td>thyroid hormone response element</td>
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<tr>
<td>tk</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
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<td>U.V.</td>
<td>ultra violet</td>
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Chapter 1

Introduction
Identification of steroid hormone receptors

Multicellular organisms have evolved with an increasing number of specialised cell types that communicate with one another to regulate their development into organised tissues and to coordinate biological functions. Cellular communication mediated by chemical secretion has been shown to occur locally and via transport in the bloodstream to tissues distributed throughout the organism. Such local chemical communication is referred to as paracrine signalling while communication between cells in more distant tissues involves a number of processes that are collectively referred to as the endocrine system. The chemical mediators which contribute to the latter class of communication include the hydrophobic steroid and thyroid hormones. These are secreted into the blood where they are solubilised by binding to specific carrier proteins and transported to different tissues in the body (King and Mainwaring 1974). Since hormones are lipophilic they are thought to enter target cells by diffusion following their release from the carrier protein. Once inside the cell, they are bound by specific proteins known as receptors which function as transcription factors to influence the expression of hormone responsive genes in the cell nucleus (reviewed in Funder et al 1993).

Many developmental and physiological processes are known to be regulated by steroid hormones, all of which are derived from cholesterol. For example, the gonadal hormones control the growth, differentiation and function of vertebrate reproductive tissues in addition to other tissues such as the bone, skin, pituitary and hypothalamus (reviewed in Jensen 1991). Steroid hormones appear to enter target cells by diffusing across the plasma membrane and yet their responses are restricted to specific tissues. Early studies at the beginning of the 1960's demonstrated that oestradiol is efficiently retained in responsive tissues, such as the uterus and vagina, in comparison to oestrone which is a weak oestrogen metabolite (Glascock and Hoekstra 1959, Jensen and Jacobson 1962). This uptake was followed using tritiated ligands and could be blocked by the anti-oestrogens clomiphene and nafoxidene providing the first indication that cells in steroid hormone responsive tissues may selectively retain specific hormones by virtue of an intracellular receptor (Emmens et al 1962).

The presence of steroid receptors was supported by subsequent fractionation studies which used radiolabelled ligands to follow the sequestration of hormones within the cell. Two types of oestrogen receptor complex were identified according to their migration in density gradients. A 9S form was detected in the cytoplasm which was found to disappear with the
concomitant formation of a 5S complex in the cell nucleus (Toft and Gorski 1966, Gorski et al 1968, Jensen et al 1968). Similar observations were made for the progesterone, androgen and glucocorticoid receptors (reviewed in King and Mainwaring 1974) and led to the proposal of a two-step model for the conversion ('transformation') of inactive receptor to an activated form in the presence of hormone (Jensen et al 1968).

**Receptor transformation**

The two-step model for receptor transformation suggests steroid receptors are maintained in the cytoplasm in an inactive multimeric complex which is dissociated upon ligand binding to give an 'activated' receptor that can translocate to the nucleus and bind to chromatin. This model is supported by several observations. For instance, the 5S complex remains the only form of the oestrogen receptor which has been detected after extracting receptor from nuclei under high salt conditions (Yamamoto and Alberts 1972, Giambiagi and Pasqualini 1990). This species can be derived from either the 9S or 4S forms of the receptor following treatment with hormone and/or elevated temperature in a process that appears to involve receptor dimerisation (Yamamoto and Alberts 1972, Miller et al 1985b, Linstedt et al 1986, Giambiagi and Pasqualini 1990). In addition, inactive 9S complexes of the progesterone, oestrogen and glucocorticoid receptors have been shown to be unstable *in vitro* and dissociate to a 4S species under conditions of high ionic strength and elevated temperature (Moudgil et al 1985, Sanchez et al 1985, Schuh et al 1985, Redeuilh et al 1987). A large amount of information regarding receptor transformation has been derived from such studies *in vitro* (reviewed in Pratt 1993) but transformation of steroid receptor complexes has similarly been observed in intact cells under physiological conditions (Munck and Foley 1979, Markovic and Litwack 1980, Holbrook et al 1983, Rexin et al 1988a, b and 1992).

The association of receptors with non-hormone binding proteins has been proposed to account for the inactive 9S species (reviewed in Pratt 1992). These large cytoplasmic complexes remained uncharacterised for almost 15 years because of their tendency to dissociate to a smaller 4S form during isolation. However, in the early 1980's inactive forms of the progesterone and glucocorticoid receptors were stabilised with a transition metal oxyanion known as molybdate and purified in the absence of ligand by affinity chromatography (Renoir et al 1982, Housely et al 1985). A major component of both complexes was a 90kd phosphoprotein which was common to molybdate stabilised

Additional proteins of 70, 56, 50, 23 and 14kd have since been detected as components of inactive receptor complexes in crosslinking and immunoassays using conditions that were designed to maintain the 9S complex intact as much as possible (Tai et al 1986, Kost et al 1989, Smith et al 1990b, Sanchez et al 1990a, Bresnick et al 1990, Alexis et al 1992). Interestingly, the 70kd and 56kd components have also been identified as heat shock proteins and correspond to hsp 70 and hsp 56 respectively (Kost et al 1989, Smith et al 1990a, Sanchez 1990). Metabolic labelling and cross-linking studies suggest the core 9S complex may contain two molecules of hsp 90 and one of hsp 56 per molecule of receptor (Mendel and Orti 1988, Rexin et al 1991, Rexin et al 1992, Alexis et al 1992). To date little information is available regarding the stoichiometry of the 50, 23 and 14kd proteins which are reviewed in Smith et al 1990a.

Hsp 70 appears to facilitate interactions between steroid receptors and hsp 90 either during or following translation (Dalman et al 1989, Smith et al 1990b, Scherrer et al 1990). The association of the glucocorticoid and progesterone receptors with hsp 90 has been reconstituted in vitro using rabbit reticulocyte lysate and is dependent on ATP and hsp 70 (Smith et al 1992). Both heat shock proteins have been found to bind directly to receptor hormone binding domains although additional regions appear to participate in the binding of hsp 90 (Denis et al 1988a, Smith et al 1990b, Chambraud et al 1990, Dalman et al 1991, Schlatter et al 1992). In contrast, hsp 56 has been shown to interact directly with hsp 90 (Renoir et al 1990) but since the protein has also been cross-linked to the glucocorticoid receptor this suggests hsp 56 may be positioned in close proximity to both the receptor and hsp 90 in the 9S complex (Rexin et al 1988b, Rexin et al 1991, Alexis et al 1992).

The role of heat shock proteins in receptor function is unclear but formation of the 9S species has been proposed to maintain steroid receptors in an inactive state in the absence of hormone and may influence the folding of the associated receptor (reviewed in Smith and Toft 1993). In the case of the glucocorticoid receptor, binding to hsp 90 appears to maintain the receptor in a conformation that facilitates high affinity ligand binding (Bresnick et al 1989, Nemoto et al 1990). A second hypothesis has been suggested more recently.
based on immunoabsorption experiments with antibodies against hsp 90 and hsp 56. These studies indicate that hsp 56, hsp 90 and hsp 70 may exist in the cytoplasm as an independent complex which is in stoichiometric excess of the steroid receptors (Sanchez et al 1990b, Perdew and Whitelaw 1991). This multimeric complex of heat shock proteins has been suggested to function as a large chaperone (called a ‘transportosome’) that is involved in escorting receptors to the nuclear membrane and facilitating their translocation into the nuclear compartment (reviewed in Pratt 1992 and Smith and Toft 1993).

Receptor localisation

Receptors were initially thought to be cytoplasmic in the absence of hormone and translocated to the nucleus following treatment with ligand based on cell homogenisation and fractionation studies (Gorski et al 1968, Jensen et al 1968). However, when monoclonal antibodies became available for the oestrogen and progesterone receptors subsequent immunocytochemical analyses revealed that the proteins are predominately nuclear even in the absence of steroid (King and Greene 1984, Perrot-Aplanat et al 1985). In contrast, glucocorticoid and mineralocorticoid receptors are predominately cytoplasmic in the absence of ligand and concentrate in the nucleus in the presence of their respective hormones (Fuxe et al 1985, Lombes et al 1990, Farman et al 1991).

The transport of proteins into the nucleus is mediated by large nuclear pore complexes which span the inner and outer nuclear membranes (Akey and Goldfarb 1989, Reichelt et al 1990). These structures contain a central pore through which small proteins of up to 60kd may pass by simple diffusion depending on their conformation (Paine et al 1975, Lang et al 1986). In contrast, larger proteins are usually recognised by virtue of a nuclear localisation sequence and then transported through the nuclear pore complexes by an active process which requires ATP. Nuclear localisation sequences generally consist of short stretches of basic amino acids, the best characterised being that of the simian virus 40 T antigen (reviewed in Kalderon et al 1984, Silver 1991).

Multiple nuclear localisation signals (NLS) have been identified within the glucocorticoid, progesterone and oestrogen receptors (Picard and Yamamoto 1987, Guiochon-Mantel et al 1989, Picard et al 1990, Ylikomi et al 1992). Individually these sequences are insufficient to direct the translocation of an associated protein to the cell nucleus (for examples see Picard et al 1990, Ylikomi et al 1992), with the notable exception of those within the
glucocorticoid receptor (Picard and Yamamoto 1987). In general, receptors possess two sets of distinct nuclear localisation signals which include a conserved hormone independent sequence near the C-terminus of the DNA binding domain and a ligand inducible NLS within the hormone binding domain (reviewed in Dingwall and Laskey 1991).

Hormone inducible nuclear localisation signals appear to have a minor role in a number of steroid receptors including the oestrogen and progesterone receptors since these proteins translocate to the nucleus in the absence of hormone (King and Green 1984, Perrot-Applanat et al 1985). In contrast, a large proportion of ligand free glucocorticoid receptor is found in the cytoplasm suggesting its hormone binding domain may mask the constitutive localisation sequence located close to its DNA binding domain. This masking activity was demonstrated by the ability of the hormone binding domain of the glucocorticoid receptor to repress the constitutive NLS of the oestrogen receptor in a chimera of the two proteins (Ylikomi et al 1992). Since only the hormone binding domain of the glucocorticoid receptor has been shown to mask a constitutive NLS this may reflect differences in the conformation of the receptor hormone binding domains. Alternatively, receptors may interact differently with secondary binding proteins such as hsp 90 whose interaction with the glucocorticoid receptor has been found to differ from that of other steroid receptors (Pratt et al 1987, Chambraud et al 1990).

Recent investigations have used immunofluorescence to determine the location of steroid receptors in intact cells and heterokaryons. These studies have revealed that the oestrogen, glucocorticoid and progesterone receptors are continually shuttling between the cytoplasmic and nuclear compartments of the cell (DeFranco et al 1991, Guiochon-Mantel et al 1991, Madan and DeFranco 1993, Dauvois et al 1993). This mechanism of nuclear localisation may explain earlier observations that oestrogen and progesterone receptors can be detected within the cytosolic fraction following homogenisation (Gorski et al 1968). Furthermore, differences in the localisation of steroid receptors could reflect the ability of the hormone binding domain to repress a constitutive nuclear localisation sequence located near the DNA binding domain (Ylikomi et al 1992) or alternatively the association of receptors with structures in either compartment of the cell. In this respect the thyroid and vitamin D receptors are thought to be retained within the nucleus in the absence of ligand by their tight association with chromatin (Walters et al 1981).
The nuclear receptor superfamily

Complementary DNA (cDNA) for individual steroid receptors has been isolated with the resultant identification of a family of related proteins which are collectively referred to as the 'nuclear receptor superfamily' (for examples see Walter et al 1985, Conneely et al 1986, Green et al 1986, Jeltsch et al 1986, Arriza et al 1987, McDonnell et al 1987, Giguere et al 1988). This family includes receptors for steroid hormones, vitamin D, retinoids and thyroid hormones in addition to an increasing number of 'orphan' receptors for which no ligand or target gene have yet been identified (reviewed in Evans 1988). The glucocorticoid receptor was the first to be cloned using a polyclonal antibody to screen an expression library prepared from a lymphoid cell line (Yamamoto 1985). The amino acid sequence for this receptor was deduced and found to contain a region that is closely related to the product of the avian erythroblastosis viral oncogene erb A. The relationship between this protein and the steroid receptors was confirmed following the isolation of genes for the oestrogen, progesterone, mineralocorticoid and vitamin D receptors (reviewed in Evans 1988). In addition, the product of the viral oncogene was identified as a receptor for thyroid hormone by two independent laboratories (Sap et al 1986, Weinberger et al 1986).

The relationship between members of the superfamily is reflected in the structural organisation of the proteins and their respective genes. Genomic clones for a number of receptors have been analysed revealing a common exon/intron organisation that is highly conserved throughout the superfamily. This suggested different receptors may have evolved from a common ancestral gene (Amero et al 1992, Laudet et al 1992). One hypothesis suggests that genes encoding the oestrogen, thyroid and retinoid receptors may have evolved through the duplication and divergence of a progenitor gene and that these in turn have been duplicated giving rise to genes for the individual receptors. Evidence for this hypothesis is derived from the similar organisation of exons within the receptor genes which have been analysed and the colocalisation of genes for isoforms of the thyroid and retinoic acid receptors on chromosomes 3 and 17 (Ponglikitmongkol et al 1988, van der Leede et al 1992). For instance, each gene is comprised of eight exons, five of which encode the hormone binding domain of the respective protein. In addition, each zinc finger in the DNA binding domain is encoded by a single exon (Huckaby et al 1987, Arriza et al 1987). However, the genes encoding some receptors have been found to differ from this organisation, such as the genes for COUP-TF and the thyroid receptors and with over thirty members of the nuclear receptor
superfamily cloned thus far the evolution of these proteins may be further elucidated (Ritchie et al 1990, Laudet et al 1991).

The isolation of cDNAs for steroid receptors has facilitated a comparison of their amino acid sequences and a dissection of the function of the different receptor domains. Based on the amino acid sequence homology between the oestrogen and glucocorticoid receptors, the proteins have been divided into six regions labelled A-F and particular functions have now been assigned to some of these regions (Krust et al 1986). For example, region C has been shown to be responsible for DNA binding while ligand binding, transcriptional activation and dimerisation functions have been identified in region E. A second transcriptional activation domain has similarly been mapped to the A/B regions of receptors. In general, the proteins have a similar structure which is comprised of a highly conserved central region (region C) that is flanked by a poorly conserved N-terminus (regions A and B) and a relatively well conserved C-terminus (region E) (Krust et al 1986, Amero et al 1992, Laudet et al 1992). The following sections describe the domains within the steroid receptors which are required for ligand binding, DNA binding and transcriptional activation and the manner in which these functions contribute to the ability of receptors to behave as a family of complex transcription factors.

Nuclear receptor DNA binding

The most highly conserved region of the nuclear receptor superfamily is Region C which is 40-90% homologous between different steroid receptors with an even higher degree of homology displayed by each type of receptor in different species (White et al 1987, Evans 1988, Ham and Parker 1989). The role of region C in DNA binding was initially implied by observations that receptors mutated in this region are unable to bind to DNA (Kumar et al 1986, Kumar et al 1987, Kumar and Chambon 1988). Direct evidence was then derived by substituting the DNA binding domains of the human oestrogen and glucocorticoid receptors using a novel strategy that is referred to as 'domain swapping'. The resultant chimeras were observed to bind with high affinity to specific DNA binding sites with a specificity that was determined by the DNA binding domain (Green and Chambon 1987, Kumar et al 1987). Portions of region C from a number of receptors have since been expressed and shown to bind DNA in a manner that is indistinguishable from the full length protein (Freedman et al 1988, Hirst et al 1992, Hu et al 1992, De Vos et al 1993).

Several amino acids in receptor DNA binding domains are invariant throughout the superfamily. These include eight cysteine residues which form
part of a sequence resembling the zinc finger motifs in *Xenopus laevis* transcription factor IIIA (TFIIIA) (Miller et al 1985a, Brown et al 1985, Evans and Hollenberg 1988). The presence of zinc fingers in steroid receptors was suggested by site directed mutagenesis studies which demonstrated that the conserved cysteine residues are required for DNA binding although some could be replaced by histidine without a loss of function (Severne et al 1988, Green et al 1988 references therein). Secondly, analysis of the purified DNA binding domain of the glucocorticoid receptor using EXAFS and visible light spectroscopy indicated that this region could preferentially ligate two zinc ions each held in a tetrahedral arrangement by four cysteine residues (Freedman et al 1988). The three dimensional structures of the DNA binding domains for the oestrogen and glucocorticoid receptors have since been resolved using nuclear magnetic resonance spectroscopy (Schwabe et al 1990, Hard et al 1990) and X-ray crystallography (Schwabe et al 1993, Luisi et al 1991) confirming the presence of zinc finger motifs in the DNA binding domains of these proteins.

The zinc finger motifs of nuclear receptors have been found to differ both structurally and functionally from those in other eukaryotic transcription factors (reviewed in Berg 1990 and Freedman and Luisi 1993). For instance, the metal chelation sites within the receptors are comprised of a zinc ion that is coordinated by four cysteine residues (for examples see Freedman et al 1988, Schwabe et al 1990, Luisi et al 1991) compared with the metal ions in TFIIIA which are tetrahedrally coordinated by pairs of histidines and cysteines (Miller et al 1985a). Analysis of the exon arrangement in the gene for TFIIIA has revealed that most of its zinc fingers are encoded by separate exons suggesting the motif may have been duplicated throughout evolution and that each zinc finger represents a single structural domain (Miller et al 1985a, Tso et al 1986). The zinc finger motifs of the steroid receptors are similarly encoded by separate exons but unlike TFIIIA, two zinc fingers fold to form a single domain which is distinct from the independent structural motifs present in TFIIIA (Ponglikitmongkol et al 1988, Hard et al 1990, Schwabe et al 1993).

The three dimensional structures of the oestrogen and glucocorticoid receptor DNA binding domains have revealed that each zinc finger contains an extended loop between the second and third chelating cysteine residues that is followed by an amphipathic alpha helix which begins after the third cysteine and extends for about ten amino acids (reviewed in Schwabe and Rhodes 1991). Previous 'finger swapping' experiments have demonstrated the N-terminal finger in each receptor monomer is responsible for recognising and distinguishing between different DNA binding sites (Green et al 1988). In
addition, mutagenesis studies have identified three residues in the N-terminal fingers of the oestrogen (Glu, Gly, Ala) and glucocorticoid (Gly, Ser, Val) receptors which are essential and sufficient to discriminate between oestrogen and glucocorticoid response elements (Mader et al 1989, Umesono and Evans 1989, Danielsen et al 1989). These residues are exposed on the surface of the alpha helix in the N-terminal zinc finger and constitute part of a region that is known as the 'P box' in both receptors (Glu-Gly-cys-lys-Ala and Gly-Ser-cys-lys-Val in the ER and GR respectively). This helix has consequently been named the 'recognition' helix and the amino acid sequence of the 'P box' correlates well with the specificity of response element recognition (reviewed in Freedman and Luisi 1993).

The role of the alpha helix in the second zinc finger was suggested from the three dimensional structures of the oestrogen and glucocorticoid receptor DNA binding domains. In each case, the C-terminal helices of two receptor monomers were found to overlap perpendicularly to form an extensive hydrophobic core that provided a rigid support structure which was important in maintaining the globular fold of the DNA binding domain. This structure has been proposed to facilitate the positioning of the recognition helices in the major grooves of the DNA (Luisi et al 1991, Schwabe et al 1993). Other DNA binding proteins, including the prokaryotic helix-turn-helix proteins and members of the eukaryote homeodomain family, have similarly been shown to contact DNA through a recognition helix that is supported by a rigid core structure (Kissinger et al 1990, Otting et al 1990, reviewed in Schwabe and Rhodes 1991). This type of organisation is therefore not restricted to members of the nuclear receptor superfamily but appears to facilitate specific DNA binding by a variety of proteins.

**Glucocorticoid response elements**

Steroid receptors regulate the transcription of a wide variety of genes by binding to specific sites referred to as hormone responsive elements (HRE) either within or upstream of the associated promoters. Response elements were first identified in the long terminal repeat (LTR) of the mouse mammary tumour virus which contains genes that are responsive to glucocorticoids, androgens and progestins (Ringold et al 1977, Grove et al 1980, Pavyar et al 1983). Binding sites for the glucocorticoid receptor were initially located between -40 and -100 of the LTR and were subsequently mapped by deletion analysis throughout this region to two distinct sites between positions -202 and -50bp upstream of the transcription start site (Scheidereit et al 1983).
ability of this region to confer glucocorticoid responsiveness to a heterologous gene was confirmed in transfection experiments (Buetti and Diggelmann 1981, Lee et al 1981, Ponta et al 1985) and the contact sites of the receptor were determined using methylation interference and DNase I footprinting (Payvar et al 1983, Scheidereit et al 1983, Scheidereit and Beato 1984). Specific binding sites for the glucocorticoid receptor have also been identified upstream of genes encoding a number of diverse proteins such as lysozyme (Renkawitz et al 1984), human metallothionein-II A (Karin et al 1984), uteroglobin (Cato et al 1984), rat tyrosine oxygenase (Danesch et al 1987) and rat tyrosine aminotransferase (Jantzen et al 1987). While the position of each site was observed to differ between the various promoters, the contacts made by the glucocorticoid receptor within the DNA binding sites are almost identical to those observed for the MMTV LTR.

Based on the above results, a consensus hormone response element for the glucocorticoid receptor was suggested to be comprised of a 15bp element containing an imperfect inverted repeat of the sequence TGTTCCT separated by three poorly conserved nucleotides (Scheidereit et al 1986). This glucocorticoid response element (GRE) was shown to be sufficient to confer hormone responsiveness to a heterologous promoter in transient transfection experiments (Klock et al 1987). Furthermore, the same DNA sequences in the MMTV LTR were observed to induce transcription in response to glucocorticoids, progestins, androgens and mineralocorticoids (for examples see Cato et al 1986, Darbre et al 1986, Arriza et al 1987, Ham et al 1988) although differences in the contact sites for the glucocorticoid and progesterone receptors have been detected using methylation interference and DNase I footprinting (von der Ahe et al 1986, Chalepakis et al 1988). A single GRE has similarly been shown to be sufficient to confer glucocorticoid, androgen and progesterone responsiveness to a heterologous promoter (Strahle et al 1987, Ham et al 1988).

Oestrogen response elements

Oestrogen response elements were first identified within promoters for genes encoding yolk proteins such as the vitellogenins and ovalbumin which are induced by oestradiol (reviewed in Wahli 1988). *Xenopus laevis* has four homologous vitellogenin genes named A1, A2, B1 and B2 which have been cloned and compared with those of the chicken vitellogenin II gene. Four regions of sequence homology were identified, one of which contained copies of a short palindromic sequence (GGTCAnnnTGACC) that was similarly found
within the promoter for apo-very low density lipoprotein (apo-VLDL) (Walker et al 1984). From these results, the authors proposed the palindromic sequence was a putative oestrogen response element (ERE). The ability of this sequence to confer oestrogen responsiveness to a heterologous promoter was supported by the mapping of similar response elements in sequences upstream of the vitellogenin A2 and B1 genes (Klein-Hitpass et al 1986, Seiler et al 1986). The ERE in the 5' flanking region of the *Xenopus laevis* vitellogenin A2 gene was identified as a single copy of the putative ERE consensus sequence GGTCAnnnTGACC (Klein-Hitpass et al 1986). Furthermore, the response element in the vitellogenin B1 promoter was found to consist of a 33bp sequence containing two 13bp imperfect inverted repeats of the putative ERE (AGTCACTGTGACC and AGTTATCATGACC) (Seiler et al 1986).

Oestrogen response elements have since been located upstream of other genes including those for prolactin (Waterman et al 1988), pS2 (Berry et al 1989) and ovalbumin (Tora et al 1988). The response element in the pS2 gene promoter is a single 13 base pair palindrome which differs from the perfect ERE upstream of the vitellogenin A gene by a single base change in both the spacer and one arm of the palindrome. This element has been observed to confer oestrogen responsiveness to a heterologous promoter but is about four fold less efficient than a perfect ERE (Berry et al 1989) which could reflect the reduced affinity of the receptor for imperfect response elements (Kumar and Chambon 1988). Interestingly, two regions upstream of the chicken ovalbumin gene were shown to be required for maximal induction of transcription which combine to form a strongly inducible promoter. The distal element is comprised of four palindrome half sites (TGACC) which function together as an ERE while the second region is located near the TATA box and consists of two GGTCA motifs (Tora et al 1988, Kato et al 1992).

**Retinoic acid, thyroid hormone and vitamin D response elements**

Response elements for a number of other classes of nuclear receptors have been identified including those for the vitamin D, retinoid and thyroid hormone receptors. It has been proposed that these receptors function by binding to direct repeats of the sequence AGGTCA spaced by 3, 4 or 5 base pairs respectively (Umesono et al 1991). Such DNA binding sites resemble the oestrogen response element which is comprised of an inverted repeat of this sequence separated by three nucleotides (AGGTCAnnnTGACCT). However, the above generalisation has been complicated by the discovery that the thyroid, vitamin D and retinoid receptors can heterodimerise with a protein
known as the retinoid X receptor (RXR).

RXR binds a naturally occurring 9-cis isomer of all-trans retinoic acid while the retinoic acid receptor (RAR) can bind both ligands (Levin et al 1992, Heyman et al 1992). The retinoic acid X receptor can itself bind to DNA as a homodimer and appears to demonstrate a preference towards direct repeats of AGGTCA spaced by either one or two nucleotides (Mangelsdorf et al 1991, Mader et al 1993). Interestingly, a number of naturally occurring retinoic acid response elements have been identified which are comprised of direct repeats of either Pu GGTCA or Pu GTTCA (Pu=purine) separated by 1, 2, 4 or 5 base pairs (for examples see Leid et al 1992, Mader et al 1993). It has been suggested that maximal transcription from direct repeats spaced by 1, 2 or 5 nucleotides may be facilitated by heterodimerisation between RAR and RXR (Mader et al 1993).

Interestingly, a number of orphan receptors also appear to heterodimerise with RXR and similarly bind to direct repeats of the sequence AGGTCA (Kadowaki et al 1992, Dryer et al 1992, Keller et al 1993). The chicken ovalbumin upstream promoter transcription factor (COUP-TF I) was one of the first orphan receptors to be identified (Wang et al 1989) and has been shown to bind several GGTCA based response elements (Cooney et al 1992, Kadowaki et al 1992). Both COUP-TF I and a related orphan receptor (ARP-1/COUP-TF II) have been shown to repress transcriptional activation by the vitamin D and thyroid hormone receptors as well as heterodimers of the retinoic acid receptor and RXR (Cooney et al 1992, Kliewer et al 1992a, Tran et al 1992). Since the orphan receptors can heterodimerise with RXR, the observed repression may be mediated by competition for the nuclear receptor binding sites or alternatively through heterodimer formation with the retinoid X receptor (Berrodin et al 1992, Kliewer et al 1992a, Widom et al 1992).

Response element recognition and specificity

Based on the studies described above, members of the nuclear receptor superfamily appear to regulate transcription by recognising and binding to three classes of response element. The first is an inverted imperfect repeat of the sequence TGTTCT which is usually referred to as a glucocorticoid response element but can be bound by progesterone, glucocorticoid, androgen and mineralocorticoid receptors. The second class is recognised by the oestrogen receptor and consists of inverted repeats of a similar sequence, TGACC, which is known as the oestrogen response element. The third class of binding site is related to the oestrogen response element and appears to be comprised of
direct repeats of the sequence AGGTCA. The latter are bound by thyroid hormone, vitamin D and retinoic acid receptors in addition to heterodimers of these proteins with the retinoid X receptor.

The similarity between the oestrogen and glucocorticoid response elements has been demonstrated by the ability to convert an ERE into a GRE by mutating one or two bases in the half sites of the response element (Klock et al 1987, Martinez et al 1987). This relationship is reflected in the structure of the DNA binding domains of the receptors which interact with them. For instance, the critical residues involved in recognising a GRE are identical between the glucocorticoid, progesterone, mineralocorticoid and androgen receptors. Similarly the oestrogen, thyroid, glucocorticoid, vitamin D and retinoic acid receptors have closely related P box sequences (reviewed in Freedman and Luisi 1993).

The nuclear receptors can therefore recognise and bind to different but related classes of response elements. However, the ability of more than one type of receptor to recognise a particular class of DNA binding site necessitates that the specificity of a hormone response be achieved by additional mechanisms in vivo other than the DNA binding specificity of the receptors. This appears to be accomplished by a combination of factors. Firstly, the amount of hormone sequestered in responsive tissues could influence the activity of nuclear receptors which are selectively expressed in different types of cells. Secondly, differences in the affinities of receptors for particular response elements may contribute to the selective induction of hormone responsive genes that may be influenced by the heterodimerisation of receptors as described for the vitamin D, retinoic acid and thyroid hormone receptors. Additional selectivity may result from the DNA environment of the response element through the positioning of nucleosomes near/over hormone response elements or alternatively by receptor induced exposure of binding sites for cell specific factors that are necessary for the induction of transcription (for examples see Philipsen et al 1988, Rigaud et al 1991). Furthermore, cooperative interactions between receptors and tissue specific transcription factors may contribute to the specificity of hormone induction.

Interactions between steroid receptors and cell specific proteins, not necessarily DNA binding factors, have been described. For example, the influence of steroid receptors on gene expression can be either stimulated or repressed by transcription factor AP-1. This protein is a heterodimer of members of the Fos and Jun families which stimulate/repress the induction of transcription by steroid receptors in a cell and promoter specific manner.
(reviewed in Angel and Karin 1991, Shemshedini et al 1991). In the case of the proliferin gene a response element has been identified which can mediate the induction or repression of transcription from a heterologous promoter in response to glucocorticoid depending on the cell type (Diamond et al 1990). Both AP-1 and the glucocorticoid receptor can bind to this response element and altering the ratio of Fos to Jun has been observed to switch the response from repression to stimulation suggesting that the hormone response is mediated by direct interactions between members of the nuclear receptor superfamily and components of AP-1 (reviewed in Miner and Yamamoto 1991, Schule and Evans 1991).

The hormone binding domain

The role of hormone in steroid receptor function is somewhat illusive but has been advanced over the years with the development of a number of synthetic receptor antagonists. These ligands compete with hormone for binding to receptor but do not elicit the normal response thereby inhibiting the effect of hormone on receptor function. Two classes of antioestrogens have been developed whose effects on the function of the oestrogen receptor appear to differ which may reflect the distinct structures of these ligands. Tamoxifen belongs to the class I family of antioestrogens and possesses a triphenyl-ethylene structure which has two rings corresponding to the A and D rings of oestradiol and an alkylamide side chain that is essential for its antagonist activity (Jordan 1984). In contrast, the structures of ligands belonging to the class II family of antioestrogens more closely resemble that of the natural agonist in that each ligand has a steroid ring system which is identical to oestradiol but with an aliphatic side chain that protrudes from position C-7 of the B ring (reviewed in Dauvois and Parker 1993). Both classes of antioestrogen have been shown to compete with oestradiol for binding to the oestrogen receptor suggesting the ligands bind to a similar region of the protein (Emmas et al 1992, Wakeling and Bowler 1992).

The binding site for oestradiol has been mapped to region E of the oestrogen receptor from the analysis of receptor deletion mutants (Kumar et al 1986). A number of studies have attempted to identify amino acids which are required for ligand binding by cross-linking the receptor with covalent agonists and antagonists in vitro. For example, treating the human oestrogen receptor with the oestrogen ketononestrol aziridine or an antioestrogen called tamoxifen aziridine covalently labels cysteine 530 within region E (Harlow et al 1989). Interestingly, this residue can be mutated without effecting the ability of the
receptor to bind agonist or antagonist suggesting that although cysteine 530 is not essential for ligand binding it may be located within/near the hormone binding pocket and therefore able to contact ligand (Reese and Katzenellenbogen 1991a). This is supported by observations that point mutations close to the corresponding cysteine (position 534) in the mouse oestrogen receptor significantly reduce hormone binding although mutation of cysteine 534 itself has no effect (Fawell et al 1990a). Further analysis of tamoxifen aziridine binding using mutants of the human oestrogen receptor, identified cysteine residues at positions 381, 417 and 447 which also appear to participate in ligand binding (Reese et al 1992). Since the four cysteine residues span a region of approximately 150 amino acids this suggested the hormone binding pocket of the oestrogen receptor is produced by the folding of a large protein domain. Similar affinity labelling studies of the glucocorticoid receptor support this suggestion since residues 622, 656 and 754 were found to participate in ligand binding and may form part of the ligand binding site of the glucocorticoid receptor spanning a region of over 130 amino acids (Simons et al 1987, Carlstedt-Duke et al 1988).

The ability of region E to form a structurally independent domain has been suggested by several observations. Firstly, deleting regions A, B and C of the oestrogen receptor generates a truncated receptor that is capable of binding oestradiol (Kumar et al 1986). In addition, irradiation and protease treatment of the receptor produces 25-28kd protein fragments which contain the hormone binding domain and retain the ability to bind agonist and antagonist (Katzenellenbogen et al 1987a, Toulas et al 1991). Similar protease resistant fragments have also been generated for other steroid receptors including the progesterone and retinoic acid receptors suggesting the ligand binding sites of nuclear receptors may be derived in an analogous manner from the folding of a large receptor domain (Allan et al 1992a). Since the homology of the hormone binding domain between members of the nuclear receptor superfamily generally parallels the structural relatedness of the hormones themselves, it is conceivable that conserved amino acids in this region may be involved in forming the structure of the hormone binding pocket while non-conserved residues participate in binding different types of ligand.

In the case of the oestrogen receptor, point mutagenesis studies of the mouse protein (Fawell et al 1990a) have identified a region located towards the C-terminus of region E that is required for dimerisation and ligand binding (between residues 507-518). Amino acids at the N-terminal end of this region were found to be essential for dimerisation while residues in the C-terminus are
required to bind ligand. Interestingly, receptors mutated at positions 525, 521 or 522 in the mouse protein have been found to interact differentially with oestrogen and tamoxifen (Danielian et al 1992). Mutating residues 529 and 531 within the hormone binding domain of the human oestrogen receptor has similarly been found to alter the affinity of the receptor for oestradiol but not 4-hydroxy-tamoxifen (Pakdel and Katzenellenbogen 1992). Thus, a number of residues located towards the C-terminus of the hormone binding domain of the oestrogen receptor appear to participate in discriminating between the binding of agonist and antagonist. A similar observation has also been made for the progesterone receptor by Vegeto and colleagues in that deleting a region at the C-terminus of this receptor was found to abolish the binding of progesterone but had no effect on the binding of the antagonist RU486 (Vegeto et al 1992).

The role of the ligand

The hormone binding domain of the oestrogen receptor has at least two other functions in addition to binding ligand. These include protein dimerisation and transcriptional activation functions as will be discussed in detail in subsequent sections. The transcriptional activation function located within region E of the receptor is referred to as TAF-2 and is dependent on agonist for activity while a second activation function located in the N-terminus of the protein is hormone independent (Kumar et al 1987, Webster et al 1988, Tora et al 1989b, Lees et al 1989). The contribution of each activation domain to transcriptional activation varies between different types of cells and promoters (Kumar et al 1987, Lees et al 1989). It has been suggested that TAF-2 is formed upon binding oestradiol as a result of a change in the conformation of the hormone binding domain which brings elements that are dispersed throughout region E together to form the transcriptional activation domain. This hypothesis is supported by the protease digestion patterns of the oestrogen receptor which have been observed to differ in the presence of oestradiol and 4-hydroxy-tamoxifen suggesting the ligands induce different conformations of the receptor (Banaihmad and Tsai 1993). Interestingly, tamoxifen is unable to elicit the activity of TAF-2 and blocks transcriptional activation of the oestrogen receptor in the presence of oestradiol which may reflect a difference in the conformation of the hormone binding domain complexed with tamoxifen compared with oestradiol (Berry et al 1990).

It is conceivable that ligand binding may also influence the dimerisation and DNA binding activities of the oestrogen receptor since it has been shown that dimerisation of the receptor stabilises its binding to DNA. Several studies
suggest oestradiol may facilitate a general stabilisation of receptor dimerisation in solution and on the DNA which results in an increase in affinity of the protein for its response element (Skafar and Notides 1985, Kumar and Chambon 1988, Sabbah et al 1989, Skafar 1991). There is also considerable evidence to suggest that hormone binding contributes to the regulation of oestrogen receptor DNA binding activity, particularly in vivo where the use of DNA footprinting has identified sites of DNase I protection in chromatin which are only detected in the presence of hormone (Bakker et al 1988, Philipsen et al 1988). Hormone dependent DNA binding activity has also been reported in vitro (Kumar and Chambon 1988, Brown and Sharp 1990) but this remains somewhat controversial since other studies have indicated that hormone-free receptor can bind to DNA (Fawell et al 1990b, Brown and Sharp 1990, Sabbah et al 1991).

**Nuclear receptor dimerisation**

The palindromic nature of steroid hormone response elements suggests the receptors may bind as dimers to their DNA recognition sites. This has been supported by observations that the oestrogen receptor binds weakly to a single ERE half site and to response elements containing mutations in one half site of the palindrome (Kumar and Chambon 1988, Brown and Sharp 1990). Dimerisation of the oestrogen, glucocorticoid and progesterone receptors has also been suggested from DNA binding, dense amino acid labelling and cross-linking experiments (Notides et al 1981, Scholl and Lippman 1984, Miller et al 1985b, Rodriguez et al 1990, Drouin et al 1992). The dimeric nature of the oestrogen receptor has been demonstrated directly in DNA binding experiments in which full-length and truncated forms of the protein were cotranslated and observed to bind as heterodimers to an ERE (Kumar and Chambon 1988, Fawell et al 1990a). More recently a number of studies have indicated that the oestrogen and progesterone receptors may exist as dimers in solution prior to binding to their response elements in vivo. In these experiments the "wild type" receptors were observed to rescue the nuclear uptake of mutant receptors which were defective in their ability to translocate to the nucleus. This restoration in nuclear uptake is thought to result from interactions between the receptors which enable the translocation deficient mutant to be transported across the nuclear membrane (Guiochon-Mantel et al 1989, Guiochon-Mantel et al 1991, Ylikomi et al 1992, Dauvois et al 1993). In the case of the progesterone receptor such protein interactions have been shown to occur between the hormone binding domains of the receptors
Mutagenesis studies of the human oestrogen receptor indicate that the major dimerisation interface is positioned within the hormone binding domain (region E) while a second weaker dimerisation function is located within the DNA binding domain. Further studies have identified a portion of the dimerisation interface in the hormone binding domain of the mouse protein between residues 478-521 and a peptide encompassing these residues was shown to be sufficient to restore DNA binding activity to a mutant receptor which was defective in its ability to dimerise (Fawell et al 1990a, Lees et al 1990). A comparison of the amino acid sequence of proteins within the nuclear receptor superfamily revealed that a sequence similar to the dimerisation region of the oestrogen receptor is conserved between different receptors suggesting this region may have a similar function in other proteins (Fawell et al 1990a). The corresponding region within the thyroid and retinoic acid receptors has been implicated in the formation of receptor heterodimers (Forman et al 1989, Glass et al 1989).

Amino acids which are critical for oestrogen receptor dimerisation have been shown to overlap residues that are essential for ligand binding as described earlier. Such positioning of the major dimerisation interface at the hormone binding pocket of the receptor suggests that dimerisation may involve a process which is mediated by hydrophobic forces. This is supported by the hydrophobic nature of the hormone binding domain which is apparent from the amino acid sequence for region E (Kumar et al 1986). Furthermore, binding of oestradiol and 4-hydroxy-tamoxifen has been observed to reduce the surface hydrophobicity of the oestrogen receptor at the hormone binding domain suggesting that hormone binding may stabilise dimerisation by a mechanism that involves hydrophobic shielding (Frötzsch et al 1992).

The requirement of ligand for oestrogen receptor dimerisation and DNA binding was initially suggested from studies of the human protein expressed in HeLa cells (Kumar and Chambon 1988). However, the receptor used in these analyses was generated from the original complementary DNA which was later found to contain a mutation at position 400 that decreases the stability of the protein in the absence of hormone (Tora et al 1989a). Subsequent experiments have found no requirement of ligand for DNA binding or dimerisation of the oestrogen receptor in vitro (for examples see Fawell et al 1990a, Sabbah et al 1991). This contrasts with studies of oestrogen responsive genes in vivo which suggest that receptor is unable to bind DNA or does so very weakly in the absence of hormone (Bakker et al 1988, Philipsen et al 1988).
The full length oestrogen receptor has been shown to dimerise independently in solution while the DNA binding domain is monomeric and dimerises after binding to a palindromic response element (Redeuilh et al 1987, Kumar and Chambon 1988, Sabbah et al 1989, Schwabe et al 1990, Schwabe et al 1993). Thus, binding of the oestrogen receptor to DNA appears to occur following dimerisation of the protein in solution at least at the hormone binding domain (Redeuilh et al 1987, Sabbah et al 1989). Two regions of the DNA binding domain have been shown to be involved in DNA binding. The first is the 'recognition helix' of each N-terminal zinc finger which become positioned in the major grooves to facilitate specific interactions between residues in the P box and bases in the response element while the second region involves the loop of the C-terminal zinc finger motif. The latter contains a sequence of five amino acids known as the D box which is exposed on the surface of the loop and directs the dimerisation of the DNA binding domains by means of interactions between the corresponding regions of two receptor monomers (Umesono and Evans 1989, Dahlman-Wright et al 1991, Dahlman-Wright et al 1993). This positions the recognition helices of both monomers in adjacent major grooves of the DNA depending on the spacing between the HRE half sites (Tsai et al 1988, Klein-Hitpass et al 1989).

The role of the dimerisation function located in the DNA binding domains of nuclear receptors has recently been demonstrated by comparing the structure of the DNA binding domain of the glucocorticoid receptor on a GRE and a mutant response element in which the spacing between the GRE half sites was increased by one base pair to give a four nucleotide gap. The monomeric DNA binding domains were observed to dimerise on the DNA and bound specifically to the GRE with each subunit positioned in adjacent major grooves. In contrast, although the DNA binding domains could dimerise on binding to the mutant response element, the subunits were positioned with one contacting a half site and the other making nonspecific contacts with a non-cognate sequence (Luisi et al 1991). In this manner, dimerisation of the DNA binding domains enables different spacings between the HRE half sites to be distinguished while the recognition helix of each receptor monomer discriminates between the related half site sequences. Interestingly, the above results also indicate that the interactions between receptor monomers are stronger than the contacts made between the nuclear receptor and its response element.

Thus, high affinity DNA binding of the oestrogen receptor and other steroid receptors appears to be dependent on both the palindromic nature of
the response element and the dimerisation functions located in the DNA binding domain and at the C-terminus of the protein. In this manner of DNA binding, the steroid receptors resemble certain prokaryotic regulatory DNA binding proteins such as the lambda repressor which binds to its palindromic response elements as a dimer using α-helical regions of the protein for DNA recognition. The N-terminal domain of this protein has been shown to contact the DNA and contains a weak dimerisation interface that is not sufficient for formation of stable dimers in solution. Furthermore, the C-terminal domain of the repressor contains a stronger dimerisation interface which facilitates the formation of stable protein dimers in solution (reviewed in Takeda et al. 1983, Pabo and Sauer 1984).

**Hormone induction of gene expression**

The mechanism by which steroid receptors stimulate transcription appears to be mediated by a number of processes which may combine to stimulate gene expression in response to hormone in vivo. Potential mechanisms include a receptor mediated change in the structure of chromatin over promoters for hormone responsive genes. Such an alteration in the organisation of chromatin could facilitate the exposure of DNA binding sites for other activators leading to the formation of the initiation complex. Another possibility is that receptors stimulate initiation complex formation by interacting with basal transcription factors either directly or through intermediate proteins (called 'adaptors') which may not necessarily bind to DNA (reviewed in Ptashne and Gann 1990, Roeder 1991).

**Steroid receptor transcriptional activation functions**

The analysis of mutant and chimeric transcription factors in transient transfection and in vitro transcription assays has identified a large number of proteins containing regions known as activation domains which are essential for stimulating transcription (reviewed in Mitchell and Tjian 1989). The activation domains initially identified were acidic in nature and have been found in a number of proteins including the yeast transcription factors GCN4 and GAL4 (Hope and Struhl 1986, Ma and Ptashne 1987). Other forms of activation domains have also been identified including those of the mammalian transcription factors SP1 and NF-1 which are rich in glutamine and proline residues respectively (Courey and Tjian 1988, Mermod et al. 1989). The transcriptional activation functions of steroid receptors have similarly been identified by studying the activity of deletion mutants and chimeric proteins.
containing fragments of receptors fused to the DNA binding domain of a heterologous transcription factor. The oestrogen receptor has been shown to contain two regions which are important for transcriptional activation, a hormone independent function located in the N-terminal A/B regions of the protein and secondly a hormone dependent activity that is located in the C-terminus within region E (Kumar et al 1987, Webster et al 1988, Lees et al 1989a, Tora et al 1989b). These transcriptional activation functions are referred to as TAF-1 and TAF-2 respectively.

The sequences which are important for oestrogen receptor transcriptional activation are beginning to be identified. Chimeric proteins were analysed containing each of the five exons of the hormone binding domain (region E) fused to the DNA binding domain of yeast transcription factor GAL4. Individually the exons encoding the hormone binding domain were found to be insufficient for transcriptional activation suggesting TAF-2 may be comprised of segments throughout region E which are brought together in the three dimensional structure of the receptor after binding oestradiol. However, sequences important for transcriptional activation have since been mapped at the C-terminus of the mouse oestrogen receptor some of which are conserved between members of the nuclear receptor superfamily and are necessary for hormone dependent transcriptional activation by both the oestrogen and glucocorticoid receptors (Lees et al 1989a, Danielian et al 1992).

The activities of the activation functions in the oestrogen receptor are dependent on both the cell type and the nature of the promoter used in transient transfection studies. For instance, deleting part or all of the N-terminal activation function (TAF-1) generates a mutant receptor which is able to stimulate transcription in HeLa cells from the *Xenopus* vitellogenin A2 gene promoter but has a fifth to a tenth of the activity of the full-length receptor from a reporter containing the promoter for the pS2 gene (Kumar et al 1987, Lees et al 1989a). In contrast, truncation of the human oestrogen receptor to remove the hormone dependent TAF-2 domain results in the production of a mutant which is constitutively active but has only 5% activity compared to the full length protein in either HeLa or COS cells (Kumar et al 1987). The same mutant was approximately 60% active compared to the intact receptor in yeast and chicken embryo fibroblasts (White et al 1988, Tora et al 1989b). Furthermore, the corresponding deletion in the mouse oestrogen receptor produces a mutant with only 3% the activity of the full length protein in 3T3 cells (Lees et al 1989a).

The constitutive activity of TAF-1 has been proposed to account for the partial agonist activity which has been observed for the class I antioestrogens
on oestrogen receptor function in vitro. Both tamoxifen and 4-hydroxytamoxifen inhibit the stimulation of transcription by the oestrogen receptor in the presence of oestradiol but in the absence of steroid they have been found to behave as agonists in some situations. The class I antioestrogens permit the binding of the oestrogen receptor to DNA thereby facilitating the stimulation of transcription in systems where TAF-1 contributes most to transcriptional activation. However, at gene promoters on which TAF-2 contributes most activity these ligands would be expected to act predominantly as antagonists of transcription (Berry et al 1990). Interestingly, mutating residues at positions 525 and/or 521 or 522 of the mouse protein was observed to abolish the ability of the protein to bind oestradiol and stimulate transcription. In contrast, these mutants could bind 4-hydroxytamoxifen and retained the partial agonist activity that is exhibited by the wild-type receptor in the presence of this antioestrogen (Danielian et al 1992). Thus, residues at the C-terminus of the oestrogen receptor appear to participate in discriminating between the binding of agonist and antagonist thereby influencing the activity of the receptor.

Both activation domains of the oestrogen receptor have been functionally compared with the acidic activation domain of VP16 in chimeras containing the DNA binding domains of either GAL4 or the ER. The ability of the fusion proteins to cooperate with themselves (homosynergise) or each other (heterosynergise) to stimulate transcription was assessed using reporters containing two GAL4 sites, two EREs or one of each DNA binding site. All three activation domains were unable to heterosynergise while the activation domains of VP16 and TAF-2 (but not TAF-1) of the ER were observed to homosynergise. These results suggest that the transcriptional activation domains of the oestrogen receptor are distinct from one another as well as the acidic activation domain of VP16 (Tora et al 1989b).

In contrast to other steroid receptors the activation functions in the glucocorticoid receptor may be similar to those of the acidic activators (Tasset et al 1990). The activation function within the hormone binding domain of the human glucocorticoid receptor has been mapped to residues 515-550 and is referred to as τ2 (Webster et al 1988, Hollenberg and Evans 1988). A second transactivation domain has also been mapped within the N-terminus of the rat and human proteins and named enh2 and τ1 respectively (Godowski et al 1988, Hollenberg and Evans 1988). However, to avoid confusion the N-terminal activation domain and the activation domain in the hormone binding domain of the glucocorticoid receptor shall be referred to in this thesis as TAF-1 and TAF-2 respectively. Additional sequences have also been found within the DNA
binding domain of the glucocorticoid receptor that can constitutively stimulate transcription. However, no such activation region has been identified in the DNA binding domains of other steroid receptors (Miesfield et al 1987).

The transcriptional activation domains of the glucocorticoid receptor have been proposed to resemble the acidic activation functions which were described earlier (Hollenberg and Evans 1988). This is supported by studies of deletion mutants of the receptor which display an inverse relationship between their net positive charge and their ability to stimulate transcription (Danielsen et al 1987). In addition, the N-terminal activation region of the receptor can be functionally replaced by an amphipathic alpha helix in a manner analogous to yeast GAL4 (Hollenberg and Evans 1988). However, results obtained more recently from transcription interference experiments suggest that the activation domains of the glucocorticoid receptor may contain more than one type of activation function (Tasset et al 1990).

Steroid receptor synergism and transcription initiation

The initiation of transcription is a sequential process in which basal transcription factors are recruited to promoter elements to form a large multimeric initiation complex that includes RNA polymerase II. Some of these transcription factors have been purified or cloned facilitating the elucidation of steps involved in transcription initiation (reviewed in Zawel and Reinberg 1992). The binding of TFIID to the TATA box element has been identified as one of the first steps in the formation of the initiation complex and is often referred to as the ‘template commitment’ step (Sawadogo and Roeder 1985, Van Dyke et al 1989). Binding of TFIID to the TATA box appears to be mediated by TFIIA and is followed by the binding of TFIIB. RNA polymerase II is then recruited to the TFIIA/D/B complex in the presence of TFIIIF before the remaining activities bind sequentially to complete formation of the initiation complex (Breathnach and Chambon 1981, Buratowski et al 1989, Flores et al 1992). Transcription appears to begin with the hydrolysis of ATP to facilitate the phosphorylation of RNA polymerase to an active hyperphosphorylated form and to allow the ATP dependent helicase activity within TFIIF to ‘melt’/unwind the DNA in front of the polymerase (Sopta et al 1989).

The elucidation of the proteins involved in the regulation of gene expression by steroid receptors has been hindered for a number of years by the lack of a suitable in vitro transcription system. However, around the early 1990's receptor mediated induction of transcription was demonstrated in cell free systems for the oestrogen, glucocorticoid and progesterone receptors
Steroid receptors have been shown to stimulate transcription from simple promoters containing only an HRE and a TATA box suggesting they are capable of interacting directly with components of the initiation complex (Klein-Hitpass et al. 1990, Tora et al. 1989b). This is supported by observations of Tsai and colleagues (1987) that a non-DNA binding protein, later identified as TFIIB, can stabilise the binding of COUP-TF to its response element resulting in the activation of transcription. TFIIB has since been shown to interact with the oestrogen, glucocorticoid and progesterone receptors and it has been suggested that this protein may stabilise the interactions of steroid receptors with their response elements leading to the formation of a more stable receptor/TFIIB/TFIID/TFIIA complex on the DNA and subsequently initiation complex formation (Ing et al. 1992). The role of a common intermediary protein in the induction of gene expression by steroid receptors is supported by transcriptional interference ('squelching') studies. Such experiments titrate the ability of two transcription factors to compete with one another for a common component that is required for transcription. In this manner both transcriptional activation functions of the oestrogen receptor have been observed to squelch the transcriptional activation of the glucocorticoid and progesterone receptors and vice versa indicating all three receptors require a common factor to stimulate transcription (Meyer et al. 1989, Tasset et al. 1990).

Sequences upstream of natural genes often contain multiple HREs and transcription factor binding sites which function together as a strongly inducible enhancer referred to as a hormone response unit (HRU). The HREs in such enhancers are generally imperfect and are unable to function on their own to confer hormone responsiveness. For example, the *Xenopus laevis* vitellogenin B1 gene contains three copies of an imperfect 13 base pair oestrogen response element at positions -555, -334 and -314 relative to the start site of transcription. These were cloned in front of a chimeric reporter gene and characterised in MCF-7 cells which are an oestrogen responsive human breast cancer cell line. The individual response elements were unable to confer oestrogen responsiveness to a heterologous promoter while combinations of two or all three palindromes stimulated transcription upon treatment with oestradiol (Martinez et al. 1987). The ability of hormone response units to function as strongly inducible enhancers is thought to be mediated by synergistic protein-protein interactions between homologous steroid receptors or between receptors and a heterologous receptor/protein. Alternatively, cooperative binding of receptors to adjacent response elements may be
involved. Synergism (cooperativity) of transcription factors describes the ability of two or more proteins to stimulate transcription to a higher degree than would be observed if their effects were additive.

Receptor synergism mediated by protein-protein interactions was suggested from studies of the glucocorticoid receptor in which the ability of the receptor to mediate transcriptional activation was assessed using different separations between a GRE and the start site of transcription. Placing a single GRE immediately adjacent to the start site of transcription was observed to be sufficient to confer hormone responsiveness in the absence of a TATA box or additional transcription factor binding sites. This suggested the glucocorticoid receptor could directly mediate formation of the preinitiation complex (Strahle et al 1988, Schule et al 1988a). In contrast, placing the response element several hundred base pairs away from the start site of transcription abolished the ability of the GRE to stimulate transcription even in the presence of a TATA box. However, hormone responsiveness could be restored by an additional transcription factor binding site which could be either a second HRE or a recognition site for a heterologous protein (Strahle et al 1988, Schule et al 1988a). Cooperative protein interactions between the glucocorticoid receptor and a CACCC binding protein have been detected (Schule et al 1988b) and similarly between the oestrogen receptor and another transcription factor known as CTF/NF 1 (Martinez et al 1991). In addition, hormone induction of the MMTV LTR has been observed to involve synergistic interactions between the progesterone and glucocorticoid receptors and NF 1 as well as the ubiquitously expressed Oct-1 (OTF 1) transcription factor (Bruggemeier et al 1991).

Cooperative DNA binding between steroid receptors was first described by Tsai and colleagues (1989) who observed that the affinity of the progesterone receptor for two adjacent HREs was a hundred fold higher than its affinity for a single response element. The glucocorticoid receptor was also found to bind cooperatively to adjacent HREs and this interaction was favoured when the response elements were located on the same face of the DNA helix (Schmid et al 1989). In contrast, cooperativity between the adjacent oestrogen response elements of the vitellogenin B1 gene was observed to be independent of their stereochemical alignment (Martinez and Wahli 1989). However, the distance separating the EREs was found to be important since insertion of 131 base pairs between the two response elements abolished their ability to confer oestrogen responsiveness (Martinez et al 1987). Ponglikitmongkol and colleagues have suggested that the stereochemical alignment of adjacent oestrogen response elements may be required for
cooperative DNA binding when the HREs are located at a distance from the promoter (Ponglikitmongkol et al 1990).

Thus, synergism between receptors and/or additional transcription factors may be achieved through the cooperative DNA binding of a steroid receptor and another protein resulting in an increase in the stability of the receptor-HRU complex formed (Martinez and Wahli 1989, Schmid et al 1989, Tsai et al 1989). This may be influenced by the distance separating the DNA binding sites and/or their stereospecific alignment on the DNA helix if cooperative binding is derived from protein interactions rather than an alteration in the conformation of the second DNA binding site. Secondly, synergism may occur in the absence of cooperative DNA binding via protein-interactions between steroid receptors and additional proteins to facilitate more efficient formation of the transcription initiation complex. This may occur between adjacent receptor and transcription factor binding sites which are positioned close to the start site of transcription or alternatively between a distant HRE and a binding site for a basal transcription factor which is located close to or within the promoter. The latter has been proposed to occur through 'looping out' of the intervening region of DNA (Ptashne 1988).

The role of chromatin in receptor mediated transcriptional activation

The influence of transcription factors on chromatin organisation is poorly understood and most studies investigating transcriptional activation have failed to consider the implications of chromatin on the accessibility of transcription factor binding sites and initiation complex formation. The fundamental structural unit of chromatin is the nucleosome in which 146 base pairs of DNA are wrapped around a protein core containing two copies of histones H2A, H2B, H3 and H4. Binding of histone H1 stabilises the nucleosome and facilitates the packing of nucleosome arrays into higher order structures leading to the formation of a more closely packed structure that can be recognised as a chromosome.

RNA polymerase has been shown to transcribe nucleosomally assembled templates. In some instances the nucleosomes appear to be removed during transcription but in other cases it has been found that the nucleosomes are displaced whilst remaining attached to the DNA (Conconi et al 1989, Lorch et al 1987, Losa and Brown 1987). Once elongation has begun the polymerase can transcribe an assembled template although the enzyme appears to 'pause' at points along the DNA (Losa and Brown 1987, Izban and Luse 1991). In contrast, positioning a nucleosome over promoter sequences...
vitro results in the inhibition of transcription initiation (Losa and Brown 1987, Knezetic and Luse 1986). Evidence that nucleosomes are inhibitory in vivo has come from the construction of yeast strains in which histone synthesis has been arrested. The resultant loss of nucleosome assembly was accompanied by the transcription of many previously silent genes (Han and Grunstein 1988). Thus a modification in the organisation of chromatin could be an important mechanism by which a transcriptional activator is able to mediate formation of the initiation complex.

The influence of steroid receptors on chromatin organisation is poorly understood and has been studied predominately for the glucocorticoid receptor. The effects of glucocorticoid on the chromatin organisation of the MMTV was investigated by exploiting the ability of the viral DNA to adopt a highly reproducible chromatin structure upon integration as a provirus in the host cell genome (Richard-Foy and Hager 1987). At least six nucleosomes become specifically positioned on the LTR of the virus with the core of the second putative nucleosome (nucleosome B) covering the DNA binding sites for transcription factor NF-1 and the glucocorticoid, androgen, and progesterone receptors (reviewed in Hager and Archer 1991). When cells containing this minichromosome are treated with hormone the chromatin structure appears to alter as detected by a change in the accessibility of the DNA to DNase I digestion. Regions of increased sensitivity to DNase I are referred to as hypersensitive regions (HSR) and are generally considered to represent a reduction in the packing of chromatin indicating the position of sites where the structure of chromatin has been altered. In this study the hormone induced HSR was positioned over the region containing the steroid receptor and NF-1 binding sites. Interestingly, addition of glucocorticoids resulted in detectable binding of NF-1 but not the steroid receptor (Cordingley et al 1987, Archer et al 1992). The results suggest the glucocorticoid receptor may induce transcription by modifying the structure of nucleosome B in such a way that the binding site for NF-1 becomes exposed. Alternatively the presence of hormone may allow rapid exchange between the two transcription factors for the HRU by increasing the dissociation rate of the receptor which would also permit multiple rounds of initiation in the continued presence of hormone. The latter is supported by the increase in the dissociation rate of the glucocorticoid receptor which has been reported in the presence of hormone (Schauer et al 1989).

Analysis of the influence of steroid receptors on chromatin organisation has not been restricted to the MMTV. Treatment of the tyrosine aminotransferase gene with glucocorticoids has been observed to result in a
similar chromatin modification near glucocorticoid response elements that facilitates the binding of another transcription factor (HNF 5) without yielding detectable binding of the glucocorticoid receptor (Grange et al 1989). This was not observed upon steroid removal or following treatment with the antagonist RU486 indicating the change in chromatin structure was dependent on the active form of the glucocorticoid receptor (Reik et al 1991). An alteration in chromatin structure over oestrogen response elements has similarly been found to be dependent on hormone. The response element upstream of the chicken vitellogenin II gene was shown to be occupied in vivo only in the presence of oestradiol and correlated with the binding of transcription factor NF-1 to a more distal binding site (Bakker et al 1988, Philipsen et al 1988). In contrast other studies in mammalian cells have suggested the oestrogen receptor can bind to DNA in vivo in the absence of ligand. For example, it has been proposed that the receptor can bind to DNA in the absence of steroid by its ability to repress the stimulation of transcription by a chimeric transcription factor presumably by competing with the protein for an ERE (Tzukerman et al 1990). Furthermore, cross linking studies of the oestrogen receptor have similarly implied that the protein may bind to chromatin in the absence of ligand since similar amounts of receptor were found to associate with DNA in the presence or absence of oestradiol (Wrenn and Katzenellenbogen 1990). The steroid receptors may therefore stimulate transcription by altering the structure of chromatin to increase the accessibility of transcription factor binding sites to promote initiation complex formation and additionally/alternatively by interacting directly or indirectly with components of the initiation complex.

**Antagonists of oestrogen receptor function**

The influence of steroid hormones on gene expression can therefore be mediated by a family of intracellular receptors which function as transcription factors in the presence of their respective ligands. Antagonists have been developed which compete with hormone for binding to receptors and may interfere with the dimerisation, DNA binding and transcriptional activation functions of the proteins. Such ligands have been useful as tools to help dissect the steps involved in receptor activation and the stimulation of transcription. Furthermore, the antagonists have a number of clinical applications including the treatment of hormone dependent cancers and as a method of controlling fertility. The use of antagonists in the former has several advantages over other treatments such as chemotherapy and endocrine gland ablation particularly because the effects of the ligands are generally restricted.
to hormone responsive tissues. Another advantage is that normal biological function can be restored to responsive tissues following treatment by withdrawal of the antagonists compared with the permanent effects that are imposed by gland ablation.

The best characterised antagonists to date are probably the antioestrogen tamoxifen and the antiprogestin/glucocorticoid RU486 which seem to interfere with the function of their respective receptors in a similar manner. These ligands do not disrupt the dimerisation or DNA binding activities of their receptors but have been found to inhibit the ability of the proteins to stimulate transcription (Berry et al 1990, Brown and Sharp 1990, Webster et al 1988, Lees et al 1989b, Meyer et al 1990). This may be a consequence of the conformation that is adopted at the hormone binding domain of the oestrogen or progesterone/glucocorticoid receptors in the presence of antagonist which may not facilitate formation of the hormone dependent transcriptional activation domain (Allan et al 1992a, Allan et al 1992b). However, both tamoxifen and RU486 have been found to behave as partial agonists since each ligand displays some agonist activity when examined by itself. Chambon and colleagues (Berry et al 1990, Meyer et al 1990) have proposed that this may be a consequence of the constitutive activation function in the oestrogen and progesterone/ glucocorticoid receptors which can stimulate transcription from promoters where TAF-1 contributes most activity as described earlier.

Tamoxifen is often used for first line endocrine therapy in the treatment of advanced breast cancer and as an adjuvant therapy to treat postmenopausal women following tumour removal. The drug has been effective as an antagonist of breast tumour growth particularly in tumours expressing oestrogen and progesterone receptors but this does not appear to be accounted for solely by an inhibition of oestrogen receptor function. For instance, tamoxifen can also inhibit the ability of epidermal growth factor (EGF) and insulin-like growth factor (IGF) to stimulate the growth of breast cancer cells in the absence of oestradiol and it has been proposed that the antioestrogen may inhibit breast cell growth by stimulating the secretion of a growth inhibitory protein such as transforming growth factor (TGFβ) (Vignon et al 1987, Freiss et al 1990). This is supported by observations that the secretion of TGF-β by breast cells is increased after treatment with tamoxifen (Knabbe et al 1991, Butta et al 1992) and that TGF-β can inhibit the growth of a number of different cell types including those of the breast (Clarke et al 1990, Jordan et al 1990). However, although tamoxifen acts predominantly as an antagonist on breast cancer cells, the ligand has been found to behave as an agonist of
endometrial cell growth (Anzai et al 1989, Jamil et al 1991). Furthermore, the partial agonist activity of the ligand reduces the efficacy of tamoxifen in endocrine therapy. In view of this, other potential antioestrogens have been developed with the aim of synthesising a drug which is devoid of agonist activity.

Members of the class II family of antioestrogens which include ICI 164,384 and ICI 182,780 appear to lack such oestrogenic activity and have been observed to completely block the oestrogen dependent growth of the rat uterus (Bowler et al 1989, Wakeling and Bowler 1992). As a result, the ligands are often referred to as 'pure' antioestrogens because they lack agonist activity in most functional assays (Wakeling and Bowler 1988a, Wakeling and Bowler 1988b, Bowler et al 1989, Thompson et al 1989, Wakeling and Bowler 1992). Interestingly, the influence of these antagonists on oestrogen receptor function appears to differ from that of tamoxifen since it has been reported that treatment of receptor with ICI 164,384 results in an inhibition in the ability of the protein to bind to DNA in vitro and in vivo (Wilson et al 1990, Reese and Katzenellenbogen 1991b, Fawell et al 1990b). Given that amino acids essential for dimerisation and ligand binding overlap in the hormone binding domain of the oestrogen receptor, it has been proposed that binding of ICI 164,384 may allow the aliphatic side chain of the antioestrogen to sterically interfere with amino acids which are required for dimerisation consequently inhibiting DNA binding (Fawell et al 1990b).

During the course of this thesis ICI 164,384 and ICI 182,780 were found to reduce the half-life of the oestrogen receptor in intact cells resulting in a decrease in the cellular content of the protein (Gibson et al 1991, Dauvois et al 1992, Reese and Katzenellenbogen 1992). In addition, the pure antioestrogens have been shown to block the translocation of the receptor to the nucleus in contrast to oestradiol and tamoxifen which stimulate its nuclear uptake (Ylikomi et al 1992, Dauvois et al 1993). It is conceivable that ICI 164,384 and ICI 182,780 may induce a sequence of molecular events by disrupting the major dimerisation interface of the oestrogen receptor which could trigger unfolding of the protein and lead to an increase in its turnover. Understanding the influence of these antagonists on the dimerisation and DNA binding activities of the oestrogen receptor could therefore help to elucidate the mechanism by which the pure antioestrogens inhibit receptor function in addition to improving the current knowledge that is available concerning the role of ligand in the dimerisation of the receptor and its ability to bind to DNA.
Chapter 2

Materials and Methods
MATERIALS

Chemicals

All chemicals and solvents used were of analytical grade and were obtained from either FSA Laboratory supplies, Loughborough, UK or Sigma Chemicals Ltd, Poole, UK with the following exceptions:

- Absolute alcohol: James Burrough (FAD) Ltd, UK.
- Acrylamide: Boehringer Mannheim, UK and National Diagnostics, USA.
- Agarose: FMC Bioproducts, USA.
- Ammonium persulphate: Bio-Rad.
- Ampicillin: Beechams Research Laboratories, UK.
- Amplify: Amersham International PLC, UK.
- Bromophenol blue: Bio-Rad.
- Coomassie Brilliant Blue R-250: Bio-Rad.
- Dithiothreitol: Bio-Rad.
- ECL western blotting detection reagent: Amersham International PLC, UK.
- Gelatin (E1A grade): Bio-Rad.
- Glycogen: Boehringer Mannheim, UK.
- Liquid scintillation fluid (Ultima gold): Amersham International PLC, UK.
- Nonidet P40: BDH Chemicals Ltd, UK.
- RNA cap structure analogue: New England Biolabs Inc., USA.
- [7mG (5')ppp (5') G]: Serva Feinbiochemica GmbH, Germany.
- SDS: Bio-Rad.
- TEMED: Bio-Rad.
- Tween-20: Bio-Rad.

Radiochemicals

All radiochemicals used were supplied by Amersham International Plc, UK.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific activity (Ci/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(^{35}\text{S})] dATP(\alpha)S</td>
<td>400</td>
</tr>
<tr>
<td>[(\alpha)-(^{32}\text{P})] dCTP</td>
<td>3000</td>
</tr>
<tr>
<td>(3\text{H}-\text{Cl}) 164,384</td>
<td>91</td>
</tr>
<tr>
<td>L-[(^{35}\text{S})] methionine</td>
<td>1000</td>
</tr>
<tr>
<td>[2,4,6,7-(^{3}\text{H})] oestradiol</td>
<td>96</td>
</tr>
<tr>
<td>[6,7-(^{3}\text{H})] oestradiol</td>
<td>52</td>
</tr>
<tr>
<td>16(\alpha)-[(^{125}\text{I})] oestradiol</td>
<td>2000</td>
</tr>
<tr>
<td>Z-4-Hydroxy [N-methyl-(^{3}\text{H})]-tamoxifen</td>
<td>71</td>
</tr>
</tbody>
</table>

**Miscellaneous**

- Amino acid mix (minus methionine): Promega, UK.
- Ampholines: Pharmacia P-L Biochemicals, UK.
- Dried milk powder: Boots Plc, UK.
- Eupergit C matrix: Rohm Pharma GmbH, Germany

**Film**

- X-OMAT: Kodak
- RX: Fuji
- 0.45\(\mu\)M filters: Millipore, UK.
- Glutathione Sepharose 4B: Pharmacia P-L Biochemicals, UK.
- Horseradish colour development powder: Bio-Rad

**Markers**

- Prestained SDS-PAGE standards: Bio-Rad
- (Low range Mr 18.5-106kd)
- Molecular weight markers for gel filtration (Mr 12.3-145.9kd): BDH Chemicals Ltd, UK.

**Membranes**

- NA-45 DEAE: Schleicher and Schuell, Germany.
- 0.45\(\mu\)M Nitrocellulose: Schleicher and Schuell, Germany.
- Oligonucleotides: Synthesised by Ian Goldsmith, ICRF.
- pGEX vectors: Pharmacia P-L Biochemicals, UK.
- Protein Dye Reagent Concentrate: Bio-Rad
- Rabbit reticulocyte lysate (nuclease treated): Promega, UK.
- Sequenase™ sequencing kit: U.S. Biochemical Corporation, USA.
- Superose 12: Pharmacia P-L Biochemicals, UK.
**Enzymes**

Restriction enzymes were routinely purchased from New England Biolabs, USA.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Calf Intestinal Alkaline Phosphatase (20u/μl)</td>
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<tr>
<td>DNase I (RNase free, 23u/μl)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>DNA polymerase I (Klenow Fragment, 2u/μl)</td>
<td>New England Biolabs</td>
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<tr>
<td>Deep Vent polymerase (2u/μl)</td>
<td>New England Biolabs</td>
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<tr>
<td>RNase A (10μg/ml)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>RNasin ribonuclease inhibitor (HPRI)</td>
<td>Amersham International PLC, U.K.</td>
</tr>
<tr>
<td>SP6 RNA polymerase (20u/μl)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>T4 DNA ligase (1u/μl)</td>
<td>Gibco BRL, UK.</td>
</tr>
<tr>
<td>Thrombin (120u/mg from human plasma)</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>T7 Sequenase version 2.0 (12u/μl)</td>
<td>U.S. Biochemical Corporation, USA.</td>
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**Cell culture media**

<table>
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<tr>
<th>Media</th>
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<tbody>
<tr>
<td>L-Broth</td>
<td>ICRF media supplies.</td>
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<tr>
<td>PBSA</td>
<td>ICRF media supplies.</td>
</tr>
<tr>
<td>Grace's insect medium (supplemented)</td>
<td>Gibco BRL, UK.</td>
</tr>
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<td>Foetal calf serum</td>
<td>Gibco BRL, UK.</td>
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**Antisera**

<table>
<thead>
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<th>Supplier</th>
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<tbody>
<tr>
<td>Peroxidase conjugated rabbit anti-rat immunoglobulins</td>
<td>Dakopatts, Denmark.</td>
</tr>
<tr>
<td>Peroxidase conjugated goat anti-rabbit immunoglobulins</td>
<td>Dakopatts, Denmark</td>
</tr>
</tbody>
</table>

MP16 is a rabbit polyclonal antibody which was raised by Dr. Steven Fawell of the ICRF Molecular Endocrinology laboratory against a peptide comprised of residues 130-142 of the mouse oestrogen receptor (Fawell et al 1990a). H222, H226 and D547 are rat monoclonal antibodies raised against the human oestrogen receptor. The epitopes for these antibodies are located within residues 463-528, 132-198 and 281-310 respectively (Green et al 1984, Kumar et al 1986). The latter were kindly provided by Dr C. Nolan, Abbot Laboratories, USA.
**Ligands**

ICI 164,384 analogues: ICI Pharmaceuticals PLC, UK.

Oestradiol: Steraloids Incorporated, USA

4-hydroxytamoxifen: Steraloids Incorporated, USA

Ligands were dissolved in absolute alcohol to $10^{-2}$M and stored routinely at -20°C with the exception of DES which was stored at a concentration of $5 \times 10^{-3}$M. To avoid any influence of alcohol on receptor function, the ligands were diluted to give no more than 1% (v/v) EtOH in a functional assay. The ICI 164,384 analogues were kindly provided by Dr A. Wakeling of ICI Pharmaceuticals PLC.

**BUFFERS**

All solutions were prepared using water that had been quartz distilled and deionised (D.W.). Solutions were stored at room temperature unless otherwise stated. Buffer recipes have been written with the pH of the overall solution stated at the end. In instances where the pH of a chemical was adjusted prior to buffer preparation, the pH has been stated directly following its chemical name.

- **Band shift buffer (2x)**
  - 200mM KCl, 80mM Hepes, 2mM DTT, 20% (v/v) glycerol, pH7.4.

- **CAP structure analogue (5x buffer)**
  - 2.5mM ATP, UTP and CTP, 250μM GTP and
  - 2.5mM m7G(5')ppp(5')G (stored at -20°C).

- **CIP buffer (10x)**
  - 0.5M Tris-HCl pH8.5, 1mM EDTA (stored at -20°C).

- **Coomassie Blue**
  - 0.25% Coomassie Brilliant Blue (R-250), 50% MeOH, 10% glacial acetic acid.

- **DEPC D.W.**
  - 0.1% (v/v) DEPC added to D.W., incubated for 18 hours at 37°C and then autoclaved for 15 minutes.

- **Destain**
  - 5% MeOH, 7.5% glacial acetic acid.

- **DCC suspension**
  - 0.025% (w/v) dextran, 0.25% (w/v) charcoal suspended in TE, pH7.4 (stored at 4°C).

- **DNA loading buffer (5x)**
  - 0.25% (w/v) bromophenol blue, 5x TBE, 25% (v/v) glycerol.

- **Gel shift buffer (2x)**
  - 200mM KCl, 80mM Hepes, 2mM DTT, 20% (v/v) glycerol, pH7.4.

- **Glutathione pH8.0**
  - 50mM Tris-HCl, 5mM or 20mM reduced glutathione, pH8.0 (prepared immediately prior to use at 4°C).
GTE 0.05M glucose, 0.025M Tris-HCl pH8.0, 0.01M EDTA.

HRCD solution (from powder) 1mg/ml 4-chloro-1-napthol, 17% (v/v) MeOH, 0.03% (v/v) H₂O₂, 17mM Tris-HCl pH7.5, 417mM NaCl. Prepared immediately prior to use by dissolving 60mg 4-chloro-1-napthol powder in 10ml ice-cold MeOH and addition of this to 50ml TBS containing 60μl of 30% (v/v) H₂O₂.

HRCD solution (from tablets) 0.6mg/ml 4-chloro-1-napthol, 1% (v/v) ethanol, 150mM NaCl, 16mM Na₂HPO₄, 4mM NaH₂PO₄, pH7.3, 0.006% (v/v) H₂O₂. Prepared immediately prior to use by dissolving 2x 30mg 4-chloro-1-napthol (4CIN) tablets in 1ml of 100% ice-cold EtOH. This was added to 99ml PBS followed by filtration through Whatmann 3MM paper and addition of 20μl of 30% (v/v) H₂O₂.

Labelling Mix 1.5μM dGTP, dCTP, dTTP.

Ligand binding buffer 10mM Tris-HCl pH7.4, 1mM EDTA pH7.0, 1mg/ml BSA (stored at 4°C).

Ligation buffer (5x) 250mM Tris-HCl, 50mM MgCl₂, 25mM DTT, 25mM ATP, pH7.5 (stored at -20°C).

NTE (1x) 0.1M NaCl, 10mM Tris-HCl pH7.5, 1mM EDTA pH8.0.

MTPBS 150mM NaCl, 16mM Na₂HPO₄, 4mM NaH₂PO₄, 10mM DTT, 1% (v/v) Triton X-100, 10mM benzamidine, 5μg/ml leupeptin, 40μg/ml PMSF, 5μg/ml pepstatin A.

PBS 150mM NaCl, 16mM Na₂HPO₄, 4mM NaH₂PO₄, pH7.3.

PBSA 140mM NaCl, 2.5mM KCl, 10mM Na₂HPO₄, 1.5mM KH₂PO₄, pH 7.2.

PBST As for PBS but with 1% (v/v) Triton X-100.

Phenol/chloroform Redistilled phenol, equilibrated in TE pH8.0, in a 50:50 mix (v/v) with chloroform (stored in the dark).

Poly [dC][dC] Dissolved in TE to give 1mg/ml by incubation for 10 minutes at 45°C (stored at -20°C).

Protease inhibitors (stored at -20°C).

Aprotinin Dissolved in DW to give a stock of 1mg/ml.
Protease inhibitors (cont) (stored at -20°C).

Bacitracin Dissolved in DW to give a stock of 0.1g/ml.
Leupeptin Dissolved in DW to give a stock of 2.5mg/ml.
Pepstatin A Dissolved in 100% EtOH to give a stock of 2.5mg/ml.
PMSF Dissolved in 100% EtOH to give a stock of 10mg/ml.

Protein loading buffer (4x) 8% (w/v) SDS, 250mM Tris-HCl and 40% (v/v) glycerol were heated to 50°C to dissolve the SDS. This was allowed to cool before adjusting the pH to pH 6.8 followed by addition of 0.1% (w/v) bromophenol blue and 4% (v/v) β-mercaptoethanol.

Repair buffer (10x) 500mM Tris-HCl, 70mM MgCl2, 10mM DTT, pH 7.4 (stored at -20°C).

Restriction enzyme buffers (10x) (all stored at -20°C).

Low salt 100mM Tris-HCl, 100mM MgCl2, 10mM DTT, pH 7.4
Medium salt As for low salt buffer but with 0.5M NaCl.
High salt As for low salt buffer but with 1M NaCl.
Very high salt As for low salt buffer but with 1.5M NaCl.

Sequenase buffer (5x) 200mM Tris-HCl pH 7.5, 100mM MgCl2, 250mM NaCl.

Sequencing loading buffer 80% (v/v) deionised 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% (w/v) sucrose, 0.1% (v/v) Triton X-100, 50mM EDTA pH 8.0, 50mM Tris-HCl pH 8.0.
T buffer (5x) 200mM Tris-HCl, 30mM MgCl2, 10mM spermidine, 25mM NaCl, pH 7.4. (autoclaved and stored at -20°C).

TBE (10x) 89mM Tris-borate, 89mM boric acid, 2mM EDTA, pH 8.0.

TBS 20mM Tris-HCl, 500mM NaCl, pH 7.5
TE 10mM Tris-HCl, 1mM EDTA, pH 8.0.
Tfb1 buffer 30mM KOAc, 100mM RbCl, 10mM CaCl2, 50mM MnCl2.4H2O, 15% (v/v) glycerol (filter sterilised and stored at 4°C).

Tfb2 buffer 20mM MOPS, 75mM MgCl2, 10mM RbCl, 15% (v/v)
Bacterial media and plates

L-plates
1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.1% (w/v) glucose, 1.5% (w/v) bactoagar.

L-broth
As for L-plates but lacking the bactoagar.

Ψa plates
2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) MgSO₄, 1.4% (w/v) bactoagar, pH to 7.6 with KOH.

Ψb
As for Ψa plates but lacking the bactoagar and filter sterilised before autoclaving.

METHODS

Bacterial transformation

Bacterial strains and their storage

The *E. coli* DH5α strain was used throughout this thesis for the propagation and purification of plasmids and for all work involving the expression of proteins using the prokaryotic GST expression system. Cells were stored routinely in L-broth containing 50% (v/v) glycerol at -20°C. All plasmids described in this thesis carry the β-lactamase gene (Amp<sup>R</sup>) which confers resistance to ampicillin. Bacterial transformants were therefore grown and maintained in media or plates containing 100µg/ml ampicillin.

Preparation of competent bacteria

The method used was that of M. Scott and V. Simanis (unpublished) which was derived from Hanahan 1983. Bacteria were streaked out on a Ψa
plate and grown at 37°C until colonies of approximately 2mm in diameter were obtained. Single colonies were then inoculated into 5ml Ψb (glassware prewashed with Ψb) and grown at 37°C for approximately 3 hours until an O.D.550 0.3a.u. was derived. This was subcultured into 100ml of pre-warmed Ψb (glassware prewashed with Ψb) and grown for 2-3 hours with continuous shaking at 37°C until the O.D.550 reached 0.48 a.u. Cells were then transferred to 50ml falcon tubes, chilled on ice for 5 minutes and pelleted by centrifugation at 4000xg for 10 minutes at 4°C. The cell pellet was gently resuspended in 2/5 original volume of ice cold Tfb1 buffer and incubated for 5 minutes on ice before being respun at 4000xg for 10 minutes at 4°C. The resultant pellet was resuspended in 1/25 original volume of chilled Tfb2 buffer and the cell suspension snap frozen on cardice as 300μl aliquots. The cells were then stored routinely at -70°C.

Transformation of competent bacteria

Competent cells were thawed and incubated on ice for 10 minutes prior to addition of DNA. Typically 50μl of cells were added to 20μl chilled DNA solution and incubated for 30 minutes on ice (maximum DNA concentration 0.5ng/μl cells). Cells were then heat shocked by incubation for 90 seconds at 37°C and allowed to recover at room temperature for 40 minutes following addition of 200μl L-broth. Cells were immediately spread on L-plates containing 100μg/ml ampicillin which were inverted and grown overnight at 37°C. Competent DH5 cells typically gave 10⁶-10⁸ bacterial colonies per μg of supercoiled DNA. (Unpublished method by M. Scott and V. Simanis).

Preparation of plasmid DNA

Small scale preparation

The method of plasmid preparation described below is often referred to as the 'boiling' method (or the 'mini-prep' method) and was used to prepare approximately 5μg of plasmid DNA for direct double stranded sequencing, diagnostic restriction enzyme digestion and to screen bacterial colonies following transformation (Del Sal et al 1988). A single colony from an L-plate was inoculated into 5ml of L-broth containing 100μg/ml ampicillin and grown overnight at 37°C with continuous shaking. The following morning 1ml of culture was removed into an eppendorf tube and the cells were pelleted by an 20 second spin in a benchtop centrifuge. The supernatant was discarded and the pellet resuspended in 350μl of STET buffer by vortexing. After adding 25μl
of 10mg/ml lysozyme (in 0.01M Tris-HCl pH8.0) the cell suspension was vortexed and boiled immediately for 40 seconds followed by centrifugation for 10 minutes at room temperature. The pellet was then removed using a toothpick and the DNA in the supernatant was precipitated by the following.; 40µl of 3M NaOAc pH7.0 and 420µl of isopropanol were added to the supernatant which was vortexed and placed on cardice for 10-20 minutes. The mixture was then spun for 20 minutes at room temperature in a benchtop centrifuge and the resultant DNA pellet resuspended in 50µl distilled water. If the DNA was not to be used for sequencing, RNA in the preparation was digested by addition of heat treated RNase A to a final concentration of 1mg/ml.

**Large scale preparation**

This method of plasmid preparation was used to prepare 0.5-5.0mg of plasmid DNA for cloning and *in vitro* translation (Ish-Horowitz and Burke 1981). A 5ml overnight culture was inoculated into 400ml of L-broth containing 100µg/ml ampicillin and grown overnight at 37°C with continuous shaking. The culture was harvested the following morning in 500ml Sorvall bottles by centrifugation at 7000xg for 10 minutes at 4°C. The bacterial pellet was then resuspended in 20ml GTE containing 50mg/ml lysozyme and incubated for 10 minutes at room temperature. To this 40ml of freshly prepared lysis buffer (0.2M NaOH, 1% SDS) was added, the suspension vortexed and incubated on ice for 5 minutes. This was followed by addition of 25ml potassium acetate pH4.8 and incubation for a further 15 minutes on ice with periodic vigorous mixing throughout the incubation. Cell debris was removed by centrifugation at 7000xg for 15 minutes at 4°C followed by filtration through four fold medical gauze.

Plasmid DNA was then precipitated by addition of 52ml isopropanol and incubated for two minutes at room temperature before being centrifuged at 7000xg for 10 minutes at 4°C. The dried pellet was resuspended in 8.5ml T.E plus 0.7ml of 0.5M EDTA pH8.0 by vortexing. To this 237.5µl of 2M Tris base was added to neutralise the mixture. Caesium chloride (10.5g) was then added and dissolved into the mixture by gentle shaking followed by addition of 50µl EtBr (10mg/ml). The resultant solution was transferred to Beckman 5/8' x 3' quick seal tubes using a syringe with a 19 gauge needle and the volume was adjusted to fill the tubes which were subsequently heat sealed and balanced. The tubes were then centrifuged at 64,000rpm for 20-24 hours at room temperature using a 70 Ti rotor and a Beckman L3-50 ultracentrifuge.
The supercoiled DNA fraction could normally be visualised by eye the following morning as a distinct orange/pink band but if yields were lower than a milligram of DNA a short wavelength U.V. light was used for visualisation. The supercoiled DNA was harvested using a syringe with a 19 gauge needle and transferred to a 50ml falcon tube where its volume was adjusted to 5ml with D.W. An equal volume of isobutanol was added to the solution which was vortexed and the solvent phase (upper layer) containing EtBr discarded. This extraction was repeated three times or until the aqueous DNA solution was free from EtBr as judged by visualisation under U.V. light. During the last round the bottom layer was discarded using a syringe and the DNA precipitated from the aqueous phase by addition of 2\(^1/2\) volumes of 100% EtOH followed by centrifugation at 10,000xg for 15 minutes at 4°C. The supernatant was discarded and the pellet washed by addition of 25ml ice cold 70% EtOH and respinning at 10,000xg for 15 minutes at 4°C. The resultant supernatant was once more discarded and the DNA pellet dried in a dessicator. The DNA was then resuspended in 0.25-1.0ml of D.W. or T.E. and the concentration and purity of the solution were determined from the O.D\(_{260}\) and O.D\(_{280}\) values as described by Maniatis et al (1982).

**DNA manipulation and subcloning**

**Agarose gel electrophoresis**

Analysis of DNA by electrophoresis was performed using the method described by McDonnell et al 1977. Agarose 1.0-2.0% (w/v) was dissolved in 200ml 1x TBE by boiling in a microwave oven. The solution was allowed to cool to approximately 50°C before adding EtBr to a final concentration of 1µg/ml. The gel was then poured and allowed to set for 30 minutes at room temperature before being transferred to a two litre flat bed electrophoresis tank and submerged in 1x TBE. Samples in 1x DNA loading buffer were loaded into the gel which was run at 0.5V/cm\(^3\) gel until the DNA fragments were well separated. DNA was visualised using a long wavelength U.V. light box and photographed with a Polaroid camera. A better image was obtained using a shorter wavelength U.V. light source but since this can result in mutation it was only used when the DNA was not required for subsequent cloning. The size of the DNA fragments was determined by comparing their mobility with DNA markers of known molecular weight. Phage \(\lambda\) DNA digested with Hind III or pAT153 vector DNA digested with Hinf I were typically used as markers for this purpose.
Restriction endonuclease digestion

Restriction enzyme digestion of DNA was performed in low, medium or high salt buffer at the appropriate temperature as specified in the suppliers instructions. DNA (2µg) was routinely digested for 2 hours using 3 fold excess of enzyme in a final volume of 20µl. The volume of enzyme did not exceed 10% (v/v) of the final reaction volume. Reactions were stopped by either precipitating the DNA for subsequent cloning or by addition of 5x DNA loading buffer in order to analyse the digestion products by agarose gel electrophoresis. Samples were routinely stored at -20°C after stopping the reaction.

DNA precipitation

An equal volume of phenol/chloroform was added to the DNA solution which was vortexed and spun for 2 minutes in a bench top centrifuge. The aqueous phase (upper layer) was then transferred to a fresh eppendorf and the DNA precipitated by addition of 2½ volumes of absolute alcohol, an equal volume of 5M ammonium acetate and 1/100 volume of 20mg/ml molecular biology grade glycogen. The mixture was vortexed, incubated on cardice for 20 minutes and then centrifuged for 20-30 minutes at room temperature. The resultant supernatant was discarded and the pellet washed by addition of 400µl ice-cold 70% ethanol and respinning for 5 minutes. The supernatant was once more discarded, the DNA pellet dried in a dessicator and then resuspended in distilled water. A bench top centrifuge was used for all spins referred to in this method.

Preparation of vector

2µg vector were typically digested with the appropriate restriction enzyme, precipitated as above and resuspended in 27µl D.W. The terminal phosphates were then removed from the digested DNA using alkaline phosphatase in order to prevent self-ligation during cloning as described below.

Alkaline phosphatase treatment of DNA

DNA was treated with alkaline phosphatase in a final volume of 30µl by addition of 3µl 10x CIP buffer and 1µl calf intestinal alkaline phosphatase (20units/µl) to 27µl DNA solution. The sample was then incubated for one hour at 37°C to remove 5' terminal phosphates from DNA fragments with a 5' overhang. Samples containing DNA fragments with a recessive overhang were initially incubated for 15 minutes at 37°C followed by addition of a further unit
Figure 2.1. Polymerase Chain Reaction Primers

(A)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHBD 330</td>
<td>5'-CGGGATCCGAATTCCATGACCAGATCTATTCTGAATATGATC-3'</td>
</tr>
<tr>
<td>NHBD 341</td>
<td>5'-CGGGATCCGAATTGAATGCAGCTTCAGTGACGCTCAATGAT-3'</td>
</tr>
<tr>
<td>NHBD 366</td>
<td>5'-CGGGATCCGAATTCCATGACCAAGAGAGTGCCAGGTTC-3'</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHBD 434</td>
<td>5'-GAACCGACTTTGACGATGC-3'</td>
</tr>
</tbody>
</table>

The PCR primers were designed in order to synthesis a DNA fragment encoding a portion of the mER hormone binding domain between the starting amino acid (330, 341 or 366 as indicated following "NHBD") and residue 434. The resultant DNA contained a Bgl II site which was unique in the mER coding sequence and was used for subsequent cloning steps. The sequences of the coding strand primers are outlined in (A) while that of the noncoding strand primer is shown in (B).
of enzyme and incubation for 45 minutes at 55°C. The DNA was recovered by precipitation and resuspended in D.W. to give a concentration of 20ng/μl for ligation.

**Polymerase chain reaction (PCR)**

DNA fragments encoding regions of the mER hormone binding domain were synthesised by PCR using Vent DNA polymerase in 100μl reactions containing either 1ng, 10ng or 100ng of template as outlined below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x Vent buffer</td>
<td>(supplied by Biolabs)</td>
</tr>
<tr>
<td>Vent DNA polymerase</td>
<td></td>
</tr>
<tr>
<td>Template pSP6 MOR 1-599</td>
<td>(White et al 1987)</td>
</tr>
<tr>
<td>Each of the four deoxyribonucleotides</td>
<td>0.2mM</td>
</tr>
<tr>
<td>Each primer</td>
<td>1.0μM</td>
</tr>
<tr>
<td>BSA</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

The samples were overlaid with mineral oil to reduce their evaporation and then subjected to 25 cycles of incubation for one minute at 94°C followed by a minute incubation at 52°C and two minutes at 70°C. The last cycle was followed by a five minute incubation at 70°C. A tenth of the PCR products were removed into 20μl of 5x DNA loading buffer and their size determined by comparing their mobility with λ DNA/Hind III markers on a 2% agarose gel. The remainder were precipitated and resuspended in D.W. for subsequent cloning.

Four 5' primers were used for PCR each possessing BamHI and EcoRI sites for cloning purposes, an initiating methionine codon and then 18-22 bp of the coding sequence for the mER beginning with the first codon of the hormone binding domain (figure 2.1.). The initiating methionine was positioned within a Kozak sequence to facilitate efficient translation of the hormone binding domain should the PCR fragment be cloned into a mammalian expression vector at a later date. A single 3' primer was used for PCR consisting of the noncoding sequence of the mER between nucleotides 1280-1317. The use of this primer facilitated the synthesis of a unique Bgl II site within the coding sequence of the hormone binding domain for cloning purposes (figure 2.1.).

**Purification of DNA fragments**

DNA was purified for ligation using the method outlined by Dretzen et al 1981. The DNA fragments were usually generated by PCR or restriction enzyme digestion of plasmid DNA and were initially run out on a 1-2% agarose
gel to check their size relative to that of DNA markers of known molecular weight (usually λ DNA digested with Hind III). Fragments of interest were then purified by inserting NA-45 DEAE membrane (pre-soaked for 10 minutes in T.E.) into a slot cut immediately in front of the band of interest in the agarose gel. An additional piece of membrane was similarly placed behind the DNA band in order to prevent contamination by other DNA fragments. The voltage, typically 0.5V/cm³ gel, was reapplied across the gel for 10 minutes allowing transfer of DNA from the gel to the membrane. The DNA was then eluted by removing the membrane into an eppendorf tube containing 250µl 1M NaCl followed by two rounds of alternate incubation at 68°C and vigorous vortexing. The purified DNA was recovered by precipitation and the DNA pellet resuspended in D.W. to give a concentration of 1ng/µl for ligation.

DNA ligation

Ligation was routinely performed using insert DNA that had been gel purified and vector which had been treated with alkaline phosphatase prior to ligation. Typically 20ng vector were incubated in a final volume of 20µl containing 1 unit of T4 DNA ligase, 2µl 10x ligation buffer and 1ng, 2ng or 5ng insert for one hour at 37°C or 2-4 hours at room temperature. The ligation reactions were then stored at -20°C until required for transformation.

DNA sequencing

The recombinant pGEX vectors were sequenced using Sequenase T7 polymerase version 2.0 and 2-5µg DNA. Plasmid DNA was denatured in a final volume of 20µl containing 0.2M NaOH and EDTA by a 20 minute incubation at 68°C. To this, 8µl of 5M ammonium acetate pH5.4 and 100µl absolute alcohol (ice-cold) were added and the DNA was precipitated by incubation for 5 minutes on cardice followed by centrifugation for ten minutes in a benchtop centrifuge. The resultant supernatant was discarded and the pellet was washed by adding 300µl of 70% ice-cold ethanol and respinning for 5 minutes at room temperature. The DNA pellet was then dried for 2 minutes in a dessicator and resuspended in 7µl D.W. To this DNA solution, 2µl of 5x Sequenase buffer (supplied by USB) and 2.5ng primer were added for the sequencing reaction.

The template and primer DNA were annealed and the sequencing reactions performed as directed in the Sequenase (USB) protocol. Regions of DNA 100-200bp from the primer were sequenced using undiluted and 1/20 dilutions of Labelling Mix respectively (see buffers). In contrast, sequencing next to the primer was achieved by doubling the amount of template and primer
in the sequencing reaction and using $\frac{1}{75-1}{100}$ dilutions of Labelling Mix. The primers that were used in the sequencing reactions are outlined below. The sequence of the coding strand primer is identical to that of the thrombin recognition site located upstream of the coding sequence for the mER within the recombinant pGEX vectors. The non-coding strand primer is identical to the non-coding sequence of the mER between nucleotides 1300-1310.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Thrombin site</th>
<th>Coding strand</th>
<th>5'CTGGTTCCGTGGATCC-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-coding strand</td>
<td></td>
<td>mER</td>
<td>nt 1310 1300</td>
</tr>
</tbody>
</table>

**Denaturing gel electrophoresis of sequencing samples**

A 6% continuous denaturing gel was prepared by dissolving 34.5g electrophoretic grade urea into 7.5ml 10x TBE, 11.25ml acrylamide solution (38% acrylamide, 2% bis-acrylamide) and 40ml D.W. by incubating the mixture for approximately 20 minutes at 37°C. The resultant solution was made up to 75ml with D.W. before adding 375μl of 10% (w/v) ammonium persulphate and 90μl TEMED. The gel was immediately poured between two glass plates (20x30cm) separated by 0.25mm spacers and the blunt edge of a comb inserted at the top. The gel was left to set for 1 hour before removing the comb and rinsing the wells with 1x TBE to remove any unpolymerised acrylamide. The sharp toothed side of the comb was then vacuum greased and inserted until the teeth protruded 1-2mm into the top of the gel.

A constant current of 45mA was applied across the gel which was prerun for 20 minutes in 1x TBE. In parallel, samples intended for electrophoresis were incubated for 2 minutes at 80°C and placed immediately on ice. The samples were then loaded onto the gel (2μl/track), the voltage reapplied and the gel run for the time required. Following electrophoresis the gel was fixed for 15 minutes in fix solution (10% v/v glacial acetic acid, 12% v/v methanol) and then transferred to Whatmann 3MM paper. The gel was dried for 30 minutes at 80°C under vacuum and the radiolabelled bands were
visualised by autoradiography using Kodak XAR film. Sequencing of large DNA fragments was improved by addition of 3M sodium acetate pH5.0 during electrophoresis to increase the length of the sequence which could be resolved as described by Del Sal et al 1988.

Protein Synthesis (in vitro)

Expression of the mER in SF9 cells

Oestrogen receptor was expressed in Spodoptera Frugiperda (SF9) insect cells by infection with a recombinant baculovirus (Autographa Californica nuclear polyhedrosis virus, AcNPV) containing cDNA for the mouse oestrogen receptor (Fawell et al 1990b). Cells were grown routinely at 27°C as monolayer cultures on T-175cm³ tissue culture flasks in Grace's medium supplemented with 10% (v/v) foetal calf serum. Cells were infected with a multiplicity of infection of approximately 5-10 plaque forming units per cell by replacing the growth medium of the cells with high titre virus (2.5x10⁸p.f.u.) followed by incubation for 20 minutes at room temperature. 20ml of medium were then added without removing the virus and the cells were incubated for up to 6 hours at 27°C. Cells were harvested by centrifugation at 1500rpm for 5 minutes in an MSE Centaur centrifuge and the supernatant was removed for storage at 4°C as virus stock. The cell pellet was then placed on cardice for 5 minutes before being thawed and resuspended in 10 volumes of whole cell extract buffer by passing the suspension through a 19-gauge needle. Debris was removed by centrifugation at 50,000xg for 20 minutes at 4°C and the cell extract was stored at -70°C following addition of glycerol to a minimum of 10% (v/v). The specific activity of the extract was typically 100-300 pmoles/mg protein with a total protein concentration of approximately 1mg/ml.

Oestrogen receptor translation in rabbit reticulocyte lysate

The vector pSP6 MOR1-599 (White et al 1987) was used as a source of template for the synthesis of complementary RNA encoding the full length mouse oestrogen receptor. The vector was digested with Hind III in order to linearise the plasmid by virtue of a unique Hind III site located downstream of the coding sequence for the receptor. DNA was recovered by precipitation and resuspended in diethyl pyrocarbonate (DEPC) treated distilled water. The template was then transcribed from a SP6 bacteriophage promoter within the vector by preparing the reaction mixtures outlined below using DEPC treated D.W. to give a final volume of 100µl. The samples were incubated for
one hour at 37°C with addition of a further 25 units of SP6 polymerase after 30 minutes of the incubation. The reactions were then stopped by addition of 5μl of RNase free DNase I (23 units/ml) followed by incubation for 15 minutes at 37°C. The RNA was recovered by precipitation (as for DNA) and the pellet resuspended in 25μl DEPC treated D.W. A yield of 2μg cRNA/5μg of linearised DNA template was typically obtained.

| 5μg | linearised template |
| 20μl | 5x T buffer |
| 20μl | 5x CAP structure analogue |
| 1μl | 1M DTT |
| 4μl | human placental ribonuclease inhibitor (HPRI) |
| 50 units | SP6 polymerase |

Oestrogen receptor was translated in vitro using the rabbit reticulocyte lysate translation system as described by White et al 1991. The lysate was prepared for translation by addition of ZnCl\(_2\) and an amino acid mix devoid of methionine (supplied by Promega) to give concentrations of 0.1 mM and 20 μM respectively. cRNA was then translated in the presence or absence of 35S-methionine by preparing samples containing the following:

| 30μl | Rabbit reticulocyte lysate (containing ZnCl\(_2\) and amino acid mix) |
| 3μl | cRNA (approximately 100-150ng/μl) |
| 1-2μl | DEPC treated D.W. |
| +/- | 1μl Ligand (diluted in 0.1% BSA) |

A small volume (typically 10μl) of each sample was transferred to a separate eppendorf to which 1μl 35S-methionine was added (see materials). In parallel, 1μl of 1mM unlabelled methionine was added to the remaining 20μl of each sample. The reactions were incubated for one hour at 30°C and then stored at -70°C following addition of 15% (v/v) glycerol. Denaturing gel electrophoresis of 1-2μl of the labelled translation products was routinely performed to assess the yield, size and purity of the receptor before studying the unlabelled protein in functional assays.

Translation reactions were performed in the presence of ligand by addition of 1μl ligand that had been diluted appropriately in 0.1% (w/v) BSA. The concentration of alcohol in the translation reaction was maintained below 1% (v/v) since the reaction was found to be sensitive to higher levels of ethanol.
Six GST-ER fusion proteins were studied in this thesis and their induction conditions optimised as described in chapter five. The GST-fusion proteins were prepared as follows with the exception of GST-ER (121-599). A 5ml overnight culture of transformed DH5 cells was inoculated into 200ml of L-broth containing 100μg/ml ampicillin and grown overnight at 37°C with continuous shaking. The following morning 50ml was subcultured into 2x 500ml cultures of L-broth containing 100μg/ml ampicillin and grown at 37°C with continuous shaking until an A600nm of 0.7a.u. was obtained. Fusion protein synthesis was then induced by addition of IPTG to 1.0mM followed by incubation for a further 4 hours at 37°C. The cells were harvested in 500ml Sorvall bottles by centrifugation at 5000xg for 5 minutes at 4°C and the bacterial pellets resuspended by vortexing in 1/100 volume MTPBS containing 15mg/ml lysozyme and 10mM DTT. The resultant suspensions were transferred to a 15ml corex tube and sonicated at 14 microns for 1 minute at room temperature using a 3mm probe (see below). Cell debris and insoluble protein were immediately removed by centrifugation at 10,000xg for 10 minutes at 4°C and the supernatant was stored at -70°C as 1ml aliquots following addition of glycerol to 10-15% (v/v).

GST-ER (121-599) was prepared as outlined above except for the following induction conditions : 2x 500ml cultures of transformed DH5 cells were prepared and induced at an A600nm of 0.7a.u. by addition of IPTG in conjunction with ZnCl2 to concentrations of 1.0mM and 0.1mM respectively. The cells were then incubated for 2 hours at 37°C with continuous shaking before preparing an extract as above. Pellets containing insoluble protein and cell debris were routinely resuspended in 4x protein loading buffer to a volume equal to that of the cell extract. The pellet was partly resuspended by stirring and then sonicated for 20 seconds at 14 microns using a 3mm probe. The pellet was subsequently boiled for 90 seconds prior to analysis on a 7% or 10% denaturing gel and fusion protein was detected by western blotting or staining with Coomassie Blue.

Small scale fusion protein synthesis

A small scale preparation method was routinely used to screen bacterial colonies for fusion protein synthesis following transformation. Single colonies were inoculated into 5ml L-broth containing 100μg/ml ampicillin and grown overnight at 37°C with continuous shaking. The following morning 1ml was subcultured into 20ml L-broth containing 100μg/ml ampicillin and grown at
37°C with continuous shaking until an A600nm of 0.7a.u. was obtained. Fusion protein synthesis was then induced by addition of IPTG to 0.1mM followed by incubation for 30 minutes at 37°C. 2ml of each culture were subsequently transferred to eppendorf tubes and extract was prepared as described above by resuspending the bacterial pellets in 50μl MTPBS plus lysozyme followed by sonication for 20 seconds at 4 microns using a 3mm probe. After removing the cell debris the resultant supematant was added to 25μl of 2x protein loading buffer and 20μl was analysed by denaturing gel electrophoresis and western blotting with a suitable antibody.

**Extract sonication**

Sonication was used to break open harvested DH5 cells using a Soniprep 150 Ultrasonic Disintegrator in conjunction with an exponential microprobe of 3mm in diameter as outlined in the Soniprep protocol. Samples with a volume smaller than a millilitre were placed in eppendorfs and sonicated by placing the tip of the microprobe below the surface of the sample until approximately a third of the liquid was above the tip of the probe. Samples of 10-20ml were placed in 15ml or 30ml corex tubes respectively and sonicated by inserting the probe into the sample until the tip of the probe was covered by approximately two thirds of the liquid. The time and power of sonication adopted varied depending of the volume and nature of the sample being treated (see above text and individual figure legends). In general, samples of less than 1ml were sonicated at 4 microns for 20 seconds while volumes of 10-20ml were treated with 14 microns for 1 minute. Sonication was performed at room temperature although samples were placed on ice for all steps before and after sonication.

**Protein purification**

**Affinity chromatography of the mouse oestrogen receptor**

Purification of the mER was performed by Dr Arnold Coffer and Jane Fellows of the ICRF Protein Isolation laboratory (data unpublished). A Eupergit C matrix was coupled to oestradiol hemisuccinate via a BSA spacer according to the manufacturers instructions and equilibrated with 4ml running buffer (20mM Hepes, 10% v/v glycerol, 0.2M NaCl, 1mM DTT, 100μM PMSF, 1μg/ml aprotinin and 1μg/ml leupeptin, pH7.4). In parallel, the mER was expressed in SF9 cells using a baculovirus expression system and 4 ml of the resultant cell
extract were incubated with 1g of affinity beads for 2 hours at 4°C. The beads were then washed with 30ml running buffer containing 1M NaCl and secondly with 30ml of running buffer alone. Retarded protein was eluted by incubating the beads with 6ml of elution buffer (running buffer containing 0.5M sodium thiocyanate and 20μM [2,4,6,7-3H] E2 at 2.5 Ci/mmol) for 25 minutes at 30°C. The eluate was collected and stored in 1 ml aliquots at -70°C following addition of glycerol to 10% (v/v).

**Affinity chromatography of GST-fusion proteins**

The purification of GST-fusion proteins by affinity chromatography was optimised as described in chapter five. A 2ml solid-phase Glutathione Sepharose 4B column was equilibrated by passing 20ml of PBS and subsequently 10ml MTPBS through the column. Extract (10ml) containing fusion protein was then loaded onto the column in 1ml steps. The column was washed twice with 10ml PBS before eluting retarded protein in 1ml steps with freshly prepared 20mM reduced glutathione pH8.0 (see buffers). 1ml aliquots of the starting material, flow-through, washes and eluate were routinely collected and stored at -70°C following addition of glycerol to 10% (v/v). All loadings and collections described above were performed manually.

**Electrofocusing**

A 50ml electrofocusing column was prepared using a peristaltic P-3 pump. A dense layer containing the anode electrolyte (0.2% v/v H₂SO₄, 55% w/v sucrose, 5% v/v glycerol) was poured into the base of the column. Above this a 0-55% (w/v) linear sucrose density gradient was prepared in a stepwise manner in 5% (v/v) glycerol containing ampholites with pH ranges of 7-9 and 3.5-10 respectively. The pure GST-ER (281-599) sample was applied to the middle of the column during gradient generation. 2ml of 2% (v/v) glycerol was then poured onto the top of the gradient to protect it from the cathode solution (0.3% w/v NaOH) which was the last fraction applied to the column. The density gradient once poured was allowed to equilibrate for 3 hours at 4°C prior to focusing the sample. Water was then applied to the column jacket and electrofocusing was performed for 18 hours at 4°C with a voltage of 300V applied across the column. The following morning the anode solution was removed using a pipette, the top of the column reconnected to the pump and 30 drop (approximately 1ml) fractions were collected at a flow rate of 25ml/hour. The pH of the individual fractions was subsequently measured on ice using a 'Coming 20' pH meter to check the linearity of the column gradient.
Protein quantitation and characterisation

The size, yield and purity of the mER and GST-fusion proteins were routinely analysed before studying the proteins in functional assays. The proteins were initially resolved on a 7-10% SDS-PAGE gel and detected using fluorography, western blotting or staining with silver or Coomassie Blue as described below.

Denaturing gel electrophoresis

Denaturing gel electrophoresis was performed using 0.75mm spaced glass plates (14x16cm) unless otherwise stated. The resolving and stacking gel solutions were prepared separately containing the chemicals listed below. To the resolving gel, 300μl of 10% (w/v) ammonium persulphate and 20μl TEMED were added and the gel was poured to within 3cm of the top of the glass plates. This was immediately overlaid with water saturated isobutanol and allowed to set at room temperature for approximately 30 minutes. The alcohol was subsequently poured off and the top of the gel was rinsed with D.W. The stacking gel was then poured to the top of the glass plates after initiating polymerisation by addition of 200μl of 10% (w/v) ammonium persulphate and 10μl TEMED. A toothed comb was immediately inserted at the top of the stacking gel which was allowed to set for one hour at room temperature before removing the comb and rinsing the wells with 1x SDS-PAGE buffer. The plates were then placed in an Atto corporation dual slab chamber and the chambers filled with 1x SDS-PAGE buffer.

Samples intended for electrophoresis were prepared in 1x protein loading buffer and boiled for 1 minute prior to being loaded into 6mm wells at the top of the stacking gel. A voltage of 250V was typically applied across the gel and electrophoresis was performed (within one hour of the stacking gel having set) in 1x SDS-PAGE buffer for approximately 90 minutes. After this, the stacking gel was removed using a scalpel and the resolving gel was fixed in 20% (v/v) methanol, 10% (v/v) glacial acetic acid for 15 minutes. Gels containing 35S-labelled protein were then incubated in Amplify for 15 minutes before drying the gel for 1 hour at 80°C under vacuum. The radiolabelled bands were visualised by fluorography using Kodak X-OMAT film. Resolving gels intended for western blotting or staining with Coomassie Blue/silver were treated as described in the following sections and were not fixed as above. It should be noted that molecular weight markers were run on each denaturing gel and that the composition of the stacking gel was constant regardless of the percentage of the resolving gel.
**Western blotting**

All protein samples analysed by western blotting were initially resolved by denaturing gel electrophoresis. The proteins were then transferred to nitrocellulose using a wet blotting method in which the gel was placed in a 'sandwich' in the following order starting at the bottom: fibrous pad, two pieces of Whatmann 3MM paper, gel, nitrocellulose, two more pieces of paper and a second fibrous pad. The gel and all components of the sandwich were first equilibrated in transblotting buffer for 10 minutes at room temperature. To remove air bubbles, the sandwich was constructed and each layer above the gel was rolled with a glass pipette. The sandwich was then enclosed in a basket and placed in a Bio-Rad blotting tank such that the nitrocellulose was positioned close to the anode. Transfer was performed at 30V overnight at 4°C and the sandwich was dismantled the following morning. The nitrocellulose membrane was then washed in D.W. with gentle shaking for 5 minutes at room temperature before incubating the gel for up to one minute with Ponceau S solution and removing excess stain with D.W. This dye stains protein on the nitrocellulose membrane allowing the efficiency of protein transfer to be determined. Stain was subsequently removed by washing the membrane in PBSA for 5 minutes. The nitrocellulose was then treated and protein was detected using one of the two methods outlined below:

**Milk method (used routinely and unless otherwise stated)**
The nitrocellulose membrane was washed, blocked and incubated with the first and second antibodies as directed in the 'western blotting detection system' protocol (Amersham Plc). The blocking solution was 2% (w/v) dried milk in PBST which was also used for all the washing steps before the second
antibody incubation after which the membrane was washed with PBST alone. The first and second antibody solutions were prepared with a final concentration of 1μg/ml antiserum in blocking solution. After the final wash, protein was detected by fluorography using Fuji RX or Kodak X-OMAT (greater sensitivity) film as instructed in the Amersham protocol stated above. Alternatively protein was detected using horseradish colour development solution (prepared from tablets, see buffers) by incubating the membrane in the development solution for 5-20 minutes until distinct brown coloured bands were obtained. The membrane was then photographed or could be stored in the dark for several days with a thin covering of water. All incubations were carried out at room temperature.

**Gelatin method**

The following method was used for figure 4.5. in this thesis. The nitrocellulose membrane was washed for 10 minutes in TBS and then incubated for one hour in blocking solution (3% w/v gelatin in TBS). This was discarded and the membrane was incubated overnight with the first antibody solution (1% w/v gelatin in TTBS containing 2μg/ml antibody) at 4°C. The membrane was then washed twice with 20ml TTBS for 5 minutes and incubated with the second antibody solution (1% w/v gelatin in TTBS containing 0.5μg/ml peroxidase conjugated antibody) for 2-3 hours. The antibody solution was once more discarded and the nitrocellulose was washed twice with 20ml TTBS for 5 minutes and once with TBS for a further 5 minutes. Protein was subsequently detected by incubating the membrane with horseradish colour development solution (prepared from powder, see buffers) for up to 1 hour. The reaction was stopped by pouring off the development solution and washing the blot with D.W. The membrane was then photographed or could be stored in the dark with a thin covering of water for up to one week. All incubations were performed at room temperature unless otherwise stated.

**Staining gels with silver and Coomassie Blue**

GST-fusion proteins was frequently detected following denaturing gel electrophoresis by staining the resultant gel with Coomassie Blue or silver. The resolving gel was transferred to a clean plastic tray without fixing and stained with Coomassie Blue by incubating the gel with approximately 5 gel volumes of Coomassie Blue stain (see buffers) for 20-40 minutes at room temperature with gentle rotation on a flat bed rotator. The gel was then rinsed in D.W. to remove any unbound dye before being incubated overnight in destain with gentle
rotation at room temperature. Tissues were placed at the corners of the container to bind dye that was removed from the gel thereby increasing the efficiency of stain removal and shortening the time required for destaining. The gels were then photographed either before or after being dried at 80°C for 1 hour under vacuum.

Silver staining was used to determine the size and purity of the mER and GST-ER (281-599) following affinity chromatography. The proteins were resolved by denaturing gel electrophoresis and the resolving gel was placed in a clean plastic container (supplied with the silver stain kit) immediately after electrophoresis. Silver staining was then performed following the instructions in the protocol provided with the silver stain kit (supplied by SIGMA).

### Protein quantitation (Bio-Rad assay)

The total protein concentration of samples was determined using a microassay procedure referred to as the Bio-Rad assay which was performed as directed by the supplier’s instructions (Bio-Rad). This was adapted from a dye binding assay published by Bradford in 1976. Duplicate 1ml reactions were prepared in disposable plastic cuvettes containing 0.8ml of sample (appropriately diluted) and 0.2ml Dye Reagent Concentrate. The two solutions were mixed by gentle inversion and then incubated for 5 minutes at room temperature before measuring their A595nm relative to a blank reaction (0.8ml sample buffer plus 0.2ml Dye Reagent Concentrate). In parallel, a series of 1ml standard reactions were similarly prepared in duplicate containing 1-18µg BSA and their A595nm measured. A graph of absorption versus the amount of protein (µg) was plotted after correcting for the ability of BSA to give an A595nm value which corresponds to twice the amount of protein actually added. The amount of protein in a sample of interest was then extrapolated from the resultant standard curve and the total protein concentration was calculated as the mean amount of protein detected per µl of sample analysed.

### Protein functional and structural assays

#### Ligand binding assays

All the ligand binding assays used in this thesis were modified from the method described by Coffer et al (1980). The single point ligand binding assay was used to quantitate the amount of mER or GST-fusion protein in crude and purified samples and secondly to screen deletion mutants of GST-ER (281-599) for their ability to bind oestradiol and 4-OH-Tamoxifen. The relative
binding affinity (R.B.A.) of the mER and GST-fusion proteins for a variety of ligands and the dissociation constant ($K_d$) for oestradiol were determined as described below:

**Single point ligand binding assay**

Aliquots of DH5 or SF9 cell extracts (appropriately diluted) were incubated in triplicate for 12-18 hours at 4°C in a final volume of 50μl containing ligand binding buffer and either 6nM $[6,7-^3H]$ E$_2$, $[2,4,6,7-^3H]$ E$_2$, 16α-$[^{125}I]$ E$_2$ or Z-4-Hydroxy-[N-methyl-$^3H$]-tamoxifen. The level of nonspecific binding was determined by inclusion of 500 fold excess of diethylstilbestrol (DES) in one of the triplicate reactions. Free steroid was then removed by addition of 50μl dextran coated charcoal (see DCC suspension under buffers) followed by a 5 minute incubation at 4°C and centrifugation for 5 minutes in a benchtop centrifuge. Of the resultant supernatant, 80μl was transferred to a 3ml scintillation vial and either counted directly (iodo-oestradiol) or following the addition of 3ml counting fluid (tritiated steroids). The nonspecific binding (counts obtained in the presence of DES) was then subtracted to give the amount of radiolabelled ligand that was specifically bound.

**Determination of R.B.A. for ligand**
The relative binding affinity (R.B.A.) of the mER or GST-fusion proteins for a particular ligand was determined using an adaptation of the single point ligand binding assay. Cell extract or pure protein was incubated with either 6nM 16α-$[^{125}I]$ E$_2$, $[6,7-^3H]$ E$_2$ or $[2,4,6,7-^3H]$ E$_2$ in triplicate for 12-18 hours at 4°C in a final volume of 50μl containing ligand binding buffer and increasing concentrations of unlabelled competitor. The level of nonspecific binding was determined by inclusion of 500 fold excess of DES in one of the triplicate reactions and the free steroid was removed as above. The nonspecific binding was then subtracted to give the amount of radiolabelled ligand which was specifically bound. This was expressed as a percentage of the amount of labelled oestradiol that was bound in the absence of competitor and plotted against the concentration of unlabelled ligand. The relative binding affinity of the competing ligand was then calculated as the ratio of the concentration of oestradiol relative to the ligand required to inhibit the binding of labelled oestradiol by 50%.

**Determination of the $K_d$ for oestradiol**
The dissociation constant of the mER and GST-ER (281-599) for oestradiol
was determined by incubating SF9 cell extract or pure fusion protein for 12 hours at 4°C in 50μl containing ligand binding buffer and increasing concentrations of [6,7-^H] E₂. Free steroid was removed and the nonspecific binding was subtracted as above. The total amount of ligand added was then determined after counting the resuspended pellets and Scatchard analysis (Scatchard 1949) was used to calculate the affinity of the protein for oestradiol. The dissociation constant (K_d) for oestradiol is the concentration at which the binding of the tritiated steroid to the protein is at half maximal.

**Gel retardation assay**

The ability of the mER and GST-ER (121-599) to bind DNA was studied routinely using a gel retardation assay, also referred to as the bandshift assay. DH5 or SF9 cell extract was appropriately diluted (see individual figure legends) and incubated in a final volume of 40μl gel shift buffer for 10-20 minutes in the presence or absence of ligand and/or antiserum. To this, 10μl radiolabelled oligonucleotide probe (0.1ng/μl) was added and the samples were incubated for 30 minutes before being loaded (15-20μl sample/track) onto a prerun 6% nondenaturing gel. Electrophoresis was then performed at 300V for 30 minutes or until the marker dye (10μl of 5x DNA loading buffer loaded into the last empty well) was approximately 1cm from the bottom of the gel. The gel was immediately fixed for 15 minutes in 10% (v/v) acetic acid, 30% methanol and dried for 1 hour at 80°C under vacuum. Radiolabelled bands were then visualised by autoradiography using either Fuji RX or Kodak X-OMAT (greater sensitivity) film. The effect of ligand on DNA binding activity was examined by addition of 0.5μl ligand (appropriately diluted in absolute alcohol) to individual samples giving 1% (v/v) alcohol in the DNA binding reaction which does not interfere with protein function.

**Preparation of nondenaturing gel**

Gel solution (25ml) was prepared containing 10ml acrylamide stock (30% w/v acrylamide, 0.8% w/v bis-acrylamide), 2.5ml 10x TBE and 37.5ml D.W. Polymerisation was initiated by addition of 700μl of 10% (w/v) ammonium persulphate and 20μl TEMED and the gel was poured between two 0.75mm spaced glass plates (14x16cm). A toothed comb was immediately inserted at the top of the gel which was allowed to set for 1 hour at room temperature. The comb was then removed, the wells rinsed with 0.5x TBE to remove any unpolymerised acrylamide and the plates placed in an Atto corporation dual slab chamber. The gel was routinely prerun for 10 minutes at 300V in 0.5x TBE.
within 30 minutes of having set. Samples intended for electrophoresis were then loaded into the 6mm wells within 15 minutes of the end of the prerun.

**Preparation of labelled DNA**

Probe was prepared by annealing oligonucleotides to form an ERE binding site by incubating the complementary oligonucleotides (1μg of each) together in a final volume of 400μl of 1x NTE for 5 minutes at 85°C and then allowing the solution to cool slowly to room temperature. The DNA was recovered by precipitation and resuspended in 50μl D.W. giving a concentration of approximately 40ng/μl. The annealed DNA (200ng) was then radiolabelled by filling in the 5’ overhanging ends in the presence of [α-32P] dCTP. DNA (200ng) was incubated in a final volume of 20μl containing 1x repair buffer, 2 units of labelling grade Klenow enzyme, 0.1mM dATP, dGTP and dTTP and 0.8mM [α-32P] dCTP. The reaction was incubated for 30 minutes at room temperature before precipitating the DNA. The resultant DNA pellet was air-dried for 5 minutes and then resuspended in 20μl D.W. to give a stock of approximately 10ng labelled DNA/μl.

The oligonucleotides which were annealed to form the binding site for the oestrogen receptor are outlined below. The ERE has been written in bold print and is the same as the sequence identified between positions -338 to -313 in the *Xenopus laevis* vitellogenin A2 gene promoter (Klein-Hitpass et al 1986).

5’-CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3’
3’-TTTCAGTCCAGTGTCACTGGACTAGTTAGATC-5’

**Filter binding assay**

The DNA binding activity of the mER in SF9 cell extracts was quantified by incubating crude cell extract (0.2μg/sample) in duplicate for 10 minutes at 25°C in a final volume of 40μl gel shift buffer in the presence or absence of ligand. This was followed by addition of 10μl radiolabelled DNA (0.1ng/μl) and incubated for a further 30 minutes at room temperature. The samples were then transferred onto individual 0.45μM filters that had been prewashed with 2x 5ml of 50mM Hepes pH7.4. The filters were incubated for 5 minutes at room temperature and the vacuum applied to filtrate the sample. The filters were then washed with 3x 5ml of 50mM Hepes pH7.4 under vacuum and air-dried for 5-10 minutes before being transferred to scintillation vials containing 5ml
Figure 2.2. FPLC calibration calculations

Marker protein (300μg) was diluted in running buffer to a final volume of 300μl and then loaded onto a Superose 12 column. Protein was eluted with a flow-rate of 0.2ml/min and 200μl fractions were collected. The protein content of the resultant fractions was then determined using a Bio-Rad assay in order to identify the peak elution fraction. The ratio of the elution volume over the void volume (i) and the capacity of the FPLC column (ii) were calculated as follows:

(i)

Fraction volume = 0.20ml
Elution volume (Ve) = Fraction volume x Peak Fraction Number
e.g. Ovotransferin = 0.20ml x 62
= 12.40ml
Void volume (Vo) = Elution volume derived for Dextran Blue
= 7.6ml
Inclusion volume (Vi) = Elution volume derived for Cytochrome C
= 15.2ml

Ratio $\frac{Ve}{Vo} = \frac{Ve}{7.6ml}$

(ii)

Column capacity = $\frac{Vi}{Vo}$
= $\frac{15.2ml}{7.6ml}$
= 2.0
counting fluid. The filters were counted to quantify the protein-DNA complexes retained. The labelled DNA was the same as that described in the gel retardation assay above. The effect of ligand on DNA binding activity was examined by addition of 0.5μl ligand (appropriately diluted in absolute alcohol) to individual samples giving 1% (v/v) alcohol in the DNA binding reaction which does not interfere with protein function.

**Fast protein liquid chromatography (FPLC)**

FPLC analysis of proteins was performed using a 25ml Superose 12 column at 4°C. All solutions and samples were pumped through the column using a peristaltic pump with a flow rate of 0.2ml/min and a back pressure that was routinely maintained below 1.6Pa. The column was initially equilibrated in 50ml running buffer (40mM Hepes pH7.4, 0.1M KCl, 10mM benzamidine, 40μg/ml PMSF, 1.0mM DTT) before loading approximately 200μg marker protein or 1μg pure GST-ER (281-599) onto the column using a 200μl loading loop. Protein was eluted with running buffer and 200μl fractions were collected using an automated fraction collector.

Calibration was facilitated by running individual marker proteins through the column and measuring the protein content of fractions collected in a Bio-Rad assay. The elution volume for each marker protein was calculated from the position of the peak fraction as outlined in figure 2.2. Dextran blue and cytochrome C were used to measure the void (Vo) and inclusion (Vi) volumes of the column respectively. The ratio of the elution volume over the void volume was then plotted against the logarithm of the molecular weight for each marker protein to generate a standard graph that could be used to extrapolate the molecular weight of proteins of interest.

**Electron microscopy**

To investigate the effect of ligand on the conformation of the hormone binding domain, increasing amounts of pure GST-ER (281-599) were incubated in the presence or absence of ligand in a final volume of 50μl of EM buffer (50mM Hepes pH7.4, 0.1M KCl, 10mM benzamidine, 1.0mM DTT and 0.5mg/ml bacitracin) for 20 minutes at 25°C. Samples were then sprayed onto freshly cleaved mica (Hall 1964) and shadowed at a fixed angle of 10° with platinum, backed by carbon and then floated onto 400 mesh copper grids. The samples was subsequently examined in a calibrated Philips 300 transmission electron microscope using an accelerating voltage of 80kV.
**Molecular modelling of ligand structures**

The structures of different analogues of ICI 164,384 were modelled using the molecular modelling package HyperChem (release 3 for Microsoft Windows from Autodesk Incorporated) on a Viglen 486 DX33. The structures of the analogues were built up atom by atom and bonds were formed between the relevant atoms. The structures of the ligands were then approximated using the model-builder function of the program and energy minimisations were performed for each analogue by placing them in a MM+ force field *in vacuo*. The energy minimisations were performed using molecular mechanics calculations initially with the Fletcher Reeves algorithm (conjugate gradient method). Local energy minima between approximately 3-40kcal/mol were obtained. The resultant structures were compared with those derived using the Polak-Ribiere algorithm and found to be the same indicating that a true local minimum energy structure had been obtained for each analogue of ICI 164,384.
Chapter 3

The effects of class II antioestrogens on mER DNA binding activity
Introduction

The aim of the study described in this chapter was two fold; firstly, to investigate the role of the aliphatic side-chain of ICI 164,384 in the inhibition of oestrogen receptor dimerisation and DNA binding and secondly, to address reported inconsistencies in the ability of the antagonist to inhibit the DNA binding activity of the receptor.

The influence of the side-chain chemical structure on the antagonism of oestrogen receptor function was investigated using a series of analogues of ICI 164,384. The results obtained demonstrate a direct correlation between the ability of the analogues to inhibit the DNA binding of the receptor in vitro and their reported antagonism in vivo (Wakeling and Bowler 1988, Bowler et al 1989, Wakeling and Bowler 1992). Inhibition of DNA binding appeared to be mediated by a disruption to receptor dimerisation rather than a direct effect on DNA binding activity per se, with maximal antioestrogen activity requiring an aliphatic side-chain in the α-orientation of 15-16 atoms in length. Evidence is presented to suggest that antagonism by ICI 164,384 is dependent on the stability of oestrogen receptor dimers and that while the antioestrogen may prevent the dimerisation of the receptor during protein synthesis, it may not necessarily disrupt the dimerisation of ‘preformed’ dimers depending on their inherent stability.

The influence of ICI 164,384 analogues on mER DNA binding

The influence of the amidoalkyl side-chain on the ability of the pure antioestrogens to inhibit the DNA binding activity of the oestrogen receptor was studied using a series of analogues of ICI 164,384. These ligands differ in the length, substitution or orientation of the side chain as shown in figure 3.1.

The affinity of the mouse oestrogen receptor (mER) for each analogue was determined by testing the ability of increasing concentrations of unlabelled ligand to compete with the binding of 6nM ³H-E₂ to the receptor (see methods). The relative binding affinity was then calculated as the ratio of the I.C.₅₀ value for E₂ relative to that of the antagonist (figure 3.2.). The analogues were found to bind specifically to the receptor with ICI 165,801 having a similar affinity to oestradiol. Analogues ICI 164,384, ICI 163,964 and ICI 182,780 were found to have a slightly reduced affinity for the mER, approximately one fifth that of oestradiol while ICI 169,784, ICI 165,889 and the long chain analogue ICI 165,375 bound relatively poorly to the receptor.

The effects of the analogues on the dimerisation and DNA binding activity of the oestrogen receptor were studied using a gel retardation assay in
Figure 3.1: ICI 164,384 analogue side chain structural data

![17β-oestradiol: steroid ring system with position of analogue side chain (R)](image)

<table>
<thead>
<tr>
<th>ICI Analogue</th>
<th>Side chain (R) structure</th>
<th>Length (atoms)</th>
<th>Side chain orientation</th>
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<td>$(\text{CH}_2)_10\text{CON}\text{CH}_3$</td>
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Figure 3.2. Affinity of baculovirus expressed mER for chemical analogues of ICI 164,384

<table>
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<th>Ligand</th>
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<tr>
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</tr>
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<td>ICI 165,801</td>
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<td>0.01</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>0.17</td>
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Receptor expressed in SF9 cells (0.2μg protein/sample) was incubated in triplicate with 6nM [6,7-3H] E2 in the presence of increasing concentrations of unlabelled analogue for 12-18 hours at 40°C. The level of nonspecific binding was determined by inclusion of diethylstilbestrol to a final concentration of 10⁻⁶ M in one of the triplicate reactions. Free steroid was removed using dextran coated charcoal and the nonspecific binding subtracted to give the specific ³H-E₂ bound. The relative binding affinity for each analogue was then calculated as the ratio of the concentration of oestradiol relative to antioestrogen required to inhibit the binding of tritiated E₂ by 50%.
conjunction with antiserum MP16. The method was based on the assumption that dimerisation is required for efficient binding of the oestrogen receptor to DNA and if disrupted both activities could be restored upon addition of MP16 to the reaction (Fawell et al 1990b). The epitope for this antiserum is located between residues 130-142 of the mER (Fawell et al 1990a).

Receptor was treated with a saturating concentration of analogue followed by incubation with a 32P-labelled ERE containing oligonucleotide in the presence or absence of MP16. The resultant DNA binding activity was then assessed by electrophoresis on a 6% nondenaturing polyacrilamide gel (figures 3.3.a. and 3.3.b.). For each analogue, a direct correlation was observed between its ability to inhibit the DNA binding of the oestrogen receptor in vitro and its reported activity as an antagonist in vivo (Wakeling and Bowler 1987, Wakeling and Bowler 1988, Bowler et al 1989, Wakeling and Bowler 1992). The effects of the analogues in vitro broadly paralleled their affinity for the oestrogen receptor. However, the short-chain analogue ICI 165,889 was unable to inhibit DNA binding despite having an affinity for the receptor only five fold lower than ICI 164,384. The ability of MP16 to restore DNA binding suggested the observed inhibition was a consequence of a disruption to the dimerisation of the receptor rather than a direct effect on DNA binding activity.

The efficiency with which each analogue could inhibit DNA binding was analysed in more detail by titrating the ability of increasing concentrations of ligand to inhibit the DNA binding activity of the oestrogen receptor in a gel retardation assay (figure 3.3.c.). The antagonism was observed to be dependent on the length and orientation of the amidoalkyl side-chain with ICI 182,780 being the most potent antioestrogen followed, in order of decreasing efficiency, by ICI 165,801, ICI 164,384, ICI 163,964 and ICI 169,784. In contrast analogues ICI 165,375 and ICI 165,889 had no effect on DNA binding even at the highest ligand concentration. Antagonism was also restricted to the α-isomer since ICI 169,784, the β-form of ICI 164,384, was unable to significantly inhibit DNA binding in vitro nor act as an antagonist in vivo (Bowler et al 1989). Thus maximal antioestrogen activity required an aliphatic side-chain in the α-orientation of 15-16 atoms in length.

Computer modelling of analogues of ICI 164,384

Computer modelling was performed for each analogue of ICI 164,384 to compare the structures of the different aliphatic side-chains. The structures of the ligands were modelled by Dr. Steven McCgary (Cardiac Medicine Department, National Heart and Lung Institute) using the molecular modelling
Figure 3.3.a. The effect of ICI 164,384 analogues on mER DNA binding activity

Receptor expressed in SF9 cells (0.2µg protein/track) was incubated in the presence (+) or absence (-) of 5x10^-7M ligand for 10 minutes at room temperature. The resultant DNA binding activity was then determined by addition of 1ng ^32P-labelled ERE containing oligonucleotide followed by incubation for 30 minutes at room temperature and electrophoresis on a 6% non-denaturing polyacrylamide gel. (Ab) indicates that antiserum MP16 (1µg protein/track) was added to the ligand treated receptor in conjunction with the labelled oligonucleotide. The arrows indicate the position of protein bound DNA while the position of free radiolabelled ERE has been shown using an asterix (*).

Dr J. Bowler and Dr A. Wakeling have investigated the ability of the ICI 164,384 analogues to behave as agonists or antagonists in vivo using a rat uterotrophic assay. Their results have been summarised above figure 3.3.a. for ease of comparison between the in vitro and in vivo data (Wakeling and Bowler 1988, Bowler et al 1989).
### Figure 3.3.a.

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<th>% Antagonism</th>
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<th>$169784_\beta$</th>
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<th>163964</th>
<th>165889</th>
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<td>-</td>
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<td>Ab</td>
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</table>

- : no Ab
+ : Ab

**Legend:**
- E$_2$: Essential element 2
Receptor expressed in SF9 cells (0.2µg protein/track) was incubated in the presence (+) or absence (-) of either 5x10^{-7}M oestradiol, ICI 164,384 or ICI 182,780 for 10 minutes at room temperature as indicated. The resultant DNA binding activity was then determined by addition of 1ng 32P-labelled ERE containing oligonucleotide followed by incubation for 30 minutes at room temperature and electrophoresis on a 6% non-denaturing polyacrylamide gel. The arrows indicate the position of protein bound DNA while the position of free radiolabelled ERE has been shown using an asterix (*). In the figure, (Ab) indicates that antiserum MP16 (1µg protein/track) was added to the receptor in conjunction with the labelled DNA.
**Figure 3.3.c. Influence of ICI 164,384 analogues on mER DNA binding**

**Figure 3.3.c.(i)**
Receptor expressed in SF9 cells (0.2 µg protein/track) was incubated in the presence of either 10^{-6}M oestradiol (lane 2), 10^{-6}M ICI 164,384 (lane 3), 10^{-6}-10^{-8}M ICI 163,964 (lanes 4-6), 10^{-6}-10^{-8}M ICI 182,780 (lanes 7-9) or in the absence of hormone (lane 1) for 10 minutes at room temperature as indicated. The resultant DNA binding activity was then determined by addition of 1 ng 32P-labelled ERE containing oligonucleotide followed by incubation for 30 minutes at room temperature and electrophoresis on a 6% non-denaturing gel.

**Figure 3.3.c.(ii)**
Receptor expressed in SF9 cells (0.2 µg protein/track) was incubated in the presence of either 10^{-6}M oestradiol (lane 3), 10^{-6}-10^{-8}M ICI 164,384 (lanes 4-6), 10^{-6}-10^{-8}M ICI 165,801 (lanes 7-10) or in the absence of hormone (lane 2) for 10 minutes at room temperature and the resultant DNA binding activity determined as described for figure 3.3.c.(i). As a control (lane 1), the effect of 1% (v/v) ethanol on oestrogen receptor DNA binding activity was assessed.

**Figure 3.3.c.(iii)**
Receptor expressed in SF9 cells (0.2 µg protein/track) was incubated in the presence of either 10^{-6}M oestradiol (lane 2), 10^{-6} ICI 164,384 (lane 3), 10^{-6}-10^{-8}M ICI 169,784 (lanes 4-6), 10^{-6}-10^{-8}M ICI 165,889 (lanes 7-9), 10^{-6}-10^{-8}M ICI 165,375 (lanes 10-12) or in the absence of hormone (lane 1) for 10 minutes at room temperature and the resultant DNA binding activity determined as described for figure 3.3.c.(i).

In the figure, protein bound DNA complexes have been displayed with the concentration (M) of the ICI analogues indicated by its exponential for ease of reference.
Figure 3.3.c. (i)

Lane: 1 2 3 4 5 6 7 8 9
ICI 163964 | ICI 182780
-6 -7 -8 | -6 -7 -8

Figure 3.3.c. (ii)

Lane: 1 2 3 4 5 6 7 8 9 10
ICI 164384 | ICI 165801
-6 -7 -8 | -6 -7 -8 -9

Figure 3.3.c. (iii)

Lane: 1 2 3 4 5 6 7 8 9 10 11 12
ICI 169784 | ICI 165889 | ICI 165375
-6 -7 -8 | -6 -7 -8 | -6 -7 -8
package HyperChem on a Viglen 486 DX33. The structures of the analogues were subjected to energy minimisation and local minimum energy conformations were derived for each ligand (see methods). The resultant structures have been presented in figures 3.4. and 3.5. so that the A ring of each ligand is in the same orientation as that of oestradiol. For each analogue of ICI 164,384, the D ring and to some extent the C ring of the steroid ring system were found to be distorted compared with oestradiol. In general, the aliphatic side-chains protrude from the B ring and appear to adopt a regular conformation with the exception of ICI 169,784 which has a more folded structure. The steroid ring systems of several analogues including ICI 164,384 (RBA 0.23), ICI 165,801 (RBA 1.00) and ICI 165,375 (RBA 0.01) have a similar structure suggesting that differences in the affinities of the ligands for the oestrogen receptor are not a result of distortions of the C and D rings of the steroid ring system and alternatively that the amidoalkyl side-chain may influence the binding of the ligands to the receptor. The more folded structure of the side-chain for ICI 169,784 may therefore interfere with the binding of this ligand to the receptor in contrast to the α-isomer ICI 164,384 which has a 20 fold higher affinity for the ER (refer to figure 3.2.).

The ligands ICI 182,780, ICI 165,801, ICI 164,384 and ICI 163,964 have been shown to act as antagonists in vitro in that order of potency (refer to figure 3.3.c.). A comparison of the structures of these ligands revealed that both ICI 182,780 and ICI 165,801 contain significant kinks in the structures of their side-chains (figures 3.4. and 3.5.). In contrast, ICI 164,384 and ICI 163,964 had a more regular conformation while ICI 164,384 was found to adopt a folded structure after the branch-point in its side-chain. No relationship was observed between the electron densities or the orbital occupancies of the analogues indicating that the potency of the compounds is not related to their charge or the electron distribution on the side-chain. It is therefore not unreasonable to conclude that the effects of the analogues on the dimerisation and/or DNA binding activity of the oestrogen receptor may reflect the bulk or hydrophobicity of their amidoalkyl side-chains. In this respect the kinks introduced into the side-chains of ICI 182,780 and ICI 165,801 may contribute significantly to the potency of these ligands.

**Stabilising the ER dimer abolishes ICI 164384 antagonism**

Oestrogen receptor dimerisation is thought to be mediated by hydrophobic interactions which would be stabilised by conditions of high ionic strength. For this reason the ability of ICI 164,384 to inhibit the DNA binding
The structures of the different analogues of ICI 164,384 were modelled using the molecular modelling package *HyperChem*. The structures of the analogues were built up using the molecular-builder function of the program and then subjected to energy minimisation using molecular mechanic calculations (see methods). Local minimum energy structures were obtained for the analogues and are presented with the A ring of the steroid ring system in the same orientation.
Figure 3.4. Comparison of the structures derived for oestradiol and ICI 164,384 by computer modelling
Figure 3.5. Comparison of the structures derived for

ICI 165801

ICI 163964

ICI 182780
analogues of ICI 164,384 by computer modelling

ICI 169784

ICI 165375

ICI 165889
activity of the mER was investigated using a range of salt conditions.

The sensitivity of ICI 164,384 to salt concentration was studied in vitro using a filter binding assay in preference to the gel retardation assay (see methods). In the latter, salt is diluted out of the reaction during the course of electrophoresis and in order to be feasible would require a separate gel be prepared, each time, with the appropriate salt concentration. A more practical approach was to use the filter binding assay which relies on the ability of Millipore HA 0.45mM filters to retain protein whilst allowing protein-free DNA to pass through the filter. Thus, the only DNA which is retained is that which is bound to protein.

Receptor was incubated in duplicate at the desired salt concentration, with or without ICI 164,384, before incubation with a 32P-labelled ERE containing oligonucleotide. The DNA binding activity of the receptor was then determined by passing the sample through a single 0.45mM filter under vacuum. Following three washes, the filters were removed into counting fluid and any radioactivity retained was quantified (figure 3.6.). Maximal DNA binding was observed at 0.1-0.12M KCl in the absence of hormone with the greatest inhibition by antioestrogen observed at the same concentration. In general very low and high salt concentrations abolished both DNA binding and the effect of the antagonist. Since high salt would be expected to stabilise hydrophobic interactions that mediate receptor dimerisation, this suggested that increasing the stability of oestrogen receptor dimers may interfere with the ability of ICI 164,384 to inhibit DNA binding. To test this hypothesis it was investigated whether stabilising receptor dimerisation by prebinding the receptor to DNA prior to incubation with ICI 164,384 could also affect antioestrogen function.

mER was treated with ligand either before or after incubation with a 32P-labelled ERE containing oligonucleotide and then studied by electrophoresis on a 6% non-denaturing gel (figure 3.7.). In the absence of hormone receptor was observed to bind efficiently to DNA giving a characteristic downshift in the presence of E2 regardless of whether receptor was bound to DNA prior to incubation with hormone. In contrast, ICI 164,384 could only inhibit DNA binding when bound to the mER prior to incubation with the DNA. Sabbah and colleges (1991) have reported that the antioestrogen can bind to the human ER-ERE complex suggesting that although ICI 164,384 could bind to the mER in the above experiment, the effect of the antagonist was abolished by prebinding the receptor to DNA.
Receptor expressed in SF9 cells (0.2µg protein/sample) was incubated in the presence or absence of 10⁻⁶M ICI 164,384 at the indicated salt concentration for 10 minutes at 25°C. The resultant DNA binding activity was then determined by addition of 1ng ³²P-labelled ERE containing oligonucleotide at the same salt concentration followed by incubation for 30 minutes at 25°C. Samples were transferred to individual 0.45µM filters under vacuum and the filters were washed with 50mM Hepes buffer pH7.4. The filters were then removed into counting fluid after being air-dried for 5-10 minutes and the radioactivity retained on the filters was counted (see methods). The results have been expressed as a percentage of the maximal DNA binding activity observed in the absence of ligand and represent the mean of three experiments.
Figure 3.7. Prebinding receptor to DNA blocks ICI 164,384 antagonism

Receptor expressed in SF9 cells (0.2μg/track) was treated with 10^{-6}M E_2 (E), 10^{-6}M ICI 164,384 (I) or no hormone (N) for 20 minutes before incubation with 1ng ^{32}P-labelled ERE containing oligonucleotide for 30 minutes at 25\(^\circ\)C (lanes 1-4). The resultant DNA binding activity was compared with receptor which had been prebound to DNA by incubation of SF9 cell extract with labelled oligonucleotide for 1 hour prior to treatment with ligand for 1-2 hours (lanes 5-10). (Ab) indicates the addition of 1μg MP16 antiserum in conjunction with the labelled ERE.
Antagonism by ICI 164384 depends on the inherent stability of ER dimers

A number of studies using alternative sources of receptor have failed to demonstrate an inhibition of oestrogen receptor DNA binding activity by ICI 164,384. (Martinez and Wahli 1989, Sabbah et al 1991) These conflicting observations may result from differences in the stability of ER dimers in different cells since conditions which favour dimer stabilisation have been shown to abolish the effect of the antioestrogen (figures 3.6. and 3.7.).

To address this issue the rabbit reticulocyte lysate in vitro translation system was used as a source of receptor. The cDNA for the full-length mouse oestrogen receptor had previously been cloned into the expression vector pSP64 as described by White et al 1987. The resultant vector, pSP6 MOR 1-599, was linearised by digestion with Hind III and used as a template for the synthesis of cRNA by bacteriophage SP6 polymerase (method described by Lees et al 1989a, modified method of Melton et al 1984). A yield of approximately 2|xg cRNA/5|ig linearised DNA template was obtained and subsequently used to direct the translation of the oestrogen receptor in rabbit reticulocyte lysate as described by White et al 1991.

ICI 164,384 has previously been shown to have no effect on mER DNA binding when added to receptor following translation in vitro (Lees et al 1989b). As an alternative it was investigated whether translation of receptor in the presence of ligand would influence the DNA binding activity of the protein. Receptor was translated in the presence of either ICI 164,384, ICI 182,780 or ICI 169,784 with the addition of 35S-methionine to one fifth of the translation reaction. The efficiency of translation was examined by analysis of an equal volume of radiolabelled translation product on a 10% SDS-PAGE gel followed by autoradiography. Little effect of ligand on the efficiency of receptor translation was observed provided the ligands were diluted in BSA to give a concentration of alcohol in the translation reaction of no greater than 0.01%. The incorporation of radiolabelled methionine was quantified from the dried gel using an Ambis Systems β-scanner in order to normalise the amount of receptor synthesised in the presence of each ligand (figure 3.8.). The effect of hormone on mER DNA binding activity was then assessed using equal amounts of receptor in a gel retardation assay (figure 3.9.).

Translated receptor was observed to bind efficiently to an ERE in the absence of hormone giving a similar level of binding following translation in the presence of oestradiol with a characteristic increase in complex migration. In contrast, translation of receptor in the presence of ICI 164,384, ICI 182,780 and ICI 169,784 resulted in a complete loss of DNA binding which was not rescued
mER was translated in the presence of either $10^{-6}$M oestradiol, $10^{-6}$M ICI 164,384, $10^{-7}$M ICI 182,780, $10^{-6}$M ICI 169,784 or no hormone (lanes 1-5 respectively) with addition of $^{35}$S-methionine to a fifth of each translation reaction. The amount of receptor synthesised was normalised and checked by electrophoresis of labelled protein on a 10% SDS-PAGE gel. As a control, translation was performed in the absence of cRNA and analysed by electrophoresis of a volume of translation product equal to that of the mER sample in the absence of hormone (lane 6).
**Figure 3.9. Antioestrogen inhibits the DNA binding activity of in vitro translated mER**

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<tr>
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<th>ICI 164384</th>
<th>ICI 182780</th>
<th>ICI 169784</th>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</table>

mER was translated in the presence of either $10^{-6}M$ oestradiol (lanes 3-4), $10^{-6}M$ ICI 164,384 (lanes 5-6), $10^{-7}M$ ICI 182,780 (lanes 7-8), $10^{-6}M$ ICI 169,784 (lanes 9-10) or in the absence of hormone (lanes 1-2) as indicated. The DNA binding activity of the receptor was then assessed using a gel retardation assay. Equal amounts of unlabelled receptor were incubated with 1 ng $^{32}\text{P}$-labelled ERE containing oligonucleotide for 30 minutes at $25^\circ\text{C}$ in the presence (+) or absence (-) of antiserum MP16 (1 $\mu$g protein/track). As a control (lanes 11-12), translation was performed in the absence of cRNA followed by analysis of a volume of translation product equal to that of the mER translated in the absence of hormone.
Figure 3.10. Normalisation of mER translated in vitro in the presence of ligand

mER was translated in the presence of either 10⁻⁶M E₂, 10⁻⁶M ICI 164,384, 10⁻⁷M, 10⁻⁸M, 10⁻⁹M ICI 182,780, 10⁻⁶M ICI 165,889, 10⁻⁶M, 10⁻⁷M ICI 169,784 or 10⁻⁶M 4-OH-Tamoxifen (lanes 2-10 respectively) with ³⁵S-methionine added to a fifth of each translation reaction. The amount of receptor synthesised was normalised and checked by electrophoresis of labelled protein on a 10% SDS-PAGE gel. As a control (lane 11), translation was performed in the absence of cRNA and analysed by electrophoresis of a volume of translation product equal to that of the mER sample in the absence of hormone (lane 1).
by addition of MP16 antiserum. The antioestrogens could therefore inhibit the DNA binding activity of in vitro translated receptor but whether this was a consequence of disrupting the dimerisation of the oestrogen receptor is unclear due to the inability of MP16 to restore DNA binding.

The ability of ICI 164,384 to prevent oestrogen receptor dimerisation during in vitro translation was then compared with its ability to disrupt the dimerisation of preformed dimers when added to receptor that had been expressed in SF9 cells (figure 3.3.). The effect of translating the mER in the presence of the class I antioestrogen 4-OH-Tamoxifen was also studied to determine if the antagonism was restricted to the class II antioestrogens.

Receptor was translated in the presence of ligand followed by normalisation and analysis using a gel retardation assay as described above (figures 3.10. and 3.11.). The receptor was found to bind efficiently to DNA following translation with either oestradiol, 4-OH-Tamoxifen or no hormone (figure 3.11. lanes 1-4 and 19-20). A slight increase and decrease in complex migration was observed with oestradiol and 4-OH-Tamoxifen respectively as previously reported (Lees et al 1989b). Thus, the antagonism appeared to be restricted to the class II antioestrogens. Translation of receptor in the presence of ICI 164,384, ICI 182,780 and ICI 169,784 was found to completely inhibit DNA binding activity while no effect was observed for the short chain analogue ICI 165,889 (figure 3.11. lanes 5-18). Antiserum MP16 was unable to restore DNA binding following translation in the presence of antagonist.

Several differences in the effects of the analogues were observed depending on whether ligand was added during translation in vitro (figure 3.11.) or following protein synthesis as previously characterised using mER expressed in SF9 cells (figure 3.3.). Translation of receptor in the presence of ICI 169,784 resulted in a complete inhibition of DNA binding (figure 3.11. lanes 15-18) in contrast to the lack of effect of this analogue upon addition to receptor which had been synthesised in SF9 cells (figure 3.3.c.). In addition translation of receptor with 10^{-8}M ICI 182,780 abolished DNA binding activity (figure 3.11. lanes 9-10) while addition of the same concentration to receptor expressed in SF9 cells resulted in approximately 50% inhibition of DNA binding (figure 3.3.c).

Thus, ICI 164,384 was able to inhibit the DNA binding activity of the oestrogen receptor following translation in the presence of the antioestrogen. This was in contrast to the lack of effect upon addition of ligand to the same source of receptor following protein synthesis suggesting the receptor is more sensitive to ICI 164,384 during translation when receptor dimers are forming.
**Figure 3.11. Influence of the class II antioestrogen side-chain on the antagonism of in vitro translated receptor**

mER was translated in the presence or absence of ligand and the resultant DNA binding activity determined using a gel retardation assay. Equal amounts of unlabelled receptor were incubated with 1ng $^{32}$P-labelled ERE containing oligonucleotide for 30 minutes at $25^\circ$C in the presence (+) or absence (-) of antiserum MP16 (1μg protein/track). The protein bound and free DNA were then separated on a 6% non-denaturing gel. As a control (lanes 21-22), translation was performed in the absence of cRNA followed by analysis of a volume of translation product equal to that of the mER translated in the absence of hormone. The concentration of ligand in the translation reactions was as follows:

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**Figure 3.11.**

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MP16: - = +

[Image of gel electrophoresis with bands at positions 1-22]
The class II antioestrogens may therefore prevent the formation of ER dimers during protein synthesis but may not be able to disrupt the dimerisation of preformed dimers depending on their inherent stability. The results imply that the ability of ICI 164,384 to inhibit the DNA binding activity of the oestrogen receptor may be dependent on the strength of the dimerisation interaction.

**Summary and conclusions**

The amidoalkyl side-chain of the class II antioestrogens was found to play a vital role in the antagonist activity of this family of ligands. A side-chain in the α-orientation of 15-16 atoms in length was required for maximal antioestrogen activity *in vitro* and correlated directly with the antagonism reported for the ICI 164,384 analogues *in vivo*. Inhibition of ER DNA binding activity appeared to be mediated by a disruption to receptor dimerisation and was abolished by conditions which stabilise the receptor dimer. The antagonism of oestrogen receptor function by ICI 164,384 was shown to be dependent on the stability of receptor dimers, being more sensitive to the antioestrogen during translation than following protein synthesis.
Chapter 4

A ligand independent loss of mER DNA binding activity
Introduction

The antioestrogen ICI 164,384 has been found to inhibit the DNA binding activity of the oestrogen receptor in a number of systems (Fawell et al 1990b, Wilson et al 1990, Reese and Katzenellenbogen 1991b). In the previous chapter, the presence of the antagonist during translation of the oestrogen receptor in vitro resulted in the production of receptor which was unable to bind to DNA. Addition of antiserum MPI 6 did not restore the DNA binding activity of this receptor suggesting that ICI 164,384 may directly affect DNA binding in addition to disrupting the dimerisation of the oestrogen receptor as implied by the results of chapter three.

The aim of the following study was to investigate whether ICI 164,384 could directly affect the dimerisation and DNA binding activities of the oestrogen receptor. Incubation of receptor in the presence or absence of hormone for increasing periods of time was observed to result in a time-dependent loss of DNA binding activity which was accelerated by ICI 164,384. A similar loss of DNA binding was also observed following the purification of receptor by affinity chromatography. Western blotting with antisera against epitopes in the N- and C-termini of the protein demonstrated that the loss of DNA binding was not a result of protein degradation. However, a monoclonal antibody known as H226, displayed a time-dependent loss of epitope recognition which paralleled the loss of receptor DNA binding activity. Since the epitope for this antisera is located close to the N-terminus of the DNA binding domain it was conceivable that a change in a covalent modification within the vicinity of the domain may influence the DNA binding activity of the receptor. The results presented suggest a role for both phosphorylation and a secondary protein(s) in stabilising the binding of the oestrogen receptor to DNA.

Time-dependent loss of mER DNA binding activity

The mouse oestrogen receptor was expressed in SF9 cells and incubated with hormone for increasing time intervals before assessing the DNA binding activity of the receptor in a gel retardation assay (figure 4.1). A time-dependent loss of DNA binding was observed in the presence and absence of oestradiol which could be restored by addition of antiserum MP16 (figure 4.1, lanes 1-3). In contrast, treating receptor with ICI 164,384 was found to inhibit DNA binding as described in chapter three (figure 4.1, lane 4). Addition of antiserum MP16 was observed to partly restore DNA binding activity to receptor treated with antagonist but this restoration decreased as receptor was incubated with ICI 164,384 for longer periods of time (figure 4.1, lane 5).
Receptor expressed in SF9 cells (0.2μg protein/track) was incubated with 10^{-6}M oestradiol (E), 10^{-6}M ICI 164,384 (I) or no hormone (N) for up to 2 hours at 25°C as indicated. The resultant DNA binding activity was determined by addition of 1ng ³²P-labelled ERE containing oligonucleotide followed by incubation for 30 minutes at 25°C and electrophoresis on a 6% non-denaturing gel. In lanes 2 and 5, antiserum MP16 (1μg/track) was added with the radiolabelled DNA.
contrasts with the observation that MP16 can restore DNA binding to the oestrogen receptor in the presence and absence of oestradiol suggesting that prolonged incubation with the pure antioestrogen may accelerate a loss of DNA binding that is observed regardless of the presence of ligand.

To determine whether oestradiol could reduce the effect of the antioestrogen, receptor was incubated in the presence or absence of hormone for 20 minutes before being treated with a second ligand for up to two hours (figure 4.2.). Binding ICI 164,384 to the receptor before addition of oestradiol was found to result in a loss of DNA binding activity at a similar rate to that observed following incubation of receptor with the antioestrogen alone (figure 4.2. lanes 4 and 6). Similarly, binding of oestradiol prior to incubation with ICI 164,384 initially blocked the effect of the antioestrogen but after one hour a loss of DNA binding was observed with a slight decrease in rate compared to treatment with ICI 164,384 alone (figure 4.2. lanes 4 and 7). The latter was thought to reflect the slower association rate of the antioestrogen rather than a direct effect of the agonist. The loss of DNA binding was found to occur during prolonged incubations of receptor in either the presence or absence of ligand since binding the receptor to an ERE containing oligonucleotide before incubation with or without hormone completely blocked any decrease in DNA binding (figure 4.3.). No change in complex formation was observed following incubation in the presence or absence of oestradiol (figure 4.3. lane 6) or ICI 164,384 (figure 4.3. lane 5) for up to four hours after binding the receptor to DNA.

The influence of phosphorylation on mER DNA binding activity

The inability of oestradiol to reverse the loss of DNA binding suggested the receptor may be degraded prior to binding to its response element. The role of ICI 164,384 as an inducer of such proteolysis was supported by the increase in oestrogen receptor turnover that has been reported in the presence of ICI 164,384 in vivo (Dauvois et al 1992). To investigate the stability of the receptor in the presence and absence of antioestrogen, aliquots of samples analysed in the gel retardation assays above were studied by electrophoresis on a 10% SDS-PAGE gel. Proteins were then transblotted onto nitrocellulose and the receptor was detected by western blotting with antiserum MP16, H226 or H222. The epitopes for these antibodies are located between residues 130-142, 136-202 and 467-532 of the mER respectively (figure 4.4.).

Equal amounts of receptor were detected after western blotting with
**Figure 4.2. Irreversible loss of mER DNA binding activity**

Receptor expressed in SF9 cells (0.2μg protein/track) was incubated with 10^{-6}M oestradiol (E), 10^{-6}M ICI 164,384 (I) or no hormone (N) for 10 minutes at 25°C (lanes 1-7). Oestradiol (10^{-6}M) was then added to track 6 and ICI 164,384 (10^{-6}M) to track 7 before incubating samples 1-7 for an additional 10 minutes, 1 hour or 2 hours at 25°C as indicated. The resultant DNA binding activity was determined by addition of 1ng ^{32}P-labelled ERE containing oligonucleotide followed by incubation for 30 minutes at 25°C and electrophoresis on a 6% non-denaturing gel. In lanes 2 and 5, antiserum MP16 (1μg protein/track) was added with the radiolabelled DNA.
Figure 4.3. **Loss of mER DNA binding activity during prolonged incubations in the presence or absence of ligand**

Receptor expressed in SF9 cells (0.2μg protein/track) was incubated with 10^{-6}M oestradiol (E), 10^{-6}M ICI 164,384 (I) or no hormone (N) for up to four hours at room temperature as indicated. The resultant DNA binding activity was then determined by addition of 1ng ^{32}P-labelled ERE containing oligonucleotide followed by incubation for 30 minutes at 25°C and electrophoresis on a 6% non-denaturing gel (lanes 1-4). This was compared with receptor which had been incubated with radiolabelled oligonucleotide prior to incubation with ligand as described above (lanes 5-6). In lane 2, antiserum MP16 (1μg/track) was added with the labelled DNA.
The epitopes for antisera H222 and H226 have been mapped between the residues outlined above (Kumar et al 1986). H222 and H226 are both rat monoclonal antisera raised against the intact human receptor (Greene et al 1984) while MP16 is a polyclonal antibody which was raised in rabbit against a peptide containing mER residues 130-142 (Fawell et al 1990a).
antibodies MP16 and H222 regardless of the presence or length of incubation with the antioestrogen (figure 4.5.). This indicates that the loss of DNA binding is not a consequence of receptor degradation. In contrast, antibody H226 displayed a time-dependent loss of epitope recognition which paralleled the loss of receptor DNA binding activity (figures 4.2. and 4.5.). Given that the epitope for H226 is located close to the N-terminus of the DNA binding domain, another possibility was that a change in a covalent modification at/near the DNA binding domain may be involved. A number of post-translational protein modifications were considered including phosphorylation, glycosylation and ribosylation. The influence of phosphorylation on the DNA binding activity of the oestrogen receptor was investigated since the epitope for antibody H226 is located between amino acids 136-202 and residues Ser 156, Ser 158 and Ser 171 appear to be phosphorylated in the mER (Hooshang Lahooti unpublished data).

Receptor was incubated in the presence or absence of a cocktail of serine, threonine and tyrosine phosphatase inhibitors for up to four hours and the resultant DNA binding activity was determined using a gel retardation assay (figure 4.6.). DNA binding was initially maintained in the presence of phosphatase inhibitors suggesting that phosphorylation was required for efficient binding of the oestrogen receptor to DNA. However, an eventual loss of DNA binding was observed albeit at a slower rate than that in the absence of inhibitors. Whether DNA binding was affected by a change in phosphorylation of the receptor itself or a secondary protein required for DNA binding was unclear due to the use of crude cell extract in the above experiment.

**DNA binding is affected by a change in oestrogen receptor phosphorylation**

To investigate whether the DNA binding activity of the oestrogen receptor is directly affected by phosphorylation, the receptor was purified using affinity chromatography by Dr. Arnold Coffer of the ICRF Protein Isolation Laboratory (data unpublished). The receptor was over-expressed in SF9 cells and then passed through an affinity column comprised of oestradiol linked via a BSA spacer to a methyl-acrylamide matrix. The purity of the receptor was determined by Jane Fellows of the same laboratory by analysing aliquots of the elution fractions on a 10% SDS-PAGE gel and staining with silver (figure 4.7.). The protein was found to be partially purified with an enrichment of protein corresponding in molecular weight to that of the oestrogen receptor.

Quantitation of the purified receptor could not be performed using a ligand binding assay due to the presence of excess steroid in the eluate. As an
Receptor expressed in SF9 cells (10μg protein/track) was incubated in the presence or absence of 10^{-6}M ICI 164,384 for up to 2 hours at 25°C. The integrity of the receptor was then assessed by electrophoresis on a 10% SDS-PAGE gel followed by western blotting with antiserum H222, H226 or MP16 as indicated. Immune complexes were detected using horseradish colour reagent (see methods).
Figure 4.6. Influence of phosphorylation on mER DNA binding activity

Receptor expressed in SF9 cells (0.2μg protein/track) was incubated in gel shift buffer in either the presence or absence of phosphatase inhibitors (2mM β-glycerophosphate, 100μM sodium vanadate and 20mM sodium fluoride) for up to 4 hours at 25°C. The resultant DNA binding activity was determined by addition of 1ng 32P-labelled ERE containing oligonucleotide followed by incubation for 30 minutes at 25°C and electrophoresis on a 6% non-denaturing gel.
Mouse oestrogen receptor expressed in SF9 cells (100pmoles/mg) was purified by affinity chromatography using a Eupergit C matrix that was coupled to oestradiol hemisuccinate via a BSA spacer. The beads were washed and retarded protein was eluted by incubating the beads with excess steroid in the presence of sodium thiocyanate for 25 minutes at 30°C (see methods). An aliquot of the starting material (3μg/track) and eluate (2μg/track) were then examined by electrophoresis on a 10% SDS-PAGE gel which was stained with silver. In the figure (Sm) and (El) denote the starting material and the eluate respectively.
alternative, the concentration of the purified protein was estimated by denaturing gel electrophoresis followed by western blotting with antibody H222. Increasing volumes of eluate and crude SF9 cell extract (of known receptor concentration) were compared and the amount of receptor in the eluate was determined by comparing the intensity of the respective immune complexes (figure 4.8.). In parallel, the total protein concentration of the eluate was measured using a Bio-Rad assay. The concentration of the purified receptor was estimated to be approximately 4pmoles/mg using this method of quantitation.

The DNA binding activity of the purified receptor was then investigated by incubating equal amounts of crude and purified receptor with radiolabelled DNA followed by nondenaturing gel electrophoresis. The purified receptor was observed to bind DNA with a reduced capacity compared with that of the crude receptor (figure 4.9.). No increase in complex formation was observed upon addition of antiserum MP16. A similar loss of DNA binding activity has also been reported by Mukherjee and Chambon (1990) following the purification of the human oestrogen receptor by affinity chromatography.

To determine whether the DNA binding activity of the mER was directly affected by phosphorylation, the purified receptor was incubated in either the presence or absence of phosphatase inhibitors for an hour followed by non-denaturing gel electrophoresis. A complete loss of DNA binding was observed in the absence of inhibitors which could not be restored by antiserum MP16. In contrast, the DNA binding activity of the receptor was maintained in the presence of phosphatase inhibitors (figure 4.10.). These results indicate that a change in the phosphorylation of the oestrogen receptor can directly affect its DNA binding activity. However, a reduction in the phosphorylation of the receptor does not appear to completely account for a loss of DNA binding activity since addition of phosphatase inhibitors did not prevent an eventual loss of DNA binding over a prolonged period of time (figure 4.6.).

Role of a secondary protein in oestrogen receptor DNA binding

The affinity purification of the human oestrogen receptor has similarly been observed to result in a loss of DNA binding activity. However, Mukherjee and Chambon (1990) observed that DNA binding could be restored by addition of crude HeLa cell extract suggesting that a secondary factor may be required for the DNA binding activity of the receptor. To examine whether a secondary factor such as an accessory protein may also be necessary to stabilise binding of the mouse oestrogen receptor to DNA, the purified mER was incubated in
Unfractionated SF9 cell extract (240pmoles mER/mg) was diluted as indicated and an equal volume of sample (1μl/track) analysed on a 10% SDS-PAGE gel followed by western blotting with antibody H222 (lanes 1-4). This was compared with 1μl affinity purified mER (lane 5) which had been diluted 15 fold in order to estimate the amount of receptor in the chromatography eluate.
The DNA binding activity of the mER expressed in SF9 cells (4fmole receptor/track) was compared before (U) and after (F) fractionation on a ligand affinity column (see methods). A 32P-labelled ERE containing oligonucleotide (1ng/track) was added to the samples followed by incubation for 30 minutes at room temperature and electrophoresis on a 6% nondenaturing gel. Antiserum MP16 (1μg/track) was added in conjunction with the labelled oligonucleotide as indicated. The DNA binding activity of mock-infected SF9 cell extract (0.2μg/track) was determined as a control (C).
Affinity purified mER (4 fmole receptor/track) was incubated in 1x dephosphorylation buffer in either the presence (lanes 5-6) or absence (lanes 3-4) of phosphatase inhibitors (2 mM β-glycerophosphate, 100 μM sodium vanadate and 20 mM sodium fluoride) for one hour at 37°C. The resultant DNA binding activity was then compared with receptor which had received no incubation (lanes 1-2) using a gel retardation assay. A 32p-labelled ERE containing oligonucleotide (1 ng/track) was added in the presence or absence of antiserum MP16 (1 μg/track) followed by incubation for 30 minutes at room temperature and electrophoresis on a 6% nondenaturing gel. Oestradiol (10⁻⁶ M) was present in each sample as a consequence of the receptor having been eluted from the affinity column with excess steroid (see methods).
Equal amounts of affinity purified mER (4fmoles receptor/track) were incubated in the presence or absence of either 30ng or 0.2µg mock-infected SF9 cell extract for 10 minutes at room temperature (lanes 5-6 and lanes 7-8 respectively). The resultant DNA binding activity of the fractionated receptor (lanes 3-8) was then determined using a gel retardation assay and compared with that of the unfractionated 'crude' receptor (4fmoles receptor/track lanes 1-2). A 32P-labelled ERE containing oligonucleotide (1ng/track) was added to each sample and incubated for 30 minutes at room temperature followed by electrophoresis on a 6% nondenaturing gel. Antiserum MP16 (1µg/track) was added in conjunction with the labelled oligonucleotide as indicated. The DNA binding activity of mock-infected SF9 cell extract (0.2µg protein/track, lanes 9-10) and MP16 (1µg/track, lane 11) were studied as controls.
either the presence or absence of mock-infected SF9 cell extract and its resultant DNA binding activity compared with that of the unfractionated receptor in a gel retardation assay (figure 4.11.). The purified protein was observed to bind DNA with a capacity significantly less than the unfractionated receptor as before. However, addition of freshly prepared SF9 cell extract was able to restore the DNA binding activity of the protein to a level similar as that observed for the unfractionated mER. No complex was observed following incubation of mock-infected SF9 cell extract or antiserum MP16 alone with the labelled DNA. This study suggests that a secondary protein may therefore be required to stabilise the binding of the oestrogen receptor to DNA and that this factor/activity appears to have been removed during affinity chromatography.

Summary and conclusions

A time-dependent loss of oestrogen receptor DNA binding activity was observed that was independent of oestradiol and was accelerated by the antioestrogen ICI 164,384. The loss of DNA binding was not a consequence of receptor degradation but appeared to involve an alteration in the phosphorylation of the receptor and/or a disruption to an interaction with an accessory protein that may be required to stabilise the binding of the oestrogen receptor to DNA.
Chapter 5

Preparation and characterisation of the purified mER hormone binding domain
Introduction

The inhibition of oestrogen receptor DNA binding activity by members of the class II family of antioestrogens has been proposed to be a consequence of a disruption to the dimerisation function located in the hormone binding domain. The aim of the following chapter was to generate a source of stable protein to test this hypothesis directly using Fast Protein Liquid Chromatography (FPLC). The results presented describe the cloning, synthesis and characterisation of two GST-fusion proteins containing different regions of the mouse oestrogen receptor. The optimal conditions for the induction and purification of these proteins have been determined and vary according to the nature of the fusion protein being expressed. The feasibility of producing sufficient amounts of pure protein for structural studies was also investigated.

Optimisation of GST-fusion protein synthesis

The hormone binding domain of the oestrogen receptor was expressed in bacteria either alone or in the presence of the DNA binding domain as a glutathione S-transferase fusion protein. This was facilitated by cloning the coding sequences for mER residues 281-599 and 121-599 into suitable pGEX expression vectors as outlined in figure 5.1. The resultant proteins were named according to the region of the oestrogen receptor that they contain, for example GST-ER (281-599) contains mER residues 281-599 fused to the C-terminus of glutathione S-transferase (figure 5.2.).

Optimisation of GST-ER (281-599) synthesis

To optimise the conditions for the synthesis of GST-ER (281-599), transformed bacteria were induced for up to six hours with increasing concentrations of isopropyl-β-D-thiogalactoside (IPTG). Extracts were then prepared and the yield of soluble fusion protein was determined by western blotting with antiserum D547 (figure 5.3.a.). GST-ER (281-599) was found to remain intact regardless of the length of incubation with peak expression detected after four hours of induction with 1.0mM IPTG. In an analogous manner the influence of cell density at the initiation of fusion protein synthesis and the extraction conditions, specifically the strength of sonication and the choice of detergent, were investigated to maximise the yield of fusion protein.

GST-ER (281-599) was found to remain intact regardless of the method of extract preparation. The density of the bacteria at the time of induction was observed to have little effect on the yield of fusion protein (figure 5.3.b.). Similarly, a modest two fold increase in yield was derived using Triton X-100.
Figure 5.1.a. Construction of pGEX-ER (121-599)

cDNA encoding mER residues 121-599 was cloned into the expression vector pGEX-3X to generate a recombinant vector for bacterial cell transformation. 2μg pJ3MOR121-599 (Fawell et al 1989) were digested with EcoRI and BamHI, the digestion products separated on a 1% agarose gel and a 1520bp insert encompassing the mER cDNA was gel purified and recovered by precipitation. In parallel, 2μg pGEX-3X were digested with the same restriction enzymes, precipitated and treated with alkaline phosphatase. The resultant vector and insert DNA were ligated and the ligation mixtures used to transform competent DH5 cells to ampicillin resistance. Resistant colonies were then screened for the presence of insert by digestion of a mini-preparation of DNA with BamHI and EcoRI.

The shading used in the figure was as follows:

- Coding sequence
- Non-coding sequence
- SV40 origin and early promoter
- SV40 T poly A site and SV40t IVS
- Fusion
Figure 5.1.a.

1. Bam HI and EcoRI digestion
2. 1% Agarose gel electrophoresis
3. Fragment purification

1. Bam HI and EcoRI digestion
2. Removal of 5' phosphates
cDNA encoding the mER hormone binding domain was cloned into pGEX-1\(\lambda\)T to generate a recombinant bacterial expression vector. 2\(\mu\)g pGEM3-MOR (281-599) were digested with EcoRI, the digestion products separated on a 2% agarose gel and a 1047bp fragment corresponding to the mER cDNA was gel purified and recovered by precipitation. In parallel, 2\(\mu\)g pGEX-1\(\lambda\)T were similarly digested with EcoRI, precipitated and treated with alkaline phosphatase. The resultant vector and insert DNA were ligated and the ligation mixtures used to transform competent DH5 cells to ampicillin resistance. Resistant colonies were screened for the presence of insert by digestion of a mini-preparation of DNA with EcoRI as above. Positive clones were then rescreened to check the orientation of the insert by digestion of a mini-preparation of DNA with BamHI and Sty I.

The shading used in the figure was as follows:

- **Coding sequence**
- **Non-coding sequence**
- **Fusion**
**Figure 5.1.b.**

1. EcoRI digestion
2. 1% Agarose gel electrophoresis
3. Fragment purification

**LIGATION**

1. EcoRI digestion
2. Removal of 5' phosphates

**pGEX-ER (281-599)**
Figure 5.2. Structural organisation of GST-ER (121-599) and GST-ER (281-599)

**GST-ER (121-599)**
Mr 79.5 kd

![Diagram of GST-ER (121-599)]

**GST-ER (281-599)**
Mr 62.4 kd

![Diagram of GST-ER (281-599)]

GST alone has an Mr 26 kd (Smith et al 1986)
Figure 5.3.a. Timecourse and titration of GST-ER (281-599) induction

One litre cultures of transformed DH5 cells were grown until an A600nm of 0.7a.u. was obtained. Fusion protein synthesis was then induced by addition of increasing concentrations of IPTG followed by incubation for 1-6 hours at 37°C as indicated. A soluble protein supernatant (10ml) was prepared in the presence of 1% Triton X-100 using 4 microns of sonication to break open the cells (see methods). An equal volume of each extract (5µl extract/track) was then analysed on a 10% denaturing gel followed by western blotting with antibody D547.
instead of NP40 during extract preparation (figure 5.3.b.). In contrast, increasing the strength of extract sonication was found to significantly influence the yield of GST-ER (281-599) depending on the length of induction (figure 5.3.c.). For instance, no effect of sonication was observed on the yield of GST-ER (281-599) following two hours of induction while a five fold increase was obtained using high powered sonication to prepare extracts after a four hour incubation with either concentration of IPTG. This presumably reflects the larger amount of fusion protein which is synthesised during the longer incubation with a larger proportion being released and solubilised during sonication in the presence of detergent.

In each experiment the yield of fusion protein was assessed in parallel using a single point ligand binding assay to quantitate the amount of protein that was in a functional form. In agreement with the western blotting data, an optimal yield of GST-ER (281-599) was derived by inducing bacteria for four hours with 1.0mM IPTG using high powered sonication to open the cells in the presence of Triton-X100. A typical yield of 2-3mg of functional GST-ER (281-599) was obtained per litre of culture with a specific activity of approximately 360pmoles/mg. To investigate the possibility of preparing larger amounts (approximately 10-15mg) of fusion protein for structural studies, a ten litre culture of transformed bacteria was subsequently induced using the optimal conditions derived above. The specific activity of the resultant supernatant was approximately ten fold higher than extracts prepared from one litre cultures of cells (figure 5.4.). A yield of almost 130mg fusion protein was obtained in a functional form which accounted for 13.5% of the total protein. This increase in yield and specific activity most likely reflects the improved bacterial aeration attained using large tanks appropriate for cultures of this volume.

**Optimisation of GST-ER (121-599) synthesis**

To synthesis GST-ER (121-599), transformed bacteria were initially induced and extract was prepared according to the conditions optimised for GST-ER (281-599) above. However, little or no fusion protein was produced under these conditions using ligand binding and western blotting assays to quantitate the yield of the fusion protein in the resultant cell extracts. As a result, it was necessary to establish different conditions to synthesise GST-ER (121-599).

The induction conditions for GST-ER (121-599) were initially studied by inducing transformed bacteria with increasing concentrations of IPTG for up to four hours in the presence of zinc in an attempt to nucleate formation of the
Figure 5.3.b. Influence of detergent and cell density on the yield of GST-ER (281-599)

One litre cultures of transformed DH5 cells were grown until an A600nm of 0.7 a.u. or 0.9 a.u. was obtained. Fusion protein synthesis was then induced by addition of 1.0 mM IPTG (+) followed by incubation for 4 hours at 37°C. Uninduced cultures were treated identically except for the addition of IPTG (-). A soluble protein supernatant (10mls) was prepared in the presence of either 1% Triton X-100 (T) or 0.5% NP40 (N) using 14 microns of sonication to break open the cells (see methods). An equal volume of each extract (5μl extract/track) was then analysed by electrophoresis on a 10% SDS-PAGE gel followed by western blotting with antibody H222.
One litre cultures of transformed DH5 cells were grown until an A600nm of 0.7a.u. was obtained. Fusion protein synthesis was then induced by addition of either 0.5mM or 1.0mM IPTG followed by incubation for 2-4 hours at 37°C as indicated. A soluble protein supernatant (10mls) was prepared in the presence of 1% Triton X-100 using either four or fourteen microns of sonication to break open the cells (see methods). An equal volume of each extract (5μl extract/track) was then analysed on a 10% denaturing gel followed by western blotting with antibody H222.
A ten litre culture of transformed DH5 cells was induced at an $A_{600\text{nm}}$ of 0.7a.u. by addition of 1.0mM IPTG followed by incubation for four hours at 37°C. Extract (20ml) was prepared from a sixth of the resultant cell pellet and the yield of GST-ER (281-599) was determined using a single point ligand binding assay. The amount of total protein was measured in parallel using a Bio-Rad assay (see methods). The results summarised in the table above have been extrapolated to give the total amount of fusion protein derived from the ten litre culture compared with an extract (10ml) derived from a one litre culture treated in an identical manner.
DNA binding domain and to stabilise the structure of the fusion protein. Bacterial cell extracts were then prepared and analysed in conjunction with the insoluble protein pellets to determine the solubility of the fusion protein. Each pellet was resuspended in a volume equal to that of the cell extract in order to maintain the original protein concentration. Aliquots of the pellets and supernatants were then studied by denaturing gel electrophoresis followed by western blotting with antiserum H222 (note that a 100 fold excess of supernatant to the volume of pellet was analysed). An inducible protein of approximately 80kd in size was detected corresponding to the expected size of GST-ER (121-599). The expression of this protein was observed to peak after two hours of induction and then fell to barely detectable levels (figure 5.5.a.). A large amount of GST-ER (121-599) appeared to become insoluble with an increase in the length of induction given that detectable protein shifted from the supernatant to the pellet where it was still strongly detected after four hours.

In an attempt to improve fusion protein solubility, the two and four hour incubations were repeated in the presence of a higher concentration of zinc chloride. Since zinc ions are required to tetrahedrally co-ordinate the cysteine residues of oestrogen receptor zinc fingers it was conceivable that addition of insufficient zinc during fusion protein synthesis may have resulted in the production of incorrectly folded protein which was unstable and/or insoluble. The role of the cation in maintaining the conformation of the DNA binding domain and the influence of this domain on the overall stability of the fusion protein was confirmed by the lack of detectable GST-ER (121-599) in the absence of zinc (figure 5.5.b.). However, increasing the concentration of zinc chloride for 0.1mM to 0.5mM during induction failed to improve fusion protein solubility since no increase in the level of GST-ER (121-599) was observed in the supernatant. Furthermore, the cell pellets obtained with the higher concentration of zinc were reduced in size suggesting the ion was detrimental to bacterial growth at the higher concentration.

Thus, optimal induction of GST-ER (121-599) was derived by inducing transformed bacteria with 1.0mM IPTG for two hours in the presence of 0.1mM zinc chloride. The conditions used to prepare extracts containing this fusion protein were not modified after considering the labile and insoluble nature of the fusion protein. Consequently a typical yield of 0.3-0.4mg GST-ER (121-599) was obtained per litre of culture in a soluble and functional form. The specific activity of the resultant extracts was approximately 40pmoles/mg which was relatively low for a bacterial expression system but was comparable to that of the full length receptor expressed in SF9 cells (see methods).
One litre cultures of transformed DH5 cells were induced (+) at an A600nm of 0.7a.u. by addition of 1.0mM IPTG and 0.1mM ZnCl₂ followed by incubation for up to 4 hours at 37°C. Uninduced cultures were treated identically except for the addition of IPTG (-). A soluble protein supernatant (10ml) and pellet of insoluble material were prepared from each culture and the pellets resuspended in 10ml of 1x protein loading buffer (see methods). Aliquots of the derived pellets (0.5μl) and supernatants (20μl) were then analysed by electrophoresis on a 7% SDS-PAGE gel followed by western blotting with antibody H222. In the figure, (P) indicates a pellet and (S) a supernatant.
One litre cultures of transformed DH5 cells were induced at an A600nm of 0.7a.u. by addition of 1.0mM IPTG and increasing concentrations of ZnCl2 followed by incubation for up to four hours at 37°C as indicated. A soluble protein supernatant (10ml) and pellet of insoluble material were prepared from each culture and the pellets resuspended in 10ml of 1x protein loading buffer (see methods). Aliquots of the derived pellets (0.5µl) and supernatants (10µl) were then analysed by electrophoresis on a 7% SDS-PAGE gel followed by western blotting with antibody H222. In the figure (P) indicates a pellet and (S) a supernatant.
Purification of GST-ER (281-599)

Glutathione S-transferase binds its substrate, the reduced form of glutathione, with high affinity enabling GST-fusion proteins to be purified by affinity chromatography using Glutathione Sepharose beads or columns. Purification of GST-ER (281-599) was optimised using a 2ml solid-phase Glutathione Sepharose column since this fusion protein could be prepared in milligram amounts and appeared to be stable in solution. The fusion protein was initially eluted from the affinity matrix with 5mM reduced glutathione as directed by the suppliers instructions but only 10-40% of the protein was recovered in the eluate with the remainder retained on the column (see methods). Increasing the concentration of reduced glutathione to 20mM was subsequently determined to be sufficient for GST-ER (281-599) elution.

Several milligrams of fusion protein were routinely purified from bacterial cell extracts and the elution fractions collected were screened for fusion protein using a single point ligand binding assay. The results of a typical purification are outlined in figure 5.6. Approximately 16% of the fusion protein was retained in the flow-through which could be reduced to 10% after being passed through the column for a second time. Trace amounts of GST-ER (281-599) were also detected in the PBS washes used to remove protein that was non-specifically bound to the affinity matrix but the remaining fusion protein was detected predominantly in elution fractions 2-4 resulting in a complete recovery of GST-ER (281-599).

The affinity column was capable of retaining up to 12mg of GST-ER (281-599), the largest amount of fusion protein applied, with relatively little collected in the flow-through or washes. However, a complete recovery of functional protein did not appear to be obtained when more than 3mg of GST-ER (281-599) was applied to the column (figure 5.7.a.). This was originally thought to be a result of protein being retained on the affinity matrix following elution. However, stripping the column with 3M salt failed to recover further protein indicating that GST-ER (281-599) had already been eluted using reduced glutathione (figure 5.7.b.). Considering a single point ligand binding assay was used to quantitate the yield of GST-ER (281-599) in the eluate, this suggested that the amount of steroid bound by the fusion protein was reduced following purification and that the yield of GST-ER (281-599) was therefore an under-estimation (figure 5.7.a.).

Fusion protein collected after purification remained intact as assessed by western blotting with an ER specific antiserum (figure 5.8.a.). The protein was typically purified about a hundred fold but was not homogeneous since the
Figure 5.6. Affinity purification of GST-ER (281-599)

Extract was prepared from a litre of transformed DH5 cells induced with 1.0mM IPTG for 4 hours at 37°C. The resultant supernatant (Sm) was applied to a 2ml solid-phase Glutathione Sepharose 4B column and the flow-through (Ft) collected. The flow-through was then reapplied and the column was washed with 2x10ml of PBS. Fusion protein was eluted in one ml steps with 20mM reduced glutathione pH8.0 (E1-5). The amount of fusion protein in the fractions collected was quantitated using a single point ligand binding assay and the total protein content estimated with a Bio-Rad assay.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg/ml)</th>
<th>Specific activity (moles/mg)</th>
<th>Yield (mg)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
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</thead>
<tbody>
<tr>
<td>Sm</td>
<td>7.5</td>
<td>11.55</td>
<td>231</td>
<td>1.25</td>
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<td>-</td>
</tr>
<tr>
<td>1st Ft</td>
<td>7.4</td>
<td>9.76</td>
<td>45</td>
<td>0.20</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>2nd Ft</td>
<td>7.4</td>
<td>9.52</td>
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<td>0.13</td>
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<td>0</td>
</tr>
<tr>
<td>Washes</td>
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<td>0.01</td>
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<td>0</td>
</tr>
<tr>
<td>Eluate</td>
<td>5.0</td>
<td>0.128</td>
<td>22102</td>
<td>1.11</td>
<td>89</td>
<td>96</td>
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<tr>
<td>Total recovery</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.45</td>
<td>116</td>
<td>-</td>
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</tbody>
</table>

GST-ER (281-599)
Figure 5.7.a. Titration of the capacity of Glutathione Sepharose for GST-ER (281-599)

A 10L culture of transformed DH5 cells was induced with 1.0mM IPTG for 4 hours at 37°C and extract was prepared from a sixth of the resultant cell pellet (see methods). Supernatant containing 4mg or 12mg of soluble GST-ER (281-599) was then applied to a pair of 2ml solid phase Glutathione Sepharose 4B columns and the flow-through collected. The columns were washed with 2x 10ml PBS and protein was eluted in one ml steps with 20mM reduced glutathione pH8.0 (E1-5). The yield of fusion protein in the collected fractions was quantitated using a single point ligand binding assay and expressed as a percentage of that applied to each column. In the table below (Sm) denotes the starting material and (Ft) the flow-through.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Column 1 GST-ER (281-599) Yield</th>
<th>Column 2 GST-ER (281-599) Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg s.e. %</td>
<td>mg s.e. %</td>
</tr>
<tr>
<td>Sm</td>
<td>4.05 0.35 100</td>
<td>11.83 0.31 100</td>
</tr>
<tr>
<td>Ft</td>
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<td>0.69 0.06 6</td>
</tr>
<tr>
<td>Washes</td>
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<td>0.35 0.03 2</td>
</tr>
<tr>
<td>Eluate</td>
<td>2.32 0.18 57</td>
<td>3.65 0.18 31</td>
</tr>
<tr>
<td>S1-5</td>
<td>- - -</td>
<td>0.00 0.00 0</td>
</tr>
<tr>
<td>Total recovery</td>
<td>2.78 0.59 68</td>
<td>4.7 0.58 40</td>
</tr>
</tbody>
</table>
Figure 5.7.b. Large scale affinity purification of GST-ER (281-599)

A 10L culture of transformed DH5 cells was induced with 1.0mM IPTG for 4 hours at 37°C and extract prepared from a sixth of the resultant cell pellet (see methods). Supernatant (7.5ml) containing approximately 12mg of soluble GST-ER (281-599) was applied to a 2ml solid phase Glutathione Sepharose 4B column which was washed twice with 10ml of PBS before eluting the fusion protein in one ml steps with 20mM reduced glutathione pH8.0 (E1-7). The column was then washed five times with 1ml of PBS containing 3M NaCl to strip the matrix of remaining protein (S1-5). Aliquots of the collected fractions (10μl of E1-7, 10μl S1-5 and 2μl of the remaining fractions) were examined by electrophoresis on a 10% SDS-PAGE gel which was stained with Coomassie Blue. In the adjacent figure (Sm), (Ft), (W1) and (W2) denote the starting material, flow-through, first column wash and second column wash respectively.
Figure 5.7.b.

Markers (kd)
-106
-80
-50
-32

pGex-ER
Figure 5.8.a. Analysis of the integrity of purified GST-ER (281-599)

A 10L culture of transformed DH5 cells was induced at an A600nm of 0.7a.u. by addition of 1.0mM IPTG and incubated for 4 hours at 37°C. Extract (20ml) was prepared from a sixth of the resultant cell pellet and an aliquot containing 2mg of soluble GST-ER (281-599) was applied to a Glutathione Sepharose 4B column. The column was washed twice with 10ml of PBS (W1 and W2) and retarded protein was eluted in one ml steps with 20mM reduced glutathione pH8.0 (E1-4). Aliquots of the collected fractions (1μl of E1-4 and 0.1μl of the remaining fractions) were then examined by electrophoresis on a 10% SDS-PAGE gel followed by western blotting with antiseraum H222. In the figure (Sm) and (Ft) denote the starting material and flow-through respectively.
eluate was often contaminated with a smear of proteins between 28-62kd and a single protein of approximately 80kd (figure 5.8.b.). Washing the column with PBS buffer at different ionic strengths and electrofocusing of the affinity chromatography eluate did not significantly improve the purity of the fusion protein (data not shown). Furthermore, the extent of contamination appeared to be independent of the amount of GST-ER (281-599) applied to the column since the ratio of fusion protein to contaminants remained unchanged in the elution fractions collected after purifying increasing amounts of protein (figure 5.8.b.). However, homogeneous fractions containing less than a milligram of pure GST-ER (281-599) were occasionally obtained and were used to analyse the fusion protein by electron microscopy and FPLC as described in the following chapter.

**Digestion of GST-ER (281-599) with thrombin**

To study the influence of hormone on the dimerisation of the ER hormone binding domain it was preferable to remove glutathione S-transferase from the N-terminus of GST-ER (281-599) to generate the free hormone binding domain. This was facilitated by a thrombin recognition site located within a ten amino acid linker that connects GST to the hormone binding domain (figure 5.2.). To elucidate the conditions required for efficient digestion, purified GST-ER (281-599) was prepared in the absence of protease inhibitors and incubated with an increasing percentage (w/w) of thrombin (ie. weight of enzyme relative to the weight of the fusion protein in the digestion reaction) for either one or two hours at 25°C. After digestion the samples were analysed by denaturing gel electrophoresis followed by western blotting or staining with Coomassie Blue (figure 5.9.a.).

Efficient cleavage of GST-ER (281-599) was observed after digesting approximately 15μg fusion protein for two hours with 0.1% (w/w) enzyme in solution. Similar treatment of GST-ER (281-599) attached to Glutathione Sepharose failed to yield detectable cleavage suggesting that the accessibility of the cleavage site for thrombin is blocked when the fusion protein is attached to the affinity matrix (data not shown). The major digestion product in solution was a protein of approximately 40kd which is heavier than the predicted molecular weight of the hormone binding domain (see figure 5.2.) This indicated that the enzyme may be cleaving the fusion protein at a nonspecific site generating a protein fragment containing regions of GST and the hormone binding domain or alternatively, like GST, the hormone binding domain migrates slightly heavier than would be expected in an SDS-PAGE gel.
A 10L culture of transformed DH5 cells was induced with 1.0mM IPTG for 4 hours at 37°C and extract was prepared from a sixth of the resultant cell pellet (see methods). Increasing volumes of supernatant containing approximately 2mg, 4mg and 7mg of soluble GST-ER (281-599) were then applied to 2ml solid phase Glutathione Sepharose 4B columns. These were washed twice with 10ml of PBS (W1 and W2) before eluting the fusion protein in one ml steps with 20mM reduced glutathione pH8.0 (E1-5). Aliquots of the collected fractions (10μl of E1-5 and 2μl of the remaining fractions) were then examined by electrophoresis on a 10% SDS-PAGE gel which was stained with Coomassie Blue. In the adjacent figure (Sm) and (Ft) denote the starting material and flow-through respectively.
Figure 5.8.b. Titration of GST-ER (281-599) affinity purification

Markers (kd)

2mg Column

4mg Column

7mg Column
Figure 5.9.a. Optimisation of GST-ER (281-599) cleavage

Purified GST-ER (281-599) (15μg protein/sample) was incubated in 25μl cleavage buffer in the presence or absence of increasing amounts of thrombin for 1-2 hours at 25°C as indicated. An equal volume of each sample (10μl/track) was then analysed by electrophoresis on a 10% SDS-PAGE gel followed by either western blotting with antibody H222 (figure 5.9.a.i.) or staining with Coomassie Blue (figure 5.9.a.ii.).
**Figure 5.9.a.(i).**

![Image of gel electrophoresis](image1)

- 1 hour
- 2 hours

<table>
<thead>
<tr>
<th>% Thrombin:</th>
<th>-</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>-</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>X10^-2 (w/w)</td>
<td>-</td>
<td>1</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
<td>-</td>
<td>1</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Markers:
- 80 kDa
- 50
- 32

**Figure 5.9.a.(ii).**

![Image of gel electrophoresis](image2)

- 1 hour
- 2 hours

<table>
<thead>
<tr>
<th>% Thrombin:</th>
<th>-</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>-</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>X10^-2 (w/w)</td>
<td>-</td>
<td>1</td>
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<td>10</td>
<td>-</td>
<td>1</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Markers:
- 106 kDa
- 80
- 50
- 32
- 28
The samples were observed to be contaminated following digestion and a large proportion of the proteins were detectable by western blotting with H222 indicating that the fusion protein and the resultant hormone binding domain were partly degraded. In an attempt to reduce the degradation of the fusion protein, GST-ER (281-599) was digested with thrombin in the presence of different protease inhibitors (figure 5.9.b.). PMSF and leupeptin were found to reduce the efficiency of digestion reflecting their classification as inhibitors of serine proteases. A useful observation was that benzamidine completely blocks thrombin digestion. This inhibitor could therefore be used to terminate digestion reactions. However no significant reduction in fusion protein degradation was observed regardless of the type or combination of inhibitor(s) added.

Thus, optimal digestion of GST-ER (281-599) was obtained in solution following two hours of digestion with 0.1% enzyme (w/w) at 25°C although the enzyme may not cut specifically at its recognition site. The ability of the enzyme to digest a larger amount (0.2mg) of fusion protein was examined using the derived conditions and the digestion reactions were terminated by addition of benzamidine. An equal volume of each sample was then analysed on a denaturing gel which was stained with Coomassie Blue (figure 5.10.). The enzyme was observed to digest microgram amounts (15μg) of fusion protein as before but increasing the amount of fusion protein in the digestion reaction ten fold to 0.2mg failed to produce a corresponding increase in the yield of the hormone binding domain (figure 5.10.). The ability of thrombin to digest GST-ER (281-599) appeared to be limited by the concentration of the protein which may reflect the accessibility of the cleavage site in this particular fusion protein.

Functional characteristics of the GST-fusion proteins

The functional properties of GST-ER (281-599) and GST-ER (121-599) were characterised to determine whether the hormone and DNA binding domains of the oestrogen receptor were similarly folded in these proteins compared with the full length receptor. If this were so then GST-ER (281-599) could be used as a source of protein to study the effect of ligand on the dimerisation of the ER hormone binding domain. Furthermore, the larger fusion protein containing the DNA binding domain could be studied as a control to compare the influence of ligand on the DNA binding activity of the mER and the bacterially expressed protein.

The affinity of GST-ER (281-599) for oestradiol was compared with that of the full length mER using tritiated steroid. A K_d 0.4nM for oestradiol was
Figure 5.9.b. The influence of protease inhibitors on GST-ER (281-599) degradation during digestion with thrombin

Purified GST-ER (281-599) (15μg protein/sample) was incubated in 25μl cleavage buffer in the presence (lanes 4-12) or absence (lanes 1-3) of 0.1% (w/w) thrombin for 2 hours at 25°C in the presence of different protease inhibitors. To determine the effect of the inhibitors on fusion protein degradation and digestion, an equal volume (5μl/track) of each sample was then analysed on a 10% SDS-PAGE gel followed by western blotting with antibody H222 using horseradish colour development reagent (see methods). As a control, fusion protein that had received no incubation at 25°C was also analysed (lane 1). The protease inhibitor(s) added were as follows:

- Lanes 1, 2+4: No inhibitors added.
- Lanes 3+5: All inhibitors added except benzamidine (see lanes below).
- Lane 6: 0.5mg/ml bacitracin and 5μg/ml aprotinin.
- Lane 7: 40μg/ml PMSF.
- Lane 8: 5μg/ml pepstatin A.
- Lane 9: 5μg/ml leupeptin.
- Lane 10: 10mM benzamidine.
- Lane 11: 40μg/ml PMSF and 5μg/ml pepstatin A.
- Lane 12: 40μg/ml PMSF and 5μg/ml leupeptin.
Figure 5.9.b.

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12

Markers (kd)

80
50
32

GST-ER

HBD
Figure 5.10. The capacity of thrombin for GST-ER (281-599)

Purified GST-ER (281-599) was incubated in 25μl cleavage buffer in the presence (+) or absence (-) of 0.1% (w/w) thrombin for 2 hours at 25°C. The reactions were terminated by addition of benzamidine to a final concentration of 10mM. An equal volume of each sample (20μl/track) was then analysed by electrophoresis on a 10% SDS-PAGE gel and fusion protein detected by staining with Coomassie Blue. The ability of the enzyme to digest 15μg and 200μg of GST-ER (281-599) was compared as indicated.
obtained for GST-ER (281-599) following Scatchard analysis (figure 5.11.). This was comparable to the mER which has a $K_d$ 0.3nM for oestradiol in vitro and is similar to that reported for the endogenous receptor (Fawell et al 1989, Gorski and Gannon 1976). The fusion protein also bound ICI 182,780 with a similar affinity to the full length receptor but had an affinity for the class I antioestrogen 4-OH-Tamoxifen which was reduced approximately a hundred fold (figures 5.12.). In contrast, the relative binding affinity of GST-ER (121-599) for E2, ICI 182,780 and 4-OH-Tamoxifen was similar to that of the full-length receptor (figure 5.13.). This implies that the conformation of the hormone binding domain in both fusion proteins is similar to that of the mER although GST-ER (281-599) does not appear to contain the complete ligand binding site for 4-OH-Tamoxifen. Alternatively, glutathione S-transferase may interfere with the binding of the class I antagonist to the hormone binding domain in GST-ER (281-599).

The DNA binding characteristics of GST-ER (121-599) were then compared with the mER using a gel retardation assay. Bacterial cell extract was treated with hormone and incubated with a radiolabelled ERE containing oligonucleotide before separating the protein bound and free DNA on a 6% non-denaturing gel (figure 5.14.a.). The fusion protein was observed to bind to DNA resulting in the formation of a doublet of protein-DNA complexes which migrated faster than that of the full length receptor. This pattern of complex formation suggested GST-ER (121-599) may be degraded since the fusion protein and oestrogen receptor have molecular weights of approximately 79kd and 67kd respectively. In addition, antiserum MP16 was able to shift both complexes to a slower migrating form indicating that GST-ER (121-599) was present in both bands of the doublet. However, western blot analysis of fusion protein incubated in parallel to the above reactions demonstrated the protein was intact immediately prior to electrophoresis (figure 5.14.b.).

The pattern of GST-ER (121-599) DNA binding was therefore distinct from that of the full-length oestrogen receptor. Treatment with hormone prior to incubation with DNA was also found to have no effect on the DNA binding activity of the fusion protein (figure 5.14.a.). The characteristic downshift in the presence of oestradiol and inhibition of DNA binding following incubation with ICI 182,780 were not observed. The results suggest there is either an abnormality in the conformation of the fusion protein on the DNA or that glutathione S-transferase can interfere with the function of the oestrogen receptor domains within the fusion protein. This was investigated by testing the ability of antibodies MP16 and H222 to supershift GST-ER (121-599) in a gel
The affinity of GST-ER (281-599) for oestradiol was determined by incubating 50-100 ng pure protein in triplicate with increasing concentrations of [6,7-^3H] E2 for 12 hours at 4°C. The level of non-specific binding was determined by inclusion of diethylstilbestrol to a final concentration of 10^-6M in one of the triplicate reactions. This was subtracted to give the amount of oestradiol which was specifically bound. Free steroid was removed using dextran coated charcoal and counted after resuspending the pellets in 100μl D.W. The relative binding curve which was derived is shown in graph (A). Scatchard analysis was then performed and the Kd of GST-ER (281-599) for oestradiol was determined from the gradient of graph (B).
The affinity of GST-ER (281-599) for ligand was determined by incubating 50-100ng pure protein in triplicate with 6nM [6,7-3H] E2 and increasing concentrations of unlabelled ligand for 12-18 hours at 4°C. The level of non-specific binding was determined by inclusion of diethylstilbestrol to a final concentration of 10^{-6}M in one of the triplicate reactions. Free steroid was removed using dextran coated charcoal and the non-specific binding was subtracted. The specifically bound tritiated oestradiol was expressed as a percentage of that bound in the absence of competitor and plotted against the concentration of unlabelled ligand (graphs I-III).

**Graph I: R.B.A of GST-ER (281-599) for oestradiol**
Figure 5.12.a.

Graph II: R.B.A. of GST-ER (281-599) for 4-OH-Tamoxifen

Graph III: R.B.A. of GST-ER (281-599) for ICI 182,780
Figure 5.12.b, Relative binding affinity of GST-ER (281-599)

The relative binding affinity of GST-ER (281-599) for oestradiol, ICI 182,780 and 4-OH-Tamoxifen was calculated as the ratio of the I.C.50 value for oestradiol relative to that of the antioestrogen. The I.C.50 for each ligand was extrapolated from the respective competition graphs in figure 5.12.a. The results for both the mER and GST-ER (281-599) have been summarised in the table below.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Relative binding affinity mER (1-599)</th>
<th>Relative binding affinity GST-ER (281-599)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oestradiol</td>
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</tr>
<tr>
<td>ICI 182,780</td>
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<tr>
<td>4-OH-Tam</td>
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Figure 5.13.a. Affinity of GST-ER (121-599) for agonist and antagonist

A litre of transformed DH5 cells was induced at an A600nm of 0.7a.u. by addition of 1.0mM IPTG and 0.1mM ZnCl₂ followed by incubation for two hours at 37°C. An aliquot of the resultant cell extract (0.4μg protein/sample) was incubated in triplicate with 6nM [2,4,6,7-³H] E₂ in the presence of increasing concentrations of unlabelled ligand for 12-18 hours at 4°C. The level of non-specific binding was determined by inclusion of diethylstilbestrol to a final concentration of 10⁻⁶M in one of the triplicate reactions. Free steroid was removed using dextran coated charcoal and the non-specific binding was subtracted. The amount of specifically bound tritiated oestradiol was then expressed as a percentage of that bound in the absence of competitor and plotted against the concentration of unlabelled ligand (graphs I-III).

Graph I : R.B.A. of GST-ER (121-599) for oestradiol

![Graph I: R.B.A. of GST-ER (121-599) for oestradiol](image)
Figure 5.13.a.

Graph II: R.B.A. of GST-ER (121-599) for 4-OH-Tamoxifen

Graph III: R.B.A. of GST-ER (121-599) for ICI 182,780
Figure 5.13.b. Relative binding affinity of GST-ER (121-599)

The relative binding affinity of GST-ER (121-599) for oestradiol, ICI 182,780 and 4-OH-Tamoxifen was calculated as the ratio of the I.C.50 value for oestradiol over that of the antioestrogen. The I.C.50 value for each ligand was extrapolated from the respective competition graphs in figure 5.13.a. The results for both the mER and GST-ER (121-599) have been summarised in the table below.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Relative binding affinity mER (1-599)</th>
<th>Relative binding affinity GST-ER (121-599)</th>
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<td>oestradiol</td>
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<td>1.00</td>
</tr>
<tr>
<td>ICI 182,780</td>
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<td>0.40</td>
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<tr>
<td>4-OH-Tam</td>
<td>1.20</td>
<td>1.50</td>
</tr>
</tbody>
</table>
Figure 5.14.a. Influence of hormone on GST-ER (121-599) DNA binding

50 fmols of GST-ER (121-599) and mouse oestrogen receptor were incubated with $10^{-6}$M oestradiol (E), $10^{-6}$M ICI 182,780 (I) or no hormone (N) for 20 minutes at room temperature. The resultant DNA binding activity was then determined by addition of $32^P$-labelled ERE containing oligonucleotide (1ng/track) followed by incubation for 30 minutes at room temperature and electrophoresis on a 6% non-denaturing gel. Antiserum MP16 was added with the radiolabelled DNA as indicated (5µg antiserum to GST-ER (121-599) and 1µg to the mER). The DNA binding activity of uninduced DH5 cell extract (1µg/track) and mock-infected SF9 cell extract (0.2µg/track) were studied as controls (C).
**Figure 5.14.b. Analysis of GST-ER (121-599) stability**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>1</th>
<th>Markers (kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
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<td>50</td>
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<tr>
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<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>

DH5 cell extract (20μg) was incubated in 1x retardation buffer devoid of BSA and poly dl-dC for one hour at room temperature in the absence of hormone. An identical sample was prepared in parallel which received no incubation. Fusion protein was then detected by electrophoresis of both samples on a 10% SDS-PAGE gel followed by western blotting with antiserum H222.
retardation assay to assess whether the N- and C-termini of the receptor were as accessible to antiserum in the fusion protein and therefore in a similar conformation to the analogous regions in the mER.

Equal amounts of receptor expressed in bacteria and SF9 cells were incubated with antiserum followed by incubation with radiolabelled DNA and electrophoresis as before. Antiserum MP16 was observed to efficiently retard both GST-ER (121-599) complexes suggesting the N-terminus of the receptor was correctly folded within the fusion protein (figure 5.15. lane 2). In contrast, antiserum H222 was unable to significantly shift either complex despite increasing the amount of antibody to a level 20 fold higher than that required to shift the mER (figure 5.15. lanes 3-5 and 7-8). Furthermore, the ability of antibody H222 to retard the migration of the mER when equal amounts of bacterial and SF9 cell extracts were incubated together indicates that the antiserum is functional in the presence of bacterial extract (figure 5.15. lane 6).

Summary and conclusions

The hormone binding domain of the mouse oestrogen receptor was expressed in bacteria as a glutathione S-transferase fusion protein termed GST-ER (281-599). Similarly, a larger fusion protein containing both the hormone and DNA binding domains of the mER was generated and is referred to as GST-ER (121-599). The conformation of the hormone binding domain in both proteins appears to be similar to that of the oestrogen receptor since GST-ER (121-599) and GST-ER (281-599) bind oestradiol and ICI 182,780 with an affinity similar to that of the full length receptor. In contrast, high affinity binding of 4-OH-Tamoxifen was only observed for GST-ER (121-599) while the affinity of GST-ER (281-599) for this antagonist is reduced approximately a hundred fold compared to the mER. This suggests the smaller fusion protein does not contain the complete ligand binding site for 4-OH-Tamoxifen or alternatively that glutathione S-transferase can interfere with the binding of this antagonist.

GST-ER (121-599) was observed to bind DNA giving a doublet of protein-DNA complexes. This was not the result of protein degradation but appeared to involve a change in the conformation of the hormone binding domain upon binding of the fusion protein to an ERE. This was implied by the reduction in the accessibility of the epitope for antiserum H222 in GST-ER (121-599) compared with the oestrogen receptor in a gel retardation assay. In addition, no effect of agonist or antagonist was observed on the DNA binding activity of the fusion protein although GST-ER (121-599) can bind these ligands in solution. Taking the functional properties of the two fusion proteins into
Figure 5.15. Analysis of GST-ER (121-599) conformation using antiserum H222

Equimolar amounts (50fmol/track) of GST-ER (121-599) (lanes 1-6) and mouse oestrogen receptor (lanes 6-8) were incubated in the presence or absence of antiserum H222 for 10 minutes at room temperature as indicated. The resultant DNA binding activity was then determined by addition of a 32P-labelled ERE containing oligonucleotide (1ng/track) followed by incubation for 30 minutes at room temperature and electrophoresis on a 6% nondenaturing gel. This was compared with the DNA binding activity of uninduced DH5 cell extract (1μg protein/track) in the presence or absence of H222 (lanes 9-10). As controls, 50fmol of oestrogen receptor and GST-ER (121-599) were added together in lane 6 and the effect of antiserum MP16 (5μg protein/track) on the DNA binding activity of the fusion protein was assessed in lane 2. In the figure, arrows have been used to indicate the position of radiolabelled DNA which is bound by GST-ER (121-599) while the asterix denotes the position of protein-DNA complexes containing antiserum.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>H222 (μg): -</td>
<td>-</td>
<td>0.1</td>
<td>1</td>
<td>2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.01</td>
<td>-</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>
account, GST-ER (281-599) appears to be more suitable as a source of protein to analyse the effect of ligand on the dimerisation of the hormone binding domain. Furthermore, GST-ER (281-599) was generally a more stable soluble protein than GST-ER (121-599) and could be purified approximately a hundred fold over Glutathione Sepharose affinity chromatography columns.
Chapter 6

The influence of ligand on the dimerisation of the hormone binding domain
Introduction

Hormone binding has been proposed to initiate the transformation of nuclear receptors to a transcriptionally active form by inducing a specific conformation of the hormone binding domain (Allan et al 1992a, Allan et al 1992b, Fritsch et al 1992). The aim of the following chapter was to investigate the effects of agonist and antagonist on the conformation and dimerisation of the hormone binding domain of the mouse oestrogen receptor using electron microscopy and FPLC. This study was initiated by deletion analysis of the hormone binding domain in order to define the minimal region that is required to form the ligand binding site.

N-terminal deletion analysis of the hormone binding domain

The C-terminal boundary of the hormone binding domain has been shown to be located between residues 522 and 538 in the mouse oestrogen receptor (Lees et al 1989a, Fawell et al 1990a). To define the N-terminal boundary of this domain, deletion mutants of GST-ER (281-599) were prepared based on the conservation and predicted secondary structure of the receptor. The mER shares 94% sequence homology with the chicken, rat and human proteins between residues 343-356 suggesting this region may play a vital role in the function of the hormone binding domain. Amino acids 281-342 are also conserved (63%) but with small pockets of variability around residues 285, 300, 330 and 340 (figure 6.1.). This reduction in sequence conservation suggested the N-terminal boundary of the hormone binding domain may be located between residues 281-342.

In order to prepare deletions which would disrupt the conformation of the hormone binding domain as little as possible, the secondary structure of the mER was predicted from the coding sequence of the receptor using the Chou and Fasman algorithm. Towards the C-terminus of the protein, the program predicted residues 281-328 would form a long alpha helix interspersed with several turns and a short region of beta-sheet structure. This was followed by a turn or loop formed by amino acids 333-343 with a long stretch of combined alpha-helix and beta-sheet structure between residues 342-367 (figure 6.2.). Although the structural predictions are not fully reliable, the structural and conservation data together suggested potential areas within the hormone binding domain which are less well conserved and if deleted may not interrupt a region of secondary structure.

To determine the N-terminal boundary of the hormone binding domain, internal deletions were therefore prepared within pGEX-ER (281-599) as
The mER amino acid sequence (White et al 1987) was compared with those of the rat (Koike et al 1987), human (Green et al 1986) and chicken (Krust et al 1986) oestrogen receptors using the PRTALN program of Lippman and Wilbur. Amino acids which are not fully conserved between the four species have been drawn in small letters using bold print. The residue numbers in the figure refer to those of the mER.

\[ \text{Figure 6.1. Conservation of mER amino acid sequence} \]

| 270-KhKRQRddle | gRnemgssgd | mAanLWpSP | LviKhtKKNS | pALSLTAadQM |
| 320-VSALLdAEPP | iiYSEYDPsR | PFsEASMMgL | LTNLADRELV | HMINWAKRVP |
| 370-GFgDLnLHDQ | VHLLECAWLE | ILMIGIVWRS | MEHPgKLLFA | PNLLLDRNQG |
| 420-KCVEGMVEIF | DMLLATssRF | RMMNLQGEEF | VCLKSIILLN | SGVYTFLSST |
| 470-LKSLEEkDhI | HRVLDKITDT | LIHLMAKaqGL | tQQQHRqRLA | QLLLILSHIR |
| 520-HMSNKGMEHL | YnMKCKNVVP | LYDLLLEMLD | AHRLHAPasR | mgvppEEpsq |
The secondary structure of the mER was predicted using the Chou and Fasman algorithm. The data for residues 270-380 has been summarised above using the letters 'A', 'B' and 'T' to indicate an α-Helix, β-sheet and a turn respectively. Five mutants of GST-ER (281-599) were prepared by deleting amino acids N-terminal to residues 313, 330, 341 or 366 in the hormone binding domain. The numbers of these residues correspond to those in the mER and have been indicated with an asterix (*).
outlined in figure 6.3. The fusion proteins were then generated by inducing transformed bacteria with IPTG (see methods) and the integrity of the proteins was confirmed by denaturing gel electrophoresis followed by western blotting with antibody H222 (figure 6.4.). To assess which of the deletion mutants could bind hormone, increasing amounts of fusion protein were incubated with either tritiated oestradiol or 4-OH-Tamoxifen in a single point ligand binding assay. Specific binding was observed for both oestradiol and 4-OH-Tamoxifen until the hormone binding domain was deleted beyond amino acid 313 (figures 6.5. and 6.6.). Increasing the volume of GST-ER (330-599), GST-ER (341-599) and GST-ER (366-599) in the assay to 50 fold in excess of GST-ER (313-599) failed to yield detectable ligand binding. Similar results were also obtained using 100-250 fold excess of these proteins (data not shown).

The affinity of GST-ER (313-599) for oestradiol, 4-OH-Tamoxifen and ICI 182,780 was then compared with that of the longer fusion proteins GST-ER (281-599) and GST-ER (121-599) which were characterised in chapter five. Crude and 'purified' GST-ER (313-599) were incubated with 6nM tritiated oestradiol in the presence of increasing concentrations of unlabelled ligand. The percentage of specifically bound tritiated steroid was then plotted against the concentration of competitor and the resultant graphs were used to calculate the relative binding affinity of the protein for each ligand (figure 6.7.). The affinity of GST-ER (313-599) for oestradiol and ICI 182,780 was found to be similar to that of the full length receptor. In contrast, the affinity of the fusion protein for 4-OH-Tamoxifen was reduced by approximately a hundred fold compared to the mER which was directly comparable to GST-ER (281-599) (figure 5.12.). Purification of GST-ER (313-599) was observed to result in a three fold reduction in the affinity of the fusion protein for ICI 182,780 suggesting the solubility of the protein may be slightly compromised by addition of this hydrophobic ligand.

The results indicate that the N-terminal boundary of the hormone binding domain for oestradiol and ICI 182,780 is located near amino acid 313 for both ligands. In contrast, the N-terminal boundary of the ligand binding site for 4-OH-Tamoxifen appears to be located more N-terminal since a significant reduction in affinity was observed upon deletion of residues 121-281 and 121-313 (figures 5.12., 5.13. and 6.7.). In the previous chapter, GST-ER (121-599) was shown to have an affinity for 4-OH-Tamoxifen similar to that of the mER positioning the N-terminal boundary for the class I antagonist between residues 121-281. Alternatively, the N-terminal boundary for 4-OH-Tamoxifen may similarly be located near amino acid 313 but binding of the antagonist is
GST-ER (281-599) was generated by cloning mER residues 281-599 into the expression vector pGEX-1λT (see chapter 5). Internal deletions were then prepared using the Polymerase Chain Reaction to remove an increasing number of residues C-terminal to amino acid 281 in the HBD. In the diagram, the residue numbers refer to positions within the mER.

The fusion proteins generated contain a linker (□□□) between glutathione S-transferase and the HBD. The structure of the linker for GST-ER (281-599) is outlined in figure 5.3, while the composition of the linker in the mutants is shown below:

\[
\text{Linker: } \text{Leu-Val-Pro-Arg-Gly-Ser-Glu-Phe-Thr-Met-Thr} \\
\hspace{2.5cm} \text{Thrombin} \\
\hspace{-1.5cm} \text{----------}
\]

**Figure 6.3.a. Structural organisation of the GST-ER deletion mutants**
Figure 6.3.a.

<table>
<thead>
<tr>
<th>NAME</th>
<th>STRUCTURAL ORGANISATION</th>
<th>Mr (kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-ER (281-599)</td>
<td>N— GST</td>
<td>62.4</td>
</tr>
<tr>
<td>GST-ER (313-599)</td>
<td>N— 313 330 GST</td>
<td>58.9</td>
</tr>
<tr>
<td>GST-ER (330-599)</td>
<td>N— 330 GST 341 GST</td>
<td>56.9</td>
</tr>
<tr>
<td>GST-ER (341-599)</td>
<td>N— 341 GST 366 GST</td>
<td>55.7</td>
</tr>
<tr>
<td>GST-ER (366-599)</td>
<td>N— 366 GST 599 GST</td>
<td>52.9</td>
</tr>
</tbody>
</table>
Constructs pGEX-ER (330-599), pGEX-ER (341-599) and pGEX-ER (366-599) were generated using the polymerase chain reaction to synthesis DNA between the starting codon of the hormone binding domain and residues 434 in the coding sequence of the mER (see methods). The resultant DNA fragments were then precipitated, digested with BamHI and Bgl II and gel purified. In parallel, pGEX-ER (281-599) (2μg) was digested with the same restriction enzymes and treated with alkaline phosphatase. The resultant vector and insert DNA were then ligated and used to transform competent DH5 cells to ampicillin resistance. Construct pGEX-ER (313-599) was prepared separately by isolating an EcoRI fragment encoding mER residues 313-599 from pGEM-MOR (313-599) which was kindly provided by Paul Danielian (ICRF). This fragment was cloned into pGEX-1λ.T in a manner identical to the synthesis of pGEX-ER (281-599) as outlined in figure 5.1.a.

The shading used in the figure was as follows:

- Coding sequence
- Non-coding sequence
- Fusion
**Figure 6.3.b.**

1. Precipitation
2. BamHI and Bgl II digestion
3. Fragment purification

1. BamHI and Bgl II digestion
2. Removal of 5' phosphates
One litre cultures of transformed DH5 cells were induced at an A600nm of 0.7a.u. by addition of 1.0mM IPTG and incubated for 4 hours at 37°C. Extract (10ml) was then prepared and the integrity of the fusion protein assessed by electrophoresis of an aliquot (0.1μl) of each extract on a 10% SDS-PAGE gel followed by western blotting with antibody H222. The extracts applied to the gel contained the following fusion proteins, lane 1 GST-ER (281-599), lane 2 GST-ER (313-599), lane 3 GST-ER (330-599), lane 4 GST-ER (341-599) and lane 5 GST-ER (366-599).
Increasing volumes of bacterial cell extract were incubated in triplicate with 6nM [2,4,6,7-3H] E2 for 12 hours at 4°C. The level of non-specific binding was determined by inclusion of diethylstilbestrol to a final concentration of 10^-6M in one of the triplicate reactions. Free steroid was then removed using dextran coated charcoal and the non-specific binding was subtracted. The total, nonspecific and specific counts obtained after removing the unbound steroid are displayed in the table above.
Figure 6.6. Ability of GST-ER (281-599) deletion mutants to bind 4-OH-Tamoxifen

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Volume (µl)</th>
<th>Total (cpm)</th>
<th>Nonspecific (cpm)</th>
<th>Specific (cpm)</th>
<th>Ability to bind OHT</th>
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</thead>
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<tr>
<td>GST-ER (281-599)</td>
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<td>6632 6699</td>
<td>3649</td>
<td>2983 3050</td>
<td>+</td>
</tr>
<tr>
<td>GST-ER (313-599)</td>
<td>0.02</td>
<td>5712 5511</td>
<td>3254</td>
<td>2458 2257</td>
<td>+</td>
</tr>
<tr>
<td>GST-ER (330-599)</td>
<td>5</td>
<td>6972 6403</td>
<td>6906</td>
<td>66 0</td>
<td>-</td>
</tr>
<tr>
<td>GST-ER (341-599)</td>
<td>5</td>
<td>6790 7098</td>
<td>7167</td>
<td>0 0</td>
<td>-</td>
</tr>
<tr>
<td>GST-ER (366-599)</td>
<td>5</td>
<td>6549 6594</td>
<td>6916</td>
<td>0 0</td>
<td>-</td>
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</tbody>
</table>

Increasing volumes of bacterial cell extract were incubated in triplicate with 6nM Z-4-Hydroxy [N-methyl-³H]-tamoxifen for 12 hours at 4°C. The level of non-specific binding was determined by inclusion of diethylstilbestrol to a final concentration of 10⁻⁶M in one of the triplicate reactions. Free steroid was then removed using dextran coated charcoal and the non-specific binding was subtracted. The total, nonspecific and specific counts obtained after removing the unbound steroid are displayed in the table above.
Crude and purified GST-ER (313-599) (0.2μg protein/sample) were incubated in triplicate with 6nM [2,4,6,7-^3H] E2 in the presence of increasing concentrations of unlabelled ligand for 12-18 hours at 4°C. The level of non-specific binding was determined by inclusion of diethylstilbestrol to a final concentration of 10^{-6}M in one of the triplicate reactions. Free steroid was then removed using dextran coated charcoal and the non-specific binding was subtracted. The amount of specifically bound tritiated oestraediol was expressed as a percentage of that bound in the absence of competitor and plotted against the concentration of unlabelled ligand (graphs I-III).

**Graph I: R.B.A. of GST-ER (313-599) for oestraediol**
Figure 6.7.a.
Graph II: R.B.A. of GST-ER (313-599) for ICI 182780

Graph III: R.B.A. of GST-ER (313-599) for 4-OH-Tamoxifen
The relative binding affinity of GST-ER (313-599) for oestradiol, ICI 182,780 and 4-OH-Tamoxifen was calculated as the ratio of the I.C.50 value for oestradiol over that of the antioestrogen (extrapolated from graphs I-III in figure 6.7.a.). The results for both the crude and purified fusion protein are summarised in the table below and compared with those of the full length mER.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>RBA mER (1-599)</th>
<th>RBA of crude GST-ER (313-599)</th>
<th>RBA of purified GST-ER (313-599)</th>
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</thead>
<tbody>
<tr>
<td>oestradiol</td>
<td>1.00</td>
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<td>1.50</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>0.42</td>
<td>0.50</td>
<td>0.15</td>
</tr>
<tr>
<td>4-OH-Tam</td>
<td>1.10</td>
<td>0.01</td>
<td>0.01</td>
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</tbody>
</table>

161
disrupted by glutathione S-transferase in fusion proteins GST-ER (281-599) and GST-ER (281-599). Thus, the ligand binding site for oestradiol and ICI 182,780 appears to be located between residues 313 and 522/538 of the mouse oestrogen receptor which may differ from that of the class I antagonist 4-OH-Tamoxifen.

Influence of ligand on the dimerisation of the hormone binding domain

Source of stable functional protein

To compare the effects of agonist and antagonist on oestrogen receptor dimerisation, FPLC and sucrose gradient ultra-centrifugation were initially used to determine the size of the mER expressed in SF9 cells. The fractions collected were analysed for receptor using western blot and gel retardation assays but the eluted protein was found to be extensively degraded which allowed only trace amounts of the oestrogen receptor to be detected (data not shown). To facilitate the investigation a source of stable protein was therefore essential and appeared to require the use of purified receptor for two reasons. Firstly, the use of pure protein would allow any detectable change in size to be attributed to the receptor itself rather than an indirect effect of a secondary protein. Secondly, crude purification of the receptor by chromatography and ultra-centrifugation may have increased the number of protease sites exposed on the surface of the protein. If so, efficient purification prior to size determination could prevent or reduce the degradation previously observed using the full length oestrogen receptor.

At the time of this investigation work on the purification of the mER had just begun. For this reason GST-ER (281-599) was used as an alternative source of protein since it could be synthesised in sufficient amounts for FPLC (approximately 1µg pure protein) and bound both oestradiol and ICI 182,780 with high affinity following affinity purification. The fusion protein was synthesised and purified over a solid phase Glutathione Sepharose column. Protein was eluted using reduced glutathione and the elution fractions were screened for GST-ER (281-599) using a single point ligand binding assay. Two peak fractions of hormone binding activity were identified with a specific activity of approximately 6nmoles/mg. The purity and integrity of the fusion protein were then determined by electrophoresis of an aliquot of the starting material and peak fractions on a 10% SDS-PAGE gel followed by silver staining or western blotting with antibody H222 (figure 6.8.). A single band of approximately 65kd was detected in the peak fractions using both techniques.
A litre of transformed DH5 cells was induced at an A600nm of 0.7a.u. by addition of 0.5mM IPTG followed by incubation for 30 minutes at 37°C. Extract (10ml) was prepared from which fusion protein was purified by affinity chromatography using a Glutathione Sepharose 4B column. The column was washed and protein was eluted with 5mM reduced Glutathione pH8.0 (see methods). The purity of GST-ER (281-599) was then assessed by electrophoresis of 200ng crude cell extract (lane 1) and two peak fractions (lanes 2-3) on a 10% SDS-PAGE gel which was stained with silver. In parallel, fusion protein integrity was determined by electrophoresis of 400ng peak fraction on a 10% SDS-PAGE gel followed by western blotting with antiserum H222 (lanes 4-5).
indicating that the fusion protein was intact and had been purified to homogeneity.

**Demonstration of FPLC as a dimerisation assay**

The analysis of proteins by FPLC was performed routinely using a 25ml Superose 12 column which was calibrated by running individual marker proteins through the column and measuring the protein content of the fractions collected in a Bio-Rad assay. The elution volume for each marker protein was then calculated from the position of the peak fraction and used to plot a calibration curve as outlined in figure 2.2. The resultant graph was linear which demonstrated that the column and conditions were suitable for the accurate separation of proteins according to their molecular weight (figure 6.9.). In addition, a 2:1 ratio was obtained for the void volume over the inclusion volume indicating the column was being used to its maximal separation capacity (figure 2.2.).

To demonstrate the feasibility of FPLC as a dimerisation assay, glutathione S-transferase was purified and passed through the calibrated column to determine the molecular weight of the protein in solution. The fractions collected were screened for protein by denaturing gel electrophoresis followed by staining with Coomassie Blue (figure 6.10.). The identification of the peak fraction was also confirmed by measuring the A595nm for each fraction in a Bio-Rad assay (data not shown). The elution volume for GST was then derived from the position of the peak fraction and used to extrapolate the molecular weight of the protein from the calibration curve. The pGEX expression vectors encode glutathione S-transferase from the helminth *Schistosoma japonicum* which has a subunit molecular weight of approximately 26kd although it should be noted that the denatured protein migrates in a 10% SDS-PAGE gel with a subunit molecular weight that is closer to 30kd (Smith et al 1986, Smith and Johnson 1988). Using FPLC, purified glutathione S-transferase was found to have a molecular weight of approximately 56kd indicating that the enzyme exists as a dimer in solution. The dimeric nature of mammalian GST iso-enzymes has similarly been reported by other groups using a variety of techniques (Parker 1990, Aniya and Anders 1992).

**FPLC analysis of purified GST-ER (281-599)**

To study the influence of ligand on the dimerisation of the hormone binding domain, purified GST-ER (281-599) was incubated with hormone and then analysed by FPLC. The fractions collected were screened for fusion
Figure 6.9. **Superose 12 calibration data and graph**

The data obtained following analysis of marker proteins by FPLC is summarised in the table (I) and was used to generate graph (II) below.

### Table (I)

<table>
<thead>
<tr>
<th>Protein marker</th>
<th>Mr (kd)</th>
<th>Log Mr</th>
<th>Peak Fraction</th>
<th>Ve (ml)</th>
<th>Ve/Vo</th>
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<tr>
<td>Blue Dextran</td>
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<td>38</td>
<td>7.60</td>
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<tr>
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<td>2.30</td>
<td>54</td>
<td>10.80</td>
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<tr>
<td>Lactate Dehydrogenase</td>
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<td>2.16</td>
<td>58</td>
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<td>Ovotransferrin</td>
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<td>12.80</td>
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<td>Carbonic Anhydrase</td>
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<td>Cytochrome C</td>
<td>12</td>
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<td>15.20</td>
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</tbody>
</table>

Graph (II): **Calibration curve for FPLC Superose 12 column**

\[
y = 2.4790 - 0.45398x \quad R^2 = 0.981
\]
Figure 6.10. FPLC analysis of purified glutathione S-transferase

Purified GST (1mg) was loaded onto a calibrated Superose 12 column and eluted with a flow-rate of 0.2ml/min. Fractions were collected and screened for protein by electrophoresis of 20μl elution fraction on a 10% SDS-PAGE gel followed by staining with Coomassie Blue. The approximate molecular weight of proteins within each fraction has been extrapolated from the calibration curve and inserted above the lanes in the figure for ease of reference.

Extrapolated

<table>
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<th>71</th>
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protein by denaturing gel electrophoresis followed by western blotting with the antibody H222 (figure 6.11.). Using this procedure, intact GST-ER (281-599) was detected and the peak fractions were identified. The molecular weight of the fusion protein was then extrapolated from the calibration curve as previously described (see methods). In the absence of hormone GST-ER (281-599) appeared to be dimeric with a molecular weight of approximately 145kd. A broad protein peak was detected which was particular to unbound GST-ER (281-599) since addition of hormone and analysis of all other proteins by FPLC yielded sharply focused peaks. This suggested the fusion protein may have a flexible conformation in the absence of hormone. Addition of oestradiol was observed to have no effect on the dimerisation of the fusion protein and appeared to restrict the conformation of GST-ER (281-599) producing a single sharply focused peak which corresponded in size to the protein dimer. In contrast, treating GST-ER (281-599) with ICI 182,780 was found to produce a change in the molecular weight of the fusion protein resulting in the formation of a protein with a molecular weight of approximately 77kd. This change in size was indicative of a disruption to the dimerisation of GST-ER (281-599) with the concomitant production of a monomeric form of the fusion protein. Thus, the hormone binding domain of the mouse oestrogen receptor appears to be dimeric in solution in the presence or absence of oestradiol while the antioestrogen ICI 182,780 is able to disrupt the dimerisation function located in this domain.

**Analysis of GST-ER (281-599) by electron microscopy**

Binding of agonist and antagonist to the hormone binding domain of the progesterone and retinoic acid receptors has recently been shown to generate different conformations of the proteins in protease mapping studies (Allan et al 1992a, Allan et al 1992b). To investigate whether oestradiol and ICI 182,780 can similarly alter the structure of the hormone binding domain of the oestrogen receptor, the fusion protein GST-ER (281-599) was incubated with ligand and examined using electron microscopy in collaboration with Dr. Richard Newman of the ICRF Protein Structure Laboratory.

GST-ER (281-599) was synthesised and purified by affinity chromatography using a Glutathione Sepharose column as before. The purity and integrity of the fusion protein were then assessed by electrophoresis of equal amounts of the crude and purified protein on a 10% SDS-PAGE gel followed by silver staining or western blotting with antibody H222 (figure 6.12.). In parallel, the specific activity of the purified protein was determined using a
Figure 6.11. FPLC analysis of purified GST-ER (281-599)

Pure GST-ER (281-599) (1µg) was incubated in 400µl FPLC running buffer in the presence or absence of 10^-6M oestradiol or 10^-6M ICI 182,780 for 20 minutes at 25°C before loading 200µl of the sample onto a calibrated Superose 12 column. Protein was eluted with a flow-rate of 0.2ml/min and 200µl fractions were collected. Fusion protein was then detected by electrophoresis of 60µl of each elution fraction on a 2mm 10% SDS-PAGE gel followed by western blotting with antiserum H222.
**Figure 6.11.**

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<th>Fraction No</th>
<th>Lactate Dehydrogenase 145kD</th>
<th>Amylase 200kD</th>
<th>Ovotransferrin 76kD</th>
<th>Ovalbumin 45kD</th>
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A litre of transformed DH5 cells was induced at an A600nm of 0.7a.u. by addition of 1.0mM IPTG and incubated for 4 hours at 37°C. Extract (10ml) was prepared from which fusion protein was purified by affinity chromatography using a Glutathione Sepharose 4B column. The column was washed and protein was eluted with 20mM reduced Glutathione pH8.0 (see methods). The purity of GST-ER (281-599) was then assessed by electrophoresis of 500ng crude cell extract and the peak fraction on a 10% SDS-PAGE gel which was stained with silver (lanes 1 and 2 respectively). In parallel, the integrity of the protein was determined by electrophoresis of equal amounts (600fmol) of fusion protein before and after purification on a 10% SDS-PAGE gel followed by western blotting with antiserum H222 (lanes 3 and 4 respectively).
single point ligand binding assay and a Bio-Rad assay to estimate the amount of fusion protein and the total protein in the eluate respectively. The fusion protein was found to be intact following affinity chromatography and had been purified to near homogeneity with a specific activity of approximately 15nmoles/mg.

To assess the effect of ligand on the conformation of GST-ER (281-599), fusion protein was incubated in a low salt Hepes buffer in the presence or absence of a saturating concentration of oestradiol or ICI 182,780. The samples were then studied by electron microscopy using the shadowing technique described by Hall 1964. This technique results in the deposition of platinum at one side of the protein which allows the structures of small proteins such as GST-ER (281-599) to be visualised more easily in the electron microscope. This shadowing can be observed in the electron micrographs presented as an area of darkness behind the protein (figure 6.13.). In these experiments, fusion protein was visualised using a calibrated Philips 300 transmission electron microscope and was observed to have a subunit structure in the presence and absence of ligand. From the electron micrographs, the average subunit molecular weight of the protein was approximately 65kd indicating that each subunit may represent a monomeric form of GST-ER (281-599). On this basis, the fusion protein appeared to be dimeric in the presence and absence of oestradiol since the protein was found to be comprised of two subunits by electron microscopy. In contrast, treating GST-ER (281-599) with the pure antioestrogen ICI 182,780 resulted in the production of a protein composed of a single subunit indicating that the fusion protein exists as a monomer when complexed with the pure antioestrogen. A more detailed analysis of the effect of ligand on the conformation of the hormone binding domain was not possible using electron microscopy due to the molecular size of the fusion protein which was at the limit of the resolution of this technique.

**Summary and conclusions**

The region of the hormone binding domain involved in binding ligand was investigated by deletion analysis of the fusion protein GST-ER (281-599). Oestradiol and ICI 182,780 were found to bind to a similar region of this domain which may be distinct from the binding site for the class I antagonist 4-OH-Tamoxifen. Ligand binding was observed to elicit different effects on the dimerisation of the hormone binding domain using electron microscopy and FPLC to examine GST-ER (281-599) complexed with agonist or antagonist.
Pure GST-ER (281-599) (40ng) was incubated in a final volume of 50µl of E.M. buffer in the presence or absence of 10^-6M oestradiol or 10^-6M ICI 182,780 for 20 minutes at 25°C. The samples were then sprayed onto freshly cleaved mica and shadowed with platinum before being floated onto copper grids for examination in a transmission electron microscope (see methods). The resultant micrographs were derived for GST-ER (281-599) in the presence of oestradiol (a), no hormone (b) and in the presence of ICI 182,780 (c) at high (2.5 x 10^6x) (I) and low (500,000x) (II) magnification. Individual protein subunits have been indicated using arrows and the electron micrographs are oriented so that the platinum deposits (dark areas) are positioned above the protein subunits (areas of white). The protein structures examined at high magnification correspond to the proteins numbered (1) in the low magnification micrographs.
Figure 6.13. Analysis of GST-ER (281-599) by electron microscopy

Figure 6.13.a.
(i) (ii)
Figure 6.13. Analysis of GST-ER (281-599) by electronmicroscopy

Figure 6.13.b.

(i)

Figure 6.13.c.

(i)
The fusion protein was found to be dimeric in the presence and absence of oestradiol and was converted to a monomeric form following treatment with ICI 182,780. Furthermore, binding of oestradiol appeared to promote a change in the conformation of GST-ER (281-599) which may reflect an agonist dependent stabilisation of the hormone binding domain.
Chapter 7

Discussion
The Role of Ligand in Oestrogen Receptor Dimerisation and DNA Binding

The class I and II antioestrogens

Antioestrogens are drugs that specifically inhibit the effects of oestrogen on cell growth and may do so by interfering with one or more functions of the oestrogen receptor. The best characterised antioestrogen to date is tamoxifen which has been used successfully as a cytostatic agent in the clinical management of advanced breast cancer (Furr and Jordan 1984). The drug is converted to 4-hydroxytamoxifen in the liver which increases the affinity of the ligand for the oestrogen receptor and consequently its potency as an antagonist (Jordan et al 1977). Studies of the growth of human breast cancer cells have found that tamoxifen inhibits the progression of cells through the cell cycle by blocking their passage through G1 phase. A close relationship was observed between the expression of the oestrogen receptor and the accumulation of cells in G1 phase suggesting that inhibitory effects of tamoxifen on cell proliferation are mediated by its interaction with the receptor (Sutherland et al 1983, Dong et al 1991). However, although tamoxifen and its more active metabolite 4-hydroxytamoxifen can antagonise the growth of breast cells, these ligands have been found to behave as partial agonists in vitro and in vivo. For example, both ligands stimulate the growth of the uterus in ovariectomised rats and mice and induce the proliferation of MCF-7 cells in the absence of oestradiol (Sonnenschein et al 1985). The partial agonist activity of the class I antioestrogens is species and tissue specific (reviewed in Pasqualini et al 1988) and similar cell type specificity has been observed in vitro (for examples see Wakeling 1989, Berry et al 1990, Pasqualini et al 1990, Jamil et al 1991). It has been proposed that tamoxifen may behave as a partial agonist depending on the ability of the constitutive activation function (TAF-1) in the N-terminus of the oestrogen receptor to stimulate transcription in particular cells or from certain promoters (Berry et al 1990). This has important implications in the treatment of breast cancer since the partial agonist activity of tamoxifen reduces its efficiency in endocrine therapy. Furthermore, these ligands have been found to stimulate the growth of human endometrial cells in vitro (Gottardis et al 1988, Anzai et al 1989, Jamil et al 1991) and a relationship has been suggested between tamoxifen treatment and the incidence of endometrial cancer (Fornander et al 1989, Gusberg et al 1990).

The net effect of an antioestrogen is therefore determined by a balance between the agonist and antagonist activities of the drug and the concentration
of endogenous oestradiol. In this respect, the term "antioestrogen" is perhaps inappropriate because such ligands often act as partial agonists. However, attempts have been made to synthesis a ligand which is devoid of oestrogenic activity. This search was advanced by the observation that a decamethylene bridging group may be accommodated in the 7α-position of oestradiol without substantially reducing its affinity for the receptor. Subsequent characterisation of a series of analogues of oestradiol with long aliphatic side-chain substituents at the 7α-position led to the identification of compounds which appear to behave as complete antagonists (Bowler et al 1989). These ligands form the class II family of antioestrogens and are often referred to as "pure" antioestrogens because of their apparent lack of oestrogenic activity in most functional systems (Bowler et al 1989, Thompson et al 1989, Wakeling and Bowler 1991).

One of the first class II antioestrogens to be identified was ICI 163,964 which was observed to function as an antioestrogen in vivo using a uterotrophic assay (Wakeling and Bowler 1987). This drug was succeeded by ICI 164,384 which is a more potent antioestrogen and more recently by ICI 182,780 which contains fluorine atoms in the aliphatic side-chain to increase the solubility of the ligand and consequently its potency as an antagonist (Wakeling and Bowler 1987, Wakeling and Bowler 1988a, Wakeling and Bowler 1988b, Wakeling et al 1991, Wakeling et al 1992). Both ICI 164,384 and ICI 182,780 were found to inhibit the oestrogen dependent growth of rat and mice uteri (Wakeling and Bowler 1987, Wakeling and Bowler 1988b, Wakeling et al 1991) and have been observed to block the growth of human breast cancer cells such as MCF-7 and ZR75 by arresting them in early G1 phase (Wakeling and Bowler 1988b, Wakeling and Bowler 1987, Wakeling et al 1989). The antagonists can also inhibit the oestrogen stimulated synthesis of progesterone receptor mRNA in human and mammary cancer cell lines (May et al 1989, Pasqualini et al 1990) and antagonise the growth of breast tumours transferred into nude mice (Wakeling et al 1991). Interestingly, the pure antioestrogens are able to block the tamoxifen stimulated growth of MCF-7 tumours implanted into ovariectomised athymic mice (Gottardis et al 1989) and antagonise both the uterotrophic effects of tamoxifen (Wakeling and Bowler 1988b) and its ability to stimulate the growth of human breast and endometrial cells (Thompson et al 1989, Jamil et al 1991).

Thus, the two classes of antioestrogens appear to inhibit the function of the oestrogen receptor by distinct mechanisms. The class I antagonists are generally thought to inhibit or fail to promote the activity of the hormone dependent transcriptional activation function within the hormone binding
domain of the oestrogen receptor although the protein can bind to DNA *in vitro* and translocates to the nucleus *in vivo* in the presence of tamoxifen (Lees et al 1989b, Ylikomi et al 1992, Dauvois et al 1993). In contrast, the class II antioestrogens appear to interfere with an earlier step in receptor function since ICI 164,384 and ICI 182,780 can inhibit the DNA binding activity of the oestrogen receptor *in vitro* (figure 3.3., Fawell et al 1990b, Wilson et al 1990, Reese and Katzenellenbogen 1991b) and block the nuclear uptake of the protein in intact cells (Dauvois et al 1993). The pure antioestrogens have also been found to reduce the cellular content of the oestrogen receptor (Gibson et al 1991, Dauvois et al 1992, Reese and Katzenellenbogen 1992) by increasing the turnover of the protein (Dauvois et al 1992).

The ability of the class II antioestrogens to inhibit the DNA binding activity of the oestrogen receptor is somewhat controversial with contradictory reports that DNA binding is inhibited using receptor from some sources of protein but not others. For example, ICI 164,384 and ICI 182,780 can inhibit the DNA binding activity of the mouse oestrogen receptor expressed in insect cells as described in chapter three (figures 3.3.a. and 3.3.b.). Similar observations have also been made using receptor that was expressed in COS-1 cells (Reese and Katzenellenbogen 1991b) or partially purified from human and pig uteri (Wilson et al 1990). In contrast, no effect of these antagonists was observed on the DNA binding activity of oestrogen receptor expressed in HeLa cells (Martinez and Wahli 1989) or partially purified from calf uteri and using chimeras of the oestrogen receptor containing the DNA binding domain of GAL4 (Webster et al 1988) or the acidic activation domain of VP16 (Pham et al 1991). It has been proposed that the pure antioestrogens may inhibit DNA binding by disrupting the dimerisation of the oestrogen receptor consequently reducing the affinity of the protein for a palindromic response element (Fawell et al 1990b). In this respect, differences in the ability of ICI 164,384 to inhibit the DNA binding activity of receptor from different sources may reflect differences in the stability of oestrogen receptor dimers in intact cells. This suggestion is supported by several observations. Firstly, conditions that stabilise the dimerisation of the oestrogen receptor such as high salt (figure 3.6.) and DNA binding (figure 3.7.) were found to block the effect of the antioestrogen on the DNA binding activity of the mER expressed in insect cells. Furthermore, the class II antagonists can inhibit the DNA binding of *in vitro* translated mER when added during translation but have no effect on the protein when added following protein synthesis (figures 3.9. and 3.11., Lees et al 1989b). These experiments suggest that the antagonism mediated by the antioestrogens *in vitro* is dependent on the stability of receptor dimers and
secondly that while the class II antagonists may prevent dimerisation during translation, they may not necessarily disrupt receptor dimers that are formed prior to treatment with the antioestrogen.

The class II antioestrogens possess a steroid ring system that is almost identical to oestradiol with a characteristic aliphatic side-chain which protrudes in the α-orientation from position C-7 of the B ring. Given the hydrophobic nature of the antioestrogen side-chain and the organisation of the major dimerisation interface at the hormone binding pocket of the oestrogen receptor, binding of antagonist may position the aliphatic side-chain in a manner which facilitates steric interference with amino acids that are critical for receptor dimerisation (Fawell et al 1990a, Fawell et al 1990b). In agreement with this hypothesis ICI 182,780 and oestradiol were found to bind to a similar region of the hormone binding domain. In addition, the antagonist activity of a number of analogues of ICI 164,384 was observed to be dependent on the structure of the aliphatic side-chain.

As described in chapter six, deletion mutants of the hormone binding domain of the mouse oestrogen receptor were expressed in bacteria as glutathione S-transferase fusion proteins (Smith and Johnson 1988) and their ability to bind agonist and antagonist was examined (figures 6.5. and 6.6.). In conjunction with previous studies, the results indicate that the ligand binding site of the oestrogen receptor resides within residues 313 to 522/538 given that further deletion and point mutagenesis within this region interfere with high affinity hormone binding (figure 6.5., Lees et al 1989b, Fawell et al 1990a). Site directed mutagenesis has similarly been used to prepare deletion mutants of the human oestrogen receptor which were tested for their ability to bind oestradiol after being transiently expressed in HeLa cells or translated in vitro using a rabbit reticulocyte lysate translation system (Kumar et al 1986). In this manner, Kumar and colleagues mapped the ligand binding site for oestradiol to residues 301-552 of the human oestrogen receptor which correspond to amino acids 305-556 of the mER.

Oestradiol and ICI 182,780 were found to bind to a similar region of the hormone binding domain given that the fusion protein GST-ER (313-599) binds both ligands with a similar affinity to the full length receptor and the binding of oestradiol was abolished by deleting beyond amino acid 313 of the mER (figure 6.5.). In contrast, a comparison of the ligand binding capabilities of fusion proteins GST-ER (121-599) and GST-ER (281-599) suggested that the N-terminal boundary of the binding site for tamoxifen may differ from that of oestradiol since deleting residues 121 to 281 reduces the affinity of the hormone binding domain for the antagonist by about a hundred fold (figures
5.12. and 5.13.). Alternatively, a more plausible explanation may be that the accessibility of 4-hydroxytamoxifen for its binding site is partly reduced when the hormone binding domain is positioned in closer proximity to glutathione S-transferase since the smaller fusion protein GST-ER (281-599) contains both the hormone binding domain and the hinge region of the oestrogen receptor (Kumar et al 1987). However, differences in the binding of oestradiol and 4-hydroxytamoxifen have been reported for both the human and mouse proteins in point mutagenesis studies which demonstrated that amino acids located at the C-terminus of both proteins discriminate between the binding of oestradiol and the class I antagonist (Pakdel et al 1992, Danielian et al 1992). In a different type of experiment, oestrogen receptor from calf uteri was labelled with tamoxifen aziridine before being subjected to limited trypsin proteolysis and similarly found that amino acids forming the interactive site of tamoxifen aziridine are positioned close to the C-terminus of the protein (Ratajczak et al 1989).

Differences in the binding sites for the two classes of antagonist presumably reflect the distinct structures of these ligands considering the partial agonists have a substituted triphenylethylene structure in contrast to the structures of the pure antioestrogens which are steroidal. The ability of ICI 182,780 and oestradiol to bind to a similar region of the receptor is perhaps not surprising since the A ring of oestradiol appears to initiate steroid binding with the hydroxyl group of this ring providing the first contact point with the receptor (Duax et al 1984, Chae et al 1991). Molecular modelling of a series of analogues of ICI 164,384 (figure 3.1.) revealed that the steroid ring systems of the class II antagonists adopt a structure which is similar to that of oestradiol apart from some distortion to the C and D rings (figures 3.4. and 3.5.). Differences in the affinities of the analogues for the mouse oestrogen receptor did not correlate with the extent of distortion to the C and D rings of the ring system implying that although the A ring of each ligand presumably contacts the hormone binding domain in a similar manner to oestradiol, the binding of these analogues is influenced by the structure of the aliphatic side-chain.

The similar binding sites for oestradiol and the pure antioestrogens supports the model proposed by Fawell and colleagues (1990b) that ICI 164,384 may inhibit DNA binding as a consequence of interfering with amino acids that are essential for dimerisation. Furthermore, analysis of the effects of the ICI 164,384 analogues on the DNA binding activity of the mouse oestrogen receptor indicate that their antagonist activity is dependent on the length of the amidoalkyl side chain with a length of 15-16 atoms being required for optimal inhibition of DNA binding (figures 3.1.-3.3.). This agrees with the in vivo effects
of these ligands which have been assessed using the rat uterotrophic assay (Bowler et al 1989, Wakeling et al 1991). In this system, the ability of a ligand to alter the oestrogen dependent growth of the rat uterus is determined and the resultant gain or reduction in the weight of the uterus relative to untreated animals is used as a measure of the agonist and antagonist activity of the ligand respectively. In this manner, analogues ICI 163,964, ICI 164,384, ICI 165,801 and ICI 182,780 were observed to function as complete antagonists in vivo which correlates with their ability to inhibit the DNA binding activity of the oestrogen receptor in vitro (Bowler and Wakeling 1987, Bowler and Wakeling 1988, Bowler et al 1989, Wakeling et al 1991). In contrast, ICI 165,889, ICI 169,784 and ICI 165,375 displayed no antagonism in vitro and have been found to act as partial agonists in vivo (Bowler et al 1989). Furthermore, the antagonist activity of the analogues was observed to be stereospecific for the α-isomers since the β-isomer of ICI 164,384 (called ICI 169,784) could not inhibit the DNA binding activity of the oestrogen receptor in vitro (figure 3.3.) or act as an antagonist in vivo (Bowler et al 1989). Similarly, the β-isomer of ICI 163,964 has been synthesised and is also devoid of antioestrogen activity (Wakeling and Bowler 1987, Wakeling and Bowler 1988a).

The effects of the analogues in vitro broadly paralleled the relative binding affinities of the ligands for the receptor. However, their antagonist activity could not be accounted for solely on the basis of affinity and was also found to involve the chemical structure of the aliphatic side-chain. This was emphasised by the behaviour of the short chain analogue ICI 165,889 in vitro. The relative binding affinity of ICI 165,889 for the mouse oestrogen receptor is approximately 20 fold less than that of oestradiol yet the analogue was unable to inhibit the DNA binding activity of the receptor at concentrations up to 10⁻⁶M ligand (figure 3.3.c.) in agreement with the finding that ICI 165,889 does not behave as an antagonist in vivo (Bowler et al 1989). However, this concentration would have been sufficient to saturate the receptor since the dissociation constant (Kd) of the protein for oestradiol is approximately 0.3nM (Fawell et al 1989, Gorski and Gannon 1976) indicating that, although ICI 165,889 can bind to the oestrogen receptor, it is unable to elicit an effect on the DNA binding activity of the protein as a consequence of the length of the aliphatic side-chain which was the shortest of the analogues studied (figures 3.1. and 3.3.c.).

A direct correlation was therefore observed between the ability of the pure antioestrogens to inhibit the DNA binding activity of the oestrogen receptor in vitro and their ability to act as antagonists in vivo. Furthermore, the aliphatic side-chain of the pure antioestrogens appears to play a vital role in
the ability of the ligands to function as antagonists both in vitro and in vivo. It was noted during the molecular modelling studies that the more potent antioestrogens ICI 182,780, ICI 165,801 and ICI 164,384 contain structural elements in their side-chains which appear to generally increase the bulkyness of the hydrophobic substituents. For instance, both ICI 182,780 and ICI 165,801 contain significant kinks in their side-chains that are introduced by the carboxy and sulfoxy groups respectively while the side-chain of ICI 164,384 folds dramatically following the branch-point in the aliphatic side-chain. The analogues ICI 165,375 and ICI 169,784 were also found to have particularly long and folded side-chains respectively but these ligands are unable to bind with high affinity to the oestrogen receptor indicating that the aliphatic side-chain of the pure antioestrogens initially influences the binding of the ligands to the hormone binding domain and subsequently determines their ability to act as antagonists. Surprisingly, the β-isomer of ICI 164,384 (called ICI 169,784) behaved as a complete antagonist towards the in vitro translated mER when added during translation but had no effect on the receptor expressed in SF9 cells following protein synthesis. This may be explained by a difference in the affinity of ligand for receptor monomers during translation compared with the dimeric receptor following protein synthesis since the ligand binding pocket appears to be positioned near the interface of two receptor monomers and may consequently be less accessible to the bulky ICI analogue in the receptor dimer.

An antiprogestin named ZK98299 has similarly been found to inhibit the DNA binding activity of the progesterone receptor (Klein-Hitpass et al 1991, Allan et al 1992b, DeMarzo et al 1992). In such studies the DNA binding activity of the receptor was shown to be dependent on progesterone and could similarly be induced by the antiprogestin/glucocorticoid RU486. In contrast, treatment with the antagonist ZK98299 failed to promote DNA binding (Klein-Hitpass et al 1991, Allan et al 1992b, Sartouris et al 1993, Beck et al 1993). Furthermore, addition of the latter was observed to reduced the amount of receptor that could bind to DNA in the presence of progesterone (Klein-Hitpass et al 1991, Allan et al 1992b). Based on these results it was proposed that ZK98299 may inhibit the dimerisation of the progesterone receptor consequently inhibiting its ability to bind to DNA (Klein-Hitpass et al 1991, Allan et al 1992b). Interestingly, the structures of RU486 and ZK98299 differ only in the composition of their aliphatic side-chains which protrude from position C-17 in both ligands. It is therefore tempting to draw parallels between the mechanisms by which the pure antioestrogens and antiprogestin ZK98299 may interfere with the function of their receptors in vitro given that both
possess a steroid ring system with a large hydrophobic substituent which may interfere with the dimerisation of the oestrogen and progesterone receptors respectively.

**The influence of ligand on oestrogen receptor dimerisation**

The oestrogen receptor has been shown to bind to DNA as a dimer from studies of the contact sites of the receptor on perfect oestrogen response elements using methylation and phosphate interference techniques (Kumar and Chambon 1988, Klein-Hitpass et al 1989, Koszewski and Notides 1991). The receptor binds with the DNA binding domain of each monomer positioned in adjacent major grooves of the DNA and makes electrostatic interactions with phosphate groups on the DNA backbone that contribute to the stability of the resultant complex (Koszewski and Notides 1991, Schwabe et al 1993). The dimeric nature of the oestrogen receptor has also been demonstrated in solution using a number of techniques such as dense amino acid labelling, crosslinking and density gradient centrifugation (Miller et al 1985b, Redeuilh et al 1987, Sabbah et al 1989, Giambiagi and Pasqualini 1990). Deletion analysis of the human protein revealed that the oestrogen receptor contains a major dimerisation interface in the hormone binding domain and a second weaker dimerisation function within the DNA binding domain (Kumar and Chambon 1988). Point and deletion mutagenesis of the mouse protein have since identified residues within the hormone binding domain which are essential for ligand binding and dimerisation (Fawell et al 1990a). An analysis of sequences within this region discovered a repeat of hydrophobic amino acids that is conserved between members of the nuclear receptor superfamily suggesting this sequence may have a similar function in other nuclear receptors.

The identification of a conserved sequence of hydrophobic amino acids at the major dimerisation interface and the general hydrophobic nature of the hormone binding domain as a whole suggests that the dimerisation of this domain may be mediated by hydrophobic interactions. In addition, positioning of the dimerisation region at the hormone binding pocket implies that hormone binding may promote or stabilise receptor dimerisation. This is supported by experiments in which the hydrophobicity of the oestrogen receptor was analysed in the presence and absence of hormone using polyethylene glycol (PEG)-palmitate affinity partitioning. In this technique, proteins interact with PEG-palmitate by virtue of hydrophobic interactions between the palmitate residues and hydrophobic domains on the surface of the protein enabling differences in surface hydrophobicity to be examined. Treating oestrogen receptor prepared from female rat uteri with oestradiol and the antioestrogen
4-hydroxytamoxifen was observed to produce a significant decrease in the surface hydrophobicity of the protein using this method (Hansen and Gorski 1986, Fritsch et al 1992). Similar observations were also made using the hormone binding domain of the human protein that was expressed in bacteria and secondly for the hormone binding domain of the rat oestrogen receptor which was generated by digestion with trypsin. These results indicate that the ligand induced change in the conformation of the receptor is localised to the hormone binding domain resulting in a decrease in its surface hydrophobicity (Fritsch et al 1992). Binding of oestradiol and 4-hydroxytamoxifen may therefore bury or mask hydrophobic surfaces of this domain resulting in a general stabilisation of the receptor.

Early investigations into the formation of the 5S oestrogen receptor complex indicated that oestradiol is required for dimerisation (Yamamoto and Alberts 1972, Notides et al 1975). Preparation of crude cell extracts under high salt conditions was observed to generate a monomeric 4S form of the oestrogen receptor which could be converted to a dimeric 5S species upon heat activation in the presence of steroid (Notides et al 1975, Giambiagi and Pasqualini 1990). Addition of sodium thiocyanate was observed to block this transformation process indicating that heat alone was not sufficient to generate the 5S species and that binding of oestradiol was necessary to promote dimer formation (Weichman et al 1977). Furthermore, treating receptor with oestrone, a weak oestrogen metabolite, failed to mimic the effects of oestradiol to activate the receptor (Yamamoto and Alberts 1972). A similar requirement of hormone for oestrogen receptor dimerisation was also demonstrated using a cotranslation assay in which full length and truncated forms of the human oestrogen receptor were observed to bind as heterodimers to an ERE in a hormone dependent manner (Kumar and Chambon 1988). However, this study was performed using receptor that was latter found to contain a mutation at position 400 (Tora et al 1989a). This mutation decreases the stability of the protein in the absence of hormone consequently resulting in a requirement of oestradiol for dimerisation and DNA binding. Subsequent studies of the human and mouse oestrogen receptors have observed no requirement of ligand for dimerisation or DNA binding using glycerol density gradients, coimmunoprecipitation and cotranslation assays (Fawell et al 1990a, Sabbah et al 1991).

It is conceivable that dimerisation of the oestrogen receptor is not dependent on hormone but binding of oestradiol to the hormone binding domain may facilitate a general stabilisation of the dimeric receptor as suggested by a decrease in the hydrophobicity of the protein following the
binding of oestradiol. In agreement with this proposal the hormone binding domain of the mouse oestrogen receptor was observed to dimerise constitutively in vitro when examined as a glutathione S-transferase fusion protein (GST-ER (281-599) by FPLC and electron microscopy (figures 6.11. and 6.13.). In contrast, treating GST-ER (281-599) with the pure antioestrogen ICI 182,780 was found to induce a change in the subunit composition of the fusion protein resulting in the generation of a monomeric protein with a molecular weight of approximately 65-76kd (figures 6.11.and 6.13.). These results demonstrate that although the hormone binding domain can dimerise in the absence of oestradiol, treatment with the antioestrogen ICI 182,780 can disrupt the dimerisation interface in the hormone binding domain in vitro. A similar effect of the antioestrogen on the dimerisation of the full length receptor was implied by studies of the antagonist on the DNA binding activity of the mER in gel retardation assays using an antiserum known as MP16 (Fawell et al 1990b).

As described in chapter three, the mouse oestrogen receptor can bind to DNA in the presence and absence of oestradiol but the DNA binding activity of the protein is inhibited following treatment with the pure antioestrogens (figure 3.3.). Addition of antiserum MP16 was observed to restore DNA binding activity in each case where receptor was treated with an analogue of ICI 164,384 that behaves as an antagonist (Figure 3.3.a.). Since this antibody has also been shown to restore DNA binding to mutants which are deficient in their ability to dimerise, this suggests that MP16 may similarly rescue the DNA binding activity of receptor treated with a class II antioestrogen by restoring the ability of the protein to dimerise (Fawell et al 1990a, Fawell et al 1990b). It is conceivable that MP16 may cross-link oestrogen receptor monomers at their N-termini by virtue of the bivalent nature of the antiserum. This could partly substitute for a disrupted dimerisation function by maintaining the DNA binding domains in close proximity to facilitate their interaction with the half sites of an oestrogen response element. The DNA binding domains may then dimerise on the DNA and stabilise the resultant protein-DNA complex in conjunction with the antibody mediated "dimerisation".

In agreement with this model the isolated DNA binding domain of the oestrogen receptor has been found to bind to palindromic response elements with an affinity approximately ten fold less than that of the full length receptor (Fawell et al 1990a, Schwabe et al 1990). Nuclear magnetic resonance spectroscopy and X-ray crystallographic analyses demonstrated that this domain is monomeric in solution and dimerises after binding to an ERE (Schwabe et al 1990, Schwabe et al 1993) in a similar manner to that
described for the DNA binding domain of the glucocorticoid receptor (Luisi et al 1991). Furthermore, mutagenesis studies of oestrogen and glucocorticoid receptors have indicated that the dimerisation function located in the hormone binding domain is required to stabilise binding of the receptors to DNA (Kumar and Chambon 1988, Fawell et al 1990a, Dahlman-Wright et al 1992). These results imply that the dimerisation function in the hormone binding domain is required to stabilise interactions between the DNA binding domains of steroid receptors and their HRE half sites. In the absence of this dimerisation function a bivalent antibody could restore DNA binding by substituting for these interactions and in turn may allow the DNA binding domains of receptor monomers to dimerise following their alignment on the DNA.

The epitope for antisera MP16 is located in the B region of the oestrogen receptor and was raised against a peptide generated from the amino acid sequence of the mouse protein (Fawell et al 1990a). The ability of MP16 to restore DNA binding to oestrogen receptor mutants which are defective in their ability to dimerise suggests that the position of the major dimerisation interface may not be critical for DNA binding since the hormone binding domain and the epitope for MP16 are located at different ends of the receptor at least in the primary structure of the protein (Fawell et al 1990a). The ability of the hormone binding domain to function as an independently folded domain has also been demonstrated directly by its ability to confer hormone regulation on to the function of a heterologous protein in several chimeras (for examples see Eilers et al 1989, Burk et al 1991). Furthermore, analogous observations have also been made for the progesterone receptor treated with the antiprogestin ZK98299 in that the DNA binding activity of the protein can similarly be restored by a series of monoclonal antibodies (Allan et al 1992b). In this study the epitopes for the PR specific antibodies were located in the N-terminus, middle and C-terminus of the protein indicating that different regions of receptors may be "crosslinked" to restore DNA binding activity provided the DNA binding domains are maintained in close proximity to one another to facilitate interactions with the HRE half sites.

More recently, studies investigating the nuclear uptake of the oestrogen receptor have provided strong evidence that the dimerisation of this receptor is similarly influenced by agonist and antagonist in vivo (Ylikomi et al 1992, Dauvois et al 1993). The oestrogen receptor is located predominately in the nucleus in the presence and absence of hormone under steady-state conditions and has been shown to shuttle continually between the nuclear and cytoplasmic compartments of the cell using immunofluorescence to monitor the transfer of receptor between nuclei in heterokaryons (Dauvois et al 1993).
During these studies it was observed that the nuclear uptake of a translocation deficient mutant could be rescued by cotransfecting cells with the "wild type" receptor. This is presumably mediated by protein interactions which facilitate cotranslocation of the mutant and "wild type" receptors to the nucleus. Although the region(s) of the oestrogen receptor required for cotranslocation remain to be identified it seems likely that the proteins may dimerise through their hormone binding domains. In agreement with this, analogous studies of the progesterone receptor have demonstrated that cotranslocation of PR deletion mutants is mediated through interactions between the hormone binding domains of the proteins (Guiocchon-Mantel et al 1992).

The ability to monitor the nuclear uptake of a mutant receptor using immunofluorescence has enabled the role of ligand in oestrogen receptor dimerisation to be studied in intact cells. For instance, the cellular localisation of translocation deficient mutants of the human and mouse oestrogen receptors has been studied in the presence of agonist and antagonist (Ylikomi et al 1992, Dauvois et al 1993). The mutant receptors were observed to be cytoplasmic in the presence and absence of hormone while cotransfection with the "wild type" receptor promoted its accumulation in the nucleus in the presence of oestradiol and 4-hydroxytamoxifen suggesting that both ligands may stabilise the dimerisation of the receptor in vivo. In contrast, ICI 164,384 had no effect on the nuclear accumulation of the mutant receptor implying that the pure antioestrogen is unable to promote the dimerisation of the oestrogen receptor in intact cells. Furthermore, Dauvois and colleagues (1993) demonstrated that ICI 164, 384 and ICI 182,780 block the nuclear uptake of the "wild type" receptor. Given that the oestrogen receptor appears to be degraded in the nucleus or the perinuclear cytoplasm (Welshons et al 1993), this inhibition in nuclear uptake may begin to explain observations that the receptor accumulates in the cytoplasm of cells treated with ICI 164,384 or ICI 182,780 (Gibson et al 1991, Dauvois et al 1992, Reese and Katzenellenbogen 1992). However, although ICI 182,780 was observed to disrupt the dimerisation of the hormone binding domain in vitro using FPLC and electron microscopy, it remains to be established whether the class II antagonists inhibit the nuclear uptake of the receptor in vivo as a consequence of interfering with receptor dimerisation.

The relationship between nuclear receptor dimerisation and DNA binding

The oestrogen receptor binds to its palindromic response element as a preformed dimer since a number of studies have indicated that the receptor is dimeric in solution (Linstedt et al 1986, Redeuilh et al 1987, Kumar and
Chambon 1988, Sabbah et al 1989, Fawell et al 1990a). This appears to be mediated by the major dimerisation interface in the hormone binding domain given that the DNA binding domain is monomeric in solution and dimersises on the DNA (Schwabe et al 1990, Schwabe et al 1993). Furthermore, the hormone binding domain released by trypsin digestion of the calf uterine oestrogen receptor has been shown to be dimeric in solution by glycerol density gradient centrifugation (Sabbah et al 1989). In contrast, the dimeric forms of other steroid receptors has been more difficult to detect in solution suggesting that oestrogen receptor dimers are inherently more stable. For example, the glucocorticoid receptor can form dimers in solution which are stable to gel filtration but these tend to dissociate during sucrose density gradient centrifugation. Maintenance of the GR dimer during centrifugation was shown to require stabilisation by either chemical cross-linking or alternatively by addition of DNA (Wrange et al 1989).

In general it appears that the stability of receptor dimers correlates with the ability of steroid receptors to bind to DNA since the DNA binding domains of both the oestrogen (Schwabe et al 1990) and glucocorticoid receptors (Hard et al 1990) have been found to be monomeric in solution and bind to DNA with an affinity approximately ten fold lower than that of the full length receptors. This implies that the major dimerisation function located in the hormone binding domains of these proteins is required to stabilise DNA binding (Fawell et al 1990a, Dahlman-Wright et al 1992). A direct correlation has been observed between the ability of deletion mutants of the human and mouse oestrogen receptors to dimerise in solution and their ability to bind to DNA (Kumar and Chambon 1988, Fawell et al 1990a). In contrast, a deletion mutant of the progesterone receptor that lacks the hormone binding domain has been observed to bind to DNA with high affinity although the protein is unable to dimerise in solution (Cohen-Solan et al 1993). This agrees with an earlier observation that this truncated protein can stimulate MMTV gene expression to approximately 60% of that of the full length progesterone receptor (Guiochon-Mantel et al 1988). Thus, the role of dimerisation in the binding of oestrogen and progesterone receptors to their response elements differs in that dimerisation appears to be a prerequisite for high affinity binding of the oestrogen receptor but not the progesterone receptor. This is supported by studies in which the cooperativity of ligand binding was used to measure the dimerisation constant for each receptor (Skafar 1992). The dimerisation constant of the progesterone receptor was found to be approximately 20 fold higher than that of the oestrogen receptor suggesting the receptors exist in different forms in solution. Since the dimerisation constant derived for the ER
was lower than the reported physiological concentration of the protein (Sasson and Notides 1983), the authors proposed that the oestrogen receptor is dimeric in solution. In contrast, the higher dissociation constant of the progesterone receptor indicated that this protein exists in solution predominately as a monomer in the absence of progesterone and correlates with the lower affinity of the PR for an HRE (Skafar 1992). Dimerisation is therefore an important regulatory function of steroid receptors which can influence their ability to bind DNA and subsequently stimulate transcription.

The influence of ligand on the DNA binding activity of the oestrogen receptor

Ligands which alter the stability of oestrogen receptor dimers may therefore influence the ability of the protein to bind to DNA. However, there are several mechanisms by which ligands may alter the DNA binding activity of the receptor. Early studies of the oestrogen receptor demonstrated that DNA binding is inhibited when the receptor is associated with heat shock proteins in the inactive heteromeric complex (see chapter one). In this respect most information is currently available regarding heat shock protein (hsp) 90 which binds to the hormone binding domain of the oestrogen receptor although several other regions also participate in binding the phosphoprotein (Chambraud et al 1990, Schlatter et al 1992). It has been shown that deletion of the hormone binding domain yields a constitutively active receptor (Kumar et al 1987) which does not bind hsp 90 (Chambraud et al 1990) whereas mutants that remain hormone inducible retain the ability to bind the heat shock protein. Interestingly, the inactivation function of the hormone binding domain can also confer hormone regulation onto the function of a heterologous protein in a fusion protein (Eilers et al 1989, Burk et al 1991) and Picard and colleagues (1988) have proposed that this is similarly due to hsp 90. It has been suggested that binding hsp 90 to the hormone binding domain of the oestrogen receptor causes the domain to adopt an "unfolded" conformation that is reversed on binding hormone as a result of a change in the conformation of the receptor that leads to dissociation of the heat shock protein. This hypothesis is supported by observations that the protease digestion patterns of the oestrogen receptor differ in the presence of agonist and antagonist (Baniahmad and Tsai 1993). In addition, binding of oestradiol has been shown to produce an increase in the migration of the ER-DNA complex in a gel retardation assay compared with the hormone free receptor (for examples see Lees et al 1989b, Fawell et al 1990b).

Another possibility is that hormone binding may induce a change in a post-translational modification of the oestrogen receptor which alters the ability
of the protein to bind to DNA or influences the stability of the resultant DNA complex. For example, dephosphorylation of the human oestrogen receptor following treatment with potato acid phosphatase has been shown to result in a reduction in the DNA binding activity of the receptor (Denton et al 1992). Similarly, addition of a cocktail of phosphatase inhibitors was found to abate a loss of mER DNA binding activity which was observed following prolonged incubations in vitro as described in chapter four. Addition of phosphatase inhibitors facilitated a similar maintenance of DNA binding using both crude (figure 4.6.) and fractionated preparations (figure 4.9.) of the mER suggesting that a reduction in the phosphorylation of the receptor itself may contribute to a loss of DNA binding. This was supported by the observation that a loss of DNA binding activity correlates with a reduction in the exposure of the epitope for the monoclonal antibody H226 in the mER (figure 4.5.). The epitope for this antiserum is located between residues 136-202 of the mER which contain several potential phosphorylation sites (Hooshang Lahooti personal communication). However, studies of oestrogen receptor mutants have indicated that although a large proportion of receptor phosphorylation occurs following binding of receptor to an ERE, DNA binding activity per se is not dependent on phosphorylation (Lahooti et al 1993). For example, a mutant with a single amino acid substitution at position 525 of the mER (G-525R) was found to be unphosphorylated and yet this protein retains the ability to bind to DNA. Furthermore, deleting residues 1 to 121 of the mouse protein was found to reduce the phosphorylation of the mER to 10-20% that of the full length receptor although this mutant can bind to DNA and stimulates transcription (Lahooti et al 1993). Although these results indicate that the DNA binding activity of the oestrogen receptor is not dependent on phosphorylation, such posttranslational modifications may increase the stability of the resultant protein-DNA complex.

A loss of oestrogen receptor DNA binding activity has also been reported by Mukherjee and Chambon (1990) following affinity chromatography of the human oestrogen receptor. In this study the authors observed that DNA binding could be restored by addition of HeLa and yeast cell extracts indicating that other factors are necessary to stabilise the binding of the oestrogen receptor to DNA. A protein called the "DNA binding stimulatory factor" (DBSF) was subsequently purified from yeast which could stabilise ER DNA binding and was found to be a single stranded DNA binding protein (Mukherjee and Chambon 1990). In chapter four, addition of mock-infected SF9 cell extract similarly restored DNA binding to the mouse oestrogen receptor following purification (figure 4.11.) suggesting that an accessory protein such as DBSF
may have been removed during affinity chromatography. Alternatively, given
the influence of phosphatase inhibitors on the DNA binding activity of the mER
and the fact that a loss of DNA binding was observed using crude and
fractionated preparations of the receptor, it is possible that addition of mock-
infected SF9 cell extract restored a kinase that was in an active form or
replaced depleted levels of ATP in the extract.

In the studies presented in chapter four, antiserum MP16 was initially
observed to restore DNA binding activity to the oestrogen receptor (figure 4.2.)
which was found to decreased with prolonged incubation. In addition, treatment
with the antioestrogen ICI 164,384 accelerated the loss of restoration by MP16.
This suggested that although the antagonist may act initially by disrupting the
dimerisation of the mER, there appears to be a subsequent modification which
also reduces the DNA binding activity of the receptor (figures 4.1. and 4.2.).
Given that oestradiol could not reduce the effect of the antioestrogen (figure
4.2.) and prebinding receptor to DNA abolishes a subsequent loss of DNA
binding (figure 4.3.), it is proposed that the oestrogen receptor may begin to
unfold during prolonged incubations and that this is promoted by the
antagonist. As a result, a reduction in the phosphorylation of the receptor may
facilitate such unfolding or alternatively dephosphorylation may occur as a
consequence of an alteration in the conformation of the receptor.

The class II antioestrogens have therefore been found to inhibit
the DNA binding activity of the mouse oestrogen receptor which appears to be
a consequence of a disruption to the dimerisation interface in the hormone
binding domain of the receptor. However, it is proposed that the production of
monomeric receptor may promote a unfolding of the oestrogen receptor that
prevents oestradiol from reversing the effects of the antioestrogen. This
unfolding may also contribute to the inability of the receptor to translocate to
the nucleus of the cell in the presence of the pure antioestrogens (Dauvois et
al 1993) if the nuclear translocation signals are no longer exposed for
recognition by components of the nuclear pore complexes. Furthermore, an
unfolding of the oestrogen receptor may partly explain observations that
treating intact cells with the pure antioestrogens produces an increase in the
turnover of the protein in vivo as an increasing number of protease recognition
sites become exposed on the surface of the receptor.

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