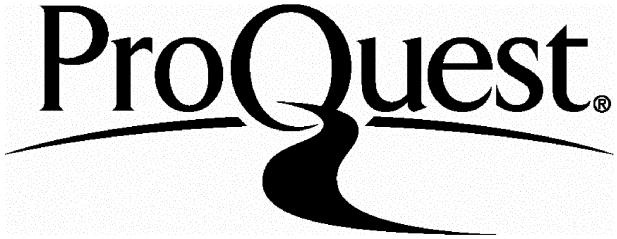
Transcriptional Activation and Dimerisation Properties of Oestrogen Receptor β

Shaun Michael Cowley

Thesis presented in partial fulfilment of the degree of
Doctor of Philosophy at the University of London
October 1998

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

Department of Molecular Pathology
University College London
Gower Street
London



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

ABSTRACT

The oestrogen receptor (ER) is a member of the nuclear receptor superfamily of ligand inducible transcription factors. In the absence of oestrogen the ER exists in the cell as part of an inactive complex with heat shock proteins. Upon binding oestrogen the heat shock proteins are displaced, and the ER is able to bind DNA as a dimer where it stimulates transcription from oestrogen responsive elements (ERE) in the vicinity of target genes. The ER exists in two forms, the classical ERα and the recently discovered ERβ, which are encoded by distinct genes. ERβ has a similar binding affinity for 17β-oestradiol as ERα and is capable of activating transcription from ERE containing promoters. When co-expressed *in vitro* or *in vivo* ERα and ERβ form heterodimers, which bind to an ERE with an affinity similar to that of ERα homodimers but greater than that of ERβ homodimers. The heterodimer, like the homodimers, are capable of binding the steroid receptor coactivator 1 (SRC1) when bound to DNA and stimulating transcription from ERE containing reporter genes in cell lines. ERα has two well characterised transcriptional activation domains, activation function 1 (AF1) in the N-terminus and activation function 2 (AF2) in the C-terminus. A comparison of the AF1 and AF2 domains from ERα and ERβ revealed that ERβ does not have an AF1 activity equivalent to that of ERα, while their AF2 activities are similar.

Nuclear receptors stimulate transcription by interacting with the SRC1 family of coactivator proteins. We have isolated a fragment of an SRC1 family member from chicken which we term, Chip1. Chip1 is highly homologous to other SRC1 coactivators and is able to interact with ERα in a ligand-dependent manner. Southern blotting analysis revealed that similar to humans, chickens contain three SRC1 coactivator genes. The conservation of sequence and number of SRC1 coactivators suggests that nuclear receptors retain a largely conserved mechanism of transcriptional activation.

ACKNOWLEDGEMENTS

Firstly I would like to thank Malcolm Parker for accepting me into his lab and for the guidance, patience and support he has shown me over the last four years. I would also like to thank past and present members of the Molecular Endocrinology Laboratory for generating a lively and enjoyable atmosphere in which to work, in particular the other members of the quietest bay in the lab, Janet Valentine and Ho Yi Mak. I would also like to thank Roger White for advice, help and the odd round of golf.

I wish to acknowledge the support of my family and friends. In particular Annabel Green and her family, who have always treated me as one of their own. To Matthew Betts, Maria Hodges, Rob Russell, Andy Saurin, Nicky Broughton and Bryony Wiseman with whom I have spent many an hour in the company of our close friend George IV. I give thanks to Alun Thomas and Ryan O'Shaughnessy for being the best two flat-mates a man could wish for. And finally, to Mum, Tim, Steven and Stacey.

CONTENTS

List of Figures

Chapter1

Chapter 3

Chapter 4

Chapter 5

ABBREVIATIONS

ACTR	activator of the thyroid and retinoid acid receptor
AD1/AD2	activation domain 1/activation domain 2
AF1/AF2	activation function 1/activation function 2
AIB1	amplified in breast cancer 1
ATP	adenosine 5' triphosphate
BES	N, N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
CBP	CREB binding protein
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CID	CBP/p300 interaction domain
CREB	cAMP response element binding protein
C-terminal	carboxy terminal
DBD	DNA binding domain
dCTP	2'-deoxycytidine-5'-triphosphate
DCC	dextran coated charcoal
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribosenucleic acid
DR+X	direct repeat with X nucleotide spacing
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
ER	oestrogen receptor
ERE	oestrogen response element
ERKO	ERα knock-out mice
FCS	foetal calf serum
GR	glucocorticoid receptor
GRIP1	GR interacting protein 1
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase

HEPES	N-2-hydroxyethylpiperazine N'-2-ethansulphonic acid
Hsp	heat shock protein
kb	kilobase
kDa	kiloDalton
K_d	dissociation constant
lacZ	β-galactosidase gene
LBD	ligand binding domain
LBP	ligand binding pocket
MR	mineralocorticoid receptor
mRNA	messenger RNA
NGFI-B	nerve growth factor inducible protein-B
NCoR	nuclear receptor corepressor
NMR	nuclear magnetic resonance
NP40	nonidet p40
NID	nuclear receptor interacting domain
NLS	nuclear localisation signal
N-terminal	amino terminal
OD_x	optical density at wavelength of x nm
p/CIP	p300/CBP cointegrator associate protein
PAGE	polyacrylamide gel electrophoresis
PBSA	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator activated protein
PR	progesterone receptor
Rac3	receptor associated coactivator 3
RAR	retinoic acid receptor
RIP	receptor interacting protein
RNA	ribonucleic acid
RXR	retinoid X receptor
S	Svedberg units
SDS	sodium dodecyl sulphate
SF1	steroidogenic factor-1
SMRT	silencing mediator for retinoid and thyroid receptors
SRC1	steroid receptor coactivator 1
TAF	TBP associated factor
TBP	TATA binding protein

TEMED	N'N'N'N'-tetramethyletylenediamine
TIF	transcription intermediary factor
TK	thymidine kinase
TR	thyroid hormone receptor
TRAM-1	TR activator molecule-1
Tris	tris(hydroxymethyl)aminoethane
Triton X-100	octyl phenoxy polyethoxyethanol
Tween-20	polyoxyethylenesorbitan monoluarate
UV	ultra violet
VDR	vitamin D receptor

Chapter 1

Introduction

Introduction

The oestrogen receptor (ER) and its hormonal ligand 17β-oestradiol play critical roles in the development of sex accessory tissues as well as the reproductive cycle, female infertility and maintenance of pregnancy (George and Wilson 1988). In addition to these well characterised roles as a sex hormone, oestrogen also has protective roles in the cardiovascular system (Grodstein *et al* 1996) and bone structure (Ettinger *et al* 1985); both of which become evident in postmenopausal women when oestradiol concentrations become lower. Although classically accepted as the female sex hormone, oestrogens also function in males. One example is the closure of the long bone epiphyses which stops the growth of bones in late puberty determining final height. Three human males have been discovered who either cannot make oestrogen or who lack ERα, all are close to seven feet tall and still growing (Carani *et al* 1997, Morishima *et al* 1995, Smith *et al* 1994). The challenge in recent years has been to link the functions of oestrogen *in vivo* to the function of the ER as a transcription factor.

This thesis describes a study of two forms of the ER, the classical ERα and the recently discovered ERβ. The aim of this chapter is to review our current understanding of the molecular mechanisms by which nuclear receptors, and in particular the ER functions within the cell.

Regulation of gene expression by oestrogens

Oestrogen acts as a potent mitogen in some target tissues, including rat uterus (Cicatielo *et al* 1993) and breast cancer cells (Dickson and Lippman 1988). This stimulation of proliferation is thought to occur by induction of proto-oncogenes, which in turn are responsible for the indirect activation of other genes. Among these proto-oncogenes are c-Fos and c-Jun (which associate to form AP-1) and c-Myc (reviewed by Weisz and Bresciani 1993). Since these proteins are themselves transcription factors, its possible that the mitogenic signal of oestrogen is

amplified, leading to a cascade of gene expression resulting in progression of cell cycle and ultimately cell division.

Not all effects of oestrogens in target tissues are proliferative. The prevention of artherscleroticplaques in the cardiovascular system occurs by inhibition of cell proliferation (Iafrati *et al* 1997). In addition, the protective effect of oestrogen in bone is thought to occur not by induction of gene expression via the ER, but rather repression (Stein and Yang 1995). Thus the ER is able to induce or repress gene expression in mediating the physiological effects of oestrogens.

The ER is a nuclear receptor

The development, growth and homeostasis of higher eukaryotes relies on the co-ordination of gene expression in response to two fundamental signal-transduction pathways. The first pathway relies on membrane bound receptors which upon binding ligand induces a kinase cascade that finally leads to the activation of a set of transcriptional enhancers. In contrast the majority of receptors for the second pathway are present in the cell nucleus, so called nuclear receptors, because their ligands are fat-soluble and so are able to diffuse across the lipid-bilayer of the cell membrane. Nuclear receptors function as ligand inducible transcription factors, modulating rates of transcription by binding DNA in the vicinity of target genes. The ER is a nuclear receptor, whose activity is regulated by its natural ligand 17β-oestradiol.

Nuclear receptor signalling is required for the regulation of many important physiological functions. The sex steroid hormones, androgens, oestrogens and progestins are required for normal development and function of the mammalian reproductive systems (Cuhna *et al* 1991). The adrenal steroid hormones glucocorticoids and mineralocorticoids regulate the enzymes of carbohydrate and mineral metabolism respectively, while vitamin D is important for bone formation and calcium metabolism (Hughes and O'Malley 1991). Retinoid and thyroid hormones are involved in growth and differentiation in mammals (Chambon 1994) and metamorphosis in amphibians (Ragsdale *et al* 1989). Signalling via nuclear receptors is not

solely the domain of vertebrates, as the insect steroid hormone ecdysone, initiates changes in development during moulting and metamorphosis (Thummel 1995).

The identification of nuclear receptors

Although oestrogens are able to permeate most cell-types, oestrogen responses are limited to a subset of tissues. The first evidence for a tissue specific ER was suggested by the observation that labelled oestradiol was retained by oestrogen responsive tissues, against a concentration gradient (Jensen and Jacobsen 1962). Cell fractionation studies initially showed that the oestrogen binding activity was present in a 9S sedimentation complex in the cytosolic fraction, which disappeared after treatment with oestradiol resulting in the stoichiometric increase of a nuclear 5S form (Jensen *et al* 1968, Toft and Gorski 1966). Similar observations were also made for glucocorticoid, androgen and progesterone receptors (GR, AR and PR respectively; reviewed by King and Mainwaring 1974). This led to the proposal of the 'two-step' model of steroid receptor signalling, in which hormone binding induces translocation of the receptor from the cytoplasm to the nucleus, where it can then bind to DNA.

Using molybdate to stabilise the unliganded complexes (Dahmer *et al* 1984) it was found that a major component of the cytosolic 9S complex was heat shock protein 90 (Hsp90; Catelli *et al* 1985, Joab *et al* 1984), and that ligand binding induced transformation of the receptor into the 5S form results in the dissociation of Hsp90 *in vitro* (Denis *et al* 1988, Sanchez *et al* 1985). To prove that the interaction with heat shock proteins was physiologically important and not an *in vitro* artefact, it was demonstrated that Hsp90 could be co-immunoprecipitated with the GR from untreated cell extracts, but not cells treated with the synthetic glucocorticoid, dexamethasone (Howard and Distelhorst 1988). In addition, GR can be cross linked to Hsp90 in cells (Rexin *et al* 1988). It eventually became apparent that Hsp 90 was not the only receptor-associated protein, the recovery of steroid receptors from cells in the absence of hormone showed that they were bound by a large number proteins including, Hsp 90 and 70, p23, p50, p54, p59 and p60 (for review

see Smith and Toft 1993). To date it seems that only steroid receptors bind heat shock proteins (Dalman *et al* 1990, Dalman *et al* 1991).

The physiological role of Hsp90 binding is not fully understood. Deletion mapping of the ER and GR showed that the main binding site for Hsp 90 is in the ligand binding domain (Chambraud *et al* 1990, Howard *et al* 1990, Pratt *et al* 1988), leading to the proposal of a chaperone role for Hsp 90 in which it maintains the unliganded receptor in a conformation ready for hormone binding. This was supported by evidence that GR expressed in a yeast strain lacking the yeast Hsp90 homologue (Hsp82), results in a significant impairment of hormone induction (Bohen and Yamamoto 1993). Chaperones in addition to Hsp 90 may also regulate hormone receptor function, as suggested by mutants of the yeast dnaJ homologue YDJ1. In contrast with yeast lacking Hsp90, mutation of one *ydj1* allele generates constitutively active ER and GR (~~Caplan *et al* 1995~~, Kimura *et al* 1995). Thus the proteins which sequester the unliganded steroid receptors may have multiple roles as chaperones, folding, repressing and maintaining the receptor in preparation for induction by hormone.

Cellular localisation of nuclear receptors

The intracellular localisation of nuclear receptors is the result of a dynamic system of shuttling between the cytoplasm and nucleus (Dauvois *et al* 1993, Guiochon-Mantel *et al* 1991, Madan and DeFranco 1993). At equilibrium the majority of receptors are nuclear (Ennis *et al* 1986, King and Greene 1984, Lin *et al* 1991, Ylikomi *et al* 1992), the exceptions being the GR (Akner *et al* 1994, Pekki *et al* 1992) for which ligand-induced nuclear localisation has been reported and also the mineralocorticoid receptor (MR, Robertson *et al* 1993). The detection of the ER and PR in the nucleus in the absence of hormone using immunocytochemistry contradicts the observations made using cell fractionation studies, in which the receptors were retained in the cytoplasm in the absence of hormone. This discrepancy may reflect receptors leaking out of the nucleus in the cell fractionation studies in the absence of ligand. Hormone receptors have multiple nuclear localisation signals (NLS), a constitutive NLS consisting of a prototypic

group of basic amino acids at the C-terminus of the DNA binding domain, and a ligand-inducible NLS in the ligand binding domain (for review see Guiochon-Mantel and Milgrom 1993). The ligand dependent nuclear localisation of GR may represent the unmasking of a constitutive NLS upon binding hormone. In contrast to nuclear import, nuclear export is apparently not an energy dependent process, since ER and PR diffuse out of nuclei from cells depleted of ATP, although export still relies on the presence of a NLS (Guiochon-Mantel *et al* 1994).

The Nuclear receptor superfamily

Since the isolation of the GR in 1984 (Miesfield *et al* 1984), approximately 70 members of the nuclear receptor superfamily have been cloned, in species ranging from nematodes to man (for review see Gronemeyer and Laudet 1995). Among these are the well-known receptors for steroid and thyroid hormones, retinoids and Vitamin D, but by far the largest group are receptors for which there is no known ligand, so-called 'orphan' receptors.

A sequence alignment of this diverse set of transcription factors reveals a modular structure, which can be divided into regions A-F, based on boundaries of homology originally observed in different species of the ER and GR (Krust *et al* 1986). The modular nature of nuclear receptors was confirmed by demonstrating that these domains and their respective functions can be swapped, resulting in receptors with chimeric function (Green and Chambon 1987). The region with highest homology amongst nuclear receptors is region C, the DNA binding domain (DBD), a unique domain which defines and distinguishes nuclear receptors from other transcription factors. Region E, which encompasses the ligand binding domain (LBD), is the next best conserved. The LBD is a complex domain which not only binds ligand, but also contains the major dimerisation interface and a ligand-dependent activation domain. Regions A/B (comprising a single domain), D and F are not well conserved. Some receptors, AR, PR, GR and RXR for instance do not have a region F. The A/B region is also highly receptor specific, for example the A/B region in the vitamin D receptor is only 23 amino acids long, whereas in receptors such as PR,

AR and MR it contains over 550 amino acids. Region D, although not well conserved, is thought to function as a flexible link between the DBD and LBD.

It is unclear whether the DBD and LBD evolved independently of each other or whether the first members of the family were simply constitutive transcription factors which developed the ability to bind ligand. If the latter is true, then orphan receptors may represent a glimpse of the ancient hormone receptors which were able to bind DNA and alter rates of transcription, but without the ability to bind ligand. Whichever is the case, the combination of DBD with a ligand binding activity has been successfully adapted for signalling by a diverse group ligands to produce the nuclear receptor superfamily (Gronemeyer and Laudet 1995).

The cloning of ERα and ERβ

The ER was amongst the first nuclear receptors to be cloned. The ER was identified using specific antibodies and oligonucleotides derived from the human ER peptide sequence (Green *et al* 1986). Expression of the ER open reading frame in HeLa cells demonstrated a protein of the same size and oestrogen binding affinity as the endogenous ER (Green *et al* 1986). Since then the ER from several species has been identified, including: chicken (Krust *et al* 1986), mouse (White *et al* 1987), Xenopus (Weiler *et al* 1987) and rainbow trout (Pakdel *et al* 1990). During the next decade it was believed that only one form of the ER existed. That was until the cloning of a second form of ER, ERβ, from rat (Kuiper *et al* 1996) and human (Mosselman *et al* 1996). ERα and ERβ have a similar affinity and specificity for oestrogens (Kuiper *et al* 1997) and both are able to stimulate transcription from reporter genes containing an oestrogen response element (Kuiper *et al* 1996, Mosselman *et al* 1996, Tremblay *et al* 1997). Other steroid receptors such as, AR, GR and PR have different isoforms of the receptor derived from alternative starts of transcription or splicing points (reviewed by Gronemeyer and Laudet 1995), however, ERα and ERβ are the only steroid receptor isoforms encoded by distinct genes. Their expression is thus regulated independently of each other, resulting in individual expression patterns (Kuiper *et al* 1997).

ERα knock-out mice

The best indications as to the role of ERα in physiological responses to oestrogen have come from the generation of ERα knock-out mice (ERKO, Lubahn *et al* 1993). Prenatal development of both male and female ERKO mice was unaffected, they appeared healthy with no obvious external abnormalities. Internally only the females showed noticeable gross differences from normal, with hypoplastic uteri and ovaries which lacked corpora lutea. A closer examination of the uterus showed the presence of all the major uterine cell types, but the stromal, epithelial and myometrial compartments were all reduced in size.

Female ERKO mice are infertile. In addition to the abnormal uteri and ovaries, they did not show any lordis posture or receptiveness to wild-type males even when treated with oestrogen. This behaviour suggests the absence of oestrogen responsiveness in the central nervous system. The ERKO males are also infertile, a study of their testes revealed normal development until puberty, when they begin to degenerate as early as 20-40 days of age, finally becoming atrophic by day 150 (Eddy *et al* 1996). The absence of ERα in the efferent ductuals results in a reduction in the reabsorption of seminal fluid with subsequent lower concentration of mature sperm and back-pressure on sertoli cells (Hess *et al* 1997a). The sperm released from ERKO mice is approximately one-tenth the wild-type control (Lubahn *et al* 1993).

The altered phenotype of the ERKO mice occurs in the presence of a functional ERβ. Lubahn *et al* (1993) expressed their surprise that the ERKO mice were viable despite the role of oestrogen in activation of blastocysts (Neider *et al* 1987). Their unaltered prenatal development could be accounted for by the presence of ERβ. ERβ is capable of mediating some of the effects of oestrogens in ERKO mice, such as inhibiting the vascular injury response, a method of artificially testing the artheroprotective effects of oestrogens (Iafrati *et al* 1997). However, the inability of ERβ to completely compensate for the loss of ERα suggests that either ERβ cannot perform all the functions of ERα, or that their pattern of expression does not precisely overlap. Which effects of

oestrogen can be prescribed to the function of ERα, ERβ or both should become clearer upon generation of ERβ knock-out mice, initially by comparison with ERKO mice, and eventually by crossing, to produce ERα/ERβ null mice.

ER function in breast cancer

The involvement of oestrogen signalling via the ER in breast cancer is based on three main lines of evidence: (i) the ability of oestrogens to generate mammary tumors in rodents (Henderson *et al* 1988); (ii) epidemiologically-derived risk factors such as the protective effect of ovariectomy and increased risk of young women given diethylstilbesterol to prevent abortion (Henderson *et al* 1988); and (iii) the mitogenic effects of oestrogens on breast cancer cell lines (Dickson and Lippman 1988) and the success of using antioestrogenic drugs such as tamoxifen in the treatment of breast tumors (Santen *et al* 1990). However, the picture is clouded by the fact that high doses of oestrogen can induce tumor regression, despite their mitogenic effects at lower concentrations.

The initiation, establishment and progression of breast tumors is a multistage process and at which point (or points) the ER functions remains to be established. Oestrogens clearly stimulate the proliferation of established breast cancer cells, but do they function as carcinogenic initiating agents? There is little evidence to suggest that they do, indeed the requirement for a classical initiating event has been questioned in breast cancers, as increased proliferation with inadequate DNA repair might lead to the accumulation of mutations which generate cancer (Preston-Martin *et al* 1990).

The greater proliferative effect of oestrogens on breast cancer cells relative to normal breast epithelium, at least in culture (Chambon *et al* 1984), is consistent with an increase in hormone sensitivity from the normal to neoplastic state. The increase in oestradiol sensitivity is probably due to increased levels of ER protein, ER positive breast tumors frequently contain more than 100 fmol ER/mg protein, whereas peak levels in normal breast are about one-tenth this value (Carpenter *et al*

1989). Breast cancers occurring in postmenopausal women are often ER positive. This may reflect the growth advantage of cells containing increased levels of ER in the low oestrogenic environment following menopause. ER positive tumors are treated with tamoxifen, which prevents ER transcriptional activity and has a success rate of approximately 50%. It is well known that the classical ER, ERα is overexpressed in breast tumours, the mRNA for the recently cloned ERβ is also present in breast tumor samples (Dotzlaw *et al* 1996), suggesting that both forms of the ER may have roles in the progression of breast neoplasia.

Classification of nuclear receptors

All nuclear receptors recognise derivatives of one of two hexameric core motifs (or half-site), 5'-AGAACA-3' recognised by AR, PR and GR and 5'-AGGTCA-3' which is recognised by all other receptors. The majority of nuclear receptors have co-evolved to optimally recognise these half-sites as dimers, arranged either as inverted or direct repeats with a receptor specific spacing in between (for review see Parker 1993) and thus can be broadly grouped into four classes based on these properties (Fig. 1.1, based on Stunnenberg 1993). Class I nuclear receptors include those of the steroid hormones, which bind palindromic half-sites with a 3 bp spacer as homodimers. Class II receptors form obligate heterodimers with RXR, and bind direct repeats with a receptor dependent spacing. These include all other receptors with a known ligand. Class III are orphan receptors which bind DNA as homodimers. Class IV receptors typically bind to extended half-sites as monomers, or dimers, in which only one subunit contacts DNA.

The DNA binding domain

The minimal nuclear receptor DBD consists of approximately 80 amino acids, characterised by two sets of four Cys residues which

The Nuclear receptor superfamily

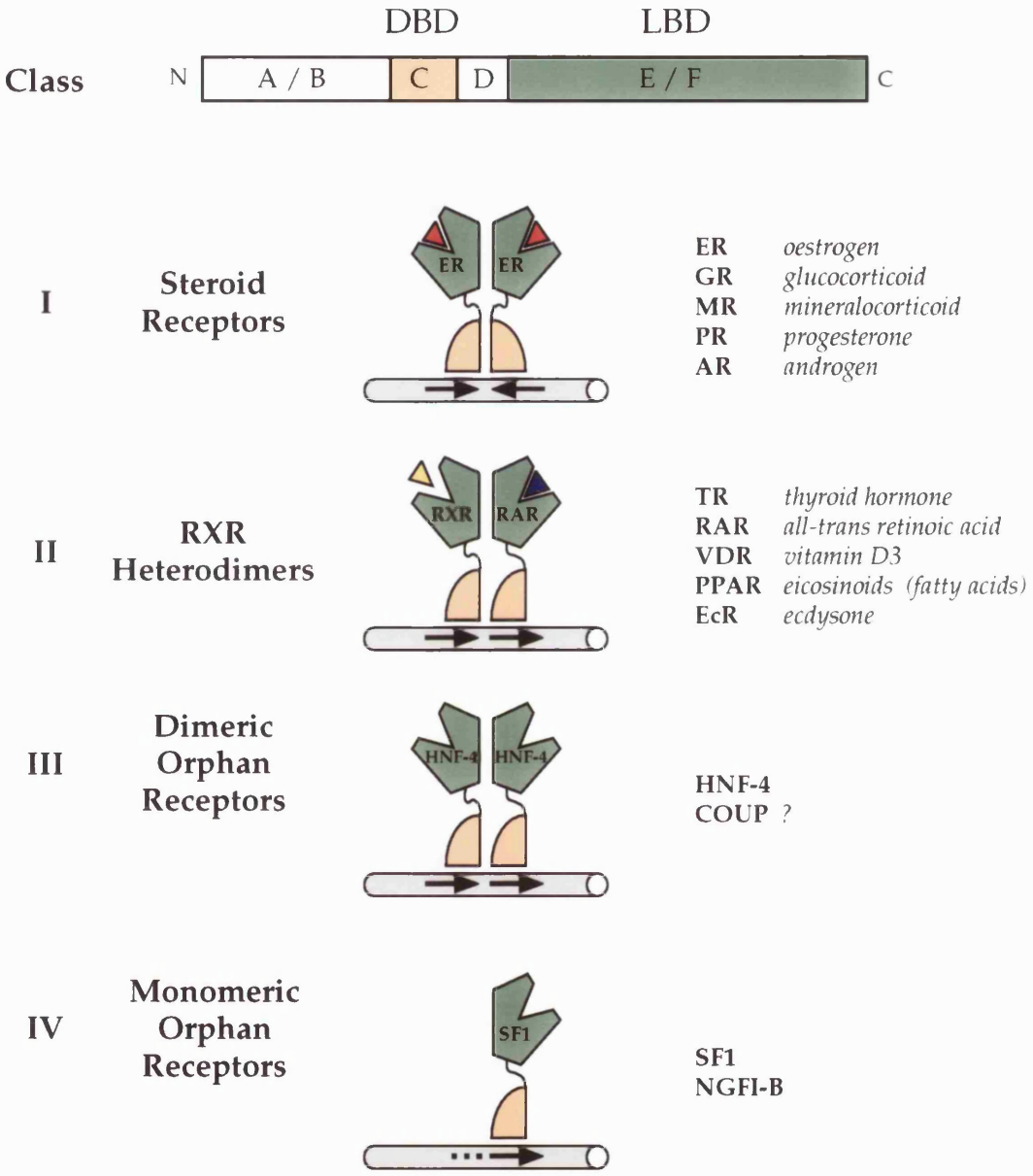


Fig. 1.1 - **Classification of the nuclear receptor superfamily.** The nuclear receptor structure can be subdivided into regions A-F, as shown above. Region C, contains the DNA binding domain (DBD), while region E, encompasses the ligand binding domain (LBD). Class I nuclear receptors include those of the steroid hormones, which bind palindromic half-sites with a 3 bp spacer as homodimers. Class II receptors form obligate heterodimers with RXR, and bind direct repeats with a receptor dependent spacing. These include all other receptors with a known ligand. Class III are orphan receptors which bind DNA as homodimers. Class IV receptors typically bind to extended half-sites as monomers. Ligands for Class I and II receptors are shown in italics.

co-ordinate two zinc ions, each with tetrahedral geometry (Hard *et al* 1990). The Cys-2-Cys-2 sequence present in the zinc ion-binding motifs has some similarity to the classical Cys-2-His-2 'zinc-fingers' described originally in the transcription factor TFIIIA, and present in other nucleic acid binding proteins (Klug and Rhodes 1987). However, the NMR structure of the ER DBD (and GR) in solution showed that the two zinc-binding motifs are folded to form a single structural unit, and are therefore distinct from the classical zinc-finger motif (reviewed by Schwabe *et al* 1993b).

The ER and GR bind DNA as homodimers, although to slightly different palindromic half-sites, 5'-AGGTCA (ERE) and 5'-AGAACA (GRE) respectively. Swapping the DBD of the ER for the GRs, Green and Chambon (1987) demonstrated that the identity of the DBD determined the half-site recognition, subsequently proving that the N-terminal zinc-binding motif was required for this distinction (Green *et al* 1988). Further mutagenesis of the N-terminal zinc-binding motif identified three residues in the ER and GR, termed the 'P' box, which if changed could alter the specificity of recognition between an ERE and a GRE (Danielsen *et al* 1989, Mader *et al* 1989, Umesono and Evans 1989). Residues in the second zinc binding-motif were implicated in protein-protein interactions of the dimeric DBD and therefore termed the 'D' box (Umesono and Evans 1989) .

The overall structure of the ER-DNA co-crystal (Schwabe *et al* 1993a) is very similar to the that of the GR bound to DNA (Luisi *et al* 1991). In each of the zinc binding-motifs the last pair of Cys residues form the start of an amphipathic α-helix, such that the zinc-binding pocket forms an N-terminal cap. The two α-helices are packed at right-angles and cross near their midpoints, hydrophobic side chains forming an extensive core between the helices. Residues from the P-box lie on the surface of the first α-helix to form the so-called DNA recognition helix. Two ER DBD molecules contact adjacent major grooves along one side of the DNA double helix. The DBD makes extensive contacts with phosphodiester back bone orientating the DBD so that the recognition helix lies inside the major groove, forming sequence specific contacts with the base pairs (See Fig. 1.2).

Residues forming the D-box are contained within a loop between the first and second Cys-zinc ligands, N-terminal to the second α-helix. On DNA the D-box forms extensive protein-protein interactions between the dimeric ER DBD molecules (Schwabe *et al* 1993a). In contrast, the ER DBD in solution is monomeric (Schwabe *et al* 1990). NMR analysis reveals that in the absence of DNA the D-box is poorly defined, suggestive of a relatively low stability (Schwabe *et al* 1990). It therefore seems possible that in solution the D-box is flexible, and only upon DNA binding is the correct conformation for the dimer interface induced, facilitating the co-operative interaction of the ER DBD monomers on binding to DNA (Schwabe *et al* 1993b).

The ER and GR belong to Class I nuclear receptors and therefore bind to palindromic half-sites, which impose a two-fold symmetry resulting in a head-to-head arrangement of DBD molecules (Luisi *et al* 1991, Schwabe *et al* 1993a). The core structure of the RXR/TR heterodimers bound to DNA is similar to ER and GR. However, the half-sites arranged as a direct repeat with a 4 base pair spacer (DR+4), creates a polar head-to-tail assembly of the two DBDs, with RXR occupying the upstream hexameric repeat (Rastinejad *et al* 1995). The asymmetric dimer interface of the RXR/TR DBDs on a DR+4 allowed Rastinejad *et al* (1995) to predict the arrangements of RXR/RAR bound to a DR+5 and RXR/VDR on a DR+3. This gave the first indications as to how Class II receptors are able to recognise DRs with a variety of spacings, although sharing a common partner, RXR. An increase in the spacer of one nucleotide causes RXR to be rotated 36° around the double helix, the result being that RXR cannot interact with its partners via a common interface. Instead RXR must have a number of nested surfaces for the interaction of its partners, which fit only the DR with a specific spacing. Furthermore, a C-terminal extension of the DBD in the TR (termed the A-box) forms extra contacts with the minor groove, these contacts sterically hinder the RXR/TR heterodimer from binding a DR with a spacer of less than 4 base pairs.

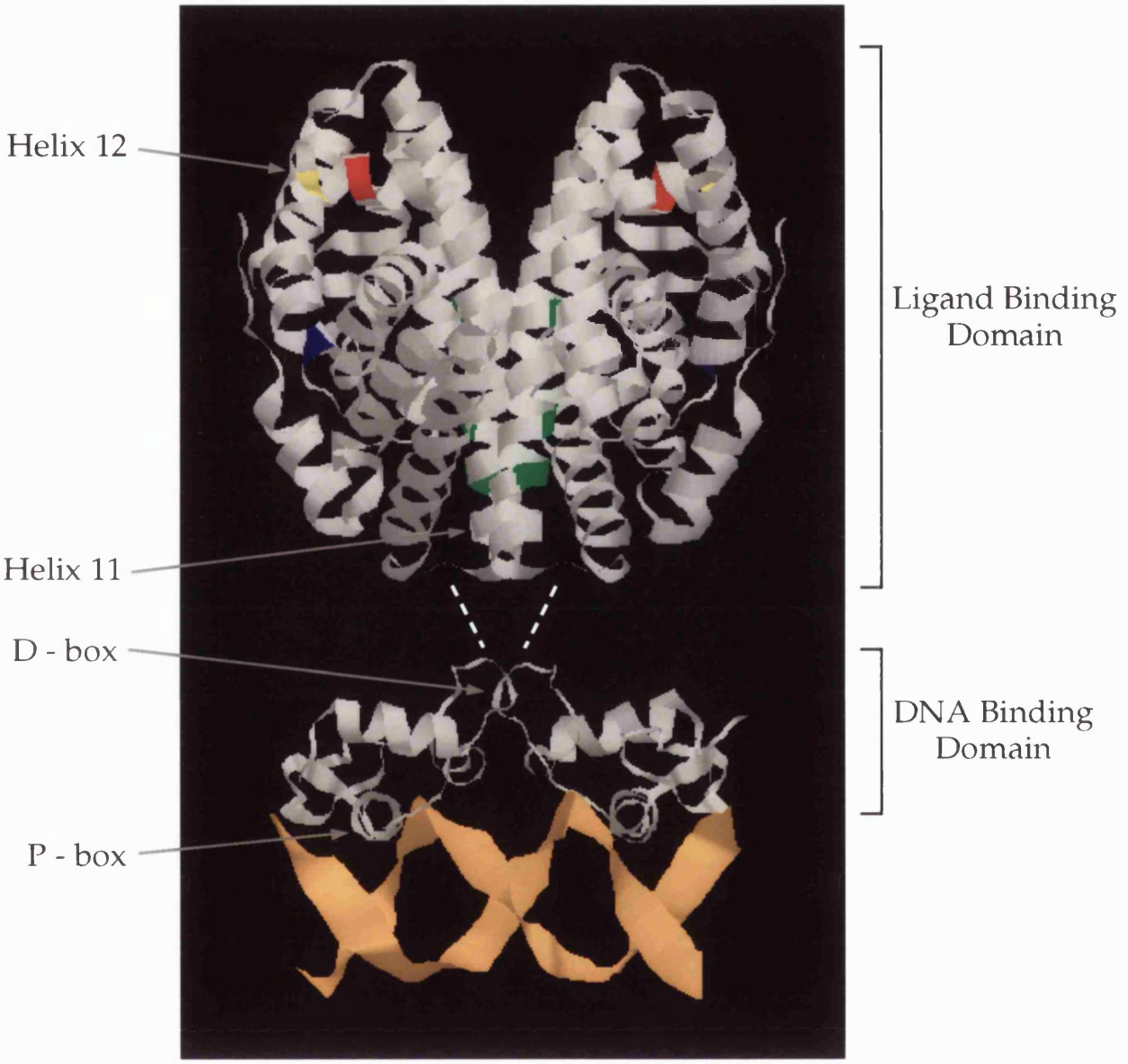


Fig. 1.2 - **The 3-dimensional structures of the ERα DNA binding and ligand binding domains**. The ERα DNA binding domain (DBD, Shwabe *et al* 1993) and the ligand binding domain (LBD, Brzozowski *et al* 1997) are linked by region D (dotted white line) whose structure is unknown. Residues in the DNA recognition helix (P-box) and dimer interface (D-box) of the DBD are shown. The critical residues in the LBD dimer interface identified by Fawell *et al* (1990) are shown in green (helix 11). Residues in AF2 which form the surface for the recruitment of coactivators: Glu 542 (Red), Leu 539 (Yellow) in helix 12 and Lys 362 (Blue) in helix 3 are also shown.

Ligand binding domain

The LBD is a complex region, encompassing not only ligand binding properties, but also the major dimerisation interface and a ligand dependent transcriptional activation domain. The first reported 3-dimensional structure of a nuclear receptor LBD was that of the un-liganded RXR (Bourguet *et al* 1995). It revealed a fold consisting of 11 α-helices (although this later became adapted to 12) and two β-turns, arranged in three layers and referred to now as an 'α-helical sandwich'. The subsequent crystallisation studies of LBDs from RAR, TR, ER and PR in the presence of ligand (Brzozowski *et al* 1997, Renaud *et al* 1995, Wagner *et al* 1995, Williams and Sigler 1998), revealed that the 'α-helical sandwich' structure is common to all these nuclear receptors. In addition, although the specificities of the holo-LBD are different, the ligand is positioned similarly at the heart of a hydrophobic core, in the centre of the LBD.

The structure of the apo-RXR and holo-RAR LBDs are similar, the main difference being that the molecule is more compact in the presence of retinoic acid and helix 12, the most C-terminal helix, instead of protruding from the core of the LBD realigns to cover the ligand binding pocket (Bourguet *et al* 1995, Renaud *et al* 1995). Helix 12 forms a lid on top of the ligand binding pocket (LBP), trapping the ligand, in what has been referred to as 'the mouse-trap model'. Exactly how the ligand enters the LBP has yet to be determined, Wagner *et al* propose that thyroid hormone enters the TR LBD via a space close to the β-turn (Wagner *et al* 1995). While this may be true for TR, the entry of ligand at this point in most nuclear receptor LBDs would disrupt multiple hydrogen bonds. Furthermore the electrostatic considerations of charged ligands such as retinoic acid would make this type of entry energetically unfavourable (Renaud *et al* 1995). The 'mouse-trap model' provides a more likely explanation, since the mobility of helix 12 allows an open channel to the LBP. The LBP in general is lined mainly with hydrophobic residues, with a few polar residues close to the β-turn which anchor the ligand. The individual anchoring residues are conserved within a particular nuclear receptor sub-type depending on the ligand.

The LBP of ERα is completely partitioned from the external environment and occupies a large proportion of the LBDs hydrophobic core (Brzozowski *et al* 1997). Hormone recognition is achieved by a combination of specific hydrogen bonds and the non-polar character of the steroidal rings. The hydroxyl group on the A-ring of 17β-oestradiol (E_2) nestles between helices 3 and 6 making direct hydrogen bonds to Glu 353 and Arg 394. The 17β-hydroxyl on the D-ring forms a single hydrogen bond with His 524 in helix 11. The rest of the molecule participates in a number of hydrophobic contacts along the remainder of the LBP. ERα is able to accommodate many non-steroidal ligands. The 'pincer like' anchoring of the ligand at the bottom of the LBP by Glu 353 and Arg 394, means that effective ligands must have at least one aromatic group, but the hydrophobic interactions along the rest of the cavity can be satisfied by a number of hydrophobic groups. This in combination with the fact that the volume of the LBP in ERα is significantly larger than its natural ligand 17β-oestradiol, allows the binding of structurally dissimilar ligands, including different oestrogens and non-steroidal compounds such as the antioestrogens, tamoxifen and raloxifene. The 3D structure of the raloxifene bound ERα LBD shows similar contacts to those of E_2, with an additional hydrogen bond formed with Asp 351. However, the greater size of the raloxifene side-chain is too large to be contained within the LBP and protrudes out, displacing helix 12 from its position as 'the lid' (Brzozowski *et al* 1997). The misalignment of helix 12 is likely to reduce, if not abolish the transcriptional activity of ERα, suggesting that this may be the molecular mechanism for its antioestrogenic function.

Dimerisation

Class I-III nuclear receptors bind to their response elements as dimers. Class I and III receptors generally bind to DNA as homodimers, while class II receptors form heterodimers with RXR (reviewed by Parker 1993). Mapping of the dimerisation interface in ERα revealed that a receptor lacking residues C-terminal of Val 538 were still able to dimerise on an ERE with high affinity, whereas a further deletion to Arg 507 reduced DNA binding to approximately 10% that of the wild-type

ERα; the residual DNA binding activity due possibly to the 'weak dimerisation function' in the DBD (Kumar and Chambon 1988). Mutational analysis of residues between 507 and 538 identified three residues at the N-terminus of this fragment: Arg 507, Leu 511 and Ile 518 which were critical for high affinity dimerisation and DNA binding (Fawell *et al* 1990). These residues are present in the majority of nuclear receptors, suggesting a conserved mechanism of dimerisation. The dimer interface identified by Fawell *et al* (1990) is in general agreement with the 3D structure of the dimeric ERα ligand binding domain (Brzozowski *et al* 1997), in so far that the residues which were critical for dimerisation of ERα *in vitro* are all present in helix 11, the helix which forms the major dimerisation interface. Although contacts between the two LBD molecules are primarily through helix 11, it was shown that dimerisation also includes contacts between helix 8 of one monomer, with helices 9 and 10 of the neighbouring monomer. Both reports are consistent with the other 3D structure of a dimeric LBD, that of the apo-RXR (Bourguet *et al* 1995).

Activation domains

Nuclear receptors contain two transcriptional activation domains, activation function 1 (AF1) in the A/B region and activation function 2 (AF2) in the LBD (Godowski *et al* 1988, Hollenberg and Evans 1988, Lees *et al* 1989, Tora *et al* 1989). In the context of the full length steroid receptors both AF1 and AF2 are activated by ligand, due to the sequestering of the receptor in the inactive heat shock protein complex in the absence of hormone. However, AF1 can function constitutively when fused to a heterologous DBD, but AF2 activity remains ligand-dependent even in such fusion proteins. The mechanism of AF1 function is poorly understood, whereas many of the underlying principles of AF2 function are now known. This is due at least in part, because although the majority of nuclear receptors have an AF1 activity they share no common homology and therefore give no clues as to their function; whereas critical residues required for AF2 activity and the structure of the LBD are conserved throughout the nuclear receptor family.

AF1 activity

AF1 is more often thought of as an activity than as a domain, because of the lack of homology in the A/B region of nuclear receptors. The AF1 region of ERα consists of only 180 amino acids, whereas the equivalent region in PR and AR has over 550 amino acids. Although they lack sequence homology, AF1 is able to synergise with AF2 in context of the full length receptor (Danielian *et al* 1992, McInerney and Katzenellenbogen 1996, Tora *et al* 1989), determining cell-type and promoter specificity in some cases (Berry *et al* 1990, Tora *et al* 1989). The basis of AF1 activity is still unclear, but may depend on phosphorylation (Kato *et al* 1995, Rochette-Egly *et al* 1997). The activity of the AF1 in ERα for instance is modulated by Ser phosphorylation at a mitogen activated protein kinase (MAPK) site, in response to growth factors (Bunone *et al* 1996, Kato *et al* 1995). A similar site is also present in the A/B region of ERβ, which is required for the stimulation of its transcriptional activity by Ras^{V12} (Tremblay *et al* 1997), however stimulation with growth factors has yet to be demonstrated.

AF2 activity

The AF2 activity of class I and II nuclear receptors is ligand dependent and, in the case of steroid receptors, can be repressed by antagonists such as 4OH-tamoxifen and RU486 (Berry *et al* 1990, Meyer *et al* 1990). A putative helix at the C-terminus of the ERα LBD domain was found to be crucial for AF2 activity (Danielian *et al* 1992). The mutation of hydrophobic residues within this helix in ERα, and GR abolished their AF2 activity. Similar results were also obtained in the RAR and TR (Barettino *et al* 1994, Durand *et al* 1994). The first 3D structure of a nuclear receptor LBD, apo-RXR, showed that the helix identified by Daniellian *et al* (helix 12) was protruding from the core of the LBD and therefore may be a target for cofactors. However, the subsequent 3D structures of the LBDs from TR, RAR, ERα and PR in the presence of hormone showed helix 12 realigned across the LBD core, adjacent to helix 3 (See Fig. 1.3, based on Brzozowski *et al* 1997, Renaud *et al* 1995, Wagner *et al* 1995, Williams and Sigler 1998). Helix 3 contains

a Lys residue conserved in the majority of nuclear receptors, which is essential for transcriptional activation, at least in the ER and TR (Henttu *et al* 1997, O'Donnell and Koenig 1990). The holo-LBD structures, in combination with the mutagenesis studies suggest that helices 3, 12 and possibly 5, form the surface for the recruitment of coactivators (Feng *et al* 1998). The 3D structure of the ERα LBD in the presence of the antioestrogen Raloxifene, shows that helix 12 is repositioned in an unfavourable position for the recruitment of coactivators, therefore abolishing AF2 activity. This may be a common mechanism for the antagonistic effects of antioestrogens (Brzozowski *et al* 1997). Thus helix 12 is able to act as a molecular switch, its realignment upon ligand binding determining the activity of AF2.

Since RXR is the only reported apo-LBD structure, it is unclear whether this is the definitive position of helix 12 in the absence of ligand. Helix 12 is a hydrophobic rich helix, it therefore seems unlikely that it would be extended from the LBD core unless it was stabilised. Renaud *et al* (1995) argued that this stabilisation may occur in RXR from interactions at the N-terminus of helix 12 with the Ω-loop between helices 2 and 3. This argument is strengthened by the observation that mutation of a Tyr residue at the N-terminus of helix 12 in ERα (and ERβ), results in ligand-independent AF2 activity (White *et al* 1997).

Transcriptional regulation

The rate of a gene's transcription is dependent on the enzymatic activity of RNA polymerase II. RNA polymerase II is a poor enzyme; in the absence of other proteins it is incapable of binding DNA, synthesising the first phosphodiester bond, finishing the synthesis of a nascent mRNA molecule without pausing or copying it without mistakes. For optimal activity RNA polymerase II relies on a large number of accessory proteins which together determine the overall rate of transcription.

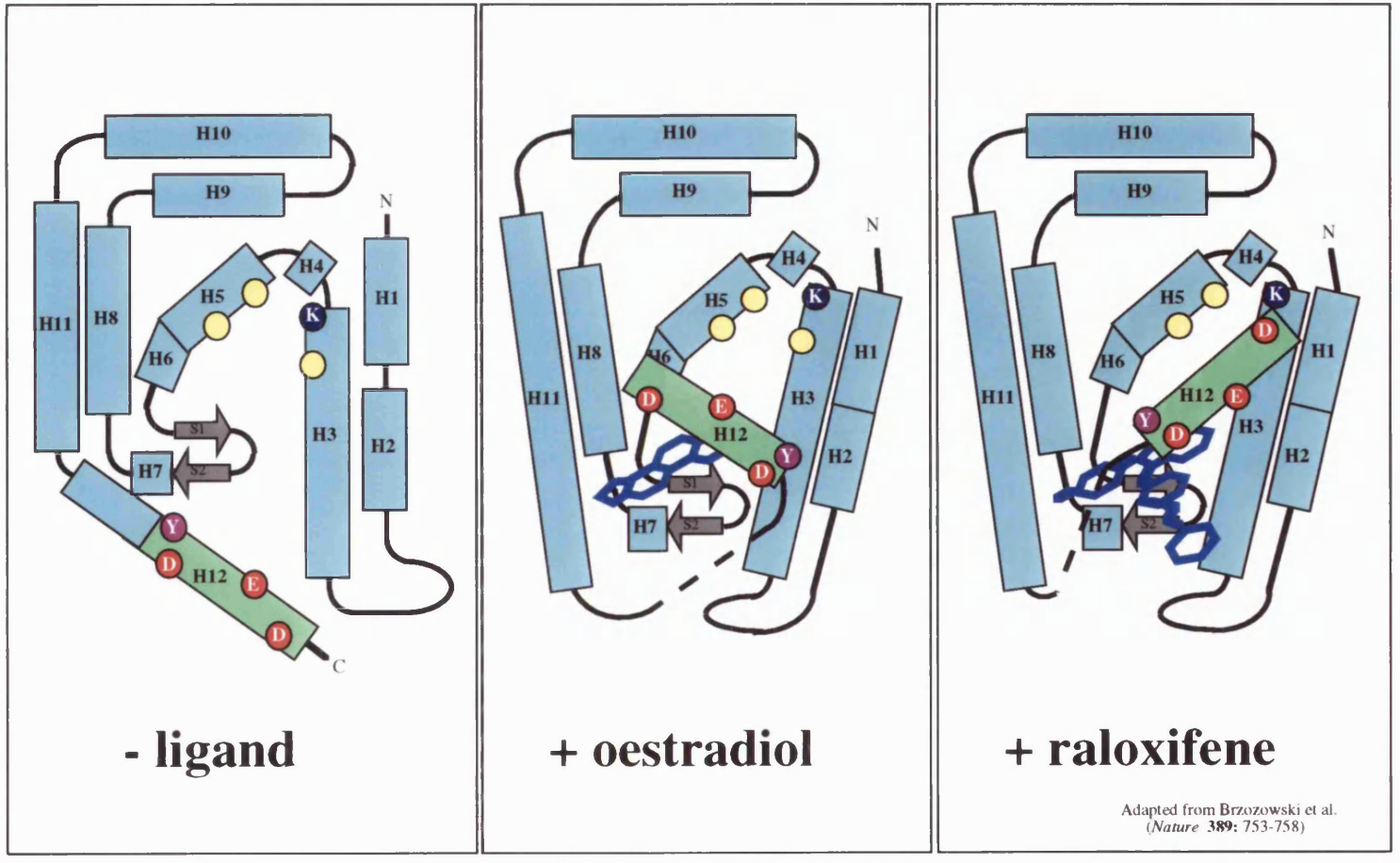


Fig. 1.3 - **Schematic representation of the changes in conformation undergone by the ERα ligand binding domain.** The ERα ligand binding domain (LBD) consists of 12 helices (H1-12) and two β-turns (S1-2). In the absence of ligand helix 12 protrudes from the core of the LBD, stabilised by Tyr 534 at its N-terminus. Upon hormone binding (+ oestradiol) helix 12 realigns across the core of the LBD, the three acidic residues in helix 12 (D538, E542, D545) and the Lys362 forming a surface for the recruitment of coactivators. Additional contacts may be made by the hydrophobic residues in helices 3 and 5, shown as yellow circles. In the presence of the antioestrogen Raloxifene, helix 12 is realigned across helix 3, in a conformation which is unfavourable for the recruitment of coactivators.

General transcription factors

Proteins involved in the initiation of transcription at the majority of genes transcribed by RNA polymerase II are called general transcription factors (GTFs). Six GTFs particularly associated with RNA polymerase II are often referred to as, TFII-A, B, D, E, F and H (reviewed by Orphanides *et al* 1996). All of these factors have been cloned and in some cases found to consist of multiple polypeptides.

TFIID consists of a core DNA binding protein either, TATA binding protein (TBP) or TBP related factor (TRF, Hansen *et al* 1997) which recognise the TATA element approximately 30 base pairs from the start of transcription, and a number of TBP-associated factors (TAFs; for review see Verrijzer and Tjian 1996). TFIID binds RNA polymerase and positions it at the TATA element, probably aided by TFIIA and TFIIB (Tang *et al* 1996). TFIIE acts by recruiting TFIIH to this pre-initiation complex. TFIIH is a highly complex GTF consisting of multiple subunits (Marinoni *et al* 1997). It contains a helicase activity, which functions in nucleotide excision repair and also transcription, possibly by unwinding the DNA, allowing access to the coding strand so that RNA polymerase II can begin to synthesise RNA (Hoeijmakers *et al* 1996). TFIIF functions in both transcriptional initiation and elongation. It dissociates after initiation of the transcript and rejoins during elongation, possibly increasing the processivity of the polymerase (Zawel *et al* 1995).

When these GTFs were first isolated, it was envisaged that they would be recruited in a 'step-wise assembly' to form a complex which could competently initiate transcription. However, recovery of RNA polymerase II from yeast showed that many of these factors existed together in a large complex (Koleske and Young 1994). A similar complex was also isolated from human HeLa cell extracts, which was capable of supporting transcription (Ossipow *et al* 1995). Thus *in vivo* the GTFs may exist as preformed preinitiation complexes even in the absence of DNA, although their exact composition still remains open to question.

Activated transcription: the role of TAFs

Reconstitution of preinitiation complexes *in vitro* from isolated GTFs, produce only low, basal levels of transcription. Transcription can be stimulated to the levels observed using cell extracts by the addition of transcriptional activators such as nuclear receptors, VP16 or p53 (Verrijzer and Tjian 1996). Transcriptional activators are thought to function *in vitro* by stabilising the preinitiation complex by interacting with GTFs, in particular TAFs. If TBP is used instead of TFIID to form a preinitiation complex lacking TAFs then VP16 is incapable of activating transcription (Goodrich *et al* 1993). VP16 interacts directly with TAFII40 and its presence is essential for transcriptional activation by VP16 *in vitro*. Different transcription factors were shown to function in a similar way, but with different TAFs, p53 activity for instance depends on TAFII40 and TAFII60 (Thut *et al* 1995). In cells, nuclear receptor activity has been shown to depend on the presence of TAFII28 and TAFII135, although no direct interaction occurs between these proteins and nuclear receptors (May *et al* 1996, Mengus *et al* 1997).

Although the activator-TAF interaction model of activated transcription appears attractive, it is not without its problems. In contrast to the examples above, efficient activation has been demonstrated in yeast and mammalian cells in the absence of TAFs, suggesting that other targets are sufficient (Koleske and Young 1995, Oelgeschlager *et al* 1998). However, these results are not inconsistent with activator-TAFs contributing to activated transcription, and that multiple activator targets exist which can function either independently or synergistically. A straight forward model in utilising activator-TAF interactions predict that TFIID should be sufficient to support activated transcription *in vitro*. The contrary is in fact the case, TFIID dependent activation *in vitro* is observed only in the presence of other 'coactivators' such as PC2 or PC4 (Burley and Roeder 1996). Finally, there is no evidence of activators interacting with TAFs in the context of the TFIID complex. It's possible that the interaction of activators with isolated TAFs may involve TAF protein surfaces which are not accessible in the TFIID complex.

Coactivators

Transcriptional activators need not bind directly to general transcription factors in the preinitiation complex. Non-TAF coactivators such as PC4 (Ge and Roeder 1994), OCA-B (Luo and Roeder 1995), CREB binding protein (CBP, Kwok *et al* 1994) and p300 (Eckner *et al* 1994), can serve as adaptors between activators and GTFs. In some cases, the DNA bound activator may function solely as a tether for the coactivator.

CBP/p300: a common coactivator for many transcription factors

CBP was originally isolated as a coactivator of the transcription factor CREB (Kwok *et al* 1994), while p300 was characterised by its ability to bind the viral oncoprotein E1A (Eckner *et al* 1994). CBP and p300 are highly homologous and generally termed CBP/p300, because of their overlapping functions. CBP/p300 function as coactivators for a large number of transcription factors including CREB (Lundblad *et al* 1995), c-Jun (Bannister *et al* 1995), c-Myb (Dai *et al* 1996), Stat2 (Bhattacharya *et al* 1996), MyoD (Puri *et al* 1997) and E2F-1 (Trouche and Kouzarides 1996, for review see Shikama *et al* 1997). By recruiting CBP/p300, a diverse set of transcription factors are able to stimulate transcription by a similar mechanism. CBP/p300 contains an intrinsic activation domain (Arany *et al* 1995) and is able to interact with TFIIB (Kwok *et al* 1994), a component of the basal transcription machinery. Since CBP/p300 acts as a common component for many transcription factors, it has been suggested that the transcriptional interference observed between many of these factors could be due to competition for limiting amounts of CBP/p300 in cells (Kamei *et al* 1996). E1A for instance inhibits the transcriptional activity of nuclear receptors and Stat1 by competing for their binding sites on CBP/p300 (Kurokawa *et al* 1998). However, mutant nuclear receptors which are unable to interact with CBP/p300 are still able to interfere with the activity of other transcription factors (Saatcioglu *et al* 1997). Thus the 'CBP/p300 competition' hypothesis may be only partially correct.

The identification of nuclear receptor cofactors

The structural similarity of different LBDs and the conservation of residues in helices 3 and 12 which are critical for AF2 function (Barettino *et al* 1994, Danielian *et al* 1992, Durand *et al* 1994, Henttu *et al* 1997), suggest a conserved mechanism of AF2 transcriptional activation. This was supported by the observation that different nuclear receptors were capable of interfering with each others transcriptional activity, presumably by sequestering a common cofactor (Meyer *et al* 1989, Tasset *et al* 1990). Mutations in helix 12 abolished transcriptional interference, suggesting that the cofactor being sequestered was specific for AF2 and not a general transcription factor (Barettino *et al* 1994). Thus began the search for proteins that could interact with the LBD of nuclear receptors, so called, receptor interacting proteins (RIPs). For a RIP to be a possible coactivator then its interaction with the nuclear receptors must fit the characteristics of their transcriptional activation. Putative coactivators should interact with the ER for example, in the presence of oestradiol, but not antioestrogens (which antagonise ER function), and not with ER mutants which abolished transcription.

The first RIPs were identified using the LBD of ERα fused to GST (GST-AF2α). GST-AF2α labelled with [^{32}P] was used to screen cell extracts in a far-western blotting analysis and RIPs of 160, 140, 80 kDa were identified (Cavaillès *et al* 1994, Halachmi *et al* 1994). These RIPs fitted the criteria of potential coactivators since their interaction with GST-AF2α was ligand dependent and abolished by mutations in helix 12.

Isolation of the first receptor interacting proteins

The first RIPs cloned were RIP140 (Cavaillès *et al* 1995) and TIF1 (Le Douarin *et al* 1995). Both proteins have a molecular weight of approximately 140 kDa, a similar size to one of the bands seen in far-western blots. RIP140 was isolated using the far-western technique to screen a ZR-75 breast cancer cell cDNA expression library. The full length RIP140 cDNA encoded a novel protein with no significant sequence homology to other proteins and no recognisable functional

domains. It interacted with ERα in a ligand dependent manner but not with ERα containing mutations in helix 12. RIP140 displayed all the characteristics of a putative coactivator, however, it stimulates nuclear receptor transcription only weakly (Cavaillès *et al* 1995). This was slightly surprising, because RIP140 contains an autonomous activation domain which stimulates transcription in both mammalian and yeast cells (L'Horset *et al* 1996). TIF1 was isolated using a yeast two-hybrid screen designed to isolate proteins which increased the transcriptional activity of RXR (Le Douarin *et al* 1995). TIF1 was also a novel protein although it contained several recognisable domains, including a RING finger, a coiled coil and bromodomain. Similar to RIP140, TIF1 interacted with nuclear receptors in a ligand dependent manner, but functioned as a poor coactivator. A subsequent yeast two-hybrid screen identified a second form of TIF1, TIF1β which also failed to potentiate nuclear receptor transcription (Le Douarin *et al* 1996).

A yeast two-hybrid screen performed by Lee *et al* (1995a) using the LBD of the TR as the bait isolated a number of partial RIP cDNAs in the presence and absence of thyroid hormone, which they termed thyroid receptor interacting proteins (TRIPs). The cDNA encoding Trip1 was found to be the human homologue of a yeast proteasomal protein, Sug1 (Lee *et al* 1995b). Sug1 was also isolated in a similar yeast two hybrid screen by vom Baur *et al* (1996) using the LBD of RXR. Sug1 is 36 kDa protein, first identified as a mutant in a yeast genetic screen which rescued defects in the transcriptional activation domain of Gal4 (Swaffield and Bromberg 1992). On the basis of these genetic studies and the direct interaction with TBP (Swaffield *et al* 1995), it was suggested that Sug1 was a coactivator. However, Sug1 stimulates nuclear receptor transcription only weakly and is thought to be more likely part of the proteasome than a coactivator (Rubin *et al* 1996). As part of the proteasomal complex Sug1 may be involved in the degradation of many proteins and therefore its interaction with nuclear receptors could be purely fortuitous.

CBP/p300

Microinjection of cells with neutralising antibodies against CBP blocks nuclear receptor transcriptional activity, suggesting that CBP (and its homologue p300) may have a role in nuclear receptor signalling (Chakravarti *et al* 1996). The interaction of CBP/p300 with nuclear receptors *in vitro* was ligand-dependent, but somewhat weak, although in cotransfection assays CBP/p300 was clearly able to stimulate the transcriptional activity of RXR and RAR (Chakravarti *et al* 1996). p300 was also shown to be a strong coactivator of ERα *in vitro* (Kraus and Kadonaga 1998). It therefore seems likely that CBP/p300 has some function in nuclear receptor transcriptional activity, similar to many other transcription factors (Shikama *et al* 1997).

The SRC1/RIP160 family

Steroid receptor coactivator 1 (SRC1) was isolated in a yeast two-hybrid screen using the LBD of the PR as bait (Onate *et al* 1995). SRC1 interacted with the PR in a ligand dependent manner, but in contrast to many RIPs cloned previously it functioned as a strong coactivator of nuclear receptors. The open reading frame of SRC1 isolated by Onate *et al* corresponded to a protein of 140 kDa, however it was subsequently discovered that this clone was lacking sequence at the 5' end. The full length SRC1 protein (including several variants termed a to e) was first reported by Kamei *et al*, who demonstrated that SRC1 was in fact a 160 kDa protein, the first RIP160 to be cloned (Kamei *et al* 1996). Antibodies raised against SRC1 were used to immunodeplete whole cell extracts, which were then used in far-western blots to show that only a fraction of the RIP160 proteins detected consist of SRC1; while other related proteins accounted for the remaining RIP160 band (Kamei *et al* 1996). One such related protein termed, transcriptional intermediary factor 2 (TIF2, Voegel *et al* 1996) in humans and GR interacting protein 1 (GRIP1, Hong *et al* 1996) in mouse was isolated soon after the discovery of SRC1. Both TIF2 and GRIP1 were shown to potentiate the activity of several nuclear receptors (Hong *et al* 1996, Voegel *et al* 1996).

The third and last member of the SRC1/RIP160 family was isolated independently by several groups who termed it, activator of TR and RAR (ACTR, Chen *et al* 1997), receptor associated co-activator 3 (RAC3, Li *et al* 1997), amplified in breast cancer 1 (AIB1, Anzick *et al* 1997), TR activator molecule-1 (TRAM-1, Takeshita *et al* 1997) in humans and p300/CBP cointegrator associated protein (p/CIP, Torchia *et al* 1997) in mouse. AIB1 was the only RIP160 protein not to be identified by yeast two-hybrid or far-western screening. Instead it was isolated in the region of chromosome 20q12, which becomes amplified in some breast cancers. AIB1 functions as a coactivator of steroid receptors, and is amplified in breast and ovarian cancer cell lines as well as breast tumor samples, suggesting a role for coactivators in the growth and progression of steroid-dependent cancers (Anzick *et al* 1997).

The three SRC1/RIP160 family members are highly related, showing an overall amino acid identity of 34%, although this is much higher within individual functional domains. All three proteins have a well conserved per/arnt/sim (PAS) basic helix-loop-helix (bHLH) at their N-terminus, the function of which is unclear. In other proteins the PAS-bHLH domain is involved in protein-protein interactions (Crews 1998), in the case of SRC1/RIP160 proteins it can be deleted without reducing the potentiation of nuclear receptor transactivation. Deletion mapping of SRC1 (Kalkhoven *et al* 1998) and TIF2 (Voegel *et al* 1998) identified two regions with transcriptional activity, the main activation domain (AD1) in the centre of the protein, and a weaker one at the C-terminus (AD2). Although ACTR/TRAM1/RAC3 and p/CIP have an AD1 activity equivalent to those of SRC1 and TIF2 (Chen *et al* 1997, Li and Chen 1998, Takeshita *et al* 1997), it is unclear whether they too have an AD2 function.

The boundaries of the AD1 activity overlaps a conserved region in all three related proteins which mediates interaction with the coactivator proteins CBP/p300 (Li and Chen 1998, Voegel *et al* 1998). Indeed co-immunoprecipitation experiments demonstrate that a large proportion of CBP/p300 in cells is complexed with p/CIP (Torchia *et al* 1997). The deletion of AD1 results in a loss of interaction with CBP/p300 and a severe reduction in the potentiation of nuclear receptor activity. It therefore seems very likely that AD1 activity is derived from CBP/p300

recruitment. The mechanism and function of AD2 transcriptional activity remains to be established.

Nuclear receptors recruit coactivators via a novel domain

Deletion mapping of SRC1 and TIF2 identified a nuclear receptor interacting domain (NID) of approximately 200 amino acids at the centre of the two proteins (Kalkhoven *et al* 1998, Voegel *et al* 1998). This region is well conserved in all three SRC1/RIP160 family members, however it has very little homology with any of the other RIPs isolated. What was lacking was a common domain in the RIPs which could explain their ligand-dependent interaction with nuclear receptors. The surface of nuclear receptor LBDs required for the recruitment of RIPs is conserved, it therefore seemed reasonable to expect a conserved domain in RIPs.

Using deletion mapping of RIP140 to identify the region which mediated the interaction with nuclear receptors, Heery *et al* (1997) found that multiple fragments of RIP140 could interact with the LBD of ERα in a ligand-dependent manner. The only homology between these fragments of RIP140 was a short motif consisting of LXXLL (L=Leucine and X=any amino acid) a derivative of the LXXLLL motif proposed by Le Douarin *et al* (1996) to mediate interactions with nuclear receptors. RIP140 contains nine copies of the LXXLL motif and each one is capable of interacting with the LBD of ERα in the presence of ligand (Heery *et al* 1997). A sequence analysis of the cloned RIPs revealed that each had at least one copy of the LXXLL motif (Heery *et al* 1997). The NID of the SRC1/RIP160 family members contains three copies of the LXXLL motif, conserved in both sequence and spacing. Because of 'the striking relatedness' of the SRC/RIP160 proteins Torchia *et al* (1997) were also able to identify the LXXLL motif, by sequence analysis.

Point mutation of a helix containing a LXXLL motif revealed that the first Leu residue could be replaced by a Val but not Ala, and that the Leu pair could not be replaced by other hydrophobic residues (Heery *et al* 1997). Replacement of the second pair of Leu residues with Ala in all three LXXLL motifs in the full length SRC1 protein resulted in a complete loss of nuclear receptor binding (Heery *et al* 1997). Mutation of

individual motifs showed that for ERα the second motif in the NID had the strongest interaction (Kalkhoven et al 1998), while the GR preferentially interacts with the third LXXLL motif (Ding *et al* 1998). This evidence suggest that although SRC1/RIP160 family members have three copies of the LXXLL motif that one may be used in preference to the others depending on the particular nuclear receptor.

Corepressors

In the absence of ligand, RAR and TR are able to actively repress transcription (Baniahmad *et al* 1992, Baniahmad *et al* 1995). This function is present in the LBD of RAR and TR, and is separable from their AF2 function. Using RXR/TR heterodimers bound to a biotinylated response element, Kurokawa *et al* (1995) were able to precipitate proteins bound to the heterodimer in the absence of ligand and then detect them using far-western blotting. This binding assay revealed that a protein of approximately 270 kDa was capable of selectively binding the unliganded TR/RXR. Addition of ligand abolished the interaction with the p270 and induced interaction with the RIP140 and RIP160 proteins (Kurokawa *et al* 1995). To isolate the cDNA encoding p270, Horlein *et al* (1995) used a yeast two-hybrid screen with the LBD of TR in the absence of ligand as bait. Only one of the seven clones isolated contained an open reading frame of sufficient length to encode a 270 kDa and this interacted with the unliganded TR more strongly than the other six. The full length open reading frame encoded a protein of 2,453 amino acids approximately the size of the p270, referred to as NCoR, for nuclear receptor corepressor (Horlein *et al* 1995). A similar protein (although only 168 kDa) was isolated by Chen and Evans (1995), which they termed silencing mediator for retinoid and thyroid-hormone receptors (SMRT). Both NCoR and SMRT interacted with the unliganded forms of TR and RAR, but not other nuclear receptors (Chen and Evans 1995, Horlein *et al* 1995). The putative roles of NCoR and SMRT as corepressors is based on two observations. The first is that mutations in the hinge region of TR and RAR which abolish interaction with NCoR/SMRT, also impairs their ability to repress transcription in cells. The second is that NCoR and SMRT contain

multiple regions capable of repressing transcription when transferred to a heterologous DBD (Chen and Evans 1995, Horlein *et al* 1995).

Unlike RAR and TR, steroid receptors are sequestered in an inactive complex with heat shock proteins in the absence of hormone. There was little reason therefore to suspect that they would interact with corepressor proteins. However, recent reports have shown that antagonist bound ERα (Lavinsky *et al* 1998, Smith *et al* 1997) and PR (Jackson *et al* 1997) are able to interact with NCoR, suggesting a mechanism whereby antagonist-occupied steroid receptors could actively repress transcription. Activation of the MAPK pathway by growth factors, or mutation of the site of phosphorylation in the AF1 domain relieves the interaction of NCoR with 4OH-tamoxifen bound ERα, converting 4OH-tamoxifen to an agonist (Lavinsky *et al* 1998). Furthermore, microinjection of cells with antibodies raised to NCoR results in conversion of 4OH-tamoxifen from an antagonist to an agonist, dependent on the integrity of the coactivator complex.

Modulation of chromatin structure during transcriptional regulation

In the past few years it has become apparent that transcription is intimately related to changes in chromatin structure. Acetylation of the amino-tails of histones is generally associated with increased accessibility of nucleosomes to DNA binding proteins and rates of transcription, while unacetylated histones are conversely associated with inaccessibility to binding proteins and transcriptional repression (reviewed in Grunstein 1997). These findings are consistent with a model in which transcriptional activators increase rates of transcription by recruiting coactivators such as p300/CBP, p/CAF and ACTR which contain intrinsic histone acetyltransferase (HAT) activity (Bannister and Kouzarides 1996, Chen *et al* 1997, Ogryzko *et al* 1996, Yang *et al* 1996 respectively), while transcriptional repressors such as pRb and Mad1 recruit histone deacetylases (HDAC, Laherty *et al* 1997, Luo *et al* 1998). The relationship between histone acetylation and rates of transcription is thus far mainly correlative. The best example of HAT activity actually effecting transcription is the demonstration that the transcriptional

activation domain of CBP is dependent on its HAT activity (Martinez-Balbas *et al* 1998).

Functional studies using frog oocytes have allowed an analysis of chromatin structure and nuclear receptor function *in vivo*. Chromatin disruption by the TR is regulated by ligand and AF2 dependent, suggesting that the activation domains are capable of recruiting chromatin remodelling activities (Wong *et al* 1997). This is supported by *in vitro* transcription studies in which p300 was only capable of potentiating the activity of ERα from chromatin, and not naked DNA templates (Kraus and Kadonaga 1998).

The nuclear receptor corepressors NCoR and SMRT, interact with Sin3, a corepressor for the Mad family of proteins (Heinzel *et al* 1997, Nagy *et al* 1997). Sin3 functions as a scaffold for the recruitment of HDAC activity (for review see Pazin and Kadonaga 1997) and accessory proteins such as SAP30 (Laherty *et al* 1998). The demonstration of a NCoR/Sin3/HDAC complex and the reduced ability of NCoR to repress transcription in the presence of HDAC inhibitors such as trapoxin, all point to deacetylation of histones as being the basis for transcriptional repression (Heinzel *et al* 1997).

A large number of diverse transcription factors may function by recruiting HAT or HDAC activities, thus altering rates of transcription by modulating local chromatin structure. Attractive as this hypothesis is however, it is by no means proven; cofactors with HAT or HDAC activity may have targets other than, and in addition to histones. Indeed acetylation of p53 has been shown to influence its DNA binding activity (Wei and Roeder 1997) and acetylation of components of the basal transcription machinery has also been demonstrated (Imhof *et al* 1997).

A current model of nuclear receptor transcriptional regulation

Based on these studies our current model of how nuclear receptors regulate transcription involves the recruitment of distinct protein complexes. In the absence of hormone steroid receptors are sequestered in an inactive complex with heat shock proteins. Retinoid

and thyroid receptors which do not bind heat shock proteins instead actively repress transcription by recruiting an NCoR/Sin3/HDAC complex, which probably represses transcription by deacetylating histones. Ligand binding results in a conformational change in the LBD, characterised by the realignment of helix 12. The repositioning of helix 12 results in the replacement of heat shock proteins and/or the NCoR/Sin3/HDAC repressor complex by a coactivator complex, consisting of one of three SRC1/RIP160 family members, CBP/p300 and possibly p/CAF, all of which contain HAT activity. The model can be extended to include 4OH-tamoxifen bound ER which is able to recruit the corepressor complex, dependent on signalling via the MAPK pathway. Thus depending on the liganded status of the LBD and the position of helix 12, nuclear receptors are able to regulate transcription by the recruitment of distinct complexes with opposing acetyltransferase properties (see Fig 1.4).

Non-genomic functions of Nuclear receptors

In addition to the well characterised DNA bound activities of nuclear receptors, they also have a number of functions which seemingly do not require DNA binding. The best characterised of these is the repression of other transcription factors, including AP-1 (Diamond *et al* 1990, Jonat *et al* 1990, Nicholson *et al* 1990), GATA-1 (Blobel *et al* 1995) C/EBP (Stein and Yang 1995), and NF-Kb (Heck *et al* 1997, Stein and Yang 1995). The repressive cross-talk of nuclear receptors with other transcription factors may be responsible for some of the observed functions of hormones *in vivo*. The protective role of oestrogens in bone for instance, may be mediated by repression of NF-κb and C/EBPβ on the promoter of the IL-6 gene by ERα (Stein and Yang 1995). IL-6 promotes bone resorption by activation of immature osteolclasts. Inhibition of IL-6 expression by ERα may therefore help maintain bone structure. Oestrogens also regulate haematopoesis by the inhibition of gene expression. Consistent with this observation is the report that ERα functions as a repressor of the erythroid specific transcription factor GATA-1 (Blobel *et al* 1995). Repression in both of these examples is thought to occur by direct protein-protein interactions. Direct interactions between NF-κb and ERα, GR and PR

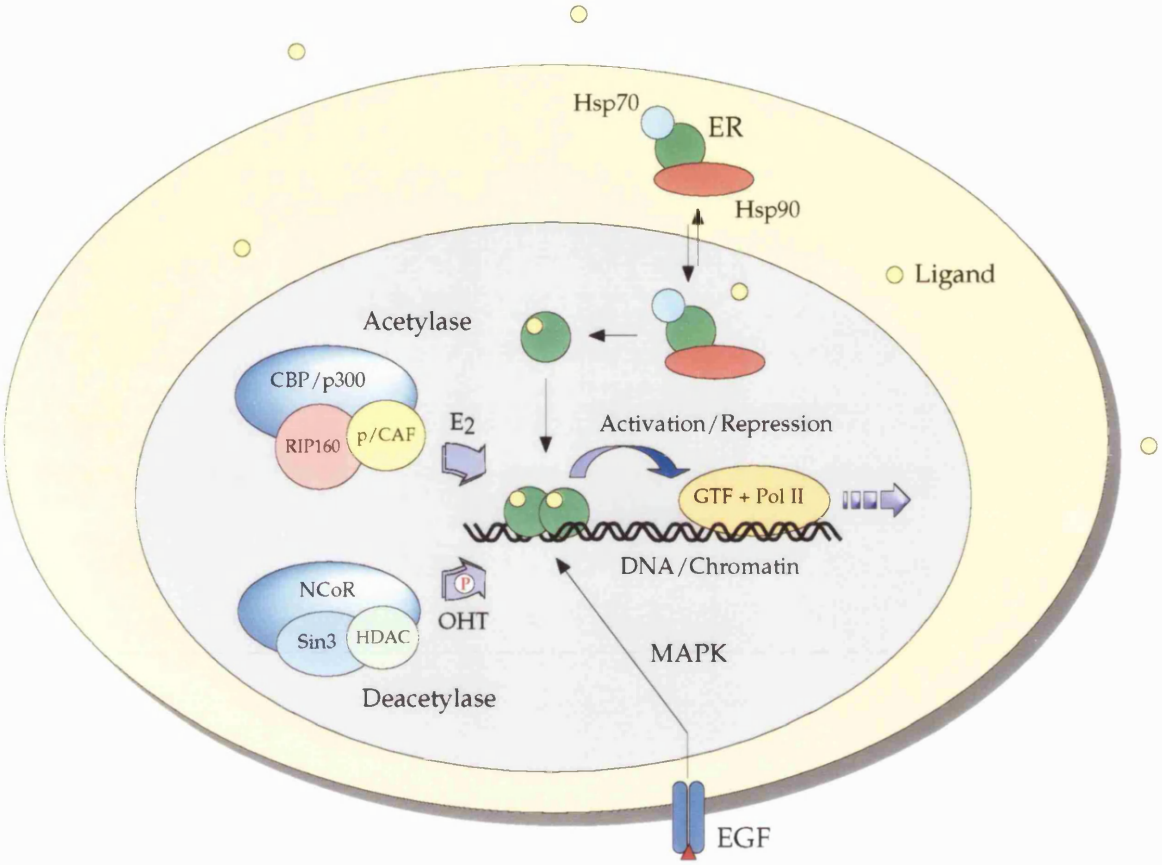


Fig. 1.4 - **Transcriptional regulation by DNA-bound ERα.** A model of our current understanding of the complexes recruited by DNA-bound ERα. In the absence of the ERα is sequestered in an inactive complex with heat shock proteins (Hsp 90, 70). Ligand binding results in a conformational change in the LBD, characterised by the realignment of helix 12. The repositioning of helix 12 results in the replacement of heat shock proteins by a coactivator complex, consisting of one of three SRC1/RIP160 family members, CBP/p300 and possibly p/CAF, all of which contain HAT activity. The model can be extended to include 4OH-tamoxifen bound ERα which is able to recruit a corepressor complex consisting of NCoR/Sin3/HDAC, dependent on signalling via the MAPK pathway. Thus depending on the liganded status of the LBD and the position of helix 12, ERα is able to regulate transcription by the recruitment of distinct complexes with opposing acetyltransferase properties.

have been demonstrated (Kalkhoven *et al* 1996, Ray and Prefontaine 1994, Stein and Yang 1995), suggesting a conserved mechanism of repression, in which there is mutual silencing of transcriptional activation domains. Alternative hypotheses have also been put forward. Repression of NF-κb by glucocorticoids has been proposed to be caused by the increased expression of Ikb (Auphan *et al* 1995). However, this may be a particular property of lymphoid cells, as other cell types show no changes in Iκb expression during GR mediated repression of NF-κb (Heck *et al* 1997).

The non-genomic effects of the GR were demonstrated *in vivo* by Reichardt *et al* (1998), who generated mice ($GR^{dim/dim}$) with a mutation in the GR gene which prevents DNA binding, but leaves the possibility of cross-talk with other transcription factors intact (Reichardt *et al* 1998). The $GR^{dim/dim}$ mice are viable, in contrast to the GR null mice (Cole *et al* 1995). Cells derived from the $GR^{dim/dim}$ mice showed that endogenous GR was incapable of activating transcription from transfected reporter genes, whereas genes such as gelatinase B and collagenase were still repressed by the addition of dexamethasone. The viability of the $GR^{dim/dim}$ mice, despite the loss of physiologically important process such as glucocorticoid-dependent apoptosis in thymocytes and proliferation of erthyroblasts, shows that DNA binding independent mechanisms of GR transcriptional regulation are sufficient for the survival of these mice.

In addition to the well documented negative aspects of nuclear receptor cross-talk there have also been examples of positive regulation by ER and GR. In contrast to other nuclear receptors the ER activates transcription from AP1 sites (Webb *et al* 1995). This activity differs between the two forms of the ER. ERα activates transcription from AP1 sites in the presence of oestradiol but not antioestrogens, whereas ERβ activates transcription only in the presence of antioestrogens (Paech *et al* 1997). The signal transducer and transcriptional activator 5 (Stat5) and GR co-operate in the induction of transcription of the β-caesin gene (Stoecklin *et al* 1997). A Stat5 response element within the β-caesin gene is sufficient for the co-operative action of Stat5 and GR, which occurs in the absence of the GR DNA binding domain. Deletion of the

transcriptional domain of Stat5 still allows co-operativity, as does removal of AF2 from GR, but not AF1. GR is therefore predicted to function as a ligand dependent coactivator of Stat5 stimulating transcription via its AF1 domain, without contacting DNA (Stoecklin *et al* 1997).

The majority of ER in cells either in the presence or absence of oestradiol is in the nucleus, as judged by immunocytochemistry. Nevertheless ERα has been implicated with the function of the membrane-bound tyrosine kinase, c-Src. Treatment of MCF7 cells with oestradiol activates the c-Src substrates Shc and p190 resulting in increased levels of GTP-bound $p21^{ras}$ and subsequent activation of the MAP kinases erk-1 and erk-2, with kinetics similar to those of ligand bound c-Src (Migliaccio *et al* 1996). These effects are dependent on the presence of ligand bound ERα, which forms a complex with c-Src, demonstrating a novel mechanism of oestrogen signalling. One interesting aspect of this novel mechanism is that the ER itself is a target for the MAP kinase pathway (Bunone *et al* 1996, Kato *et al* 1995) creating a possible positive regulatory loop.

Chapter 2

Materials and Methods

MATERIALS

Chemicals and solvents

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

Absolute Alcohol	Hayman Ltd. Witham, UK
Acrylamide	National Diagnostics, U.S.A
Agarose	Gibco BRL, UK
Ammonium persulphate	Bio-Rad
Ampicillin	Beecham Research Laboratories, UK
Amplify	Amersham International plc, UK
Bromophenol blue	Bio-Rad
Coomassie Brilliant Blue R-250	Bio-Rad
DEAE-dextran	Pharmacia Biotech, UK
Dextran-T70	Pharmacia Biotech, UK
ECL western blotting detection reagents	Amersham International plc, UK
Guanidine HCl	Calbiochem, UK

Liquid scintillation fluid (Ultima Gold)	Amersham International plc, UK
Nucleotide triphosphates	Pharmacia Biotech, UK
Poly ([dI-dC])·([dI-dC])	Pharmacia Biotech, UK
TEMED	Bio-Rad
Tween-20	Bio-Rad

Radiochemicals

All radiochemicals were supplied by Amersham International plc, UK.

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

Enzymes

Restriction enzymes were routinely supplied by New England Biolabs, U.S.A.

Calf Intestinal Alkaline Phosphotase (CIP)	Boehringer Mannheim
DNA polymerase I (Klennow fragment)	Boehringer Mannheim

eLONGase	Gibco BRL, UK
Heart Muscle Kinase (catalytic subunit of PKA)	Sigma
RNase A	Boehringer Mannheim
T4 polynucleotidekinase	Pharmacia Biotech, UK

Miscellaneous

Film: ECL	Amersham International
RX	Fuji
X-OMAT	Kodak
Filtration Units	Nalgene
Galacto-light-β-gal kit	TROPIX Inc
Geneclean Spin columns	BIO 101
Gene Pulser cuvettes (0.4 cm)	Bio-Rad
Glutathione Sepharose 4B	Pharmacia Biotech, UK
Hybond N+ nylon membranes	Amersham International plc, UK
kilobase DNA markers	Stratagene
Luminometer cuvettes	Labsystems Group, UK
Nitrocellulose Membranes	Schleicher and Schuell, UK
Peroxidase conjugated	Dakopatts, Denmark

anti-rabbit immunoglobulins	
Oligonucleotides	synthesised by I. Goldsmith, ICRF
Peptides	synthesised by N. O'Reilly, ICRF
Powdered milk	TESCO
Protein A sepharose	Pharmacia Biotech, UK
Maxiprep kit	Qiagen
Miniprep kit	Qiagen
Rainbow protein markers	Amersham International plc, UK
TNT coupled reticulocyte lysate *in vitro* translation kit	Promega
TRIzol Reagent	Gibco BRL, UK

Plasmids

pSG5-HEGO	Dr. P. Chambon Instituit de Génétique et de Biologie Moléculaire et Cellulaire CNRS, Strasbourg, France
pNGV-ERβ	Dr. S. Mosselman Organon Oss, Netherlands
p2ERE-PS2-CAT	Dr. B. Katzenellenbogen University of Illinois, Urbana Illinois, U.S.A

3ERE-TATA-luc	Dr. B. van der Berg, Hubrecht University Utrecht, Netherlands

Antibodies

Monoclonal antibody H226	Dr. C. Nolan Abbot Industries

Buffers

All solutions were made with water that was distilled and deionised. Solutions were stored at room temperature unless otherwise stated.

BBS (2x)	50 mM BES adjusted to pH 6.95 with 1 M NaOH, 280 mM NaCl, 1.5 mM Na_2HPO_4 (filtered sterilised, stored at -20^{o}C)
CIP buffer (10x)	0.5 M Tris-HCl pH 8.5, 1 mM EDTA (stored at 4^{o}C)
Denaturing solution	1.5 M NaCl, 0.5 M NaOH
DCC suspension	0.025% (w/v) Dextran-T70, 0.25% (w/v) charcoal, suspended in TE pH 7.4 (stored at 4^{o}C)
Digestion buffer	100 mM NaCl, 10 mM Tris-HCl pH 8.0 25 mM EDTA, 0.5% (w/v) SDS 0.1 mg/ml proteinase K
DNA loading buffer (5x)	0.25% (w/v) bromomphenol blue, 5x TBE, 25% (w/v) glycerol

Far-western transfer buffer	25 mM Tris base, 192 mM glycine, 0.01% (w/v) SDS
Formaldehyde gel running buffer (10x)	200 mM MOPS, 10 mM EDTA, 50 mM NaAc. Adjust pH to 7.0 with NaOH
Galacto-light reaction dilutent buffer	100 mM sodium phosphate pH 8.0, 1 mM $MgCl_2$ (stored at 4^oC)
Gelshift buffer (2x)	100 mM KCl, 40 mM HEPES pH 7.4, 2 mM β-mercaptoethanol, 40% (w/v) glycerol (stored at 4^oC)
HBB (10x)	250 mM HEPES pH 7.7, 250 mM NaCl 50 mM $MgCl_2$
HMK kinase buffer (10x)	200 mM Tris-HCl pH 7.5, 1 M NaCl 120 mM $MgCl_2$
HMK stop buffer (1x)	10 mM sodium phosphate pH 8.0 10 mM sodium pyrophosphate 10 mM EDTA, 1 mg/ml BSA
Hyb-75	20 mM HEPES pH 7.7, 75 mM KCl 0.1 mM EDTA pH 8.0, 2.5 mM $MgCl_2$ 1% (w/v) powdered milk 0.05% (v/v) NP40
Hybridisation Buffer (Church and Gilbert)	0.2 M sodium phosphate pH 7.2, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS , 15% (v/v) deionised formamide
Kinase Buffer (10x)	0.5 mM Tris-Cl pH 7.4, 100 mM $MgCl_2$, 1mM EDTA pH 8.0 (stored at -20^oC)

Labelling buffer (5x)	1 mM dATP/dGTP/dTTP, 250 mM Tris-HCl pH 7.4, 35 mM $MgCl_2$ (stored at -20^{o}C)
Ligation Buffer (10x)	0.5 M Tris-HCl pH 7.8, 0.1 M $MgCl_2$, 0.1 M DTT, 10 mM ATP, 250 µg/ml BSA (stored at -20^{o}C)
Luciferase reaction buffer	25 mM glycylglycine pH 7.8, 5 mM ATP pH 8.0, 150 mM NaCl (stored at -20^{o}C)
NETN	100 mM NaCl, 1mM EDTA, 20 mM Tris-HCl pH 8.0, 0.5% (v/v) NP40, 1mM DTT, 0.5 mg/ml bacitracin, 40µg/ml PMSF, 5µg/ml pepstatin, 5µg/ml leupeptin (stored without protease inhibitors and DTT)
Neutralising Solution	1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA
PBSA	140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2
Phenol/Chloroform	Redistilled phenol, equilibrated in TE pH 8.0, in a 50:50 mix (v/v) with chloroform (stored at 4^{o}C)
Protein Loading Buffer (2x)	4% (w/v) SDS, 20% (v/v) glycerol, 50 mM Tris-HCl pH 6.8, 0.1% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol
Qiagen buffers :	
P1	50 mM Tris-HCl pH 8.0, 10 mM EDTA 100 µg/ml RNase A (stored at 4^{o}C)

P2	200 mM NaOH, 1% SDS
P3	3.0 M KAc pH 5.5 (stored at 4^{o}C)
QBT	750 mM NaCl, 50 mM MOPS pH 7.0 15% isopropanol, 0.15% Triton X-100
QC	1.0 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol
QF	1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol

Restriction enzyme buffers :
(stored at -20^{o}C)

NEBuffer 1 (yellow)	10 mM Tris Propane-HCl pH 7.0, 10 mM $MgCl_2$, 1 mM DTT
NEBuffer 2 (blue)	10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$ 50 mM NaCl, 1 mM DTT
NEBuffer 3 (red)	50 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT
NEBuffer 4 (green)	20 mM Tris-acetate pH 7.9, 10 mM $MgCl_2$, 50 mM KAc, 1 mM DTT
RNA loading buffer	1x formaldehyde gel running buffer 9% (v/v) formaldehyde, 60% deionised formamide
SDS-PAGE buffer (10x)	250 mM Tris base, 1.9 M Glycine, 1% (w/v) SDS
SSC (20x)	3 M $NaCl_2$, 0.3 M sodium citrate

STET buffer	8% (w/v) sucrose, 0.1% (v/v) Triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0
SM buffer	0.1 M NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl pH 7.5, 2% (w/v) gelatin
Stripping buffer	100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.7
TBE (10x)	0.9 M Tris-Borate, 20 mM EDTA pH 8.0
TE buffer (1x)	10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0
Tfb1	30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2.4H_20$, 15% (v/v) glycerol. Adjust pH to 5.8 with acetic acid and then filter sterilise. (stored at 4^oC)
Tfb2	10 mM MOPS, 75 mM $MgCl_2$, 10 mM RbCl, 15% (v/v) glycerol. Adjust pH to 6.5 with KOH and filter sterilise. (stored at 4^oC)
Transfection lysis buffer	0.65 % (v/v) NP40, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0 150 mM NaCl
Western transfer buffer	25 mM Tris Base , 192 mM glycine 20% (v/v) MeOH at pH 8.3
Whole cell extract buffer	0.4 M KCl, 20 mM HEPES pH 7.4 20% (v/v) glycerol, 1 mM DTT

	0.5 mg/ml bacitracin, 40μg/ml PMSF, 5μg/ml pepstatin, 5μg/ml leupeptin (stored without protease inhibitors and DTT at 4^{o}C)

Bacterial media and agar

All the organic compounds listed below were supplied from Difco Laboratories.

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

Cell culture media

All media was stored at -20^{o}C with the exception of PBSA.

Chick Serum	Sigma
Dulbecco's modified Eagle's medium (DMEM)	ICRF media supplies
Foetal bovine serum	Gibco BRL, UK
PBSA	ICRF media supplies
Trypsin	ICRF media supplies, 0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.01% (w/v) Na_2HPO_4, 0.01% (w/v) streptomycin, 0.25% (w/v) trypsin, 100 U/ml penicillin, phenol red
Versine	ICRF media supplies, 0.02% (w/v) EDTA in PBSA, phenol red

METHODS

Storage of bacteria

The *Escherichia coli* strain DH5α was used for propagation of all plasmids. These bacteria were stored in L-broth containing 50% (v/v) glycerol at -20^{o}C. All plasmids described in this thesis carry the β-lacatamase gene (Amp^r) which confers resistance to ampicillin, transformed bacteria were grown in L-broth containing 50-100 μg/ml ampicillin.

Preparation of competent bacteria

This is the unpublished method of M. Scott and V. Simanis, derived from Hanahan (Hanahan 1983). All glassware was prewashed with ψ broth before use. Bacteria were streaked out on ψ agar plates and incubated at 37°C overnight or until the colonies reached 2 mm in diameter. Single colonies were used to inoculate 5 ml of ψ broth and incubated with vigorous shaking at 37°C until the OD_{550} reached 0.3 (approximately 3 hours). This was then subcultured (1:20) into 100 ml of prewarmed ψ broth and incubated as before until the OD_{550} was 0.48 (approximately 2 hours). The bacteria were then placed on ice for 5 minutes and pelleted by centrifugation at 4000 x g for 5 minutes at 4°C. The cell pellet was resuspended in 40 ml (2/5 original volume) of ice cold Tfb1 and incubated for 5 minutes on ice. The cells were then respun for 4000 x g for 5 minutes at 4°C and then resuspended in 4 ml (1/25 original volume) of ice cold Tfb2 and left on ice for a further 15 minutes. The cell suspension was snap frozen on cardice in aliquots of 0.3 ml and then stored at -70°C.

Transformation of competent bacteria

Competent cells were thawed on ice and typically, 50µl of cells were added to 20µl of prechilled DNA solution (<1 ng DNA /µl of cells). After 30 minutes on ice the cells were heat shocked at 37°C for 90 seconds and then placed on ice for a further 2 minutes. Following the addition of 4 volumes of L-broth the cells were incubated at 37°C for 30-50 minutes. The cells were then spread on L-agar plates containing 50-100 mg/ml ampicillin, inverted and incubated overnight at 37°C. Competent DH5α cells typically gave between 10^6 and 10^8 colonies per µg of supercoiled plasmid DNA.

Preparation of DNA

Small scale preparation of plasmid DNA (miniprep)

Boiling point method

The 'boiling point' miniprep was routinely used ~~for~~ to prepare between 1-5 µg of plasmid DNA for diagnostic restriction enzyme digest and direct double stranded sequencing. Single bacterial colonies were used to innoculate 5 ml of L-broth containing 50-100 µg/ml of ampicillin and incubated overnight at 37^{o}C with vigorous shaking. 1 ml of the overnight culture was spun in a microcentrifuge and the cell pellet resuspended in 350 µl of STET buffer. After addition of 25 µl of 10 µg/ml of lysozyme (in STET buffer) and mixing, the suspension was placed in a boiling water bath for 40 seconds and then spun in a microcentrifuge for 10 minutes. The pellet was removed and the DNA was precipitated by adding 40 µl of 3 M NaAc pH 7.0 and 420 µl of isopropanol, mixing and then incubating at -70^{o}C for 10 minutes. The DNA was pelleted by centrifugation for 10 minutes. After removal of the supernatant and drying the DNA was resuspended in distilled water.

Qiagen miniprep kit

The Qiagen miniprep kit was routinely used to prepare 2-10 µg of high quality plasmid DNA for cloning and automated sequencing. 1 ml of overnight culture (prepared the same as above) was pelleted in a microcentrifuge and then resuspended in 250 µl of buffer P1. 250 µl of buffer P2 was added to the suspension, mixed and then incubated at room temperature for 5 minutes. After the addition of 350 µl of buffer N3 the tubes were spun for 10 minutes and the supernatant added to a miniprep column. The columns were spun for 1 minute to bind the DNA, 750 µl of PE buffer was added and then spun again to wash the column. The DNA was eluted by adding 50 µl distilled water to the column and spinning into a fresh eppendorf tube.

Large scale preparation of plasmid DNA

The Qiagen maxiprep DNA purification system (Qiagen) was used to prepare between 0.1-0.5 mg of plasmid DNA. Plasmid DNA prepared

by this method was used for cloning, transient transfection, *in vitro* transcription/translation and sequencing. Single colonies or 10 µl of bacterial culture were used to inoculate 150 ml of L-broth containing 50-100 µg/ml of ampicillin and incubated overnight at 37°C with vigorous shaking. The bacteria were harvested in 500 ml sorval bottles by centrifugation at 6000 x g for 5 minutes at 4°C. The bacterial pellet was resuspended in 10 ml buffer P1, then 10 ml buffer P2 was added with thorough mixing and incubated for 5 minutes. After addition of 10 ml of buffer P3 the suspension was spun at 6000 x g for 30 minutes at 4°C. Whilst the suspension was spinning 10 ml of QBT buffer was used to equilibrate a Qiagen-tip 500. The supernatant was then carefully filtered through medical gauze into the Qiagen-tip 500 and washed twice with 30 ml of buffer QC. The DNA was eluted by addition of 15 ml of buffer QF and then precipitated by adding 10.5 ml of isopropanol. The DNA was pelleted by centrifugation at 20000 x g at 4°C for 30 minutes. The pellet was washed with 5 ml of 70% ethanol and then centrifuged again at 20000 x g for 15 minutes. After removing the supernatant and drying the DNA pellet was resuspended in distilled water (0.2-0.5 ml). The DNA concentration was determined by measurement of the OD_{260} and OD_{280} (Sambrook *et al* 1989). The DNA was run on an agarose gel and typically found to be 80% supercoiled.

DNA manipulation and subcloning

Restriction endonuclease digestion

Restriction enzyme digests were performed at 37°C in unique, low, medium or high salt buffers as recommended by the supplier. DNA was digested with a 3-5 fold excess of enzyme in a volume not exceeding 10% (v/v) of the total. For analysis of digestion products by gel electrophoresis the digestion was stopped by the addition of DNA loading buffer to 25% (v/v). Alternatively digestions were stopped by extraction with an equal volume of phenol/chloroform and the aqueous phase transferred to a fresh tube. The DNA was precipitated by addition of 1/50 volume of 5 M NaCl and 2.5 volumes of ethanol. After incubation at -70°C for 10 minutes the tube was spun for 10 minutes in a

microcentrifuge, washed with ice cold 70% ethanol and then spun for a further 5 minutes. After removal of the supernatant and drying, the pellet was resuspended in distilled water.

Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose 0.8-2% (w/v) in 1x TBE by heating the suspension in a microwave oven. The solution was allowed to cool and then ethidium bromide was added to 1 µg/ml before casting in a gel mould. Once set the gel was submerged in 1 x TBE buffer in a gel tank and the DNA samples, containing 25% (v/v) DNA loading buffer, loaded into the wells. Electrophoresis was performed typically with a potential difference of 100 V until the DNA fragments were well resolved. The DNA was visualised by illumination with a long wave UV light box and photographed. The size of the DNA fragments was determined by comparing their mobility relative to that of restriction fragments of known size, typically 1kb markers (Stratagene).

Purification of restriction fragments

Restriction fragments were purified from agarose gels using Geneclean Spin columns (BIO 101). The fragment of interest was cut from the gel using a scalpel and placed into an eppendorf tube. The gel slice was dissolved by adding 400µl of SPIN GLASSMILK and incubating at 55^{o}C for 5 minutes. The solution was then added to a SPIN FILTER and spun in a microcentrifuge for 1 minute and the flow through discarded. The SPIN FILTER was washed by adding 500 µl of SPIN NEW WASH and spinning for 1 minute, the flow through was discarded and then spun once more to remove the last trace of SPIN NEW WASH. The SPIN FILTER was transferred to a clean eppendorf tube and the DNA eluted by adding 25 µl of distilled water and spinning for 1 minute. This was repeated once more to give a final eluted volume of 50 µl.

Preparation of vectors

Routinely 1-2 µg of plasmid DNA was digested with the appropriate restriction enzyme(s) and then extracted with phenol/chloroform, ethanol precipitated, washed with 70% ethanol and resuspended in 43 µl distilled water. After addition of 5 µl of 10x CIP buffer and 1 µl (20U) of CIP the solution was incubated at 37°C for 30-60 minutes. CIP removes the 5' terminal phosphates from the cut end of the vector so that self ligation is prevented. After incubation the DNA was re-extracted with phenol/chloroform, ethanol precipitated, washed with 70% ethanol and resuspended in distilled water at a final concentration of 10-50 ng/µl.

Oligonucleotide kinasing and annealing

Oligonucleotides were synthesised by I Goldsmith (ICRF) with hydroxyl groups at both 5' and 3' termini. In order to ensure efficient ligation the 5' ends were kinased prior to annealing. 100 ng of oligonucleotide was incubated with 1x ligase buffer and 1 µl (approximately 10U) of T4 polynucleotide kinase in a final volume of 20 µl at 37°C for 30 minutes. After kinasing complementary oligonucleotides were annealed by mixing together and heating to 90°C for 5 minutes and then allowed to cool slowly to room temperature.

Ligations

Ligations were performed with 10-50 ng of vector DNA and a 1:1 and 1:5 molar ratio of vector to insert DNA (either a purified DNA fragment, or a pair of annealed oligonucleotides). All ligation reactions were performed in a final volume of 20 µl which contained 2 µl of 10x ligase buffer and 1 µl T4 DNA ligase (1 unit), these were then incubated at either room temperature for >2 hours or overnight at 16°C.

Polymerase chain reaction

Polymerase chain reactions (PCR) were performed using eLONGase DNA polymerase (Gibco BRL) in 100 µl reactions that consisted of 1x eLONGase buffer (buffer A and buffer B in a 1:1 ratio), 0.25 mM dNTP's, 100 ng of each primer, 1-100 ng template DNA and 1 µl of eLONGase. Mineral oil was used to cover the reaction mixture to prevent evaporation during thermo-cycling. PCR reactions typically consisted of 30 thermo-cycles at the following temperatures: 60 seconds at 94^{o}C, 60 seconds at the appropriate annealing temperature (5^{o}C below Tm of the primers) and 2 minutes at 68^{o}C. The last cycle was followed by 5 minutes at 68^{o}C. A portion of the final product (typically 10%) was analysed by gel electrophoresis, the remainder was extracted with phenol/chloroform, precipitated with ethanol, washed, dried and resuspended in distilled water.

DNA sequencing

Maxi- or mini- preparations of DNA were sequenced by a linear PCR method using dye-labelled terminators from Perkin Elmer. The sequencing reactions consisted of 0.3-0.5 µg of plasmid DNA, 8 µl of Terminator Ready Mix (containing the Taq polymerase and the dye-labelled terminators) and 30 ng of primer made up to 20 µl with distilled water. PCR reactions typically consisted of 25 thermo-cycles at the following temperatures: 30 seconds at 94^{o}C, 15 seconds at the 50^{o}C and 4 minutes at 60^{o}C. Following the PCR, to remove the excess dye-terminators, the DNA was precipitated by adding 2 µl of 3 M NaAc pH 5.2 and 50 µl of 95% ethanol followed by incubation on ice for 15 minutes. The DNA was pelleted by centrifugation for 20 minutes and then air-dried. The sequence reactions were run-out on a polyacrylamide gel by G. Clark, ICRF.

Genomic DNA preparation

One T175 flask of the human breast cancer cell line, ZR-75 or chicken embryo fibroblasts were grown in cell culture to approximately 80% confluence. The cells were trypsinised and pelleted by centrifugation at 500 x g for 5 minutes. The cell pellet was washed twice by resuspending in ice cold PBSA and then centrifuging at 500 x g for 5

minutes. The cells were then resuspended in 0.5 ml of digestion buffer and incubated at 50^{o}C with shaking for 12-18 hours. The solution was mixed with an equal volume of phenol/chloroform and then centrifuged for 10 minutes at 1700 x g in a swing bucket rotor. The aqueous layer was retained and the DNA precipitated by adding 1/2 volume of 7.5 M NH_4Ac and 2 volumes of ethanol. The DNA was pelleted by centrifugation at 1700 x g for 2 minutes, washed with 70% ethanol, dried and then resuspended in distilled water.

Southern Blotting

Aliquots containing 10 µg of genomic DNA were digested overnight at 37^{o}C with the appropriate restriction enzymes. The digested DNA was then run-out on a 1% agarose gel for approximately 4 hours at 100 V. The resolved DNA was transferred for 1 hour onto a hybond-N+ membrane using a vacuum blotting apparatus (Pharmacia). The DNA was cross linked to the membrane by transferring in 0.2 M NaOH. The membrane was prehybridised for at least an hour at 60^{o}C with gentle shaking. Radiolabelled probe generated by random priming was then added (10^5 cpm/ml) and the incubation continued overnight. Following hybridisation the membrane was washed with 2x SSC at 60^{o}C for 3 periods of 15 minutes, placed in saran wrap and autoradiographed.

RNA preparation

Approximately 0.5 g of chicken tissues were homogenised with 5 ml of TRIzol Reagent and then incubated at room temperature of 15 minutes. After the addition of 1 ml chloroform and mixing the samples were centrifuged at 12,000 x g for 15 minutes at 4^{o}C. The aqueous layer was retained and the RNA precipitated by the addition 2.5 ml of isopropanol. The RNA was then pelleted by centrifugation at 10,000 x g for 10 minutes at 4^{o}C. The pellet was washed with 70% ethanol, centrifuged at 7,500 x g , dried and then resuspended in distilled water.

Northern blotting

Samples containing 10 µg of total RNA were run on a 1.5% agarose gel containing 1x northern running buffer and 7% formaldehyde for 4 hours at 120 V. The resolved RNA was transferred for 1 hour on to a hybond-N+ membrane using a vacuum blotting apparatus (Pharmacia). Following transfer the membrane was placed on 3MM paper soaked in 0.4 M NaOH for 5 minutes to cross link the RNA to the membrane. The membrane was prehybridised for at least an hour at 60^{o}C with gentle shaking. Radiolabelled probe generated by random priming was then added (10^5 cpm/ml) and the incubation continued overnight. Following hybridisation the membrane was incubated with wash buffer at 60^{o}C for 3 periods of 15 minutes, placed in saran wrap and autoradiographed.

In Vitro protein analysis

In Vitro protein synthesis

A coupled rabbit reticulocyte lysate system was used to synthesise proteins *in vitro.* A typical reaction contained 25 µl of TNT lysate, 2 µl of TNT buffer, 1 µl amino acid mix lacking methionine, 1 µl of RNasin, 2 µg of plasmid DNA and 1µl of TNT RNA polymerase (T7 or SP6), made up to 50 µl with distilled water. 3 µl of [^{35}S]-methionine was added to the mix if the proteins were to be radiolabelled, 1 µl of amino acid mix lacking leucine was added instead if the proteins were to be unlabelled. The translation reactions were incubated at 30^{o}C for 1 hour, glycerol added to 10% (v/v) and stored at -70^{o}C. Radiolabelled translations were used for pulldown and immunoprecipitation assays, unlabelled translations were used for gelshift assays.

SDS polyacrylamide gel electrophoresis

Proteins were analysed on discontinuous polyacrylamide gels using the ATTO Corporation AE-6220 dual slab chamber. Gels were prepared from two solutions to form the stacking and resolving gels respectively. The resolving gel routinely contained 10% acrylamide (30%

acrylamide, 0.8% bis-acrylamide stock), 375 mM Tris-HCl pH 8.8 and 1% SDS in a final volume of 20 ml. The stacking gel contained 4% acrylamide, 125 mM Tris-HCl pH 6.8 and 1% SDS in a final volume of 10 ml. Polymerisation of the resolving gel was initiated by the addition of 12 µl of TEMED and 200 µl of ammonium persulphate. The solution was then poured between the plates to within 5 cm of the top and overlaid with distilled water. Once polymerisation was complete (approximately 20 minutes) the distilled water was poured off. Polymerisation of the stacking gel was initiated by the addition of 10 µl of TEMED and 100 µl of ammonium persulphate, the solution was then poured on top of the resolving gel and a comb inserted. Once the stacking gel had set (approximately 15 minutes) the comb was removed and the wells were rinsed with distilled water. Protein samples containing 50% protein loading buffer were first boiled for 2-3 minutes before loading into the 6 mm wells. RAINBOW markers (Amersham) were added to a well adjacent to the samples to estimate the size of the proteins resolved. The gel was run in 1x SDS-PAGE buffer at 250 V for
90 minutes.

Determination of protein concentration

A dye binding assay first described by Bradford (Bradford 1976) was used to determine the protein concentration of cell extracts. The dye concentrate, supplied by Bio-Rad was used according to the manufacturers instructions. Typically, 2-5 µl of cell extract was added to 1 ml of Bradford reagent diluted 1:5 with distilled water. After mixing the samples were transferred to 1 ml polystyrene cuvettes and their OD_{595} measured using a spectrophotometer. The protein concentrations were determined by extrapolation from a standard curve made using BSA samples of known concentration. In this assay BSA binds twice as much dye compared to other proteins and this was taken into account when calculating the concentration of protein extracts.

Purification of GST fusion proteins

The fusion proteins GST-AF2α, GST-AF2β and GST-SRC1(570-780) were expressed and purified from *Escherichia coli.* Bacteria transformed with the aforementioned GST fusion proteins were grown overnight at 37^{o}C in L-broth/ampicillin with vigorous shaking. The culture was diluted 1:10 in 100 ml of fresh media and grown until the OD_{600} reached 0.7-0.8. IPTG was then added to a final concentration of 0.1 mM and the culture grown for a further 3 hours with shaking. The cells were harvested by centrifugation in 50 ml falcon tubes at 5,000 rpm for 5 minutes. The pellets were resuspended in 1/10 volume of ice cold NETN (containing protease inhibitors) and the cells lysed by sonicating for three periods of ten seconds at 4^{o}C using a Soniprep 150 Ultrasonic Disintegrator with a 3 mm probe. The sonicates were then centrifuged at 10,000 x g for 5 minutes. The supernatant was incubated with Glutathione sepharose beads (equilibrated in NETN) for 1 hour at 4^{o}C with constant mixing. The beads were then washed three times with NETN to remove non specific proteins. The GST fusion protein was eluted by incubation with 1 ml of 20 mM glutathione, 100 mM Tris-HCl pH 8.0 and 120 mM NaCl for 30 minutes at 4^{o}C with constant mixing. The beads were removed by centrifugation, the supernatant carefully removed and made to 10% (v/v) glycerol and stored at -70^{o}C.

Pull Down assay

GST fusion proteins were expressed and purified as described above and then left attached to the Glutathione sepharose. The beads were resuspended in 1 ml of NETN and then incubated with 5-10 µl of *in vitro* synthesised protein with or without ligand overnight at 4^{o}C with constant mixing. The beads were washed three times with 1 ml of ice cold NETN to remove unbound proteins, dried under vacuum in a speedivac, then resuspended in 30 µl of 2x protein loading buffer. The sample was boiled for 3 minutes and analysed by SDS-PAGE.

Immunoprecipitation

Equal amounts of [^{35}S]-methionine labelled receptors and 1 µl of immune sera were diluted in 200 µl of 1x gelshift buffer in an eppendorf

tube and incubated for 2 hours at 4^{o}C. 50 µl of Protein A sepharose (equilibrated in 1x gelshift buffer) was added to each sample and incubated for 30 minutes at 4^{o}C with gentle agitation, to precipitate the immunocomplexes. The mix was spun for 5 minutes in a microcentrifuge at 4^{o}C and the supernatant removed by aspiration. The beads were washed three times with 500 µl of 1x gelshift buffer and then resuspended in 30 µl of 2x protein loading buffer. The samples were boiled for 3 minutes and analysed by SDS-PAGE.

Gel shift assay

Aliquots of 1-5 µl of *in vitro* translated receptor or 2-4 µg of whole cell extract was preincubated with or without antisera, purified GST-SRC(570-780), or oestradiol in 1x gel shift buffer, 0.5% BSA and 1 µg of poly ([dI-dC])-([dI-dC]) for 5 minutes at room temperature. 1 ng of the appropriate [^{32}P]-labelled oligonucleotide probe was added and the reactions incubated for a further 25 minutes. The samples were run on a 6% polyacrylamide (30% acrylamide, 0.8 bis-acrylamide stock) 0.5x TBE non denaturing gel, which had been pre-run for 30 minutes at 100 V. The protein DNA complexes were loaded on the gel and run in 0.5x TBE at 220 V for 60-90 minutes. Gels were fixed for 15 minutes (10% acetic acid and 30% methanol), dried under vacuum at 80^{o}C for 40 minutes and visualised by autoradiography.

Probes were prepared by annealing complementary pairs of oligonucleotides to form the binding site and 5' overhanging end. The oligonucleotides were labelled by filling in the overhanging ends in the presence of α–[^{32}P]-dCTP. Typically, 500 ng of annealed oligonucleotide was incubated in a final volume of 50 µl containing 1x labelling buffer, 5 µl α-[^{32}P]-dCTP and 2 µl klennow enzyme at 37^{o}C for 1 hour. The probe was purified by elution through a G-50 Sepharose column with TE buffer.

Far-Western blotting

Protein samples were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane using the wet blotting method (Sambrook *et al* 1989), except that far-western transfer buffer was used instead of methanol. The gel was placed in a 'sandwich' of a fibrous pad, two pieces of Whatman 3MM paper, the gel, a piece of nitrocellulose, two pieces of Whatman 3MM paper and another pad. To remove air bubbles the 'sandwich' was constructed submerged in transfer buffer and each layer above the gel rolled with a glass pipette. The 'sandwich' was placed in a basket and placed in far-western transfer buffer in a blotting tank (Bio-Rad) with the nitrocellulose towards the anode and the gel towards the cathode. The transfer was performed at 30 V overnight or 100 V for 1 hour at $4^{o}C$.

The membrane was then incubated in a blocking solution of 1x HBB, 5% (w/v) powdered milk, 1 mM DTT and 0.05% (v/v) NP40 with gentle shaking for 1 hour at $4^{o}C$. The proteins on the membrane were denatured by incubating in 100 ml of 1x HBB, 6 M GdHCl, 1 mM DTT for 10 minutes with gentle rocking. This was repeated with the denaturing solution diluted 1:2 by adding 1 volume of 1x HBB and then incubated for a further 10 minutes. This was repeated four more times giving a serial dilution of GdHCl of 3 M, 1.5 M, 0.75 M, 0.375 M, 0.187 M respectively; slowly renaturing the proteins on the membrane. The membrane was incubated in blocking solution of 1x HBB, 5% (w/v) powdered milk, 1 mM DTT and 0.05% NP40 with gentle agitation for >1 hour at $4^{o}C$ before the addition of the probe.

The probe, GST-AF2α, was expressed in bacteria and purified (as described above for purification of GST fusion proteins). The purified GST-AF2α was radiolabelled with ^{32}P by phosphorylation at a consensus PKA site. The kinasing reaction was performed in a total volume of 30 µl, consisting of 3 µl 10x HMK buffer, 2 µl (20 µCi) [^{32}P]-γ ATP and 1 µl HMK for 30 minutes at $4^{o}C$. The reaction was stopped by adding 1 ml of HMK stop buffer. The labelled GST-AF2α was washed 5 times with NETN and then eluted from the GST beads by adding 20 mM glutathione.

The membrane was incubated overnight with the probe in 50 ml of Hyb (75) buffer containing 1% (w/v) powdered milk, 10^{-6} M oestradiol

and 2 ml of cleared sonicate from bacteria expressing GST. The membrane was removed, washed three times for periods of 10 minutes with 200 ml of Hyb (75) and 1% (w/v) powdered milk, placed in saran wrap and then autoradiographed.

Far-western screening of a cDNA library

The cDNA library used was a λgt11 chicken embryo library (oligo (dT) primed) obtained from B. Venstrom (Karolinska Institute, Sweden). The Y1090 host cells used were prepared as follows. A colony from a fresh (1-2 day) L-agar/tetracycline plate was used to inoculate 50 ml of L-broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$. The culture was grown for 6 hours at 37^{o}C with vigorous shaking. The cells were harvested by centrifugation at 3,000 rpm for 5 minutes, then resuspended in 0.5 volume of 10 mM $MgSO_4$ and stored at 4^{o}C. For infection the cells were diluted with 10 mM $MgSO_4$ to an OD_{600} of 0.5 and incubated with phage at 37^{o}C for 15 minutes. To titer the library, 200 µl Y1090 cells were infected with serial dilutions of the library phage, added to 3 ml of top agar and plated out on 9 cm L-agar plates. Once the titer had been ascertained the library was plated out with 2 ml host cells and 30 ml top agar/plate on 22 cm x 22 cm L-agar plates to approximately 250,000 plaques/plate, Approximately $2x10^6$ plaques were screened in total. The plates were incubated at 37^{o}C for 4 hours until the plaques were just visible.

An ECL nitrocellulose filter was soaked in 0.1 mM IPTG for 5 minutes, dried and then placed carefully on the surface of the plate. A sterile needle was used to orientate the filter. The plate and the filter were incubated at 37^{o}C for a further 4 hours. The IPTG induces the expression of the fusion proteins contained within the phage. The first filter was carefully removed, washed in 1x HBB to remove bacterial debris and then placed in block (5% (w/v) powdered milk, 1x HBB, 1 mM DTT) and incubated at 4^{o}C overnight with gentle agitation. A second duplicate filter (similarly soaked in 0.1 mM IPTG) was placed on the plate and incubated at 4^{o}C overnight. The second filter was removed, washed in 1x HBB and then placed in block with the first filter, for an hour. The filters were then denatured/renatured, probed, washed and

autoradiographed (as described above for Far-western blotting). Plaques of interest (those that gave signals on both duplicate filters) were identified, plugged from the agar plate and eluted into 1ml of SM buffer containing 100 µl chloroform. The eluted phage were rescreened, as before except on a smaller scale. Typically, 200 µl Y1090 cells were incubated with 1-10 µl of phage (diluted 1:100 with SM buffer) added to 3 ml of top agar and plated out on 9 cm L-agar plates. Phage from plaques that made it through secondary and tertiary screens were isolated and the cDNA insert amplified by PCR. The PCR product was digested with EcoR1 endonuclease and then subcloned into the pBluescript plasmid (Stratagene) for analysis and DNA sequencing.

Cell Culture

Maintenance of cell stocks

Cells were grown as a monolayer on 175 cm^2 tissue culture flasks (Nunclon) at 37^oC in a humidified atmosphere maintained at 10% (v/v) CO_2. Cells were maintained in DMEM with 10% (v/v) foetal bovine serum (FBS). Chicken embryo fibroblasts (CEF) cells (primary cell cultures provided by ICRF Central Services, Cell Production) were further supplemented with 1% chick serum. Cell cultures were subcultured once or twice a week depending on their growth rate. The media was removed and the cells were washed twice with 25 ml of PBSA and then incubated for approximately 5 minutes with 8 ml of prewarmed trypsin/versine mix (1:8). The flask was gently agitated until all the cells were detached, 8 ml of serum containing culture media was then added to neutralise the trypsin. The 16 ml of cell suspension was transferred to a sterile universal bottle and then spun at 1,200 rpm in an MSE bench top centrifuge for 5 minutes. The pellet was gently resuspended in fresh growth media and subcultured at a suitable dilution.

Storage of cell stocks

Subconfluent monolayer cultures were trypsinised and pelleted as above. The cell pellet was then resuspended in 4.5 ml of DMEM containing 10% FBS and 0.5 ml DMSO. 1 ml aliquots were wrapped in tissue and frozen at -20^{o}C for 2 hours then placed at -70^{o}C overnight before being transferred to liquid nitrogen for long term storage.

Charcoal treatment of serum

Foetal bovine serum contains endogenous steroids hormones that might mask the effect of exogenously added ligand in transfections experiments. Serum used for transfections was therefore pre-treated with dextran coated charcoal (DCC) which removes all small molecules from the serum, including steroid hormones. A dextran coated charcoal suspension consisting of 5 g of activated charcoal, 0.5 g dextran T70 and 5 ml Tris-HCl pH 7.4 made up to 500 ml with distilled water was divided equally among four 250 ml disposable centrifuge bottles and spun at 2,000 x g for 5 minutes at 4^{o}C. The supernatant was removed and the pellet was resuspended with 250 ml of foetal bovine serum. The suspension was shaken vigorously at 55^{o}C for 30 minutes and then respun. The serum was decanted into bottles containing a fresh dextran coated pellet, re-incubated and respun as above. The serum was then filtered sterilised twice using 0.45 µm and 0.22 µm Nalgene filter units respectively, aliquoted and stored at -20^{o}C.

Transient transfection

Calcium phosphate precipitation

HeLa and COS-1 cells were routinely transfected by a calcium phosphate / DNA coprecipitation method modified from the method of Chen and Okayama (1987). Cells were plated at 40-60% confluency in 24-well micotitre plates (Falcon) by diluting the required number of cells in DMEM containing 5% (v/v) DCC treated FBS and adding the appropriate volume to each well. The cell media was replaced 24 hours after plating. The DNA to be transfected (1.5 µg of supercoiled DNA per well) was mixed with a solution containing 1x BBS and 125 mM $CaCl_2$,

then incubated at room temperature for 15 minutes. The precipitate was mixed again and then 100 µl was added dropwise to the well. Typically 2-4 wells were treated with the same precipitate to generate duplicate data points. After incubation for 12-16 hours at 37°C, 5% CO_2 the media was removed and the cells were washed twice with 1 ml of DMEM containing 2% DCC treated FBS to remove the residual precipitate. The cells were then refed with fresh DMEM containing 5% DCC treated FBS with or without the appropriate ligand and incubated at 37°C, 10% CO_2. The cells were routinely harvested 22-28 hours after the addition of ligand.

Electroporation

To overexpress proteins in mammalian cells for analytical purposes, COS-1 cells were transfected with SV40 based expression vectors by electroporation. The cells were grown to approximately 75% confluency in 175 cm^2 flasks, trypsinised (as described above for maintenance of cell stocks), recovered by centrifugation and the cell pellet resuspended in 1 ml of PBSA/175 cm^2 flask (~10^6 cells /ml). 0.95 ml of the cell suspension was added to 10 µg of plasmid DNA in a 0.4 cm electroporation cuvette (Bio-Rad), mixed and incubated on ice for 10 minutes. The cells were then electroporated using a Bio-Rad gene pulser, at 450 V and 250 µF giving a time constant of 4.6-5.2 mS. The cuvettes were then placed back on ice for a further 10 minutes. The cell suspension was mixed with 20 ml of DMEM containing 10% FBS and plated down in 15 cm^2 tissue culture plates (Falcon) and incubated at 37°C and 10% CO_2. After 12-18 hours the growth media was replaced, to remove dead cells killed by the electroporation. The cells were then harvested 48 hours after transfection.

Harvesting transiently transfected monolayers

The method used for harvesting cells is based on that of S. Goodbourn and P. King (Gene Expression Laboratory, ICRF). The growth media was removed and the cells were washed twice with 1 ml of PBSA. 50 µl of lysis buffer was then added to each well and incubated until only

the nuclei were visible under the microscope (approximately 2 minutes). The lysate was transferred to a pre-chilled eppendorf tube and then spun in a microcentrifuge for 1 minute to pellet the cell debris. The supernatant was then used for luciferase, CAT and β-galactosidase assays. Due to the short half life of luciferase in cell extracts its activity was assayed as soon as possible after harvesting. Both CAT and β-galactosidase are stable in cell extracts for weeks when stored at -20^{o}C.

Luciferase activity assay

The relative amount of luciferase activity in cell extracts was determined using an assay based that of de Wet *et al*, (1987). 20 µl of cell extract was added to 0.35 ml to luciferase reaction buffer in a luminometer cuvette. The samples were then loaded into an LKB luminometer, 33 µl of 3 mM luciferin was injected, mixed and the peak light emission from the sample recorded. The assay was linear up to 4000 units (personal observations) and extracts that gave higher values were diluted in lysis buffer and reassayed as soon as possible. Control extracts from untransfected cells gave peak activities of 0.5-0.8 units, whereas transfected cells typically gave peak activities of 20-1000 units. The β-galactosidase activity of each sample was used to correct for transfection efficiency.

CAT activity assay

The extracts to be assayed were heated to 65^{o}C for 5 minutes to denature endogenous deacetylases. The method used is based on that of Sleigh *et al* (1986) and measures the transfer of the 1-[^{14}C]-acetyl group from 1-[^{14}C]-acetyl coenzyme A to chloramphenicol. Chloramphenicol and its acetylated derivatives are soluble in organic solvents whereas acetyl coenzyme A is not. The acetylated chloramphenicol products therefore can be separated from the labelled substrate with ethyl acetate, allowing the direct quantitation of CAT activity without the use of chromatographic separation techniques. Typically, 10 µl of extract was added to 90 µl of mix that contained, 20 µl 8 mM chloramphenicol, 7.5 µl 1 M Tris-HCl pH 7.8, 10 µl of lysis buffer and 20 µl of coenzyme A mix

(0.1 mCi 1-[^{14}C]-acetyl coenzyme A in 0.5 mM unlabelled acetyl coenzyme A). This mixture was incubated for 1 hour at 37^{o}C and then stopped by adding 200 µl of ice cold ethyl acetate. The solution was mixed thoroughly and then spun in a microcentrifuge for 2 minutes. 150 µl of the organic phase containing the labelled chloramphenicol was transferred to a scintillation vial containing 5 ml of scintillation fluid (Ultima Gold). The vials were loaded into a scintillation counter and the number of counts per minute recorded for each sample. The assay was linear up to 100,000 cpm (personal observation) and extracts that gave higher values were diluted in lysis buffer and reassayed. Control extracts from untransfected cells were assayed and gave background values of 150-300 cpm, the mean background value was subtracted from the counts obtained for the transfected cell extracts prior to correction for transfection efficiency with β-galactosidase activity.

β-galactosidase activity assay

β-galactosidase activity was measured using a Galacto-light kit, supplied by TROPIX Inc. The Galacton substrate was diluted 1:100 with Galacto-light reaction buffer dilutent to make the reaction buffer. Typically, 5 µl of extract was added to 100 µl of reaction buffer in a luminometer cuvette and incubated at room temperature for 1 hour. After the addition of 150 µl of Accelerator II the samples were incubated for 15 minutes before the samples were loaded into an LKB luminometer and the light emission of each sample recorded. The assay was linear up to 5000 units (personal observations) and extracts that gave higher values were diluted in lysis buffer and reassayed. Control extracts from untransfected cells gave background values of 1-3 units, whereas transfected cells gave 20-2000 units. The β-galactosidase activity of transfected cell extracts was used to correct the luciferase and CAT activity values for transfection efficiency.

Preparation of whole cell extracts

Cells on 15 cm^2 plates were washed three times with ice cold PBSA and harvested with a rubber policeman in 10 ml of ice cold PBSA.

After centrifugation at 1,200 rpm for 5 minutes the cell pellet was snap frozen at -70^{o}C. Cell pellets were thawed on ice into approximately 5 volumes of whole cell extract buffer and passed through a 21 gauge needle at least six times. Insoluble material was removed by centrifugation at 48,000 x g for 20 minutes at 4^{o}C. Aliquots of the supernatant were stored at -70^{o}C.

Chapter 3

The identification of nuclear receptor coactivators in chicken

Introduction

When the ligand binding domain of the oestrogen receptor (ER) was first used to identify receptor interacting proteins (RIP) in far-western blotting experiments, two predominant bands of 160 and 140 kDa were observed (Cavaillès *et al* 1994, Halachmi *et al* 1994). Among the first RIPs cloned was RIP140, a protein with a molecular weight of 140 kDa, corresponding to the lower of the two bands seen in far-western blots (Cavaillès *et al* 1995). RIP140 was a novel protein with no significant homology to other proteins and no recognisable functional domains. In addition, although RIP140 interacted with nuclear receptors in a ligand dependent manner, consistent with its putative role as a coactivator, it did not stimulate the transcriptional activity of nuclear receptors. In the absence of sequence homology and a functional role in transactivation, it was difficult to make an informed decision on where to begin a detailed analysis of such a large protein. We therefore decided to isolate a RIP140 clone from a different species, namely, chicken. In theory, regions that were well conserved between the human and chicken RIP140 might have some functional importance, which could then be studied in greater detail, whilst excluding other non-conserved regions.

The work described in this chapter was begun in May 1995, before the cloning of the SRC1/RIP160 family of nuclear receptor coactivators. The order of experiments is chronological and hopefully placed in context of what was known about nuclear receptor coactivators at the time they were performed.

Expression of RIPs in different cell lines

To establish the existence of RIPs in chicken cells, we used the ligand binding domain of ERα fused to GST (GST-AF2α) to screen several cell lines, including chicken embryo fibroblasts (CEF) in a far-western blot analysis (Fig. 3.1). The far-western blot revealed two bands of approximately 140 and 160 kDa, whose interaction with GST-AF2α was ligand dependent, since no bands were detected in the absence of

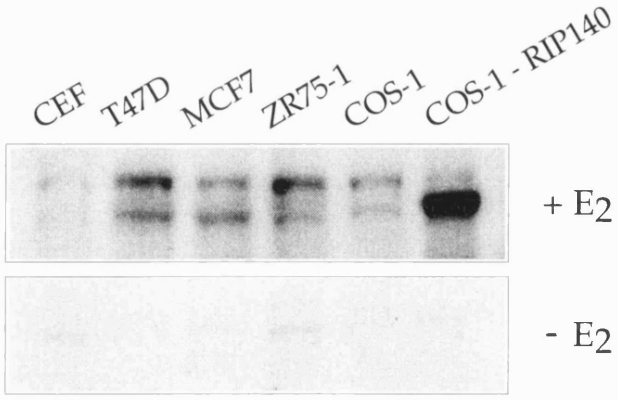


Fig. 3.1 - **Far-western blot analysis of cell lines.** Whole cell extracts of chicken embryo fibroblasts (CEF), T47D, MCF7, ZR75-1, COS-1 (50 µg of protein) and COS-1 cells overexpressing RIP140 (20 µg of protein) were separated by 10% SDS-PAGE and transferred onto nitrocellulose filters. After denaturation-renaturation, filters were probed with [^{32}P]-labelled GST-AF2α in the presence and absence of E2 (10^{-6} M), washed and exposed for autoradiography. COS-1 cells overexpressing RIP140, show the relative positions of RIP140 and RIP160 proteins.

oestradiol (Fig. 3.1, compare blots with and without oestradiol). COS-1 cell extracts overexpressing RIP140 were used as a positive control and show the relative position of RIP140 and RIP160 proteins. The same size bands were detected in all the cell types used (although these bands were somewhat weaker in CEF cell extracts), suggesting that RIP140 and RIP160 proteins are ubiquitously expressed.

Since the proteins detected were of 140 kDa or greater, we decided to use lower percentage polyacrylamide gels than were regularly employed, to improve the transfer of high molecular weight proteins from the gel to the nitrocellulose filter. When we did this, far-western blotting with GST-AF2α revealed three bands, corresponding to RIP140, RIP160 and a RIP of approximately 220 kDa in CEF cells, which had previously been undetected (Fig. 3.2). The RIP220 did not appear be in any of the other cell lines tested. COS-1 cells overexpressing RIP140 shows the relative positions of the three bands. There is a low amount of RIP140 detected in this experiment although generally it is easily detectable (See Fig. 3.1). Thus CEF cells have been demonstrated to contain RIPs of 140, 160 and 220 kDa.

Identification of a RIP from a CEF cDNA library

To isolate a chicken RIP140 orthologue we decided to use the same far-western screening approach employed by Cavaillès *et al* (1995) to clone human RIP140. Using the far-western blotting conditions determined above we screened an oligo(dT) primed cDNA expression library derived from CEF cells. Two positive clones were isolated and then purified by three further rounds of screening. To establish the authenticity of these clones, the filter lifts taken from the fourth round screen were cut in half and screened either in the presence or absence of oestradiol (Fig. 3.3). The purified clones interacted with GST-AF2α only in the presence of oestradiol, suggesting that they were bona fide RIPs. The cDNA inserts of both clones were subcloned into the EcoRI site of pBluescript and then DNA sequenced. The two clones contained the same 726 bp open reading frame, encoding a novel protein fragment of 242 amino acids (Fig. 3.4), which we termed Chicken Interacting Protein 1 (Chip1).

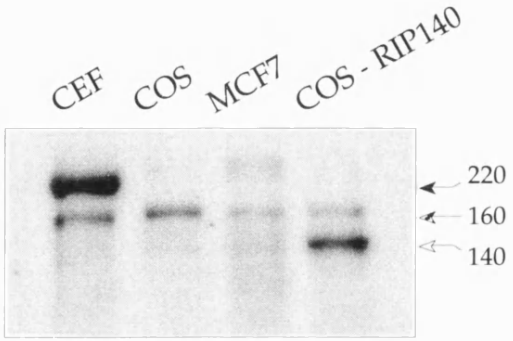


Fig. 3.2 - **Far-western blot analysis of cell lines.** Whole cell extracts of chicken embryo fibroblasts (CEF), COS-1, MCF7 (50 µg of protein) and COS-1 cells overexpressing RIP140 (20 µg of protein) were seperated by 7.5% SDS-PAGE and transferred onto nitrocellulose filters. After denaturation-renaturation, filters were probed with [^{32}P]-labelled GST-AF2α in the presence of E2 (10^{-6} M), washed and exposed for autoradiography. The arrows on the right-hand side indicate the position of endogenous receptor-interacting proteins of 140, 160 and 220 kDa.

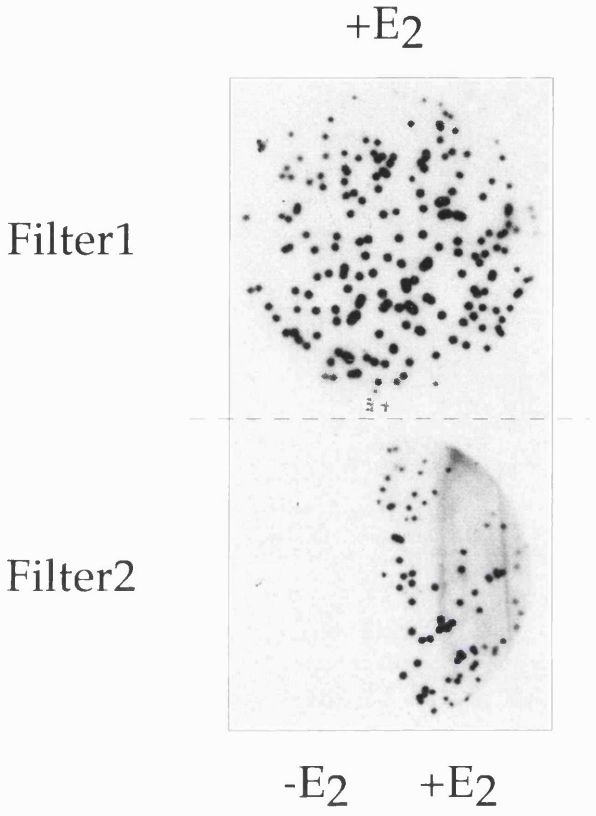


Fig. 3.3 - **The isolated clones interact with GST-AF2α in a ligand dependent manner.** The filters-lifts taken from the purified interacting clones were incubated with [^{32}P]-labelled GST-AF2α. The lower filter was cut in half and screened either in the presence or absence of oestradiol (10^{-6} M).

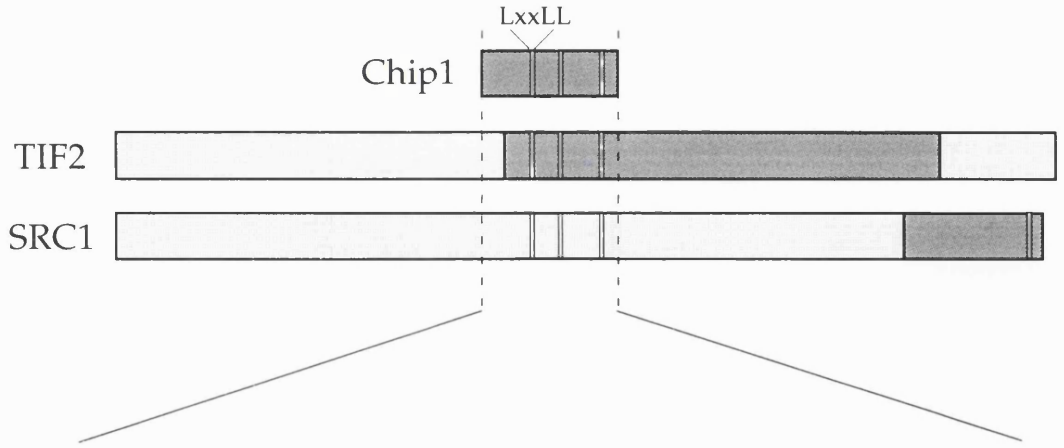
To obtain the full length Chip1 open reading frame we used the 732 bp Chip1 cDNA, labelled with [^{32}P]-dCTP, to screen the CEF cDNA library for overlapping clones. However, this resulted in the identification of seven copies of the same cDNA clone. Subsequent screening of several other CEF cDNA libraries produced only small fragments of Chip1, one of which resulted in a further 120 bp of extra sequence at the 3' end of the existing Chip1 clone. During this period two RIP160 proteins: SRC1 (Onate *et al* 1995) and TIF2/GRIP1 (Hong *et al* 1996, Voegel *et al* 1996) were isolated, both of which have homology with Chip1 (Fig. 3.4). From the homology with these two proteins it seemed likely that Chip1 was a RIP160 protein with an open reading frame of approximately 5 kb. After several failed attempts to isolate more of the Chip1 cDNA and left with the prospect of having to clone at least 4 kb more, it was decided to drop this project and instead work with the newly discovered ERβ.

Chip1 interacts with nuclear receptors in a ligand dependent manner

To add support to the idea that the Chip1 fragment contained a nuclear receptor interacting domain (NID), we fused the 242 amino acids of Chip1 to GST (GST-Chip1) and tested its ability to interact with ERα (Fig. 3.5). GST-Chip1 interacted with ERα in an oestradiol dependent manner, there was no interaction in the presence of the antioestrogens: 4OH-tamoxifen and ICI 182780.

The boundaries of a minimal NID, as defined by the Chip1 fragment, is present in the centre of SRC1 and TIF2 (Fig. 3.4). This region was contained within the first fragment of TIF2 isolated, but not that of SRC1, suggesting the presence of two NIDs in SRC1. Subsequent deletion mapping of both SRC1 (Kalkhoven *et al* 1998) and TIF2 (Voegel *et al* 1998) revealed that this region does indeed contain a NID and that SRC1 has an extra NID at the C-terminus. Chip1 and the equivalent regions in SRC1 and TIF2 function as NIDs because they contain three copies of the LXXLL motif (Fig. 3.4), which are necessary and sufficient for the binding of nuclear receptors (Heery *et al* 1997, Torchia *et al* 1997).

Fig. 3.4 - **Alignment of Chip1 with the nuclear receptor interacting domains of SRC1 and TIF2**. The primary sequence of the isolated Chip1 fragment was aligned to the equivalent regions in SRC1 and TIF2. The Chip1 sequence contains the original 732 bp, plus the extra 120 bp of 3′ sequence isolated. A schematic above shows the relative size and position of Chip1. The open boxes indicate the presence of LxxLL motifs, capable of mediating interactions with nuclear receptors. The shaded areas represent the boundaries of the original isolated fragments. There is a nuclear receptor interacting domain in the centre of SRC1 and TIF2 equivalent to that of Chip1. Each protein has three copies of the LxxLL motif, underlined in the sequence alignment.

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Chip1    1:~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TPVFSS.YRYALSNGICQQ..HQQQ:  22
TIF2   477:MNPGQP.......TSMLSPRHRMSPGVAASPRIPPSQFSPA.GSLHSPVGVCSS..TGNS:  526
SRC1   472:LNNSPMEGTGIALSQFMSPRRQANSGLATRARMSNNSFPPNIPTLSSPVGITSGACNNNN:  531

Chip1   23:H.FSSSSLSALQAISEGVGTSLLSTLSSPGPKL....DSSPNVSIAQQNKANNQDSKSPS:  77
TIF2   527:HSYTNSSLNALQALSEGHGVSLGSSLASPDLKMGNLQNSPVNMNPPPLSKMGSLDSKDCF:  586
SRC1   532:RSYSNIPVTSLQGMNEGPNNSVGFSAGSPVLRQMSSQNSPSRLSMQP.AKAESKDSKEIA:  590

Chip1   78:GLYCEQN.....QVESSICQSNSRDVLSEKDSKDGSLDASESQRGQSESKGHKKLLQLLT:  132
TIF2   587:GLYGEPSEGTTGQAESSCHPGEQKETNDPNLPPAVSSERADGQSRLHDSKGQTKLLQLLT:  646
SRC1   591:SILNE.....MIQSDNSDNSANEGKPLDSGLLHN.NDRLSEGDSKYSQTS..HKLVQLLT:  642

Chip1   133:CSSDERGHSTASNSPLDSNCKESSTNVTSPSGVSSSTSGGVSSSSNVHGSLLQEKHRILH:  192
TIF2    647:TKSDQMEPSPLASSLSDTN..............KDSTGSLPGSGSTHGTSLKEKHKILH:  691
SRC1    643:TTAEQQ....LRHADIDTSCKDVLSCTGTSSSASSNPSGGTCPSSH...SSLTERHKILH:  695

Chip1   193:KLLQNGNSPAEVAKITAEATGKDTYHDTSNTVPCGES....TVKQEQLSPKKKE..NNAL:  246
TIF2    692:RLLQDSSSPVDLAKLTAEATGKDLSQESSSTAPGSEV....TIKQEPVSPKKKE...NAL:  744
SRC1    696:RLLQEG.SPSDITTLSVEPEKKDSVPASTAVSVSGQSQGSASIKLELDAAKKKESKDHQL:  754

Chip1   247:LRYLLDKDD.....VKDPLSKELKPKVENVIIKWDNAVVLLFPRA~~~~~~~~~~~~~~~:  286
TIF2    745:LRYLLDKDD.....TKDIGLPEITPKLERLDSKTDPASNTKLIAMKTEKEEMSFEPGDQP:  799
SRC1    755:LRYLLDKDEKDLRSTPNLCLDDVKVKVEKKE.QMDPCNTNPTPMTKPAPEEVKLESQSQF:  813
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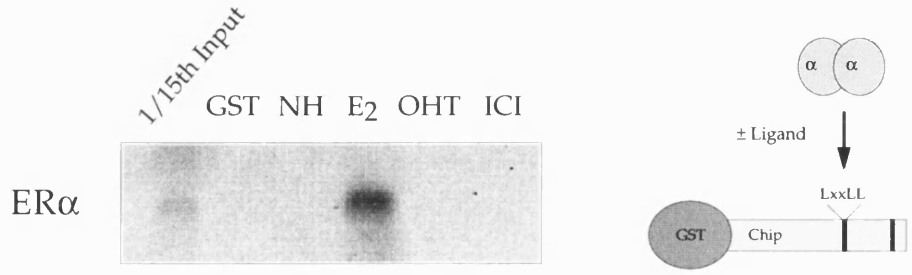


Fig. 3.5 - **Chip1 interacts with ERα in a ligand dependent manner.** Binding of [^{35}S]-methionine labelled ERα with GST-Chip1 was analysed in the presence or absence of 17β-oestradiol (10^{-6} M), 4OH-tamoxifen (10^{-6} M) or ICI 182 780 (10^{-6} M) as indicated. Bound ERα was eluted and analyzed by SDS-PAGE followed by autoradiography.

Expression of Chip1 mRNA in chicken tissues

We used Northern blotting to examine the expression of Chip1 mRNA in a number of different chicken tissues (Fig. 3.6). Allowing for the variations in the amount of RNA loaded, it appears that the Chip1 mRNA (~8 kb in length) is expressed highly in ovary, spleen, and CEF cells, while there was moderate expression in oviduct and brain. There does not appear to be any expression of Chip1 mRNA in heart or thyroid.

Chickens have three distinct SRC1/RIP160 genes

The high sequence homology of Chip1 with SRC1 and TIF2 suggested that Chip1 might be the chicken orthologue of one of these two human proteins. We therefore used southern blotting to determine whether chicken and human DNA encodes two or three RIP160 genes. If a southern blot probed with the Chip1 cDNA recognised the same restriction pattern as either SRC1 or TIF2 we could assume that Chip1 was indeed their chicken orthologue. Aliquots containing 10 μg of genomic DNA digested with either EcoRI, PstI or both in combination were run out on a 1% agarose gel. The Chip1 cDNA sequence contains a single PstI restriction site, thereby ensuring a cut in the region of interest. As a positive control 50 pg of Chip1 cDNA, representing the approximate amount of a single copy gene in 10 μg of genomic DNA, was also run out.

The southern blot was first screened with the Chip1 cDNA (Fig. 3.7). In chicken genomic DNA digested with EcoRI and PstI the Chip1 probe detected a small band of 0.8 kb which has approximately the same signal strength as the 50 pg of Chip1, suggesting that Chip1 is a single copy gene. Since we know that the PstI restriction site lies at the 3' of the Chip1 cDNA, the size and intensity of the 0.8 kb band suggests the presence of an EcoRI site 0.2 kb 5' of the Chip1 fragment. Using this prediction and the size and intensity of the other bands detected, we were able to draw a possible restriction map of the Chip1 gene (Fig. 3.7).

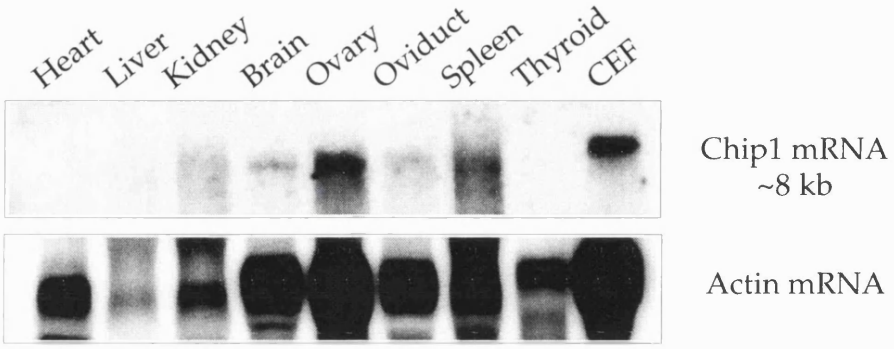


Fig. 3.6 - **Expression of Chip1 mRNA in chicken tissues.** 20 µg of total mRNA isolated from the tissues indicated above, was run out on a 1% agarose gel and then transferred to an Amersham N+ filter. The blot was probed using the 732 bp Chip1 cDNA labelled with [^{32}P]-dCTP.

The southern blot was next screened with unique probes (corresponding to the boundaries of Chip1) from SRC1 (Fig. 3.8) and TIF2 (Fig. 3.9). In each case the probes detected a unique restriction pattern. The bands detected were specific because there was no cross-reactivity of the SRC1 and TIF2 probes with the 50 pg of Chip1 cDNA. The three unique patterns of restriction fragments detected by the three different cDNA probes demonstrates that Chip1 is not a chicken SRC1 or TIF2 orthologue but a distinct gene, and that human and chicken DNA contains at least three members of the RIP160 family of nuclear receptor coactivators. Subsequently full length Chip1, the third RIP160 protein was cloned by several groups from human, Rac3 (Li *et al* 1997), ACTR (Chen *et al* 1997), AIB1 (Anzick *et al* 1997), TRAM-1 (Takeshita *et al* 1997) and mouse, p/CIP (Torchia *et al* 1997).

RIPs are not overexpressed in transformed CEF cells

Previous work by Vivanco *et al* (1995) showed that nuclear receptors had greater transcriptional activity in transformed cell lines than in primary cells. One explanation for this observation is that transformed cells may overexpress coactivator proteins relative to untransformed cells. To test this hypothesis we used far-western blotting to compare the level of RIPs in untransformed CEF cells with those transformed with either v-Myc, v-Src or v-Jun (Fig. 3.10). The level of RIPs detected in all the cell extracts was similar, with only a slight variation in the amount of RIP140 and RIP160 proteins in CEF cells and those transformed with v-Myc. Since there are no gross differences in the level of RIPs in transformed versus untransformed CEF cells, we conclude that coactivators are unlikely to be responsible for the increased activity of nuclear receptors in transformed cell lines.

Chip1 orthologues

Since the publication of the full length Chip1 homologues Rac3/ACTR/AIB1/TRAM-1/pCIP, Ho Yi Mak (Molecular Endocrinology Laboratory) using primers designed from the Chip1 sequence in

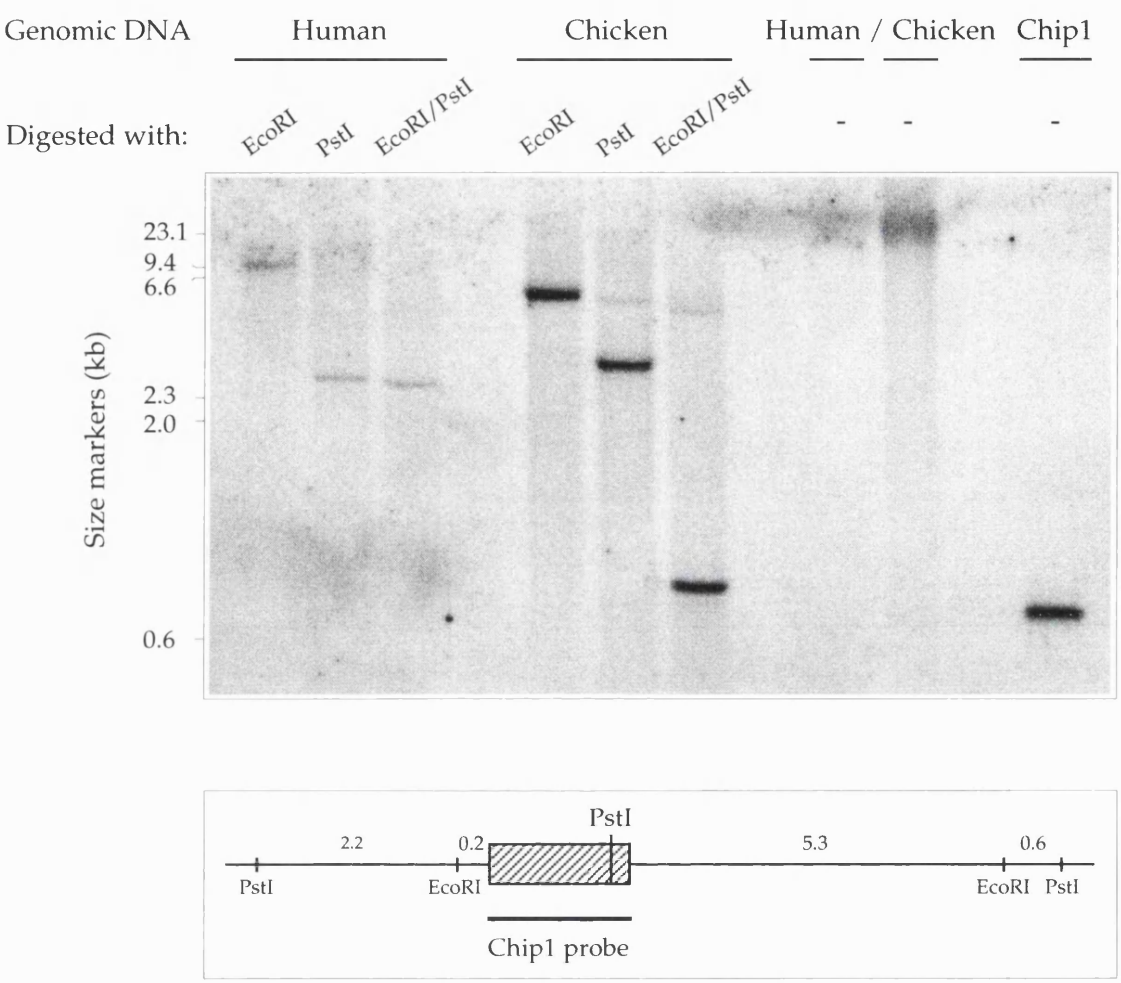


Fig. 3.7 - **Genomic organisation of the Chip1 gene.** 10 µg of human or chicken genomic DNA was digested with either EcoRI, PstI or the two enzymes in combination. The digested DNA was run out on a 1% agarose gel, transferred to an Amersham N+ filter and then probed using the 732 bp cDNA of Chip1 labelled with [^{32}P]-dCTP. As a positive control 50 pg of the Chip1 cDNA was also run out. A schematic representation of the organisation of the Chip1 gene is shown below. The shaded area represents the Chip1 cDNA fragment.

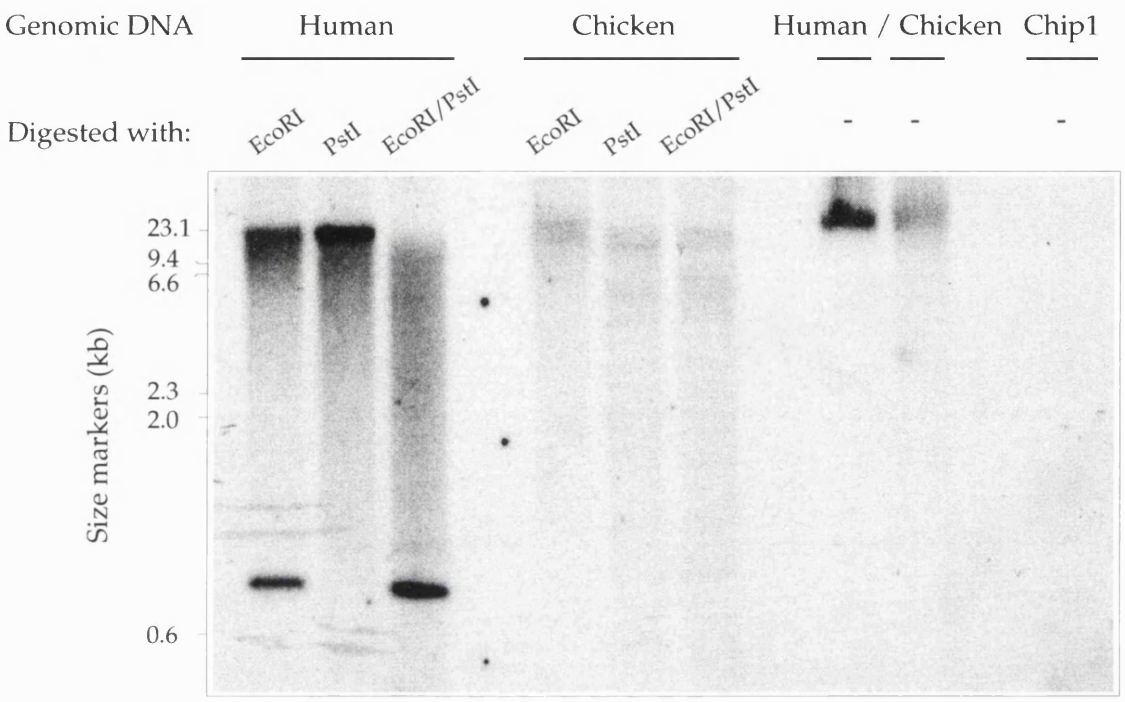


Fig. 3.8 - **Genomic organisation of the SRC1 gene.** 10 µg of human or chicken genomic DNA was digested with either EcoRI, PstI or the two enzymes in combination. The digested DNA was run out on a 1% agarose gel, transferred to an Amersham N+ filter and then probed using a fragment of the of SRC1 cDNA equivalent to Chip1 labelled with [^{32}P]-dCTP. As a positive control 50 pg of the Chip1 cDNA (Chip1) was also run out.

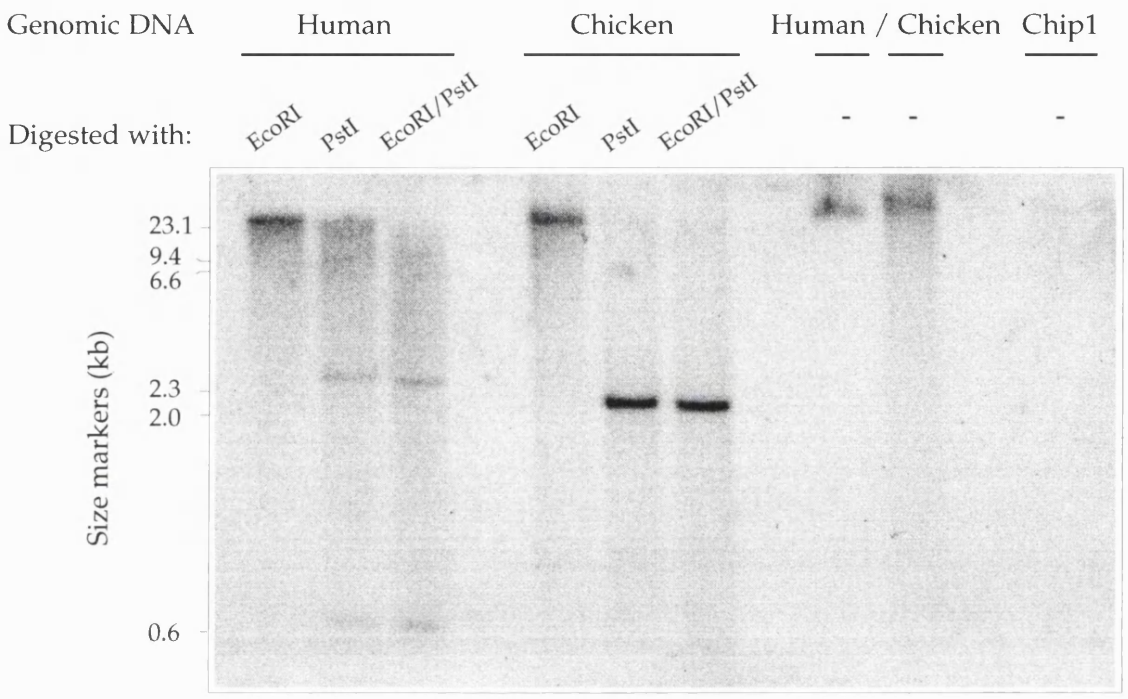


Fig. 3.9 - **Genomic organisation of the TIF2 gene.** 10 µg of human or chicken genomic DNA was digested with either EcoRI, PstI or the two enzymes in combination. The digested DNA was run out on a 1% agarose gel, transferred to an Amersham N+ filter and then probed using a fragment of the of TIF2 cDNA equivalent to Chip1 labelled with [^{32}P]-dCTP. As a positive control 50 pg of the Chip1 cDNA (Chip1) was also run out.

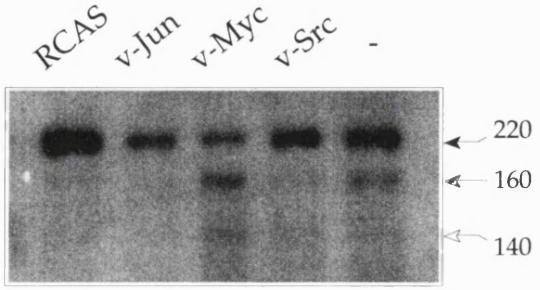


Fig. 3.10 - **Far-western blot analysis of transformed CEF cells.** Whole cell extracts were prepared from chicken embryo fibroblasts (CEF) which were either untransformed (-), transformed with v-Jun, v-Myc, v-Src or transfected with the empty retroviral vector (RCAS). 20 µg of the protein extract was then separated by 7.5% SDS-PAGE and transferred onto nitrocellulose filters. After denaturation-renaturation, filters were probed with [^{32}P]-labelled GST-AF2α in the presence of oestradiol (10^{-6} M), washed and then visualised by autoradiography. The arrows on the right-hand side indicate the position of endogenous receptor-interacting proteins of 140, 160 and 220 kDa.

combination with degenerate primers predicted from the RAC3 sequence, has been able to generate much of the missing Chip1 cDNA by PCR. Orthologues of Chip1 have now been cloned from four species, including human (RAC3/ACTR/AIB1), mouse (p/CIP), chicken (Chip1) and Xenopus (xSRC-3, Kim *et al* 1998). The human and mouse sequences are so similar as to be uninformative, but alignment of the human, chicken and xenopus sequences shows patches of homology in regions of known function (Fig. 3.11). The NID region with the three copies of the LXXLL motif are well conserved, as is the region known to bind the cointegrator proteins CBP and p300 (Fig. 3.11). It is now possible to identify residues conserved within these functional domains and study the effects of their point mutation, thus fulfilling the original aim of the project.

Summary and Conclusions

Far-western blotting analysis of several cell lines using GST-AF2α as a probe produced three predominant bands of 140, 160 and 220 kDa. The RIP140 and RIP160 bands consisting of a number of proteins of a similar size. When these RIPs were first cloned it was found that they lacked sequence homology to other proteins, so to aid their characterisation we tried to clone RIPs from a different species. The project to clone chicken RIPs was only partially successful since we were only able to clone part of a single protein, Chip1, due in part to the lack of a good quality chicken cDNA library. However, characterisation of Chip1 revealed it was capable of interacting with nuclear receptors in a ligand dependent manner and that it had significant sequence homology to the two SRC1/RIP160 coactivator proteins cloned at that time: SRC1 and TIF2. Southern blotting analysis of chicken and human genomic DNA revealed that Chip1 was not an SRC1 or TIF2 chicken orthologue but a unique gene which in all probability was a third member of the SRC1/RIP160 coactivator family. This indeed proved to

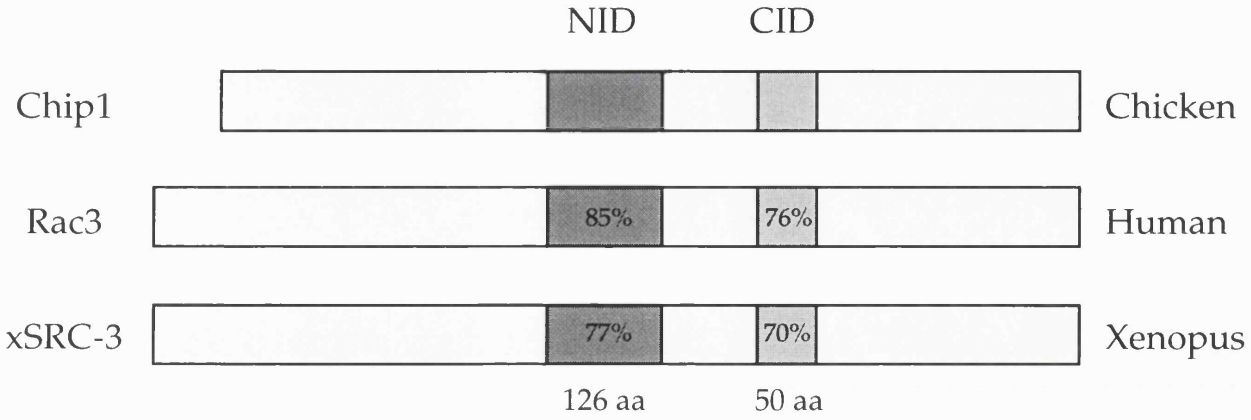


Fig. 3.11 - **Alignment of Chip1 orthologues**. Sequence conservation in the nuclear receptor interacting domain (NID) and CBP/p300 interacting domain (CID) of Chip1 orthologues from human (Rac3) and xenopus (xSRC-3) expressed as a percentage relative to Chip1.

be the case, when in course Chip1 was cloned by several other groups. The demonstration that chickens and humans have the same number of well conserved coactivator proteins suggests that nuclear receptors retain a largely conserved mechanism of transcriptional activation.

Chapter 4

ERα and ERβ form heterodimers

Introduction

It is well established that ERα functions as a transcriptional activator when bound to DNA as a homodimer. The major dimerisation interface in ERα is contained within the ligand binding domain. Helix 11 makes the main surface contacts, with smaller contributions from helices 8, 9 and 10 (Brzozowski *et al* 1997). These helices are well conserved in ERβ, as are the individual residues first identified by Fawell *et al* (1990) as being essential for homodimerisation of ERα. Given the conservation of helix 11 in ERβ, not only is it likely to form the dimerisation interface for ERβ homodimers, but also possibly for heterodimers between ERα and ERβ. If ERα and ERβ form viable heterodimers this might have major implications for oestrogen signalling, since its conceivable that ERα/ERβ heterodimers differ in their DNA binding and transcriptional activation properties from those of ERα and ERβ homodimers. This chapter describes the comparison of the DNA binding and dimerisation properties of ERα and ERβ homodimers with ERα/ERβ heterodimers.

To express ERα and ERβ *in vitro* their full length cDNAs were subcloned into pSP65 (Promega) and synthesised using the TNT reticulocyte lysate kit (Promega). Proteins were labelled by addition of [^{35}S]-methionine to the reaction. ERα and ERβ were coexpressed (ERα/ERβ) by adding equal amounts or varying ratios of both expression plasmids in the same reaction. The ERβ constructs used in this chapter contain an open reading frame of 477 amino acids (Kuiper *et al* 1996, Mosselman *et al* 1996). Subsequently ERβ was found to contain an extra 53 amino acids at the N-terminus, however the absence of these extra residues does not effect DNA binding or dimerisation.

ERα and ERβ form heterodimers in solution

To test the ability of ERα and ERβ to dimerise in solution, equal amounts of labelled *in vitro* synthesised receptor were incubated with the antisera MP15 (raised to the N-terminus of ERα) and subjected to

Ogawa et al (1998)

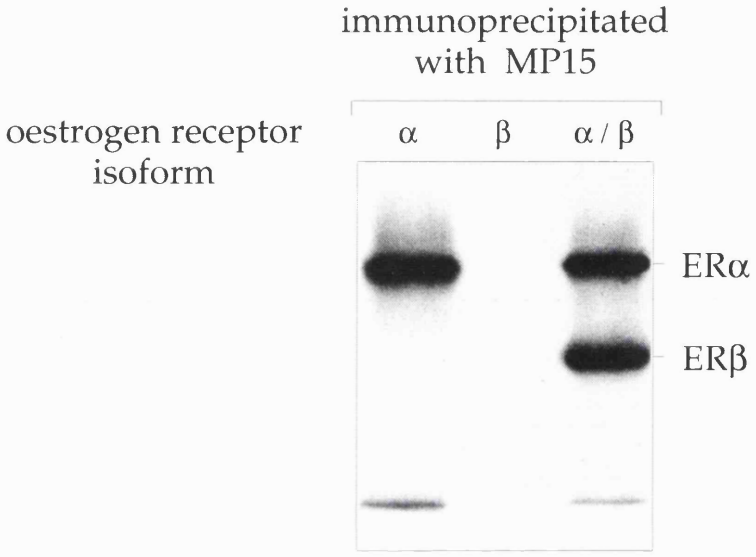


Fig. 4.1 **ERα and ERβ expressed *in vitro* form heterodimers in solution.** Equal amounts of *in vitro* translated ERα, ERβ or ERα/ERβ were incubated with 1 µl of MP15 antisera and subjected to immunoprecipitation analysis. The position of ERα and ERβ are indicated on the right hand side.

immunoprecipitation analysis. MP15 efficiently immunoprecipitated ERα but not ERβ (Fig. 4.1), showing a specificity for ERα. When MP15 was used to immunoprecipitate ERα/ERβ both ERα and ERβ were immunoprecipitated, since MP15 is specific for ERα, ERα and ERβ must be able to form dimers in solution.

ERα and ERβ form heterodimers on DNA

We then tested the ability of ERα and ERβ to form heterodimers on DNA. The DNA binding activity of ERα and ERβ was tested using *in vitro* translated receptors and a consensus estrogen response element (ERE) in a gel shift assay. Both ERα and ERβ bound to the ERE (Fig. 4.2), it was observed that ERα, the larger of the two proteins migrated more slowly than ERβ and that the mobility of ERα but not ERβ was retarded by a monoclonal antibody (H226) raised to the ligand binding domain of ERα. When the two receptors were cotranslated, an intermediate complex corresponding to ERα/ERβ heterodimers was detected, in addition to ERα homodimers. Both complexes were retarded by the addition of H226, consistent with the presence of ERα in both complexes. ERβ homodimers were not detected when cotranslated with ERα at any of the ratios used. In general, ERβ homodimers gave a weaker, less concise signal than ERα homodimers. This led us to examine the relative DNA binding affinities for ERα, ERβ homodimers and ERα/ERβ heterodimers.

The DNA binding affinities of ERα, ERβ homodimers and ERα/ERβ heterodimers were determined by performing gelshift experiments over a wide range of probe concentrations (Fig. 4.3). We found that ERα homodimers and ERα/ERβ heterodimers had a similar K_d for an ERE of approximately 2 nM, whereas that of ERβ homodimers was about 9 nM. This difference in DNA binding affinity could account for the weaker shifted complex observed with ERβ homodimers in comparison to those of ERα.

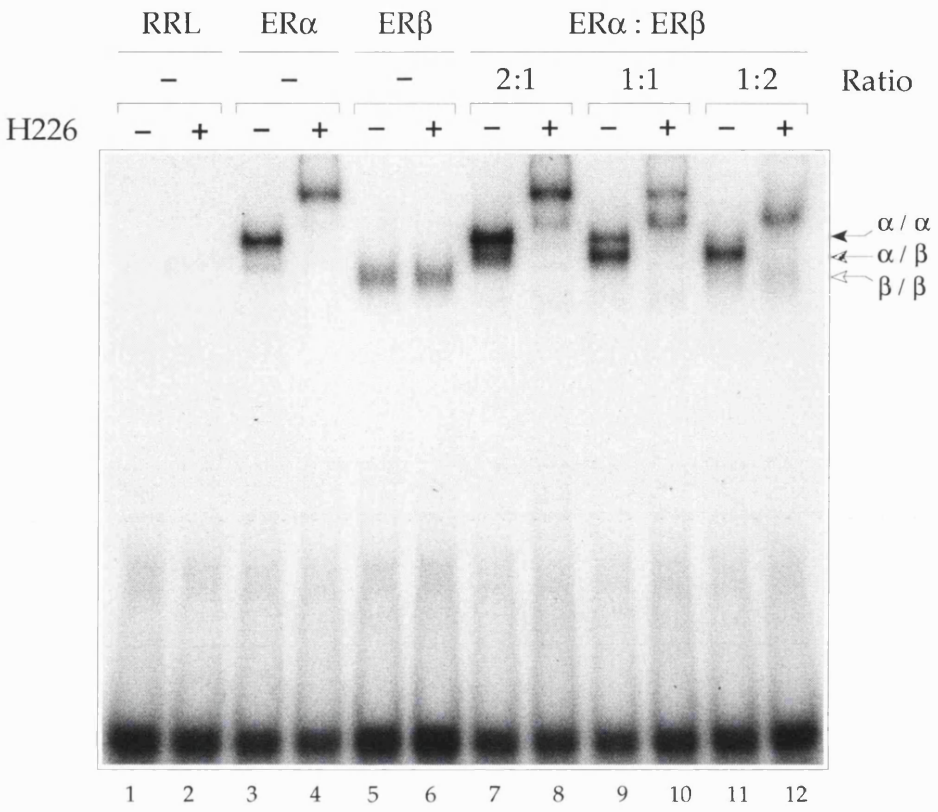
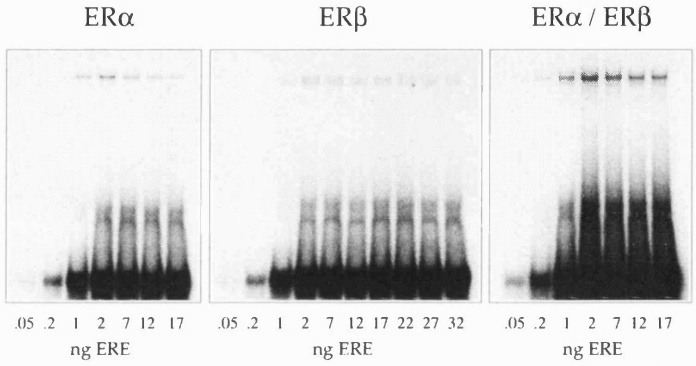
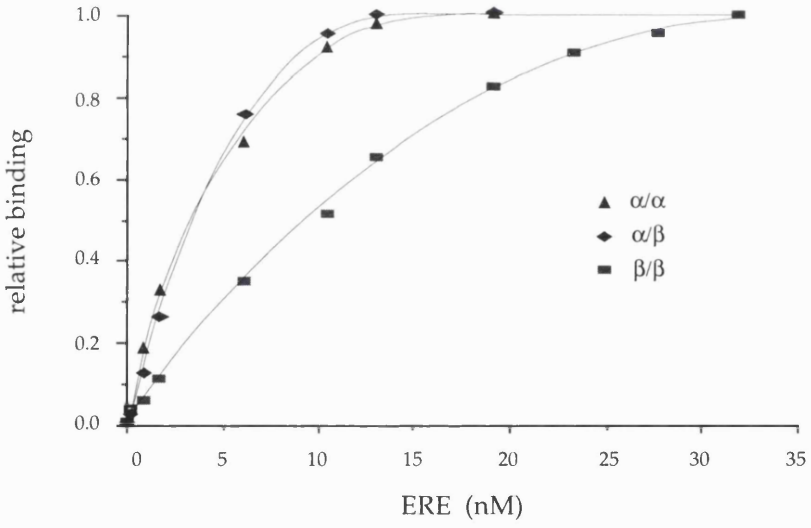


Fig. 4.2 - **DNA binding activity of ERα, ERβ heterodimers and ERα/ERβ heterodimers.** Equivalent amounts of *in vitro* translated ERα, ERβ or different ratios of the two, as indicated, were incubated with a [^{32}P]-labelled ERE in the presence or absence of ER antibody H226. As controls, the unprogrammed lysate was analysed (tracks 1 and 2). The position of the α/α homodimer, α/β heterodimer and β/β homodimer is shown on the right hand side.

Fig. 4.3 - **DNA binding affinity of ERα, ERβ homodimers and ERα/ERβ heterodimers.** *In vitro* translated ERα, ERβ or both (at a ratio of 2:1 in favour of ERβ) were analysed for DNA binding activity in a gel shift assay over the range of DNA concentrations indicated. Radiolabelled probe alone was used up to a concentration of 6 nM (2ng) and higher concentrations were achieved by diluting the labelled probe with nonlabelled oligonucleotide, and the resultant reduction in specific activity taken into account in the calculations. Gels were then scanned with a PhosphorImager and the relative binding of the different dimeric states was plotted against the concentration of ERE.


ERα
ERβ
ERα / ERβ
.05 .2 1 2 7 12 17
ng ERE
.05 .2 1 2 7 12 17 22 27 32
ng ERE
.05 .2 1 2 7 12 17
ng ERE


relative binding
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
ERE (nM)
α/α
α/β
β/β

We next tested the ability of ERα and ERβ expressed in COS-1 cells to bind an ERE. ERα expressed in COS-1 cells is prone to N-terminal proteolysis, which results in two shifted complexes, the major upper complex being the wild-type homodimer (Fig. 4.4a). ERα and ERβ gave shifted complexes similar to those observed with *in vitro* translated receptors (Fig. 4.2) and as expected H226 retarded ERα homodimer complexes but not those of ERβ. Cotransfection of equivalent amounts of ERα and ERβ expression vectors resulted in predominantly ERα/ERβ heterodimer complexes with some ERα homodimer, but no ERβ homodimers. The same extracts were preincubated with 17β-oestradiol and 4OH-tamoxifen to test the effect of ligands on the DNA binding ability of the three dimeric forms of receptor (Fig. 4.4b). As previously demonstrated for ERα (Lees *et al* 1989), the DNA binding activity of ERβ homodimers and ERα/ERβ heterodimers was unaffected by ligand binding, but their mobilities were slightly increased in the presence of 17β-oestradiol (Fig. 4.4b, tracks 2, 5 and 8). From these experiments we conclude that ERα and ERβ expressed in intact cells can form heterodimers and that ERα homodimers and ERα/ERβ heterodimers are formed preferentially above ERβ homodimers.

Analysis of the of ERα/ERβ heterodimer interface

Previous work with a series of ERα point mutants identified a region within the ligand binding domain which is required for both receptor dimerisation and high affinity DNA binding (Fawell *et al* 1990). We used these mutants to determine whether the region required for formation of ERα homodimers, is necessary to form heterodimers with ERβ. We compared the ability of a full length ERα point mutant to dimerise with either a truncated version of ERα (ERα 182-599) or ERβ in a gelshift assay. If the point mutant is able to dimerise then three bands should be observed, corresponding to the homodimers of the full length and truncated ERα, and the heterodimer of the two in a 1:2:1 ratio. As previously demonstrated (Fawell *et al* 1990) the ERα point mutants R507A, L511R and I518R are unable to form homodimers (Fig. 4.5 tracks 4,6 and 7). These point mutants were then tested for their ability to dimerise with ERβ. ERα R507A was also unable to dimerise with ERβ,

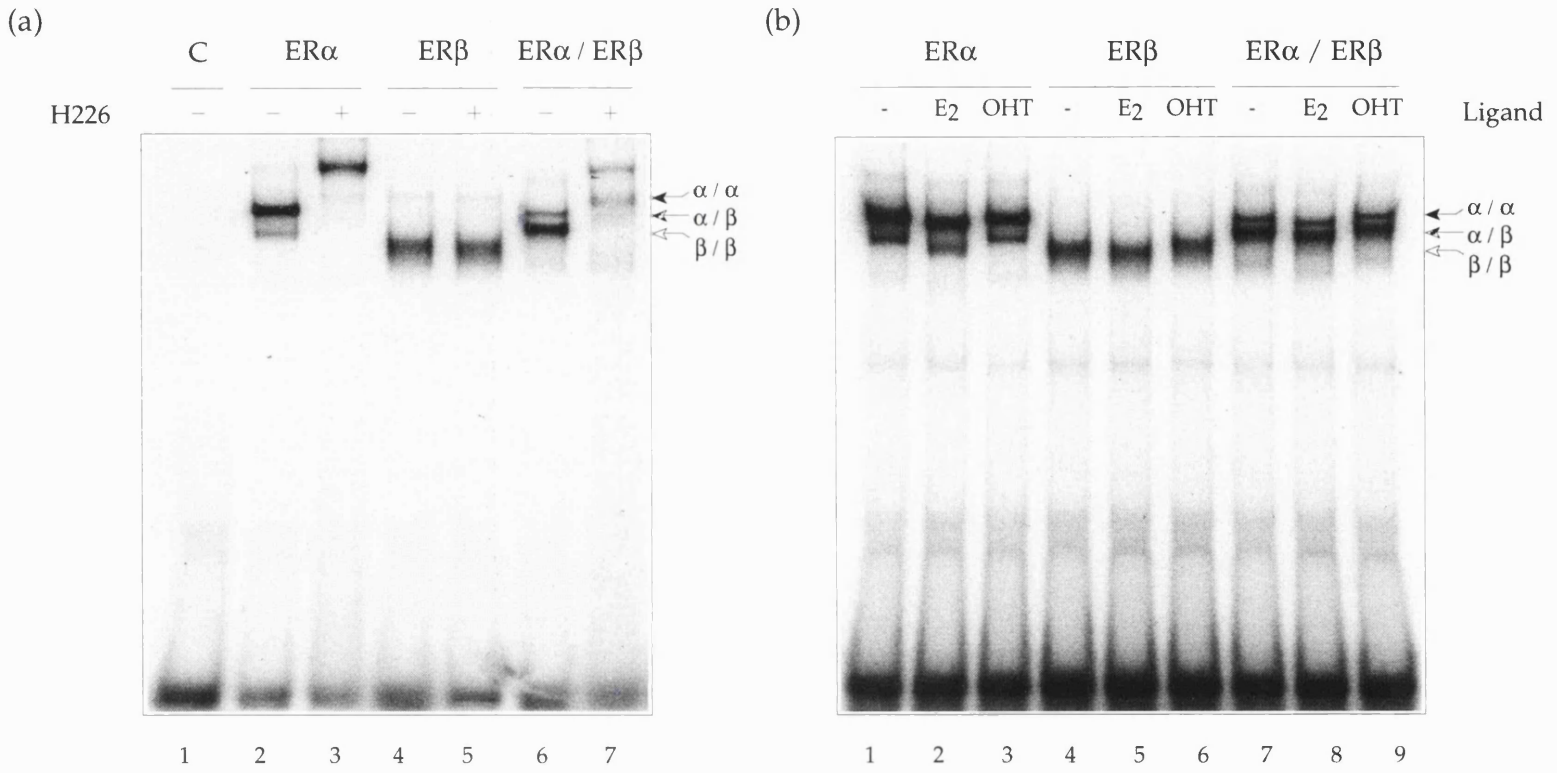


Fig. 4.4 - **ERα and ERβ, expressed in Cos-1 cells, bind as heterodimers to DNA**. Equal amounts of whole cell extracts, prepared from cells expressing ERα, ERβ or both, were incubated with a [^{32}P] labelled ERE in the presence or absence of H226 antibody (a) or different ligands (b). 17β-oestradiol or 4OH-tamoxifen were tested at 2 x 10^{-8} M and 1 x 10^{-6} M, respectively. When ERα alone was expressed, we observed two complexes, a major upper complex, corresponding to the ERα homodimer, and an additional complex that is probably generated by proteolysis. It seems to lack N-terminal sequences since it is recognised by a monoclonal antibody specific for the C-terminal F region (Le Douarin *et al* 1995). The position of the α/α homodimer, α/β heterodimer and β/β homodimer is shown on the right hand side.

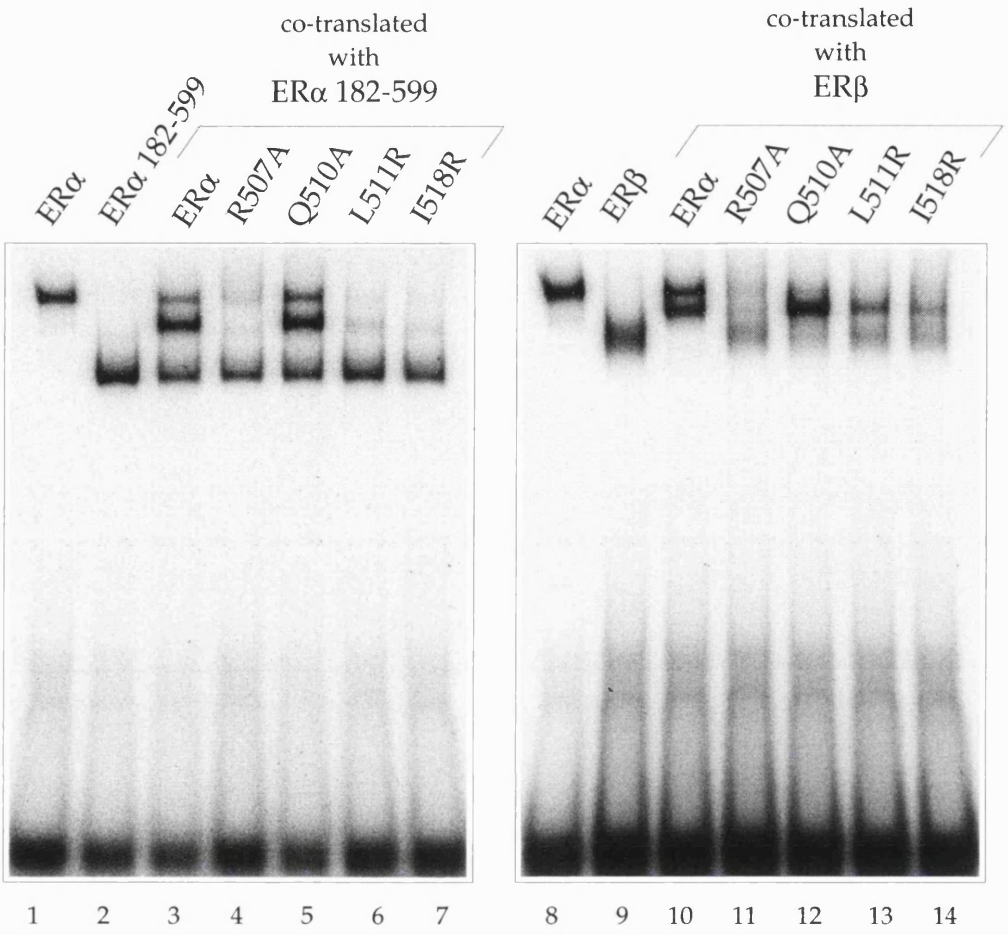


Fig. 4.5 - **DNA binding activity of wild-type, mutant ERα and ERβ**. The wild type mouse ERα, and a series of mutants were cotranslated with either ERα 182-599 or ERβ and tested for DNA binding activity using the gel shift assay. As controls, the wild type ERα (tracks 1 and 8), truncated ERα 182-599 (track 2) and ERβ (track 9) were analysed individually.

in contrast L511R and I518R were able to form weak heterodimers (Fig. 4.5, tracks 11, 13 and 14). ERα Q510A a mutant that forms homodimers like wild-type had no effect on heterodimerisation with ERβ (Fig. 4.5, tracks 5 and 12). A series of other mutations in this region were screened in an attempt to identify additional residues which could discriminate between homo- and heterodimerisation, but all the mutants tested retained their ability to form heterodimers with ERβ (Fig. 4.6). This suggests that the dimerisation surfaces of ERα and ERβ overlap, but they are not identical.

Transient transfection assays

We next compared the transcriptional activity of ERα, ERβ homodimers and ERα/ERβ heterodimers in transiently transfected COS-1 cells using the pERE-BL-CAT reporter gene. ERα and ERβ expression vectors were tested individually or in combination at a ratio of 1:1 or 1:2, conditions under which COS-1 extracts contained predominantly ERα/ERβ heterodimers (Fig. 4.4a). The ability of ERβ to stimulate transcription was approximately 50% that of ERα (Fig. 4.7a), coexpression of ERα and ERβ resulted in an intermediate level transcription that was blocked by the addition of antioestrogens 4OH-tamoxifen and ICI 182 780. Similar results were also obtained in HeLa cells (Fig. 4.7b). Since the heterodimer is the major dimeric form of the receptor under these conditions, it appears to retain its ability to stimulate transcription. The intermediate level of transcription suggests an additive combination of ERα and ERβ activation domains.

ERα/ERβ heterodimers interact with SRC1 on DNA

To support the idea that ERα/ERβ heterodimers are able to stimulate transcription we tested their ability to bind the coactivator SRC1, as previously demonstrated for ERα (Kalkhoven *et al* 1998, White *et al* 1997) and ERβ homodimers (Tremblay *et al* 1997). This was achieved by analysing the ability of receptors bound to DNA, to interact

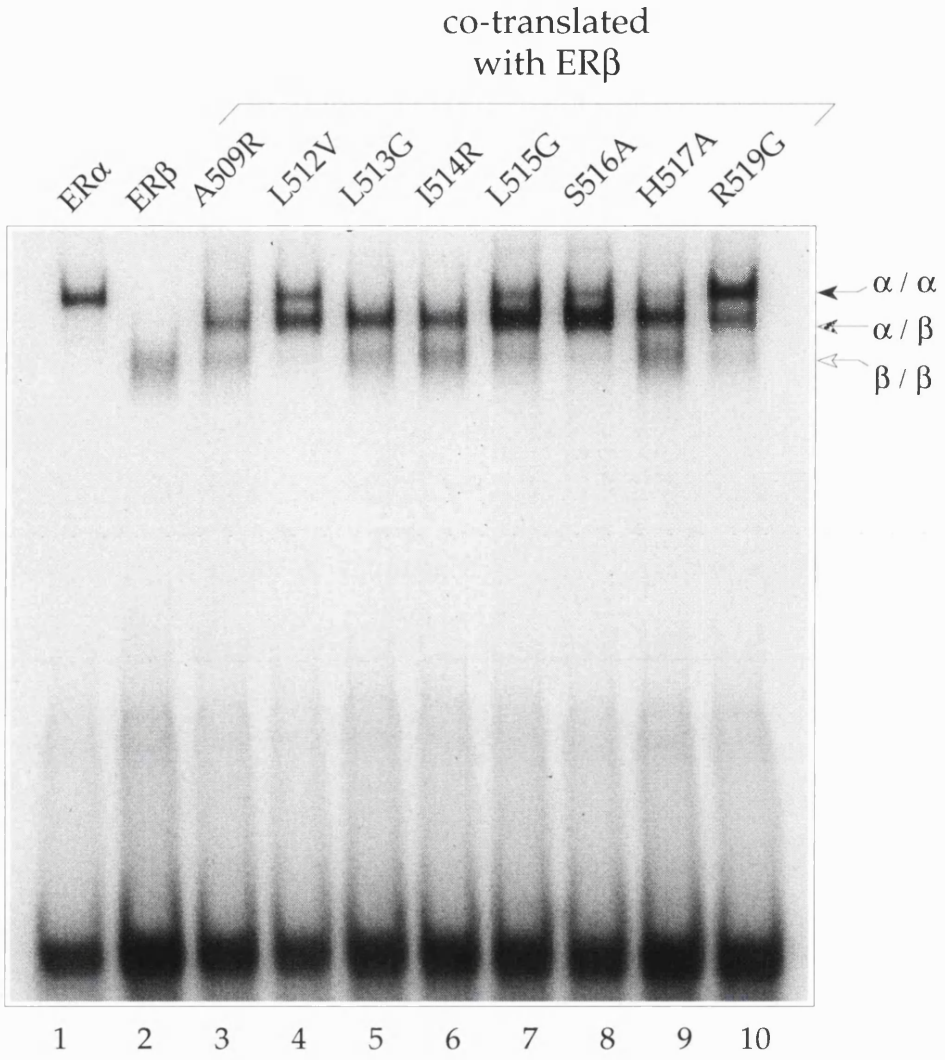


Fig. 4.6 - **DNA binding activity of wild-type, mutant ERα and ERβ**. The wild type mouse ERα, and a series of mutants were co-translated with ERβ and tested for DNA binding activity using the gel shift assay. As controls, the wild type ERα (track 1) and ERβ (track 9) were analysed individually. The position of ERα, ERβ homodimers and ERα/ERβ heterodimers are shown on the right hand side.

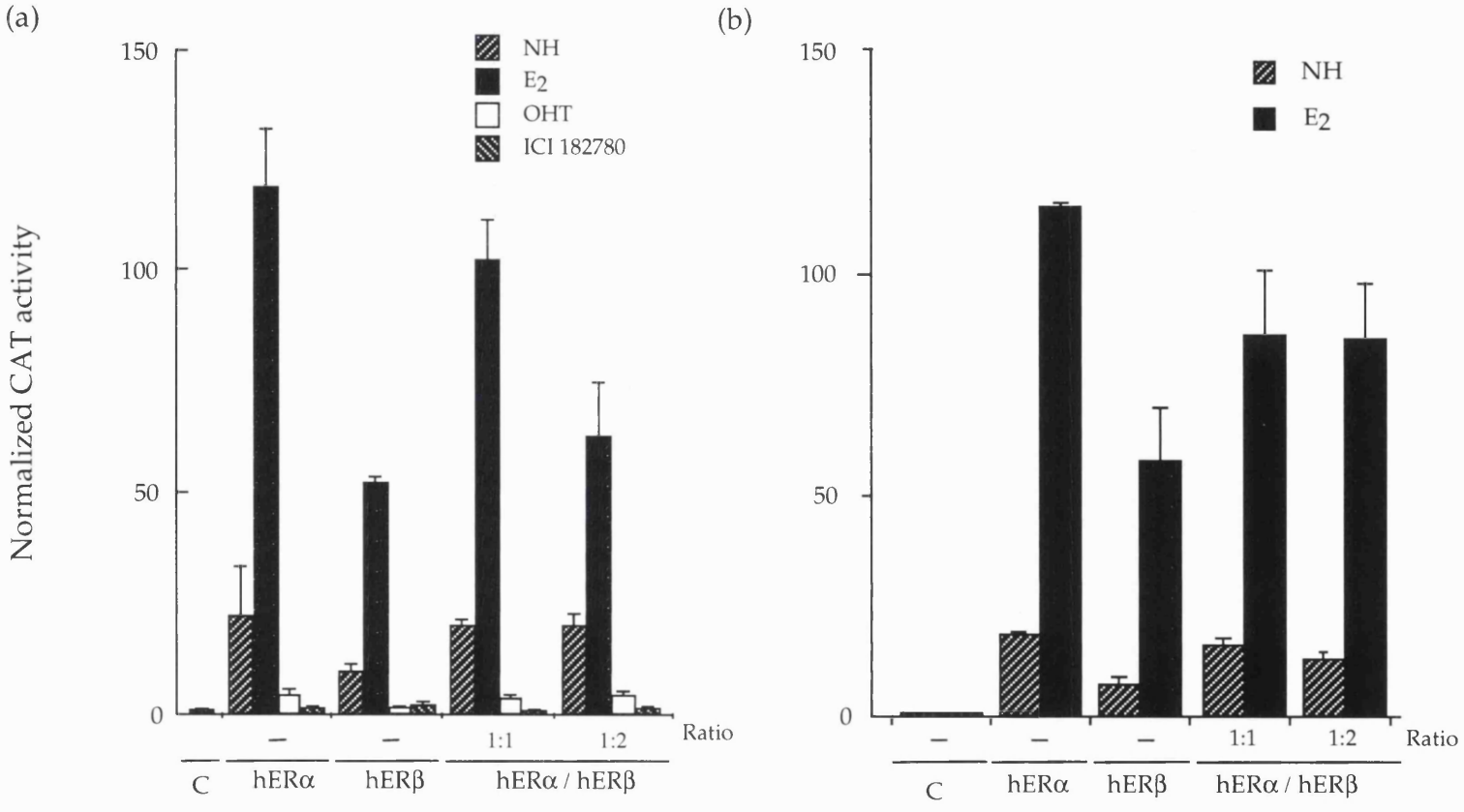


Fig. 4.7 - **Transcriptional activity of coexpressed ERα and ERβ**. The ability of ERα and ERβ expressed individually or in combination to stimulate transcription of a reporter gene pERE-BL-CAT was tested by transient transfection in Cos-1 (a) and HeLa cells (b). Transcriptional activity was determined in the absence or presence of 1 x 10^{-8} M 17β-oestradiol (E_2), 1 x 10^{-7} M 4OH-tamoxifen and 1 x 10^{-7} M ICI 182780 and corrected for the activity of the internal control pJ7lacZ. Activity of the reporter was also analysed in the absence of transfected ER as a control (c). The error bars represent standard errors determined from two transfection experiments each carried out in duplicate.

with a fragment of SRC1 (residues 570-780) known to bind nuclear receptors in a ligand dependent manner (Kalkhoven *et al* 1998). When ERα, ERβ or ERα/ERβ were prebound to DNA and then incubated with increasing amounts of purified GST-SRC(570-780) we could detect an additional 'supershifted' complex above the receptor dimers (Fig. 4.8). The amount of the 'supershifted' complex was greatly increased upon the addition of 17β-oestradiol, although there was an appreciable interaction between SRC1 and ERβ even in the absence of ligand (compare tracks 7 and 8 with 13 and 14). The interaction of the ERα/ERβ heterodimer with SRC1 is comparable to that of the ERα and ERβ homodimers, suggesting that the heterodimer forms a competent surface for the binding of coactivators and is therefore very likely to stimulate transcription when bound to DNA.

Summary and Conclusions

The sequence conservation of Helix 11 between ERα and ERβ suggested that the two receptors might be capable of forming heterodimers. ERα and ERβ expressed *in vitro* are capable of forming heterodimers in solution and on DNA. The DNA binding affinity of ERα homodimers and ERα/ERβ heterodimers (~2 nM) is greater than ERβ homodimers (~9 nM), which may explain the lack of ERβ homodimers in gelshifts when ERα and ERβ are coexpressed. The reason for ERβs reduced DNA binding affinity is not easily apparent, since the gelshift assay does not distinguish differences between DNA binding and dimerisation. The high conservation (96%) of the ERα and ERβ DNA binding domains, perhaps makes a reduced dimerisation affinity the more likely explanation. Heterodimerisation studies with a series of ERα point mutants does suggest that although the dimer interfaces are similar they are not identical.

Cotransfection of ERα and ERβ in COS-1 and HeLa cells resulted in an intermediate level of transcriptional activity to that of ERα and ERβ homodimers, suggesting that ERα/ERβ heterodimers are transcriptionaly active. However, the presence of ERα or ERβ

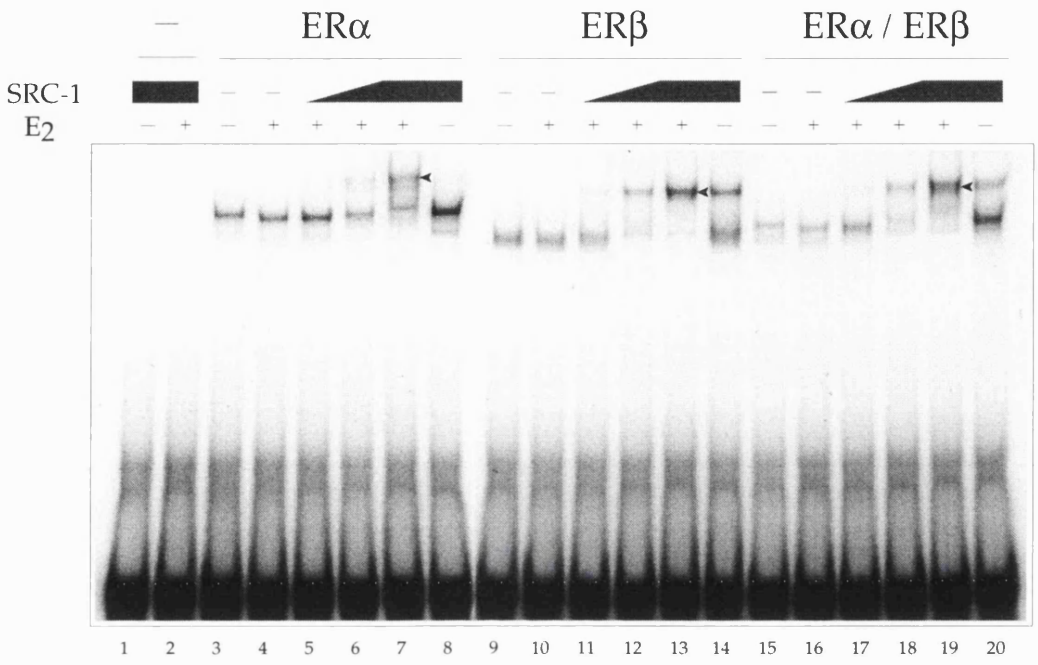


Fig. 4.8 - **SRC-1 interacts with ERα/ERβ heterodimers bound to DNA.** Equal amounts of ERα, ERβ and ERα/ERβ heterodimers were incubated with increasing amounts of GST-SRC(570-780) in the presence or absence of 2×10^{-8} M 17β-oestradiol. As controls, GST-SRC(570-780) was tested for its DNA binding activity in the absence of receptor. The position of receptors complexed with GST-SRC is indicated by solid arrow heads.

homodimers in this assay cannot be completely ruled out. We therefore showed that ERα/ERβ heterodimers could interact with the coactivator SRC1 whilst bound to DNA, similar to ERα and ERβ homodimers. It thus seems very likely that ERα/ERβ heterodimers are transcriptionally active.

Chapter 5

Transcriptional activation by ERα and ERβ

Introduction

ERα has two well characterised activation domains (Lees *et al* 1989, Tora *et al* 1989), activation function 1 (AF1) in the N-terminus, and activation function 2 (AF2) in the C-terminus. AF1 is nominally termed a constitutive activation domain, since when fused to a heterologous DNA binding domain it stimulates transcription in the absence of oestradiol. However, as part of the intact receptor, AF1 still requires oestrogen to dissociate it from the inactive complex with heat shock proteins, allowing receptor dimerisation and DNA binding. In contrast, AF2 is wholly dependent on oestrogen binding for it to function as a transcriptional activation domain. Sequence comparison of ERα and ERβ (Fig. 5.1) shows that the conservation of AF1 between the two receptors is low (<20%), whilst AF2 is quite high (58%). This chapter compares the transcriptional activities of ERα and ERβ using various reporter genes and cell types.

The ERβ cDNA used in constructs for this series of experiments is longer than the previously published form (~~Lees *et al* 1989~~, Mosselman *et al* 1996). It contains an extra 53 amino acids at the N-terminus, to give a full length protein of 530 residues (see Fig. 5.1), in agreement with a recently published report on the cloning of human ERβ (Ogawa *et al* 1998).

The transcriptional activity of ERβ is promoter and cell type specific

We began our study by comparing the ability of ERα and ERβ to stimulate transcription from a series of oestrogen response element (ERE) containing reporter genes, in chicken embryo fibroblasts. The transcriptional activity of ERβ on a reporter containing one copy of an ERE upstream of the thymidine kinase (TK) promoter (ERE-TK-luc) was approximately 50% that of ERα (Fig. 5.2). TK has a complex promoter containing binding sites for several other transcription factors. When ERα or ERβ were cotransfected with a reporter plasmid containing a simple promoter ERβ was much less active than ERα, approximately 20% using 3ERE-TATA-luc and 10% with 2ERE-PS2-CAT (Fig. 5.2).

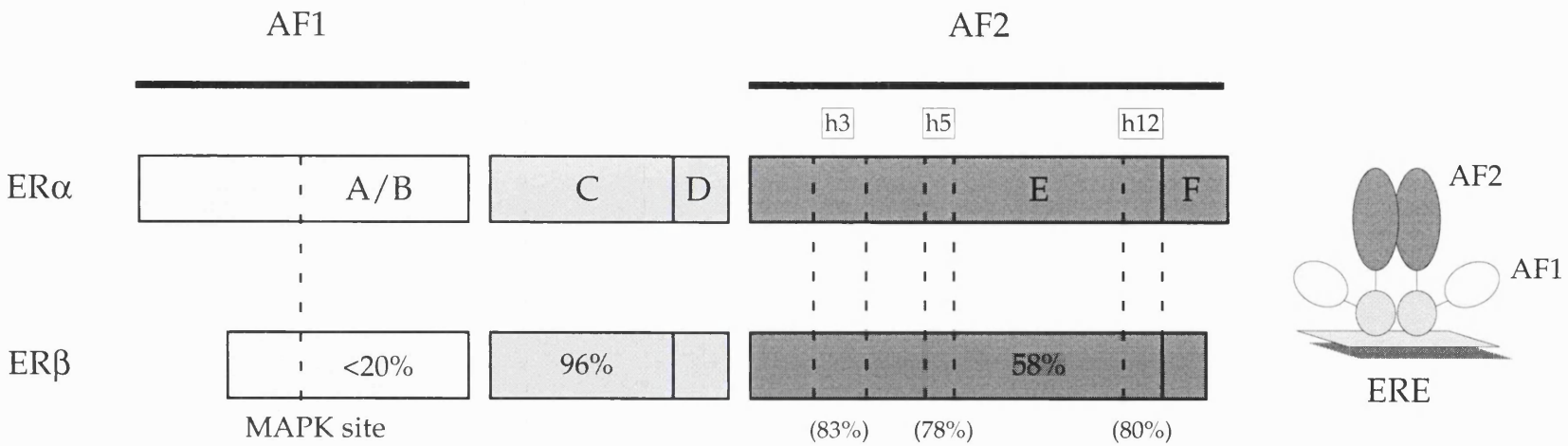


Fig. 5.1 - **Schematic representation of the activation domains of ERα and ERβ.** Oestrogen receptors (ER) have two activation domains: Activation function 1 (AF1) at the N-terminus and Activation Function 2 (AF2) at the C-terminus. The sequence conservation of AF1 and AF2 in ERα and ERβ is denoted as a percentage. The AF1 domain of ERα and ERβ both contain a mitogen activated protein kinase (MAPK) site reported to be essential for its transcriptional activity. Helices 3, 5 and 12 of the ERα ligand binding domain are likely to form the surface of AF2 required to bind coactivators, their position and relative homology to ERβ are also marked.

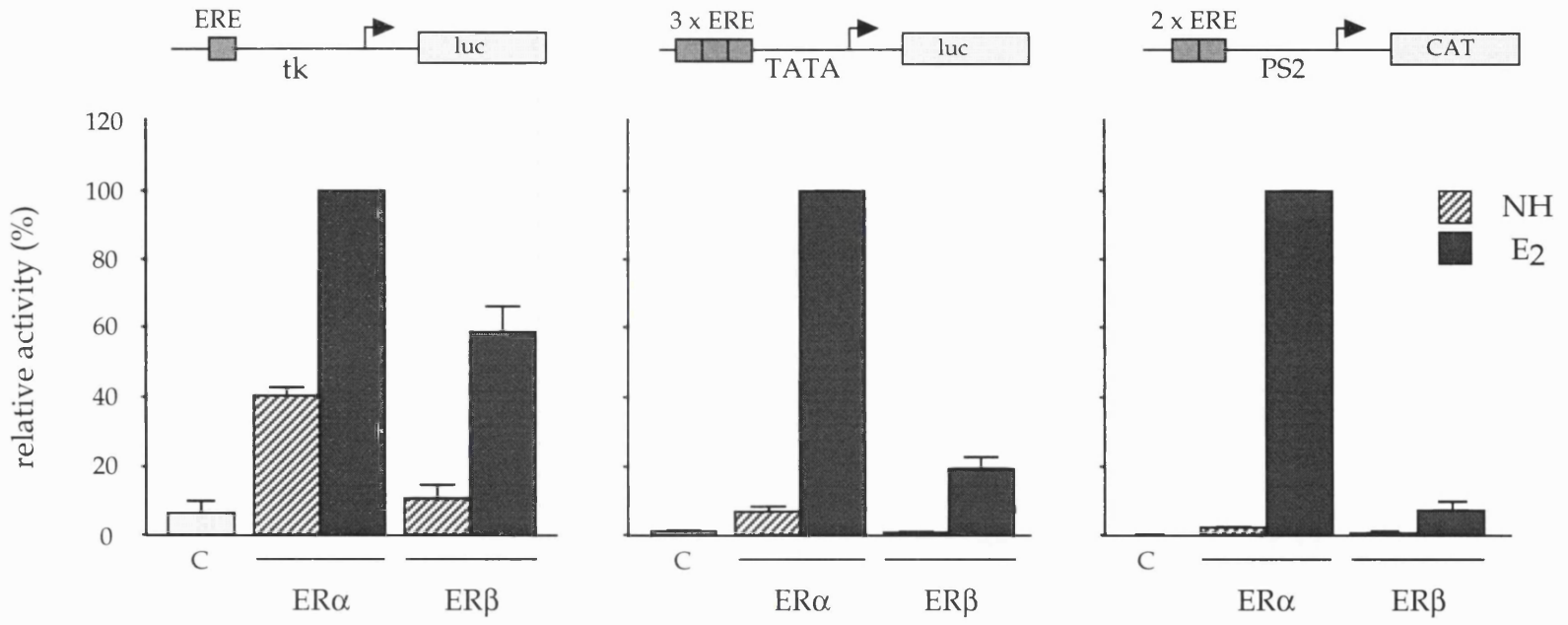


Fig. 5.2 - **Transcriptional activation of reporter genes by ERα and ERβ**. Transactivation by the human ERα and ERβ was tested by transiently transfecting chicken embryo fibroblasts (CEF) with pSG5HEGO or pSG5ERβ respectively, a reporter gene as indicated and pJ7*lacZ* internal control. Cells were treated for 24 hours in the presence or absence of $1x10^{-8}$ M 17β-oestradiol, harvested and the extracts tested for reporter activity. After correcting for transfection efficiency using the internal control, transcriptional activity from each reporter was determined relative to that of ERα in the presence of oestradiol, background activity (without transfected receptor) is denoted by C. The error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

Thus, ERα seems able to activate transcription from weak promoters much more effectively than ERβ.

Having discovered that ERβ activates transcription effectively on only a subset of promoters, we tested the ability of ERβ to stimulate transcription in different cell types. The relative transcriptional activities of ERα and ERβ were measured in HeLa, COS-1 and HepG2 cells using the ERE-TK-luc reporter, since this was the reporter on which ERβ had optimal activity (Fig. 5.3), at least in CEF cells. ERβ had approximately 50% the activity of ERα in HeLa cells, less than this in COS-1 cells, and little or no activity in HepG2 cells. ERα seems to have more activity than ERβ in all cell types tested, in no cell type have we observed ERβ to have more than half the activity of ERα.

What is the reason for these apparent differences in promoter and cell type specificity? The affinity of the ERβ homodimer for a consensus ERE is slightly lower than an ERα homodimer (Cowley *et al* 1997). This may explain why ERβ is only 50% as active as ERα, even on complex promoters, however the ERE remains the same in all experiments and so is unlikely to explain any differences in specificity. A more likely explanation is that differences in the activation domains of ERα and ERβ are responsible for their differential activity. We therefore decided to compare the AF1 and AF2 activities of ERα and ERβ, as components of the full length ER, or individually, when fused with the DNA binding domain of Gal4.

ERβ lacks a functional AF1 domain

We began the comparison of AF1 function by cotransfecting either ERα or ERβ with 2ERE-PS2-CAT, a reporter on which ERα has far more transcriptional activity than ERβ (Fig. 5.4). The activity of ERα is abolished by the removal of AF1 (ERα 182-599), demonstrating the requirement of AF1 on this reporter. The failure of ERβ to stimulate transcription from this reporter may be due to the absence of an AF1 activity equivalent to that found in ERα. The full activity of AF1 in ERα has been shown to depend on serine phosphorylation at a

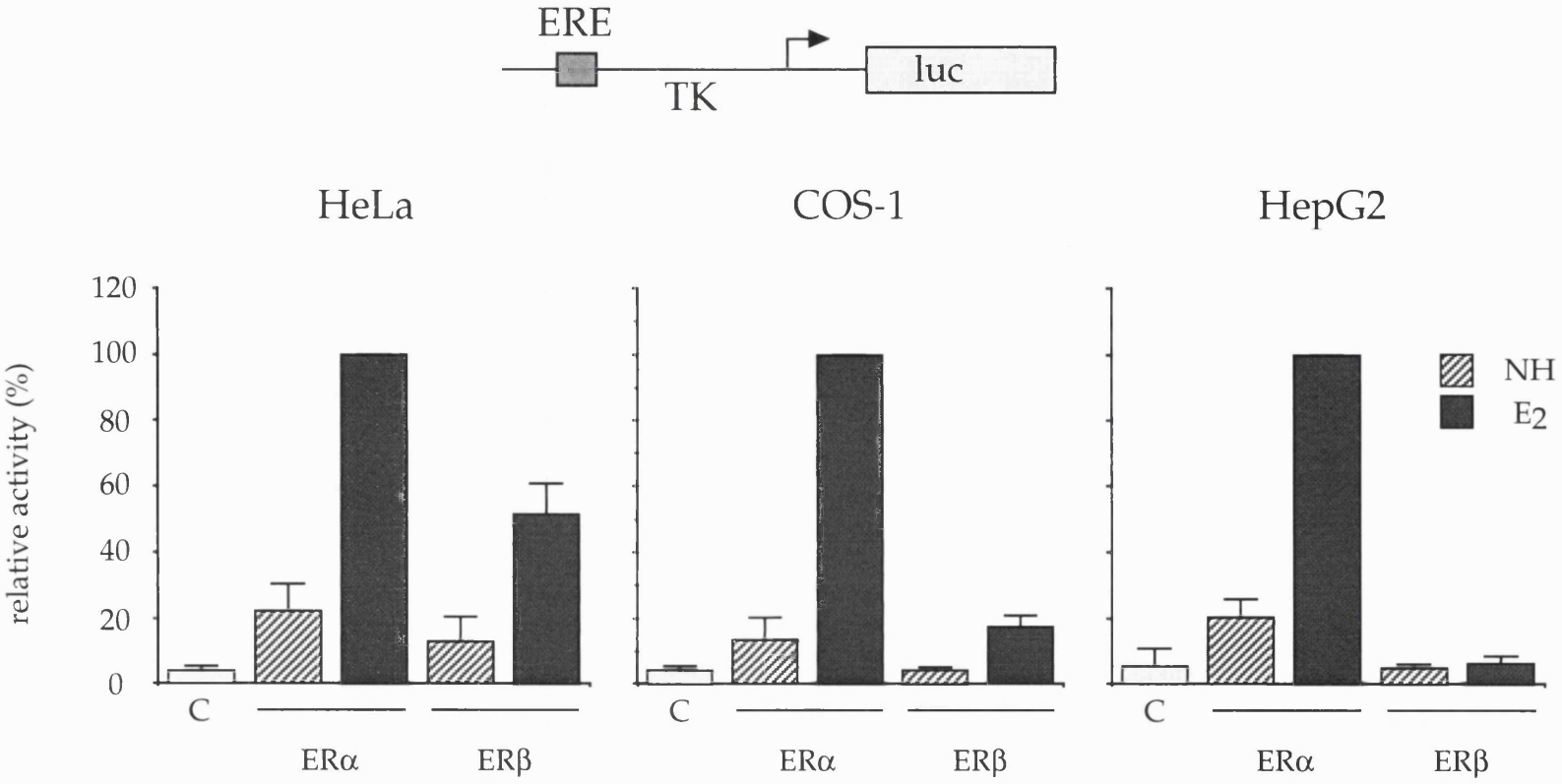


Fig. 5.3 - **Transcriptional activation by ERα and ERβ in different cell lines**. Transactivation by the human ERα and ERβ was tested by transiently transfecting HeLa, COS-1 and HepG2 cell lines with pSG5HEGO or pSG5ERβ respectively, ERE-TK-luc reporter gene and pJ7*lacZ* internal control. Cells were treated for 24 hours in the presence or absence of 17β-oestradiol (10^{-8} M), harvested and analysed as described in Fig. 5.2. Background activity (without transfected receptor) is denoted by C. The error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

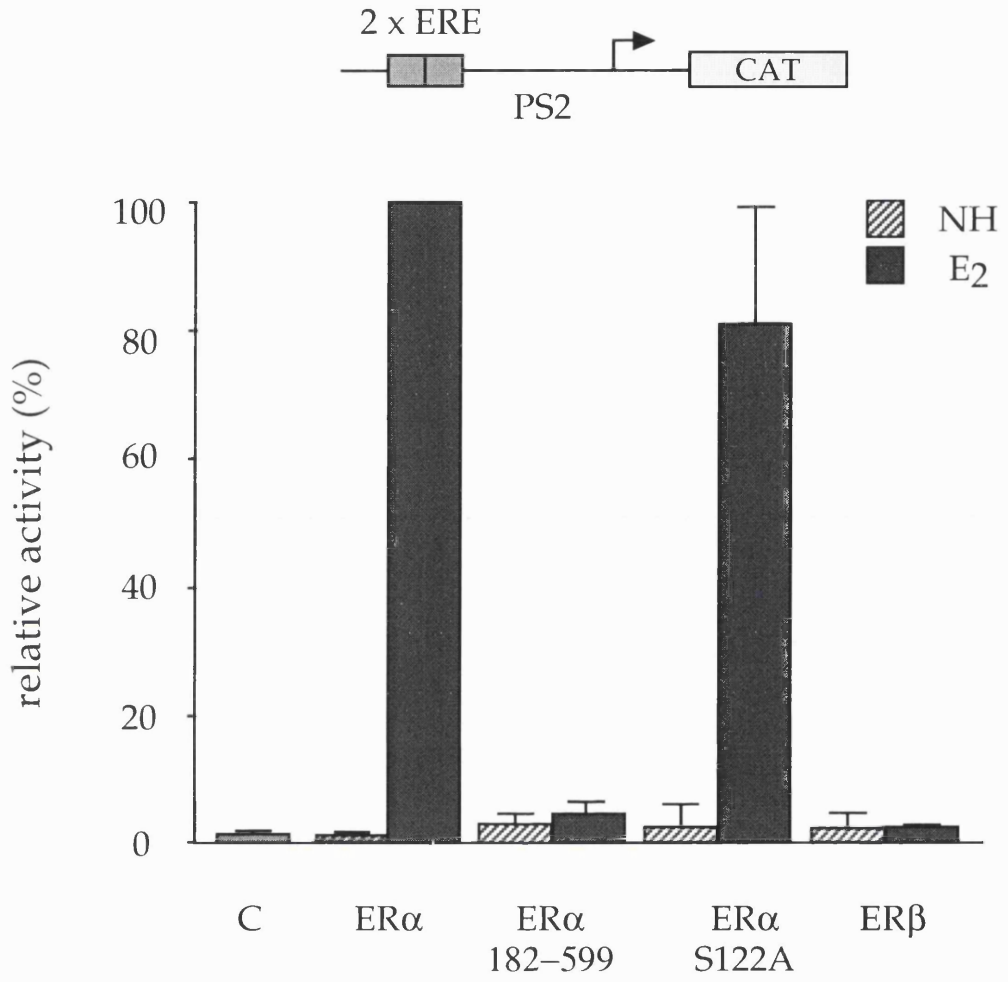


Fig. 5.4 - **ERα and ERβ differ in their AF1 activities**. The ability of ERα, ERβ, or mutant versions thereof, to stimulate transcription from 2ERE-PS2-CAT was determined in HeLa cells. Cells were treated for 24 hours in the absence or presence of 17β-oestradiol (10^{-8} M) as indicated and then harvested and analysed as described in Fig. 5.2. Background activity (without transfected receptor) is denoted by C and error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

mitogen activated protein kinase site (MAPK, Bunone *et al* 1996, Kato *et al* 1995), this site is conserved in ERβ. However, the MAP kinase site is not required for AF1 activity on this reporter since mutation of the phosphorylation site, Ser 122 to Ala in ERα, had no effect on transcriptional activity.

To compare the transcriptional activity of the isolated AF1 domains, HepG2 cells were transfected with the AF1 region from either ERα or ERβ fused to the DNA binding domain of Gal4 (Fig. 5.5). Gal4AF1α activated transcription strongly, the level of which increased proportionally with increasing amounts of transfected expression vector. Gal4AF1β activated transcription weakly in comparison to Gal4AF1α, with some slight activity observable only at the highest amount of transfected expression vector.

The inability of ERβ to stimulate transcription from an AF1 dependent reporter, in combination with the lack of transcriptional activity from Gal4AF1β, suggest that ERβ does not contain an AF1 activity comparable with that of ERα.

ERα and ERβ have a functionally similar AF2 domain

We next compared the transcriptional activity of the isolated AF2 domains of ERα and ERβ expressed as a fusion with the DNA binding domain of Gal4. Gal4AF2α and Gal4AF2β activated transcription to a similar extent and in an oestradiol dependent manner (Fig. 5.6). Two types of Gal4 reporter plasmid were used, identical, except that one has a simple TATA promoter (5xGalRE-TATA-luc) and the other is a TK based promoter (5xGal4RE-TK-luc). When using the 5xGalRE-TATA-luc reporter plasmid Gal4AF2β had approximately 50% the maximal activity of Gal4AF2α, whereas on the 5xGal4RE-TK-luc reporter Gal4AF2β had similar activity to Gal4AF2α. Although the reduction in activity is quite small, approximately 50%, it appears that the AF2 of ERβ is less efficient at stimulating transcription on weak promoters than that of ERα, while on strong promoters such as TK their activity is more or less the same.

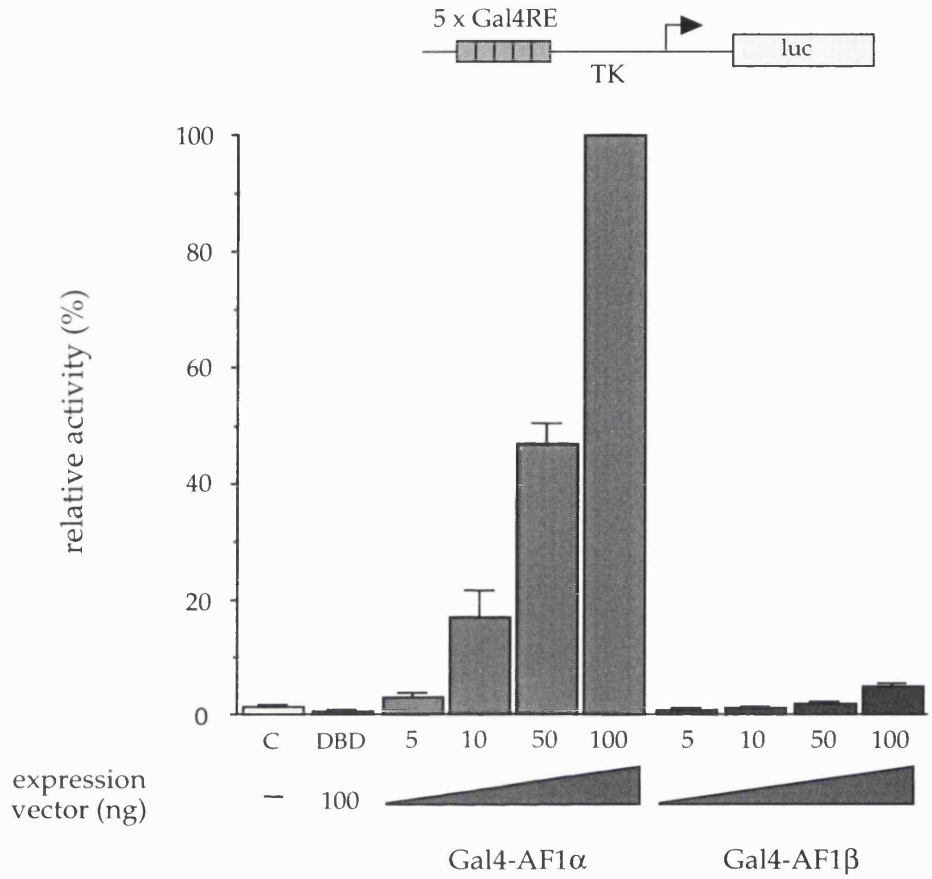
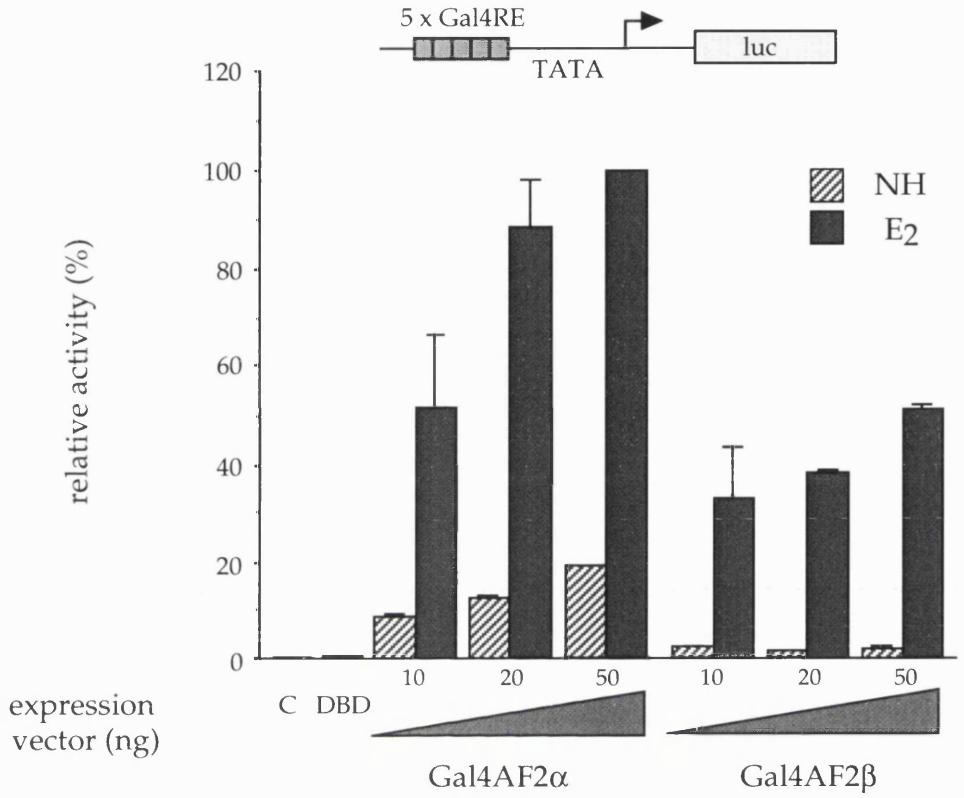


Fig. 5.5 - **ERα and ERβ differ in their AF1 activities**. The AF1 activity of ERα and ERβ was analysed by determining the ability of Gal4AF1α or Gal4AF1β to stimulate transcription from Gal4RE-TK-luc in HepG2 cells. Cells were harvested 48 hours after transfection and reporter activity measured in the extracts. After correcting for transfection efficiency using the internal control the relative reporter activity was determined. As a control the activity derived from Gal4 DNA binding was measured (DBD), background activity (without transfected receptor) is denoted by C. Error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

Fig. 5.6 - **ERα and ERβ have similar AF2 activities.** AF2 activity of ERα and ERβ was analysed by testing the ability of Gal4-AF2α or Gal4-AF2β to stimulate transcription from either 5Gal4RE-TATA-luc or 5Gal4RE-TK-luc as indicated in HeLa cells. Cells were treated for 24 hours with or without 17β-oestradiol (10^{-8} M), harvested and the extracts tested for reporter activity. Values for reporter activity were corrected for transfection efficiency using the internal control. Background activity (without transfected receptor) is denoted by C and error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.


5 x Gal4RE
TATA
luc
120
100
80
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relative activity (%)
NH
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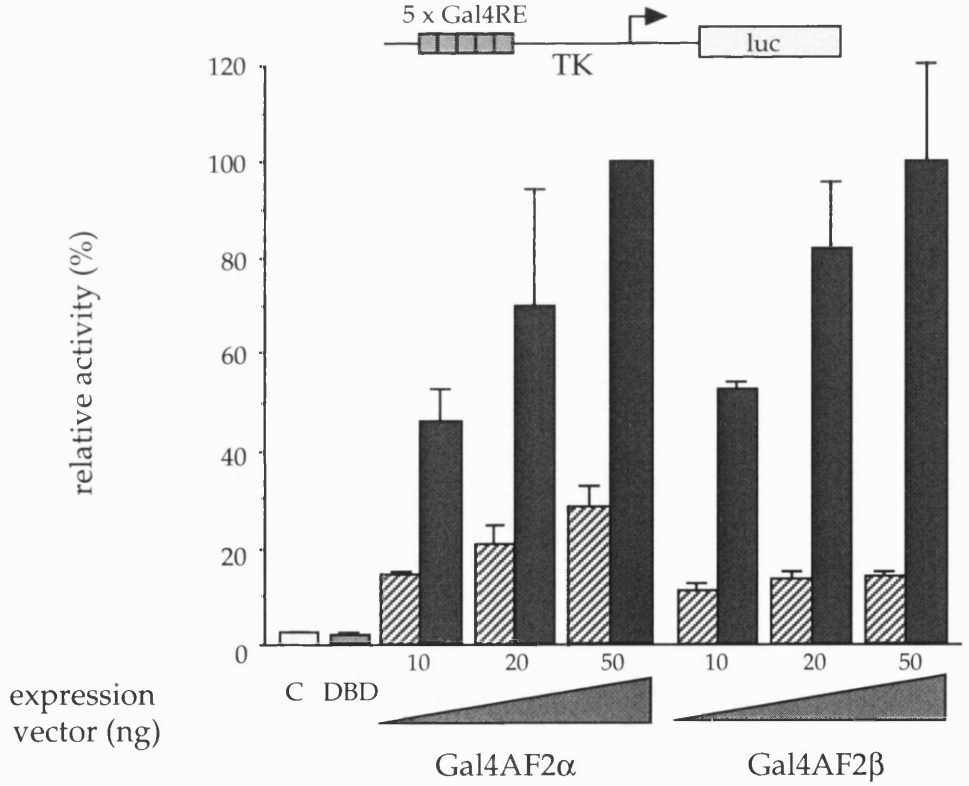
5 x Gal4RE
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10
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C
DBD
expression vector (ng)
Gal4AF2α
Gal4AF2β

We next tested the ability of Gal4AF2α and Gal4AF2β to interact with SRC1 whilst bound to DNA (Fig. 5.7). COS-1 cell extracts expressing Gal4AF2α and Gal4AF2β were used in a gelshift assay with a [^{32}P]-labelled consensus Gal4 response element (Gal4RE). Gal4AF2α and Gal4AF2β have approximately the same DNA binding activity. The Gal4AF2/Gal4RE complex was then 'super-shifted' by the addition of purified GST-SRC1(570-780), a region of SRC1 known to interact with nuclear receptors in a ligand dependent manner (Kalkhoven *et al* 1998). Titrating in an increasing amount of GST-SRC1(570-780) resulted in similar amounts of 'super-shifted' complex for both Gal4AF2α and Gal4AF2β, suggesting that they have a similar affinity for coactivators.

To test the interaction of the AF2 domains with full length coactivators, GST fusions of AF2α and AF2β were constructed (GST-AF2α and GST-AF2β) and used in a pulldown assay with *in vitro* translated SRC1e (Fig. 5.8). The interaction of SRC1e with both GST-AF2α and GST-AF2β was dependent on the addition of oestradiol, there was no interaction at all in the absence of ligand or in the presence of the antioestrogens: 4OH-tamoxifen and ICI 182780.

The interaction of ERα with SRC1 is unaffected by salt concentration (Kalkhoven *et al* 1998), suggesting that hydrophobic interactions form the major contacts between nuclear receptors and coactivators. We therefore wanted to test whether this was also true for ERβ. The interaction of GST-SRC1(570-780) with *in vitro* translated ERα and ERβ at various salt concentrations was tested in a pulldown assay (Fig. 5.9). The concentration of KCl regularly used in a pulldown assay is 200 mM, below this concentration there is an appreciable oestradiol-independent interaction of GST-SRC1(570-780) with ERα and ERβ. Increasing the KCl concentration from 200 mM to 500 mM had little effect on the interaction; not until the KCl concentration was increased to 1000 mM was there a slight reduction in the binding of ERα, while that of ERβ was still unaffected, even at the highest salt concentration of 1500 mM.

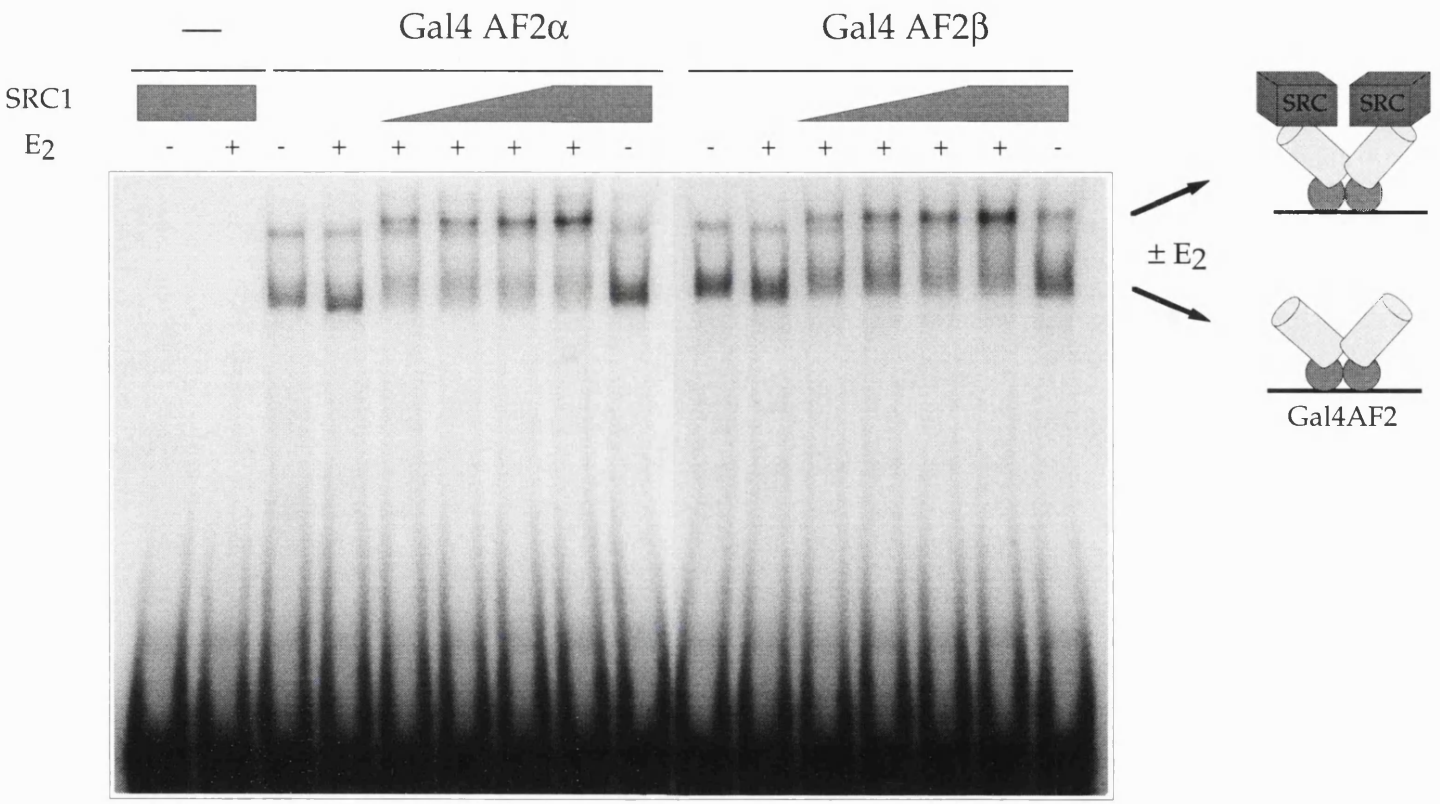


Fig. 5.7 - **The ligand binding domains of ERα and ERβ bind SRC1 similarly**. COS-1 cell extracts expressing either Gal4AF2α or Gal4AF2β were prebound to a [^{32}P]-labelled Gal4RE and incubated with increasing amounts of GST-SRC1(570-780) in the presence or absence of 17β-oestradiol (2.5×10^{-7} M) as indicated. The predicted positions of Gal4AF2-DNA and SRC1 bound complexes are shown on the right hand side.

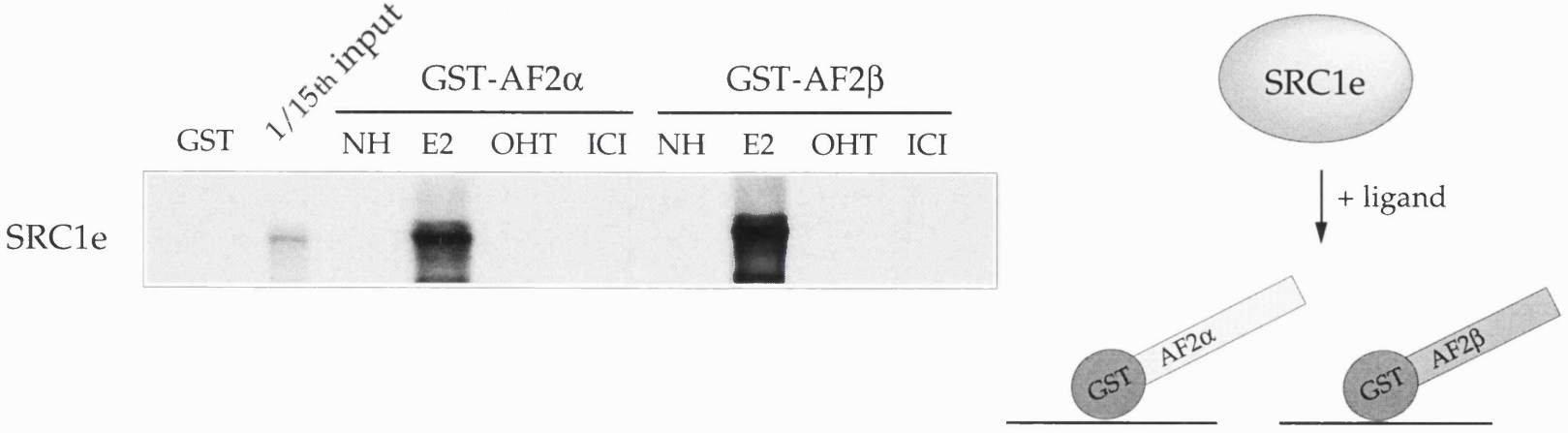


Fig. 5.8 - **The ligand binding domains of ERα and ERβ bind SRC1 similarly**. Binding of [^{35}S]-methionine labelled SRC1e with GST, GST-AF2α or GST-AF2β was analysed in the presence or absence of 17β-oestradiol (10^{-6} M), 4OH-Tamoxifen (10^{-6} M) or ICI 182780 (10^{-6} M) as indicated. Bound SRC1 was eluted and analysed by SDS-PAGE followed by autoradiography.

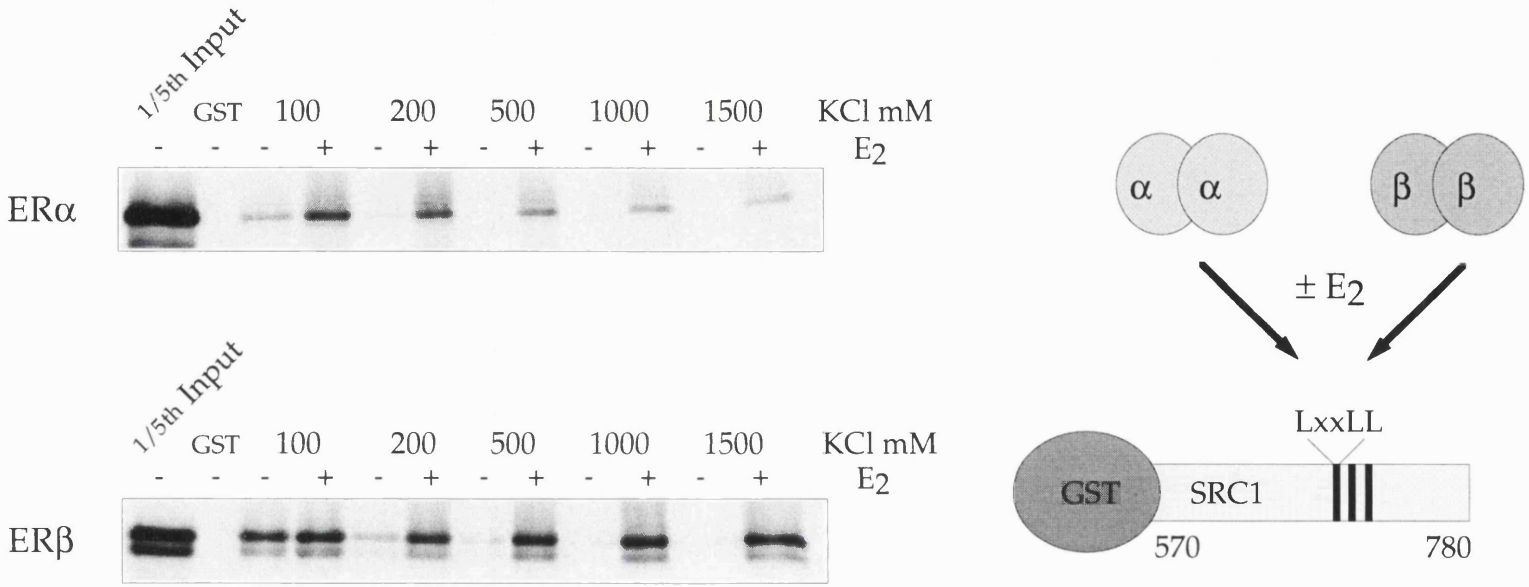


Fig. 5.9 - **Salt concentration does not effect the interaction of SRC1 with ERα and ERβ.** The ability of [^{35}S]-methionine labelled ERα and ERβ to bind GST-SRC1(570-780) at increasing salt concentration was tested in a pulldown assay, in the presence or absence of 17β-oestradiol (10^{-6} M) as indicated. Bound ERα and ERβ was eluted and analysed by SDS-PAGE followed by autoradiography.

From these experiments we conclude that the AF2 domains of ERα and ERβ are functionally similar. They activate transcription to approximately the same level and bind coactivators with a similar specificity and affinity; suggesting that the surface of AF2 which binds coactivators is conserved in both receptors.

Differential effects of mutations in helix 12 of ERα and ERβ

From mutational analysis studies (Danielian *et al* 1992, Henttu *et al* 1997) and the x-ray crystal structure (Brzozowski *et al* 1997) of the ERα ligand binding domain, we predict that the coactivator binding surface is likely to consist of helices 3, 12 and possibly from its position nearby, helix 5. A sequence comparison of ERα and ERβ shows that these three helices are well conserved, particularly helix 12. In the absence of ligand helix 12 protrudes from the core of the ligand binding domain, creating an unfavourable surface for the binding of coactivators (Bourguet *et al* 1995). Upon ligand binding helix 12 realigns (Brzozowski *et al* 1997, Renaud *et al* 1995), adopting a favourable surface for coactivator recruitment (in conjunction with helices 3 and 5), thus facilitating ligand dependent transcriptional activation. Furthermore, misalignment of helix 12 has been implicated as the molecular basis for antagonism by the antioestrogen, Raloxifene (Brzozowski *et al* 1997). Given the crucial role of helix 12 we decided to make two mutations of conserved residues within this helix in ERβ.

We made the following substitutions in helix 12 of ERβ: E493A and M494A/L495A. These residues are conserved not only in helix 12 of ERα (Fig. 5.10), but many other nuclear receptors (Danielian *et al* 1992). Their mutation in ERα proved to be detrimental to transcriptional activation (Danielian *et al* 1992, and Fig. 5.10), E546A reduces maximal transcription to approximately 60% of wild-type, while M547A/L548A reduces activity to less than 10%. ERβ M494A/L495A has little or no activity, similar to ERα. In contrast, ERβ E493A has much lower activity compared to wild-type (<20%) than its ERα counter-part E546A. The DNA and ligand binding properties of the ERβ mutants was tested (Fig. 5.11) and found to be similar to those of wild-type ERα.

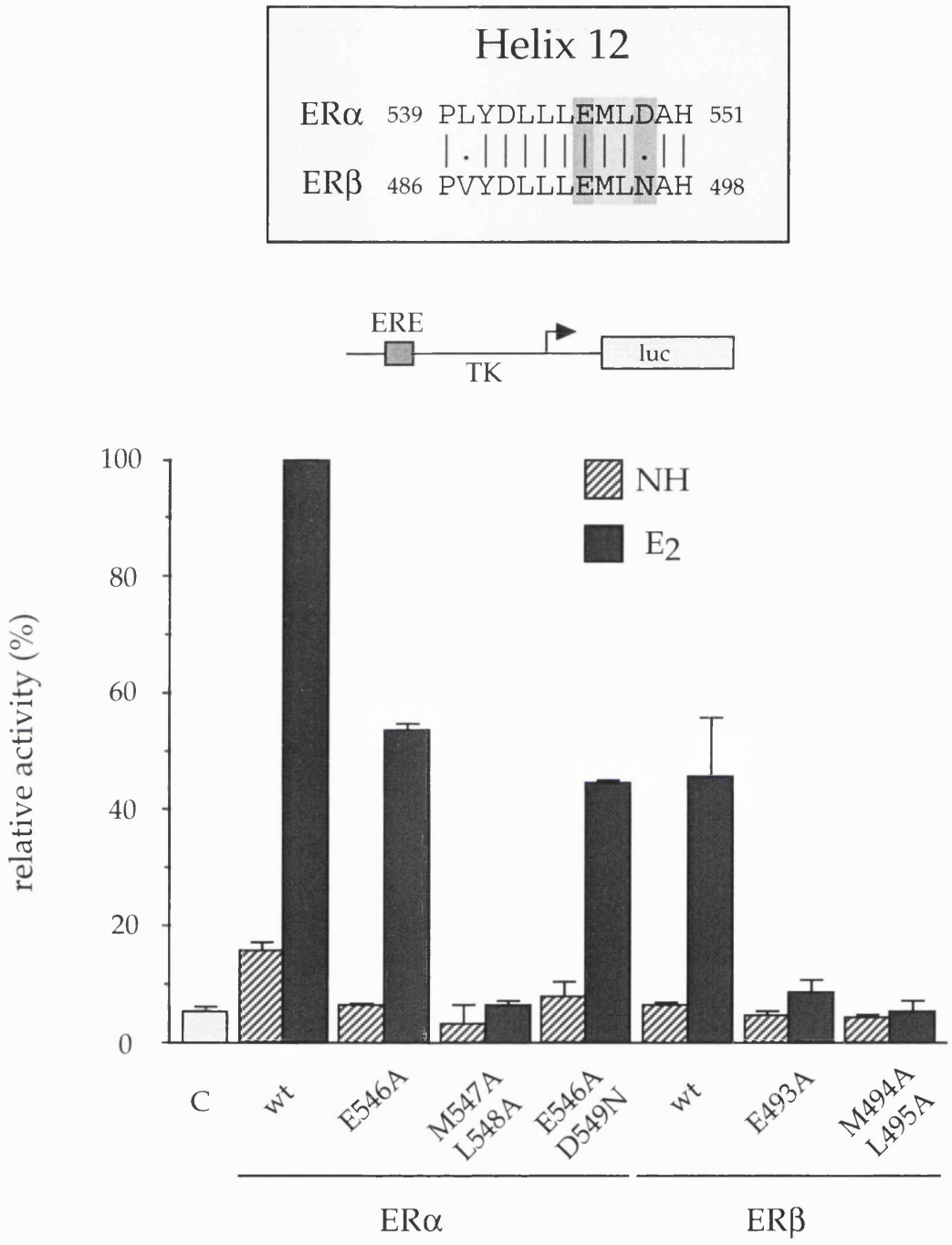


Fig. 5.10 - **Mutations in helix 12 of ERα and ERβ differentially effect their ability to activate transcription**. HeLa cells were transiently transfected with ERα, ERβ or mutant versions thereof. Cells were treated as shown in Fig. 4.2 and after correction for transfection efficiency using the internal control, relative reporter activities are presented. Background activity (without transfected receptor) is denoted by C. Error bars represent the standard deviation of values from at least two separate experiments performed in duplicate. A sequence comparison of helix 12 from ERα and ERβ is shown above, mutated residues are shaded.

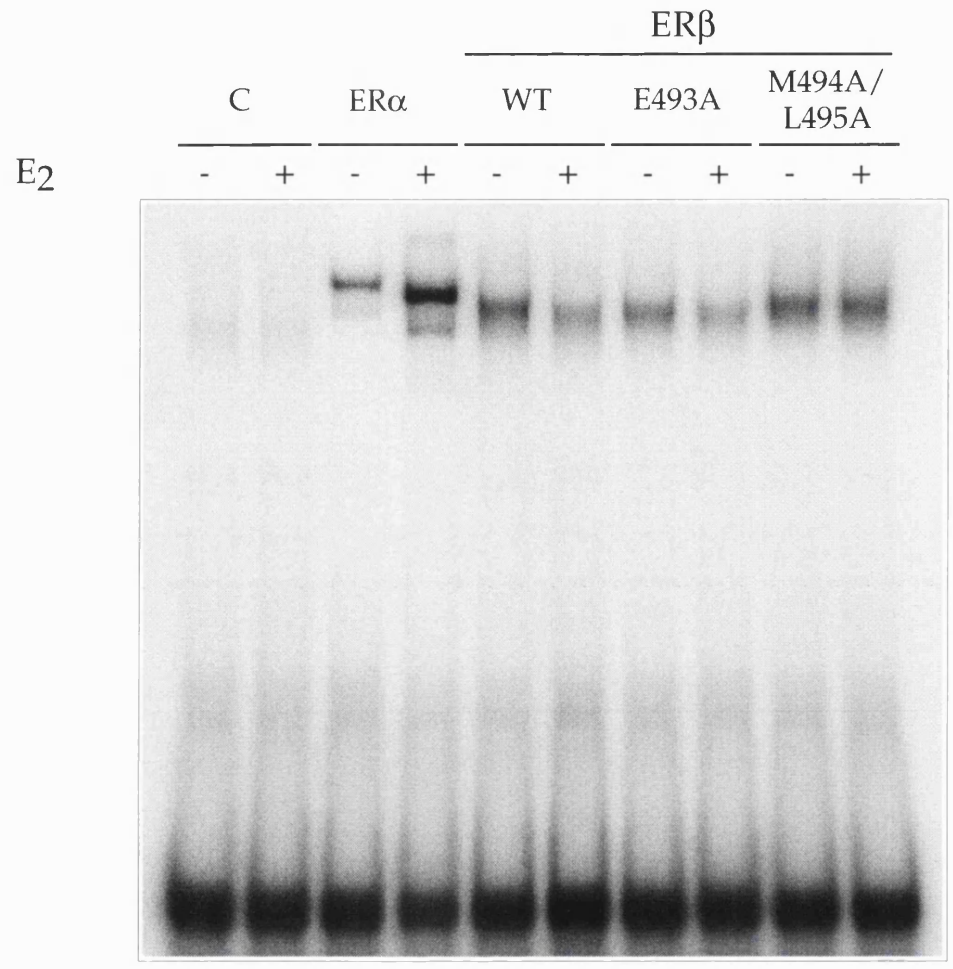


Fig 5.11 - **ERβ mutant receptors bind DNA and Oestradiol with similar affinities as the wild-type receptor**. Wild type ERα, ERβ and mutant forms of the full length ERβ were transiently expressed in Cos-1 cells. Equal amounts of receptor were analysed for DNA binding activity in a gelshift assay using a [^{32}P]-labelled oligonucleotide containing a single oestrogen response element. Prior to electrophoresis the samples were treated with either ethanol vehicle alone, or oestradiol (E2) at 1 x 10^{-7} M. Protein-DNA complexes were resolved on 6% polyacrylamide gels and detected by autoradiography. The same extracts were also tested for their ability to bind [^{3}H]-labelled oestradiol at 1 nM relative to wild-type ERβ.

A similar but clearer result was observed when the same AF2 mutations were transferred to the DNA binding domain of Gal4, using conditions in which wild-type Gal4AF2α and GalAF2β have similar transcriptional activity (Fig. 5.12). Gal4AF2α containing the E546A mutation has ~60% wild-type activity, whereas Gal4AF2β E493A has ~10%. The DNA binding of the Gal4AF2 fusion proteins was tested and found to be similar (Fig. 5.13).

Why does point mutation of the conserved Glu residue in helix 12 have a greater deleterious effect in ERβ than in ERα? One explanation may be that in helix 12 of ERα, E546 is one of three acidic residues: Asp 542, Glu 546 and Asp 549. The third of which, Asp 549 is not present in ERβ where the residue is an Asn (Fig. 5.10). Mutation of two or three of these residues together in ERα results in a further reduction of activity, more apparent in the absence of AF1 (Danielian *et al* 1992). Since ERβ lacks a third acidic residue, E493A is equivalent to an E546A/D549N mutant of ERα. We constructed ERα E546A/D549N to see whether the loss of the third acidic residue could account for the reduced transcriptional activity observed with ERβ E493A. As a mutation of full length ERα, or Gal4AF2α fusion the double mutation of E546A/D549N had approximately the same transcriptional activity as the point mutation of E546A alone, and much more than ERβ E493A (Fig. 5.10 and 5.12). Mutation of D549N in ERα does not have an additive effect to the mutation of E546A, ruling out the possibility that it is the lack of a third acidic residue in the helix 12 of ERβ which makes the mutation of E493A so much more deleterious than the equivalent mutation in ERα. Since the rest of helix 12 is identical between ERα and ERβ, its possible that variations in the residues of helices 3 and 5 which make ERβ more susceptible to the loss of the conserved Glu in helix 12.

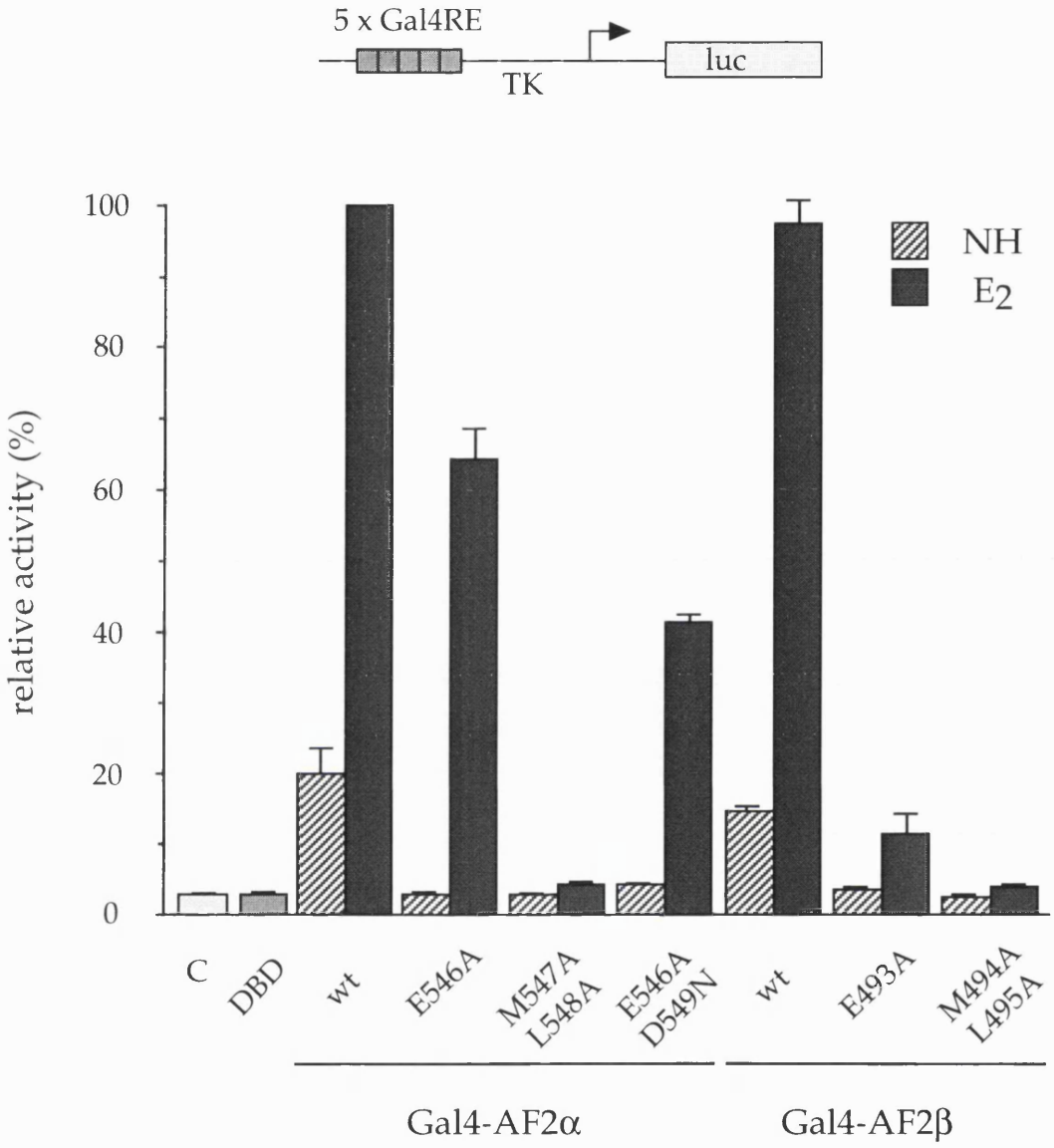


Fig. 5.12 - **Mutations in helix 12 of Gal4AF2α and Gal4AF2β differentially effect their ability to activate transcription.** HeLa cells were transiently transfected with Gal4-AF2 wild-type or mutant versions thereof. Cells were treated as shown in Fig. 5.2 and after correction for transfection efficiency using the internal control relative reporter activities are presented. Background activity (without transfected receptor) is denoted by C and error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

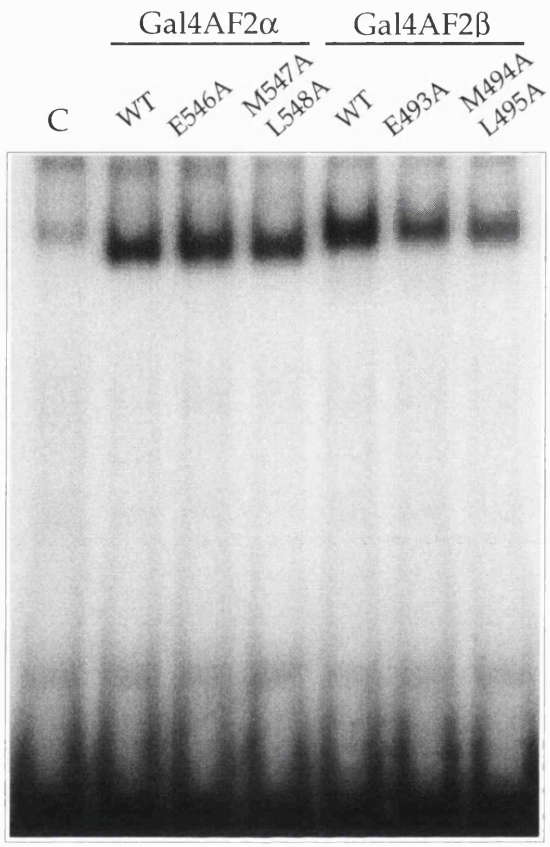


Fig. 5.13 - **Mutant and wild-type Gal4AF2 proteins bind DNA with similar affinities**. Equal amounts of COS-1 extracts containing wild type Gal4AF2 or mutant version thereof were analysed for DNA binding activity in a gelshift assay using a [^{32}P]-labelled Gal4 response element. Protein-DNA complexes were resolved on 6% polyacrylamide gels and detected by autoradiography

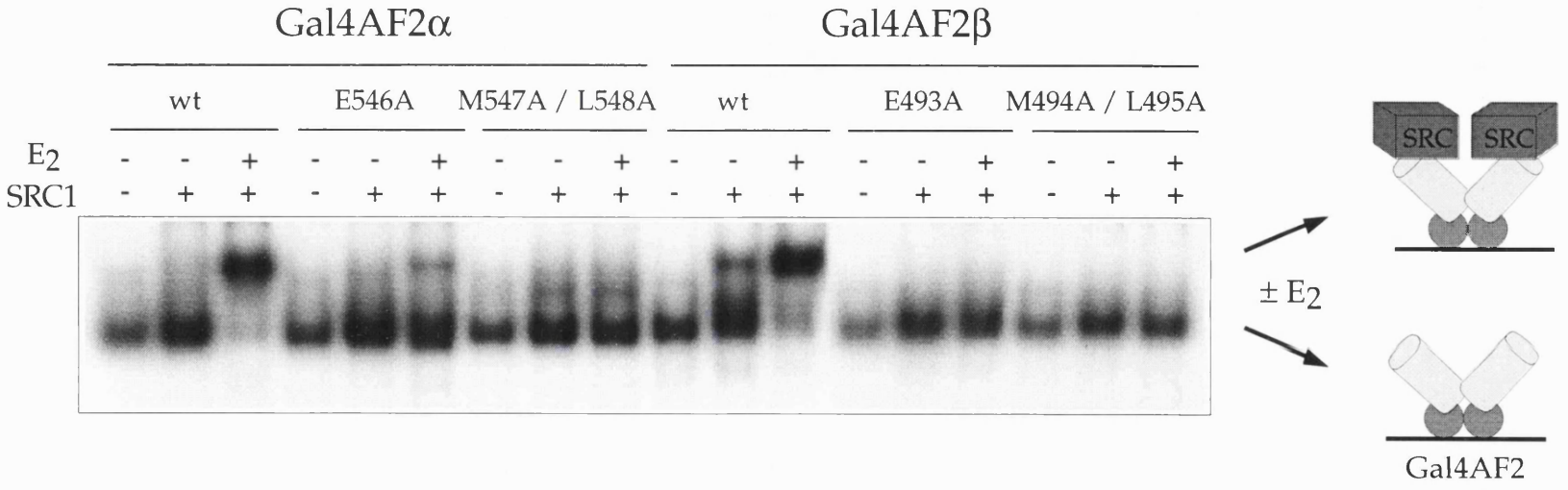


Fig. 5.14 - **Mutations in helix 12 of ERα and ERβ differentially effect their ability to bind SRC1**. COS-1 cell extracts, expressing either Gal4AF2α or Gal4AF2β, or mutant versions thereof, were prebound to a consensus Gal4 response element and incubated with GST-SRC1(570-780) in the presence or absence of oestradiol (2.5 x 10-7 M). The predicted position of DNA-bound complexes is shown on the right hand side.

Interaction of ERα and ERβ helix 12 mutants with SRC1

The differential ability of the Gal4AF2 helix 12 mutants to activate transcription might be explained by their capacity to bind coactivators. We therefore compared the Gal4AF2 mutants ability to activate transcription with coactivator binding. The Gal4AF2 fusion proteins expressed in COS-1 cells were pre-bound to a [^{32}P]-labelled Gal4RE and their ability to bind GST-SRC1(570-780) tested (Fig. 5.14). Gal4AF2α wild-type interacted with SRC1 in a strict oestradiol dependent manner (lanes 1-3), whereas there was an appreciable oestradiol-independent interaction between Gal4AF2β and SRC1; however, this interaction was greatly increased by the addition of oestradiol (lanes 10-12). The apparent difference in transcriptional activity between the analogous mutants Gal4AF2α E546A and Gal4AF2β E493A can be explained by coactivator binding, since ERβ E493A does not bind SRC1, while ERα E546A retains some ability to interact with SRC1 (compare lanes 4-6 with 13-15). The double mutants, Gal4AF2α M547A/L548A and Gal4AF2β M494A/L495A neither of which has much transcriptional activity, did not bind to SRC1, thus demonstrating a good overall correlation between SRC1 binding and transcriptional activity.

Summary and Conclusions

A comparison of transcriptional activation by ERα and ERβ revealed that ERβ had much less activity than ERα on weak promoters and in HepG2 cells, possibly due to the lack of a functional AF1 activity. The AF2 domains of ERα and ERβ stimulate transcription to a similar extent, although AF2α seems better at doing so on weak promoters than AF2β. This, plus the difference in transcriptional activity between ERα E546A and ERβ E493A, suggests that although the surface of AF2 required to bind coactivators is very similar in the two receptors, it is not identical.

Chapter 6

Discussion

The cloning of a second oestrogen receptor

For a decade after the cloning of the oestrogen receptor (ER) it was generally assumed that only one ER existed. This contrasted with other members of the nuclear receptor superfamily which have multiple forms *e.g.* TRα/β and RARα/β/γ (reviewed by Gronemeyer and Laudet 1995). The isolation of a second form of the ER, termed ERβ, therefore surprised a nuclear receptor field used to thinking in terms of a single ER. ERβ was isolated initially from rat prostate (Kuiper *et al* 1996) and human testis (Mosselman *et al* 1996), tissues not generally considered to be major targets of oestrogen signalling. The abundance of ERβ in non-classical oestrogen target tissues may explain why it eluded researchers for so many years.

The discovery of a second ER has led to the revaluation of results obtained over the previous decade, in addition to raising many new questions. The ERα knock-out (ERKO) mouse for instance was realised to contain wild-type ERβ, possibly explaining why the mice were viable, against expectation (Lubahn *et al* 1993). However, even the presence of ERβ was not able to fully compensate for the inactivation of the ERα gene. ERKO mice of both sexes are infertile (Hess *et al* 1997, Lubahn *et al* 1993), suggesting that either ERβ regulates a distinct (although possibly overlapping) subset of genes to ERα, or that their pattern of expression is different.

A sequence comparison of human ERα and ERβ reveals that they are highly homologous, particularly in the DNA binding and ligand binding domains (96% and 58% amino acid identity respectively, see Fig. 6.1). The similarity of these functional domains is represented in their similar ligand binding properties (Kuiper *et al* 1997) and ligand-dependent transcriptional activity (Kuiper *et al* 1996, Mosselman *et al* 1996, Tremblay *et al* 1997). However, their N-terminal domains which encompass activation function 1 are quite different, suggesting that their transcriptional activity on a subset of oestrogen responsive genes may be different.

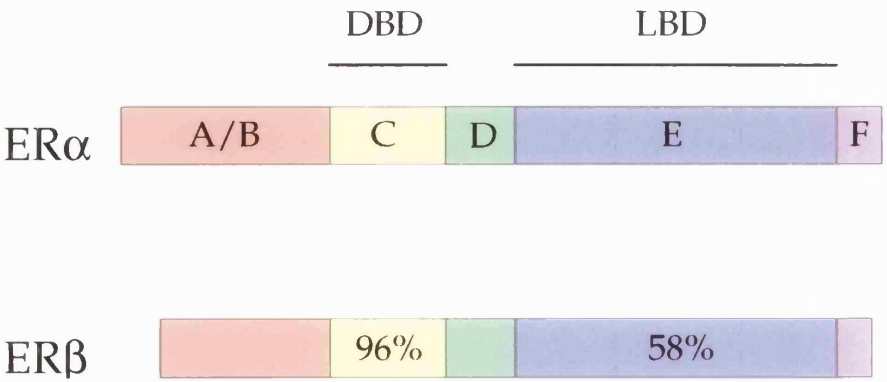
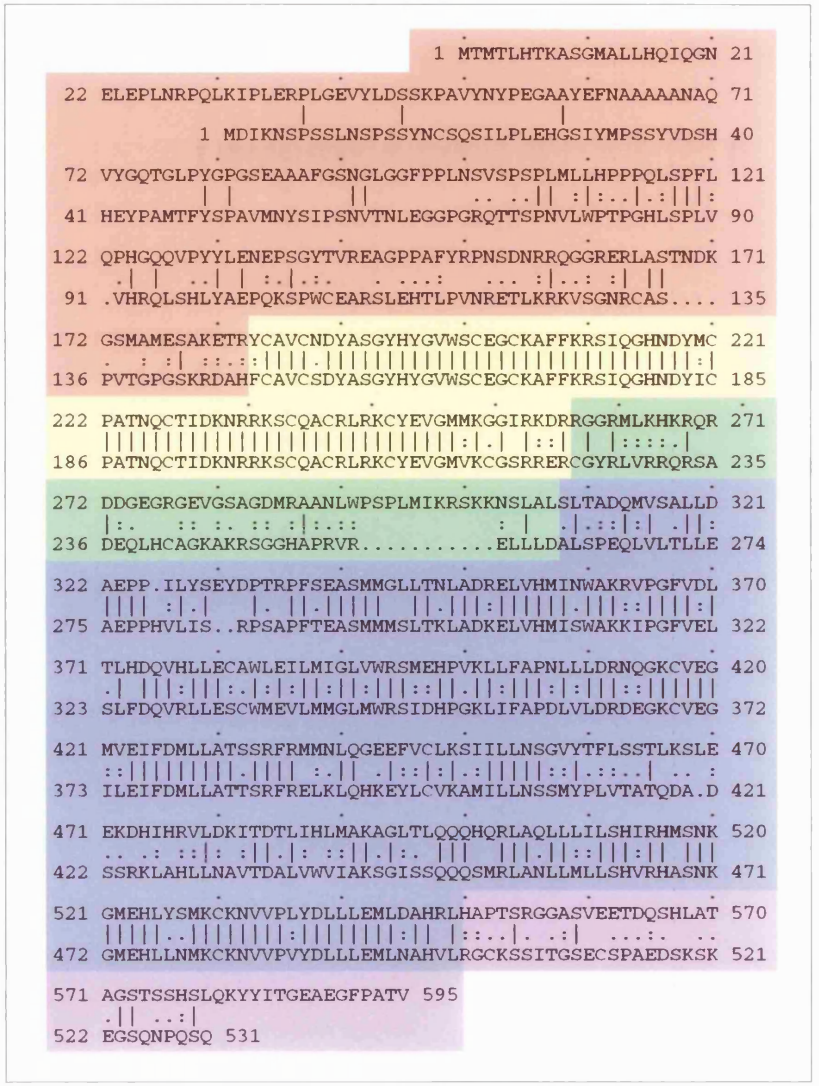


Fig. 6.1 - **Sequence alignment of ERα and ERβ**. Primary sequence alignment of the two human oestrogen receptors (ER). The ER can be subdivided into regions A-F, the boundaries of which are outlined above. Region C, contains the DNA binding domain (DBD), while region E, encompasses the ligand binding domain (LBD). Conservation of these functional domains is denoted as a percentage of amino acid identity. The short form of ERβ, first published by Mosselman *et al* (1996) begins at Met 53.



Do ERα and ERβ function independently, or together? Since ERβ retains many of the functional properties of ERα, much will depend on their relative expression. Specific antibodies for the monitoring of ERβ proteins will be of paramount importance for a precise comparison of ERα and ERβ expression and also for resolving which form of ERβ exists in cells. Two ERβ cDNAs have been isolated, which differ in the length of their N-terminal domains. The longest human ERβ clone contains 530 amino acids (Ogawa *et al* 1998, S. Mosselman - Organon), 53 amino acids more than the cDNA originally published by Mosselman *et al* (Mosselman *et al* 1996). Its possible that both forms of ERβ could exist in cells, although definitive proof, or what functional significance this has remains to be determined.

ERα and ERβ form heterodimers on DNA

After the cloning of ERβ, there was much speculation as to whether the two ERs would heterodimerise. In general class I nuclear receptors bind to DNA as homodimers, with the odd example of heterodimers, such as GR and MR (Trapp *et al* 1994). In contrast, class II receptors form obligate heterodimers with RXR (reviewed by Parker 1993). A comparison of their respective ligand binding domains reveals that the residues identified by Fawell *et al* (1990) which were required for ERα homodimerisation are also conserved in ERβ, as is the majority of helix 11. It therefore seemed probable that they would heterodimerise; and in fact they do, expressed both *in vitro* and *in vivo* (Figs. 4.2 and 4.4). Whole cell extracts containing ERα and ERβ used in gelshifts resulted in a shifted complex with an intermediate mobility to that of ERα and ERβ homodimers, corresponding to ERα/ERβ heterodimers. Addition of the ERα specific monoclonal antibody H226, super-shifted the two upper bands consistent with the presence of ERα in both complexes. The upper complex, corresponding to ERα homodimers has a larger super-shift than the heterodimer, probably due to the presence of two epitopes in the homodimer whereas there is only one in the ERα/ERβ heterodimer. When extracts containing equal amounts of ERα and ERβ were used in gelshift experiments, we observed ERα homodimers and ERα/ERβ heterodimers, but not ERβ homodimers. If

ERα and ERβ had the same DNA binding affinity, when expressed at a 1:1 ratio we would expect to see a 1:2:1 ratio of homodimer/heterodimer/homodimer complexes, similar to those observed for wild-type and a truncated ERα (Fig. 4.6), that we do not suggests that ERβ homodimers have a lower DNA binding affinity than those of ERα. In addition, ERβ homodimers gave a weaker, more diffuse complex in gelshift experiments than ERα homodimers. When we measured the DNA binding affinities of the ERα, ERβ homodimers and ERα/ERβ heterodimers, it was found that ERα homodimers and ERα/ERβ heterodimers had a similar affinity for an ERE with a k_d of 2 nM, whereas the affinity of ERβ homodimers was approximately four times lower (k_d ~9 nM). A qualitative reduction in the DNA binding capacity of ERβ homodimers compared to those of ERα, was also observed by Tremblay *et al* (1997).

The reason for the reduced DNA binding activity of ERβ homodimers is not immediately obvious, because the gelshift assay does not distinguish differences in DNA binding and dimerisation. The sequence of the ERα and ERβ DNA binding domains (DBDs) differ by only two residues; this high degree of homology allowed us to examine their position using the 3D structure of the ERα-DBD bound to DNA (Schwabe *et al* 1993). The first difference, Asn 190 in ERα is a Ser in ERβ and is positioned in the loop of the first zinc binding-motif, the second, a conservative change from Met 220 in ERα to an Ile in ERβ, is adjacent to the first Cys in the second zinc binding-motif. Neither residue is in a position to contact DNA or form part of the hydrophobic core, however Met 220 may participate in the dimer interface via an ordered water molecule. It seems unlikely that either of the these changes is responsible for the reduced DNA binding activity of ERβ homodimers. Furthermore, both DBDs are required for the ERα/ERβ heterodimer to bind an ERE (Pettersson *et al* 1997), since ERα/ERβ heterodimers have the same affinity for an ERE as ERα homodimers, this suggests that the DBDs of ERα and ERβ are equivalent. A more likely explanation for the reduced DNA binding of ERβ homodimers, is a reduced dimerisation capacity in comparison to that of ERα homodimers and ERα/ERβ heterodimers. We thus examined the ERβ dimer interface by testing its ability to heterodimerise with the series of ERα dimerisation mutants characterised by Fawell *et al* (1990, Fig. 4.5). Consistent with its inability

to homodimerise, ERα R507A was also unable to heterodimerise with ERβ, in contrast ERα L511R and I518R were able to form weak heterodimers with ERβ. The ability of ERα homodimerisation deficient mutants to heterodimerise with ERβ suggests that although the dimer interfaces are similar they are not identical, and that these differences may account for the reduction in DNA binding of ERβ homodimers.

ERα/ERβ heterodimers stimulate transcription

We next attempted to ascertain whether the ERα/ERβ heterodimers were transcriptionally active. ERβ had previously been shown to stimulate transcription in a ligand dependent manner (Kuiper *et al* 1996, Mosselman *et al* 1996) and was therefore unlikely to act as a dominant negative component in the ERα/ERβ heterodimer. We tested the ability of ERα and ERβ to stimulate transcription from a reporter gene separately or together, at ratios of expression plasmid which gave predominantly ERα/ERβ heterodimers in gelshifts (Fig. 4.4). COS-1 and HeLa cells cotransfected with ERα and ERβ had a level of activity intermediate to that of ERα and ERβ alone. Although it is impossible to rule-out the existence of ERα and ERβ homodimers in the cotransfected cells, if the majority of ER species is ERα/ERβ heterodimer, it must be transcriptionally active otherwise we would expect to see a much lower level of transcriptional activity. To support this conclusion we were able to show that ERα/ERβ heterodimers bound SRC1 in a ligand dependent manner comparable to that of ERα and ERβ homodimers, demonstrating that the activation domains of the heterodimer have a competent surface for the recruitment of coactivators and are therefore very likely to activate transcription.

What is the functional significance of ERα/ERβ heterodimers?

In general nuclear receptors heterodimerise to increase binding affinity and specificity for their respective ligands and DNA. In the case of ERα/ERβ heterodimers however, both forms of the ER have a similar ligand affinity and specificity (Kuiper *et al* 1997), while the ERα/ERβ

heterodimer has a similar DNA binding affinity to ERα homodimers (Cowley *et al* 1997). Since these characteristics are almost identical any unique properties of the ERα/ERβ heterodimer may derive from differences in their activation domains. We demonstrated that ERα/ERβ heterodimers have a level of transcriptional activity intermediate to those of ERα and ERβ homodimers (Fig. 4.7), when using a strong promoter in HeLa and COS-1 cells. However, as discussed in greater detail further on, the transcriptional activity of ERβ is promoter and cell type specific. The ability of ERα/ERβ heterodimers to stimulate transcription from different promoters, in varying cell types has not yet been addressed.

Do ERα/ERβ heterodimers exist *in vivo* ?

The determining factor for the formation of ERα/ERβ heterodimers *in vivo* is their expression patterns, if ERα and ERβ are not expressed in the same cells then they cannot form heterodimers. ERβ was initially isolated from rat prostate (Kuiper *et al* 1996) and human testis (Mosselman *et al* 1996), neither of which had been considered targets for oestrogen signalling. The first northern blotting and RT-PCR experiments suggested that ERα and ERβ had distinct but partially overlapping expression patterns (Kuiper *et al* 1997, Mosselman *et al* 1996). ERα was predominantly expressed in tissues such as mammary gland, uterus, pituitary and liver, while ERβ was mainly expressed in prostate, ovary, bladder and lung. Tissues such as testis and bone contained equivalent amounts of both ERα and ERβ mRNA. Using a polyclonal antibody raised against region D of rat ERβ, Saunders *et al* (1997) were able to demonstrate the presence of ERβ protein in the ovary, testis, heart ventricle and the epithelial layers of uterus, lung, seminal vesicle and bladder as well as the nuclei of their surrounding smooth muscle. The main targets for ERα function are mammary gland, uterus, bone and testis, all of which are effected by the targeted disruption of the ERα gene (Lubahn *et al* 1993, Hess *et al* 1997). The following pieces of evidence suggest that these tissues also contain ERβ.

ERα has for a long time been implicated in the development and progression of breast cancer. The level of its expression is routinely used

in the prognosis of tumors into those which overexpress ERα (ER positive) and those which do not (ER negative). Dotzlaw *et al* (1996) using RT-PCR were able to show the presence of both ERα and ERβ mRNA in a number of human breast cancer biopsy samples. ERβ mRNA was also present in the ER positive breast cancer cell line, T-47D as well as MDA-MB 231 and MCF 10A1, cell lines previously described as ER negative.

Using antibodies raised to rat ERα and ERβ it was demonstrated that both forms of the ER are present in the epithelial and stromal cells of the uterus (Saunders *et al* 1997). In addition, although their pattern of expression differs somewhat in testis, both receptors were also present in leydig cells (Saunders *et al* 1998) and efferent ductules (Hess *et al* 1997b). However, the presence of ERβ alone in efferent ductules is not sufficient for normal testis function, as the lack of ERα reduces the ability of the ducts to reabsorp seminal fluid, resulting in low sperm concentrations and subsequent male infertility (Hess *et al* 1997a).

Oestrogens help protect bone structure by preventing osteoclastic bone resorption, possibly by reducing the levels of bone reabsorping cytokines such as Interleukins -1 and -6. Onoe *et al* (1997) using RT-PCR were able to show that rat primary osteoblasts contained predominantly ERβ mRNA, while rat osteosarcoma cells contain both forms of the receptor. Culturing of primary osteoblastic cells for 28 days in the presence of ascorbic acid and dexamethasone results in their differentiation into mature osteoblasts, which form bone nodules. The level of ERβ mRNA was consistently high during the whole 28 days, while ERα expression was induced only after day 7, increasing until day 28. ERβ mRNA was detected in lumbar vertebrae, cancellous bone and at lower levels in cortical bone; similar levels and distribution were observed in both male and female rats.

Although ERβ is present in mammary gland, uterus, testis and bone the absence of ERα in the ERKO mouse, results in defects in all of these tissues, suggesting that ERβ is incapable of compensating for the loss of ERα. The roles of ERα and ERβ in these tissues and whether they function together or independently, will hopefully become clearer when mice lacking ERβ are generated. One target of ER function unaffected by

the ERα gene disruption is the smooth muscle of the coronary artery. Oestrogens help protect against coronary heart disease by preventing the formation of artherosclerotic plaques, progestins also have a similar effect (Lee *et al* 1997). ERKO mice are still able to inhibit the proliferation of smooth muscle cells after vascular injury, in an oestrogen dependent manner (Iafrati *et al* 1997), consistent with the presence of both ERα and ERβ in the coronary artery (Register and Adams 1998).

The data thus far undeniably demonstrates a distinct expression pattern of ERβ from that of ERα. Many tissues contain one predominant form, ERα and ERβ presumably functioning as homodimers in these cells. Other tissues such as testis, uterus and bone, contain both ERα and ERβ, however, it is also important to show they exist in the same cells. The examples listed above suggest that in a subset of cells ERα and ERβ are coexpressed, given what we know of their respective properties there is a strong possibility that ERα/ERβ heterodimers exist *in vivo.*

ERα and ERβ transcriptional activation

Whilst large advances have been made in the understanding of AF2 function, the mechanism by which AF1 activates transcription is still unclear. The identification of critical residues required for AF2 function and the ligand dependent nature of its transcriptional activity, has made the identification of proteins which interact with AF2 - possible coactivators - much easier to identify than a constitutive activation domain such as AF1. The AF2 domains of the majority of nuclear receptors interact with the SRC1/RIP160 family of coactivators, which in turn recruit the cointegrator proteins CBP/p300 (Torchia *et al* 1997, Kalkhoven *et al* 1998, Voegel *et al* 1998). This activation complex stimulates transcription, probably via the activation domain in CBP/p300, which is dependent on its acetyltransferase activity (Martinez-Balbas *et al* 1998). There are no known coactivators of AF1 function, however it is possible that AF1 also interacts with the SRC1/RIP160 proteins (Lavinsky *et al* 1998). The binding of SRC1/RIP160 coactivators by both AF1 and AF2 could explain the synergy observed between the two activation domains in the context of the full length receptor (Danielian *et al* 1992, Tora *et al* 1989). The ER in

particular requires the combination of AF1 and AF2 for optimal activity on a subset of reporter genes (Berry *et al* 1990, Tora *et al* 1989). We thus tested the ability of ERα and ERβ to stimulate transcription from a series of ERE containing reporter genes.

Differential ability of ERα and ERβ to stimulate transcription from simple promoters

We showed that ERα and ERβ stimulated transcription from complex promoters with similar activity, while on simple promoters ERα was much more active than ERβ (Fig. 5.2). Transfecting ERα and ERβ into different cell types we also observed a reduction in the activity of ERβ compared to ERα in COS-1 and HepG2 cells (Fig. 5.3). What is the reason for these apparent differences in promoter and cell type specificity? One possible reason is that the affinity of the ERβ homodimer for a consensus ERE is lower than an ERα homodimer (Cowley *et al* 1997). This may explain why ERβ is only 50% as active as ERα, even on complex promoters; however the ERE remains the same in all experiments and so is unlikely to explain any differences in specificity. A more likely explanation is that differences in the activation domains of ERα and ERβ are responsible for their differential activity. Sequence comparison of ERα and ERβ shows that in common with other nuclear receptors their AF1 domains have very little homology, whereas AF2 is highly conserved, suggesting that the differential activity most likely derives from AF1 rather than AF2.

ERα and ERβ differ in their AF1 activities

To study the function of their respective AF1 domains, we tested the ability of ERα and ERβ to stimulate transcription from 2ERE-PS2-CAT, an AF1 dependent reporter gene. We demonstrated that the ability of ERα to stimulate transcription from the 2ERE-PS2-CAT reporter was much greater than ERβ (Fig. 5.4), and that ERα activity was dependent upon the presence of AF1. Thus the inability of ERβ to stimulate transcription from the 2ERE-PS2-CAT reporter maybe due to differences in its AF1 activity. Furthermore, a comparison of the AF1 domains of

ERα and ERβ fused to Gal4 revealed that the intrinsic transcriptional activity of AF1β was negligible compared to the that of AF1α (Fig. 5.5). Both experiments suggest that ERβ lacks an AF1 activity equivalent to that found in ERα. Although AF1 is less well defined than AF2, it is necessary to have some idea of its function before we can address the differences.

Analysis of the AF1 domain of ERα suggests that it comprises at least three distinct features. Firstly, a region between residues 41 and 150 is required for AF1 activity (McInerney and Katzenellenbogen 1996, Metzger *et al* 1995). Secondly, a region between residues 91 and 121 is required for synergy with the ligand binding domain to generate optimum transcriptional activity (Danielian *et al* 1992) that may reflect their interaction (McInerney *et al* 1996). Thirdly, AF1 is a target for phosphorylation by the MAPK pathway in response to growth factors (Ali *et al* 1993, Bunone *et al* 1996, Kato *et al* 1995). A consensus MAPK phosphorylation site is also present in the AF1 domain of ERβ, in fact this represents the only significant homology between ERα and ERβ in this region. Similar to ERα, the MAPK site in ERβ is required for the stimulation of transcriptional activity produced by dominant positive Ras (Tremblay *et al* 1997). However, stimulation of ERβ with growth factors, which function via the endogenous MAPK pathway has not been shown. Since Gal4AF1β has little transcriptional activity compared to Gal4AF1α, it would appear that the presence of the MAPK site is not sufficient for the N-terminal domain of ERβ to stimulate transcription. Furthermore, substitution of the conserved Ser residue to Ala in the MAPK site of ERα (Fig. 5.4 ERα S122A) did not effect its ability to stimulate transcription from the 2ERE-PS2-CAT reporter. It thus appears that there are two functions within AF1. The first, dependent upon Ser phosphorylation at the MAPK site, is conserved in both ERα and ERβ. The second, 'MAPK independent' function may rely on sequences outside of the MAPK site; since the sequence conservation in this region is negligible it is conceivable that ERβ lacks this second 'AF1α' function. The absence of an AF1 activity in ERβ may also explain the lack of agonist activity of antioestrogens such as 4OH-tamoxifen (Watanabe *et al* 1997), which is mediated via AF1 in ERα (Berry *et al* 1990, McInerney *et al* 1996).

Given this evidence we would predict that on complex promoters ERα and ERβ would have approximately the same activity, because AF1 is not required, while on simple promoters where AF1 is essential, ERα would have much more activity than ERβ. This is precisely what we observed, namely, transcription from ERE-TK-luc is stimulated by both ERα and ERβ, whereas ERα is much more active than ERβ on -TATA and -PS2 based reporters which require both AF1 and AF2 (Fig. 5.2). Why ERβ stimulates transcription differentially on the same complex promoter in different cell types (Fig. 5.3) is unclear, the only explanation would seem that ERE-TK-luc although not requiring the presence of AF1 activity in HeLa cells does so in HepG2 cells.

ERα and ERβ have similar AF2 activities

In contrast to AF1, the transcriptional activity of the AF2 domains in ERα and ERβ, when fused to the Gal4 DNA binding domain, is similar (Fig. 5.6). The ability of Gal4AF2β to stimulate transcription from the TATA based reporter was slightly less than Gal4AF2α, whilst on a TK based promoter their activity was more or less the same. Although the difference in activity between Gal4AF2α and Gal4AF2β on TATA and TK promoters was only small, it demonstrates once more that ERα seems better adapted to stimulate transcription from simple promoters than ERβ. The AF2 domains interacted with the coactivator SRC1, with a similar affinity (Fig. 5.7) and ligand specificity (Fig. 5.8), consistent with their similar transcriptional activity.

The equivalent SRC1 binding properties of ERα and ERβ suggests that the surface of the ligand binding domain (LBD) required for coactivator recruitment is similar. Residues required for AF2 function in ERα, namely, a Lys residue in helix 3 (Henttu *et al* 1997) and hydrophobic residues in helices 3, 5 and 12 (Danielian *et al* 1992, Ho Yi Mak - Molecular Endocrinology Laboratory) are also conserved in ERβ. It seems likely that these residues form the surface which contacts the conserved LXXLL (L=Leucine, X=any amino acid) helices that mediate the binding of coactivators to nuclear receptors (Heery *et al* 1997, Torchia *et al* 1997). To strengthen the hypothesis that nuclear receptor/coactivator interactions are of a hydrophobic nature, it was

demonstrated that salt concentration had no effect on the binding of SRC1 to ERα (Kalkhoven *et al* 1998), this is also the case for ERβ (Fig. 5.9).

Mutations in helix 12 of ERα and ERβ differentially effect their ability to activate transcription

In the absence of hormone helix 12 protrudes from the core of the LBD (Bourguet *et al* 1995). Upon ligand binding helix 12 realigns across the hydrophobic core of the LBD (Brzozowski *et al* 1997, Renaud *et al* 1995, Wagner *et al* 1995) forming a competent surface for the recruitment of coactivators, thus facilitating ligand dependent transcriptional activity. The ER antagonist, Raloxifene is thought to function by misaligning helix 12 in a position which is unfavourable for coactivator binding, thus preventing transcriptional activation (Brzozowski *et al* 1997). Given the crucial role of helix 12 we decided to make two mutations of conserved residues within this helix in ERβ. We constructed two ERβ mutants: ERβ E493A and ERβ M494A/L495A, equivalent mutations to those first characterised in ERα by Danielian *et al* (1992). Similar to ERα M547A/L548A, ERβ M494A/L495A has very little transcriptional activity (Fig. 5.10), consistent with their inability to bind SRC1 (Fig. 5.14). In contrast to this result, the ability of ERα E546A to stimulate transcription was greater than the equivalent mutation in ERβ, ERβ E493A. The explanation for this observation is that ERβ E493A does not bind SRC1, while the interaction of SRC1 with ERα E546A although reduced, is still detectable (Fig. 5.14). However, the reason for the point mutation of the conserved Glu residue in helix 12 being more detrimental in ERβ than ERα is not immediately obvious. Danielian *et al* (1992) observed that the mutation of E546A in ERα did not reduce its ability to stimulate transcription compared to wild-type, but when they removed AF1 the reduction of activity was much more marked. As discussed above, ERβ lacks an AF1 activity equivalent to that present in ERα, this might explain why ERβ E493A has less activity than ERα E546A. However when these mutant AF2 domains were compared for their ability to stimulate transcription as Gal4 fusions, Gal4AF2α E546A still had more activity than Gal4AF2β E493A (Fig. 5.12). A comparison of helix 12 from ERα and ERβ shows that ERα has three acidic residues in

helix 12 while ERβ has only two. Mutation of two or three of these acidic residues in ERα reduces the transcriptional activity in an additive manner (Danielian *et al* 1992). Therefore since ERβ has one acidic residue less than ERα in helix 12, the mutation of E493A in ERβ is equivalent to the double mutation of E546A/D549N in ERα (Fig. 5.10). However, the double mutant ERα E546A/D549N still had more activity than ERβ E493A (Fig. 5.10 and 5.12), thereby all but ruling-out the theory that lack of a third acidic residue in helix 12 of ERβ makes the point mutation of ERβ E493A more detrimental than the equivalent mutation in ERα. The remainder of helix 12 is identical in both ERα and ERβ. It's possible therefore that it is non-conserved residues in helices 3 and 5 which make ERβ more susceptible to the mutation of the conserved Glu residue in helix 12. The equivalent mutation in TR (Barettino *et al* 1994) and RAR (Durand *et al* 1994) causes a severe reduction in their transcriptional activity, similar to that observed with ERβ E493A. Although this does not explain the discrepancy in transcriptional activation observed with ERα and ERβ, it does show that the effect of this mutation in ERβ is more akin to other nuclear receptors, than ERα.

Why is the transcriptional activity of ERα greater than ERβ?

In the series of experiments comparing transcriptional activation by ERα and ERβ we have never observed ERβ stimulate transcription more than ERα. There are three reasons why this might be the case. The first is that ERβ homodimers have a lower affinity for an ERE than ERα homodimers, so that even if their respective transcriptional activation domains were identical, ERβ would have reduced transcriptional activity compared to ERα. The second is that ERβ lacks an AF1 activity equivalent to that found in ERα. This means that on simple promoters and cell lines where an AF1 activity is essential, ERα stimulates transcription much more than ERβ. The third and perhaps least consequential is that on simple promoters the AF2 domain of ERβ stimulates transcription slightly less well than the AF2 domain of ERα. On complex promoters only the first reason is relevant and so ERβ stimulates transcription to approximately 50% the level of ERα, equivalent to the difference in their relative DNA binding affinities (Fig.

4.3). On simple promoters it is a combination of all three which makes ERβ a poor transcriptional activator in relation to ERα.

This poses two questions: why does ERβ lack a functional AF1 domain? and what is the role of the AF1 domain in ERα? Unfortunately there are no definitive answers to either question, but from what is known we are able to hypothesise about the possible functions of AF1 (summarised in Fig. 6.2).

As discussed there is little sequence homology in the AF1 domains of ERα and ERβ, with the exception of the consensus MAPK site, however this site does not seem sufficient for AF1 activity in ERβ. The lack of homology suggests one of two things, either AF1 in ERβ has no function and a lack of evolutionary pressure has caused the divergence with ERα, or the opposite is true, and ERβ has a different role to that of ERα which at the moment is not apparent. As for the role of AF1, on complex promoters such as ERE-TK-luc, AF1 is not required at all (Fig. 6.2A). However this represents an idealised situation of a perfect ERE upstream of strong promoter and is probably less representative of physiological conditions than a simple promoter such as 2ERE-PS2-CAT, where AF1 is essential. On simple promoters AF1 and AF2 are able to synergise (Danielian *et al* 1992, Berry *et al* 1990, Tora *et al* 1989), exactly how they synergise is unclear; in light of the present evidence its possible for AF1 to do two things. In addition to the activity of AF2 it could act as an independent activation domain (Lees *et al* 1989, Tora *et al* 1989), or interacting with the SRC1/RIP160 coactivator proteins (Lavinsky *et al* 1998), these two explanations are not necessarily mutually exclusive and both are consistent with the observed activity of AF1α (Fig. 6.2B). AF1 is a target for growth factors which stimulate the activity of ERα (Ignar-Trowbridge *et al* 1996). It is conceivable that phosphorylation at the conserved MAPK site could activate either of these functions. For ERα to stimulate transcription from simple promoters then one of these activities must be present, evidence suggests that neither is present in ERβ and therefore it necessarily has low transcriptional activity (Fig.6.2 C).

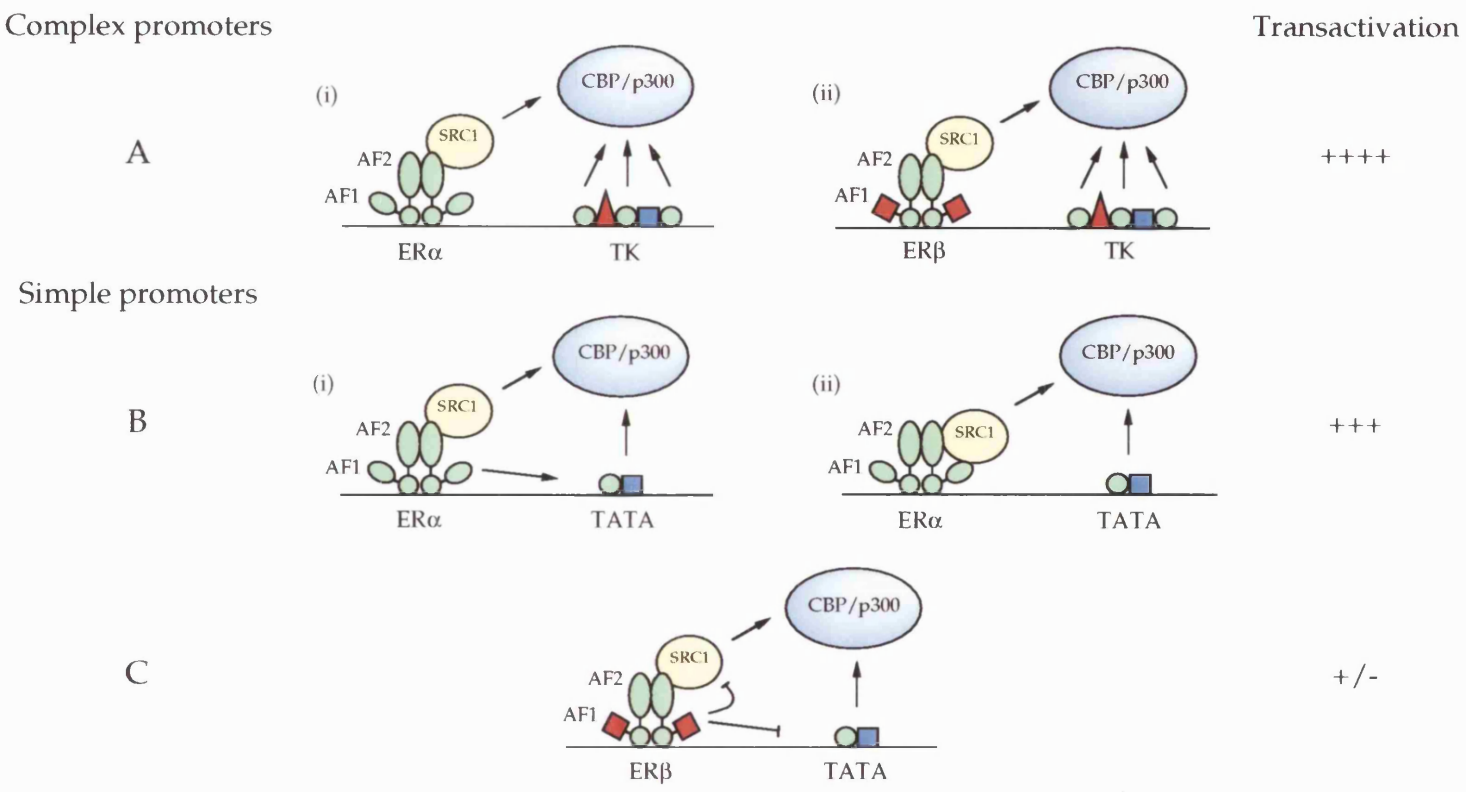


Fig. 6.2 - **Model of ERα and ERβ transcriptional activation on complex and simple promoters.** A, On complex promoters AF1 is not required, therefore ERα (i) and ERβ (ii) have approximately the same activity. B, On simple promoters where AF1 is required, ERα is able to activate transcription, because AF1 functions as either (i) an independent activation domain or (ii) in combination with AF2 to recruit coactivators, these mechanisms are not necessarily mutually exclusive. C, ERβ stimulates transcription poorly from simple promoters due to the lack of an 'AF1α like' activity. The arrows indicate interactions with the cointegrator proteins CBP/p300, other transcription factors are represented by ▫, ○ and ▲ .

Isolation of nuclear receptor coactivators

In the two year period following the first demonstration of RIP140 and RIP160 proteins (Halachmi *et al* 1994, Cavaillès *et al* 1994), a plethora of RIPs were cloned (see introduction and review by Glass *et al* 1997), either using far-western or yeast two-hybrid screening methods. The challenge since then has been to decide which of these RIPs are bona fide. This problem was highlighted by the discovery that a helix containing a short motif consisting of just LXXLL was sufficient for a ligand dependent interaction with nuclear receptors (Heery *et al* 1997, Torchia *et al* 1997). The majority of the RIPs isolated have one or more of these motifs, begging the question: are these proteins physiologically relevant, or do they simply interact with nuclear receptors because they fortuitously contain one or more copies of the LXXLL motif? RIP140 for instance has nine copies of the LXXLL motif, each one individually has the ability to interact with the LBD of ERα (Heery *et al* 1997); RIP140 does not seem to function as coactivator, so what are we to conclude from its interaction with nuclear receptors? RIPs such as TRIP1/Sug1, TIF1α and RIP140 fall into this category (Cavaillès *et al* 1995, Le Douarin *et al* 1995, vom Baur *et al* 1996). In the absence of a functional assay we will only have a better idea of their function when these genes are deleted in mice. The most convincing putative coactivators amongst the RIPs isolated are those of the SRC1/RIP160 proteins: SRC1 (Kamei *et al* 1996, Onate *et al* 1995), TIF2/GRIP1 (Hong *et al* 1996, Voegel *et al* 1996) and Rac3/AIB1/pCIP/ACTR (Anzick *et al* 1997, Chen *et al* 1997, Li *et al* 1997, Torchia *et al* 1997) which have a similar domain structure and all stimulate the transcription of nuclear receptors. Inevitably most attention has focused on the SRC1/RIP160 family members, not only because they stimulate nuclear receptor activation, but because they also interact with the homologous cointegrator proteins CBP and p300 (Anzick *et al* 1997, Chen *et al* 1997, Li *et al* 1997, Torchia *et al* 1997). Leading to the current model of a nuclear receptor activator complex consisting of the nuclear receptor, one of the SRC1/RIP160 proteins and CBP/p300.

Identification of an SRC1/RIP160 family member from chicken

At the height of the search for coactivators, we attempted to clone nuclear receptor coactivators from chicken. The theory being that regions of homology between the human and chicken RIPs would highlight regions of functional importance. Using far-western screening we were able to show the presence of RIP 140, 160 and 220 proteins in chicken embryo fibroblasts (CEF), similar to those seen in human breast cancer cells (Fig. 3.1 and 3.2). Although the far-western assay is not strictly quantitative, there appears to be more RIP 220 in CEFs than any other cell line. The only RIP cloned which approximates to this size is Trip230, a protein which stimulates the activity of the TR and also interacts with the retinoblastoma protein, pRb (Chang *et al* 1997). If the RIP220 observed is Trip230, the explanation for the increased amount in CEF cells is unclear from what we know currently of Trip230 function. Using the far-western assay to screen a CEF cDNA expression library we isolated a partial protein fragment capable of interacting with GST-AF2α in an oestradiol dependent manner (Fig. 3.4), which we termed Chip1. Chip1 had homology to both SRC1 and TIF2, the only RIP160 proteins cloned at that time. The region of homology coinciding with the published nuclear interaction domain (NID) of TIF2 (Voegel *et al* 1996), but not SRC1 (Onate *et al* 1995). The original fragment of SRC1 isolated in the two-hybrid screen with the LBD of PR was at the C-terminus of the protein. Subsequent deletion mapping demonstrated that SRC1 also has a NID domain in the centre of the protein equivalent to those of TIF2 and Chip1, and that the second NID at the C-terminus is present only in SRC1a and not the variant SRC1e. The reason for this became obvious after the identification of the LXXLL motif. The NID domain in the centre of both isoforms of SRC1, TIF2 and the fragment of Chip1 contains three LXXLL motifs (Fig. 5.4), while SRC1a (the isoform cloned by Onate *et al* 1995) has an extra LXXLL at the C-terminus. The explanation for SRC1a having an extra NID at the C-terminus is unclear, not only is it dispensable but the same region suppresses the ability of SRC1a to stimulate the activity of ERα (Kalkhoven *et al* 1998).

Chickens and humans contain three RIP160 genes

Chip1 has high sequence homology to the NIDs of SRC1 and TIF2. Since Chip1 is a chicken protein and SRC1 and TIF2 are human, there was a possibility that Chip1 was an SRC1 or TIF2 orthologue. We used equivalent cDNA probes from Chip1, SRC1 and TIF2 to probe human and chicken genomic DNA in a southern blot analysis. Each probe gave a unique pattern of bands in human and chicken DNA demonstrating that both genomes contain at least three RIP160 genes and that Chip1 was not a chicken SRC1 or TIF2 orthologue, but a unique gene. The full length Chip1 cDNA was subsequently cloned by several groups from human, Rac3 (Li *et al* 1997), ACTR (Chen *et al* 1997), AIB1 (Anzick *et al* 1997), TRAM-1 (Takeshita *et al* 1997) and mouse, p/CIP (Torchia *et al* 1997). The conservation of the number and sequence of coactivators from human to chicken suggests that the mechanism of nuclear receptor transcriptional activation is highly conserved.

Why do nuclear receptors require three highly conserved coactivators? are the functions of the SRC1/RIP160 proteins degenerate, or do they have unique roles? This of course assumes that all three SRC1/RIP160 coactivators are present in all cells. Although their expression has not been extensively mapped, northern blotting experiments suggest that all three proteins are ubiquitously expressed, at least in cell lines (Anzick *et al* 1997, Kamei *et al* 1996, Voegel *et al* 1996). The most surprising aspect of the SRC1/RIP160 coactivators function is that there is no specificity of interaction with nuclear receptors, if it interacts with one nuclear receptor it interacts with them all. This is probably a consequence of the conserved structure of nuclear receptor LBD's (reviewed by Moras and Gronemeyer 1998) and their method of recruiting coactivators through the LXXLL motif. This does however ~~does~~ remove one possible mechanism of regulation and suggests that all nuclear receptor irrespective of ligand specificity activate transcription via the same coactivators.

SRC1 and TIF2 have two activation domains, AD1 which overlaps their CBP/p300 interaction domain, stimulating transcription via the recruitment of p300/CBP (Voegel *et al* 1998), and AD2 in their

C-terminus whose function is unclear. p/CIP/AIB1/ACTR/RAC3 also have an equivalent AD1 activity but do not appear to have AD2. ACTR and SRC1 have also been shown to have histone acetyltransferase activity in their C-termini, however the boundaries of this activity in SRC1 do not coincide with those of AD2 activity. At face value the three coactivators seem to function similarly, particularly SRC1 and TIF2; if there is a black sheep amongst the family then it is possibly p/CIP/AIB1/ACTR/RAC3/TRAM-1.

p/CIP is a poorer coactivator of nuclear receptors than SRC1 and TIF2, and yet counter-intuitively it seems to be associated with the majority of CBP in cells (Torchia *et al* 1997). Microinjection of cells with antibodies raised against either SRC1 or TIF2 prevents transcriptional activation by nuclear receptors. This block can be removed by overexpression of SRC1 or TIF2. This is not the case for p/CIP, the addition of p/CIP antibodies prevents nuclear receptor transcription, but the activity is rescued only if p/CIP and CBP are coexpressed. The authors argue that p/CIPs stronger association with CBP/p300 may make it indispensable for transcriptionally active nuclear receptor complexes. However, why Torchia *et al* should find that p/CIP is a weaker coactivator is unclear, because ACTR, RAC3 and TRAM-1 all stimulate nuclear receptor transcription to a similar extent as SRC1 and TIF2 (Chen *et al* 1997, Li *et al* 1997, Takeshita *et al* 1997). In addition, p/CIP appears to function as a coactivator of STAT1 transcription, again this may be an effect of being stably complexed with CBP/p300 (Torchia *et al* 1997).

The region of chromosome 20q12 can become amplified in some breast cancers. While examining this region for candidate genes, Anzick and co-workers discovered a gene with homology to SRC1 and TIF2, which they termed amplified in breast cancer 1 (AIB1, Anzick *et al* 1997). AIB1 functions as a coactivator of ERα and since the ER itself has been implicated in breast cancer Anzick *et al* studied the expression and copy number of AIB1 gene in breast cancer cells. In 60% of established breast cancer cell AIB1 mRNA is expressed at much higher levels than SRC1 and TIF2, this maybe because the AIB1 gene is present in multiple copies, some MCF7 cells for instance contain ~20 copies. The overexpression of AIB1 in breast cancer cells could promote the activity

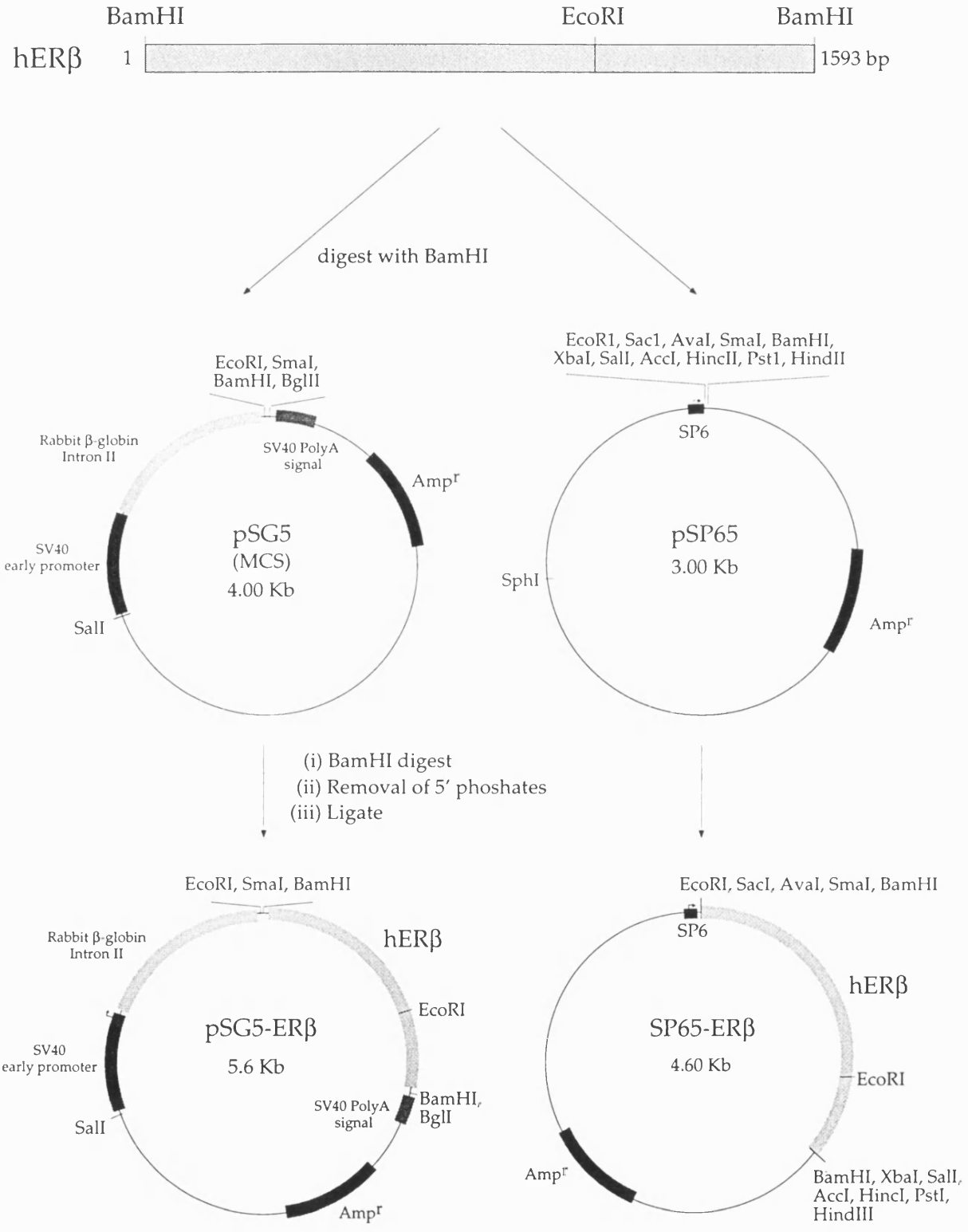
of ERs, increasing their growth and possibly explaining why nuclear receptors are more active in transformed cells than primary cells (Vivanco *et al* 1995).

The generation of mice lacking p/CIP will hopefully help determine whether it has unique functions from those of SRC1 and TIF2. Some degeneracy certainly exists in the function of SRC1, since mice of both sexes lacking SRC1 are viable and fertile. Although they do have reduced growth and development of steroid hormone targets such as uterus, prostate, testis and mammary gland (Xu *et al* 1998). The expression of TIF2 mRNA is elevated in SRC1 null mice compared to wild-type, perhaps partially compensating for the lack of SRC1; although presumably to do this they must perform the same functions.

APPENDIX

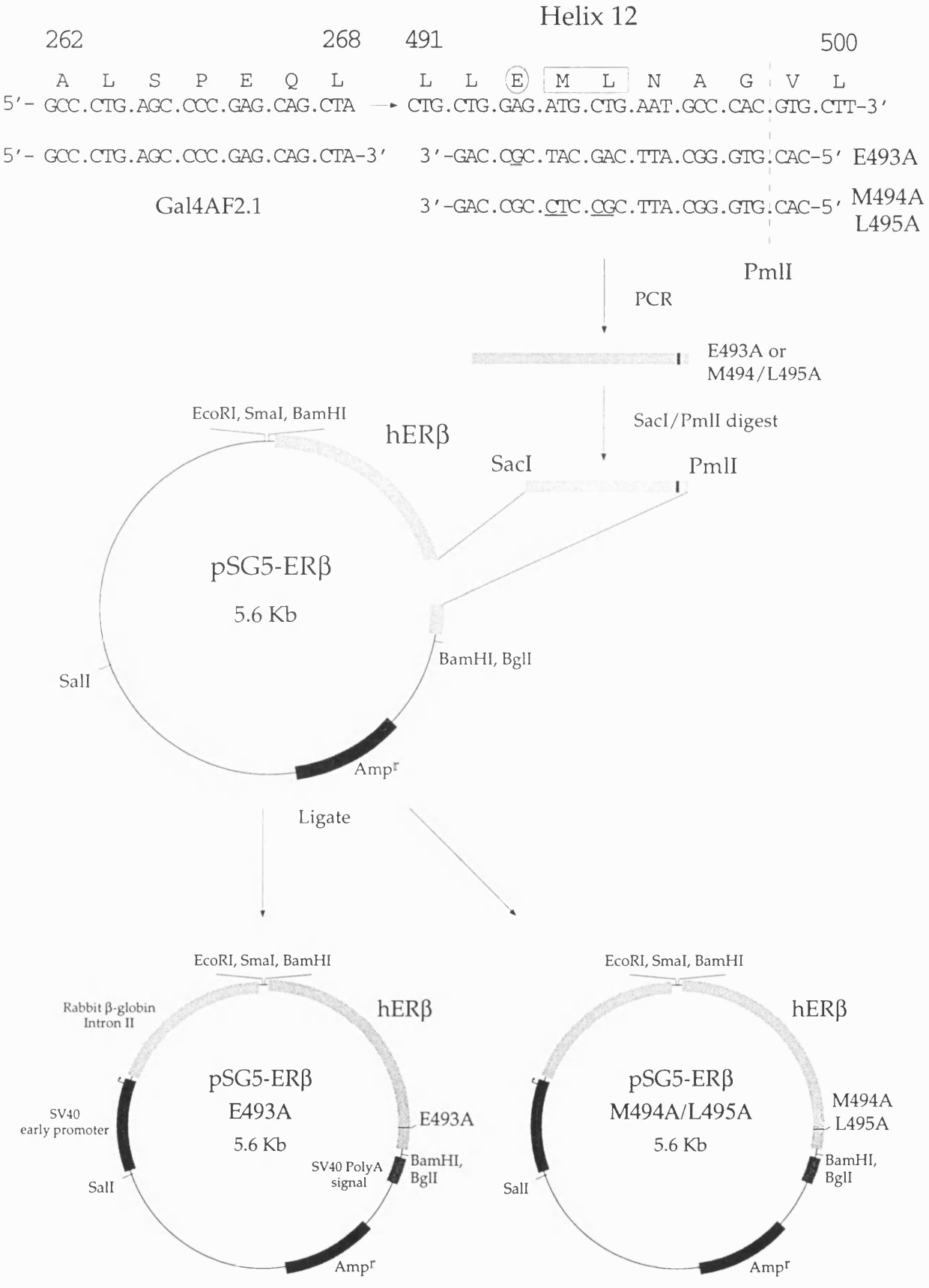
A1 Construction of pSG5ERβ and pSP65ERβ

The 1593 bp open reading frame of ERβ was removed from pNGV-ERβ (S. Mosselman - Organon) by digestion with BamHI followed by gel purification. The vectors, pSG5 (3μg) and pSP65 (3μg) were prepared by BamHI digest followed by treatment with calf intestianl phosphatase (CIP), to remove 5' phosphates. The 1.6 kb ERβ fragment was subcloned into these vectors, resulting in pSG5-ERβ and pSP65-ERβ respectively. Correct recombinants were identified by digestion with EcoRI and then verified by DNA sequencing.


BamHI
EcoRI
BamHI
hERβ
1
1593 bp
digest with BamHI
EcoRI, SmaI, BamHI, BglII
Rabbit β-globin Intron II
SV40 PolyA signal
Ampr
pSG5
(MCS)
4.00 Kb
SV40 early promoter
SalI
EcoR1, Sac1, AvaI, SmaI, BamHI, XbaI, SalI, AccI, HincII, Pst1, HindII
SP6
pSP65
3.00 Kb
SphI
Ampr
(i) BamHI digest
(ii) Removal of 5′ phoshates
(iii) Ligate
EcoRI, SmaI, BamHI
Rabbit β-globin Intron II
hERβ
EcoRI
pSG5-ERβ
5.6 Kb
SV40 early promoter
SalI
SV40 PolyA signal
BamHI, BglI
Ampr
EcoRI, SacI, AvaI, SmaI, BamHI
SP6
hERβ
SP65-ERβ
4.60 Kb
EcoRI
Ampr
BamHI, XbaI, SalI, AccI, HincI, PstI, HindIII

A2 Constructioin of pSG5-ERβ helix 12 mutants: E493A and M494A/L495A

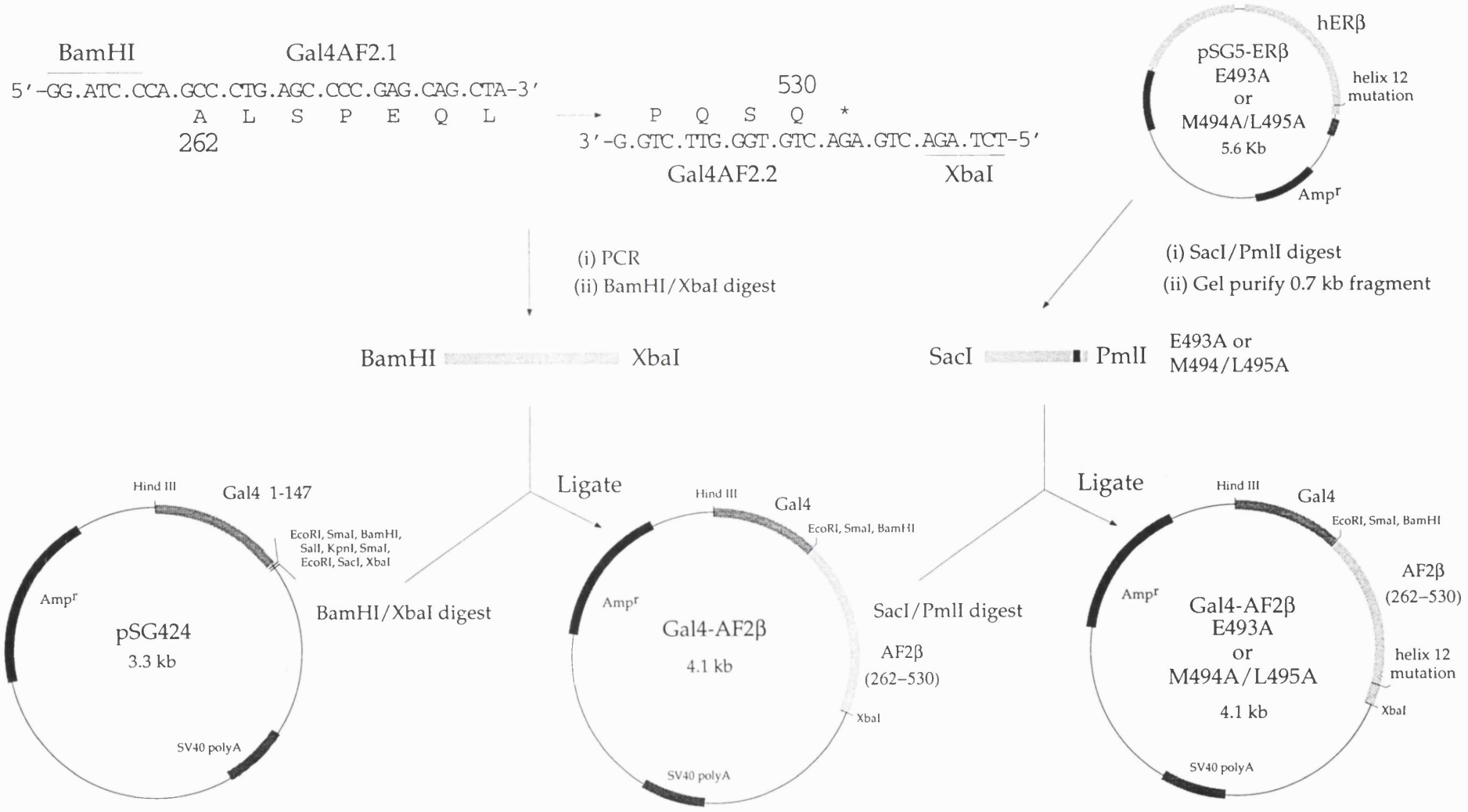
The helix 12 mutations were generated by PCR. The 5' primer, Gal4AF2.1 was used in conjunction with either ERβ.E493A or ERβ.M494/L495A primers, to generate a 714 bp ERβ fragment. This was then digested with SacI/PmI to generate the specific sites and then gel purified. These inserts were subcloned into the vector, pSG5-ERβ (3 μg) which had been digested with SacI/PmI. The recombinants were identified by DNA sequencing.


Helix 12
262
268
491
500
A L S P E Q L
L L E M L N A G V L
5′- GCC.CTG.AGC.CCC.GAG.CAG.CTA → CTG.CTG.GAG.ATG.CTG.AAT.GCC.CAC.GTG.CTT-3′
5′- GCC.CTG.AGC.CCC.GAG.CAG.CTA-3′
3′-GAC.CGC.TAC.GAC.TTA.CGG.GTG.CAC-5′ E493A
Gal4AF2.1
3′-GAC.CGC.CTC.CGC.TTA.CGG.GTG.CAC-5′ M494A L495A
PmlI
PCR
E493A or M494/L495A
SacI/PmlI digest
EcoRI, SmaI, BamHI
hERβ
SacI
PmlI
pSG5-ERβ
5.6 Kb
SalI
BamHI, BglI
Ampr
Ligate
EcoRI, SmaI, BamHI
Rabbit β-globin Intron II
hERβ
pSG5-ERβ
E493A
5.6 Kb
SV40 early promoter
E493A
SV40 PolyA signal
BamHI, BglI
SalI
Ampr
EcoRI, SmaI, BamHI
hERβ
pSG5-ERβ
M494A/L495A
5.6 Kb
M494A
L495A
BamHI, BglI
SalI
Ampr

A3 Construction Gal4-AF2β, wild-type and helix 12 mutants

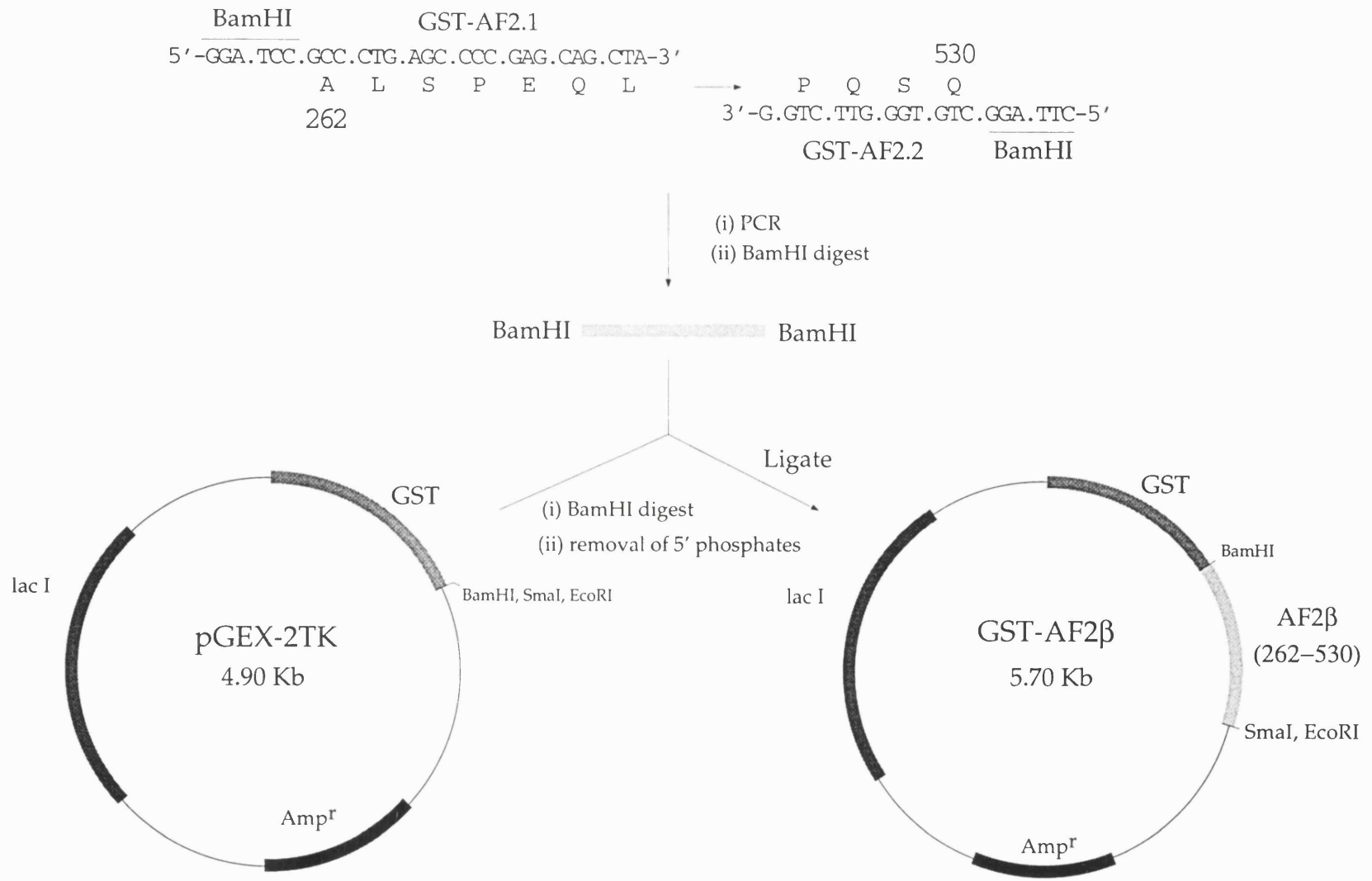
The AF2 domain of ERβ (residues 262-530) was generated by PCR, using Gal4AF2.1 and Gal4AF2.2 primers. The synthesised fragment was digested with BamHI/XbaI to obtain cohesive ends and then gel purified. This fragment was then subcloned into the vector, pSG424 (3 μg) digested with BamHI/XbaI, resulting in Gal4-AF2β. Correct recombinants were identified by digestion with EcoRI and DNA sequencing of the Gal4-AF2 fusion point.

To construct Gal4-AF2β helix 12 mutants, pSG5ERβ E493A or M494/L495A was digested with SacI/PmlI and the 0.7 kb band gel purified. This fragment was then subcloned into Gal4-AF2β digested with SacI/Pml. Recombinants were identified by DNA sequencing.


BamHI
Gal4AF2.1
5′-GG.ATC.CCA.GCC.CTG.AGC.CCC.GAG.CAG.CTA-3′
A L S P E Q L
262
530
P Q S Q *
3′-G.GTC.TTG.GGT.GTC.AGA.GTC.AGA.TCT-5′
Gal4AF2.2
XbaI
hERβ
pSG5-ERβ
E493A
or
M494A/L495A
5.6 Kb
helix 12
mutation
Amp^r
(i) PCR
(ii) BamHI/XbaI digest
(i) SacI/PmlI digest
(ii) Gel purify 0.7 kb fragment
BamHI
XbaI
SacI
PmlI
E493A or
M494/L495A
Ligate
Ligate
Hind III
Gal4 1-147
EcoRI, SmaI, BamHI,
SalI, KpnI, SmaI,
EcoRI, SacI, XbaI
Amp^r
pSG424
3.3 kb
SV40 polyA
BamHI/XbaI digest
Hind III
Gal4
EcoRI, SmaI, BamHI
Amp^r
Gal4-AF2β
4.1 kb
AF2β
(262–530)
XbaI
SV40 polyA
SacI/PmlI digest
Hind III
Gal4
EcoRI, SmaI, BamHI
AF2β
(262–530)
Amp^r
Gal4-AF2β
E493A
or
M494A/L495A
4.1 kb
helix 12
mutation
XbaI
SV40 polyA

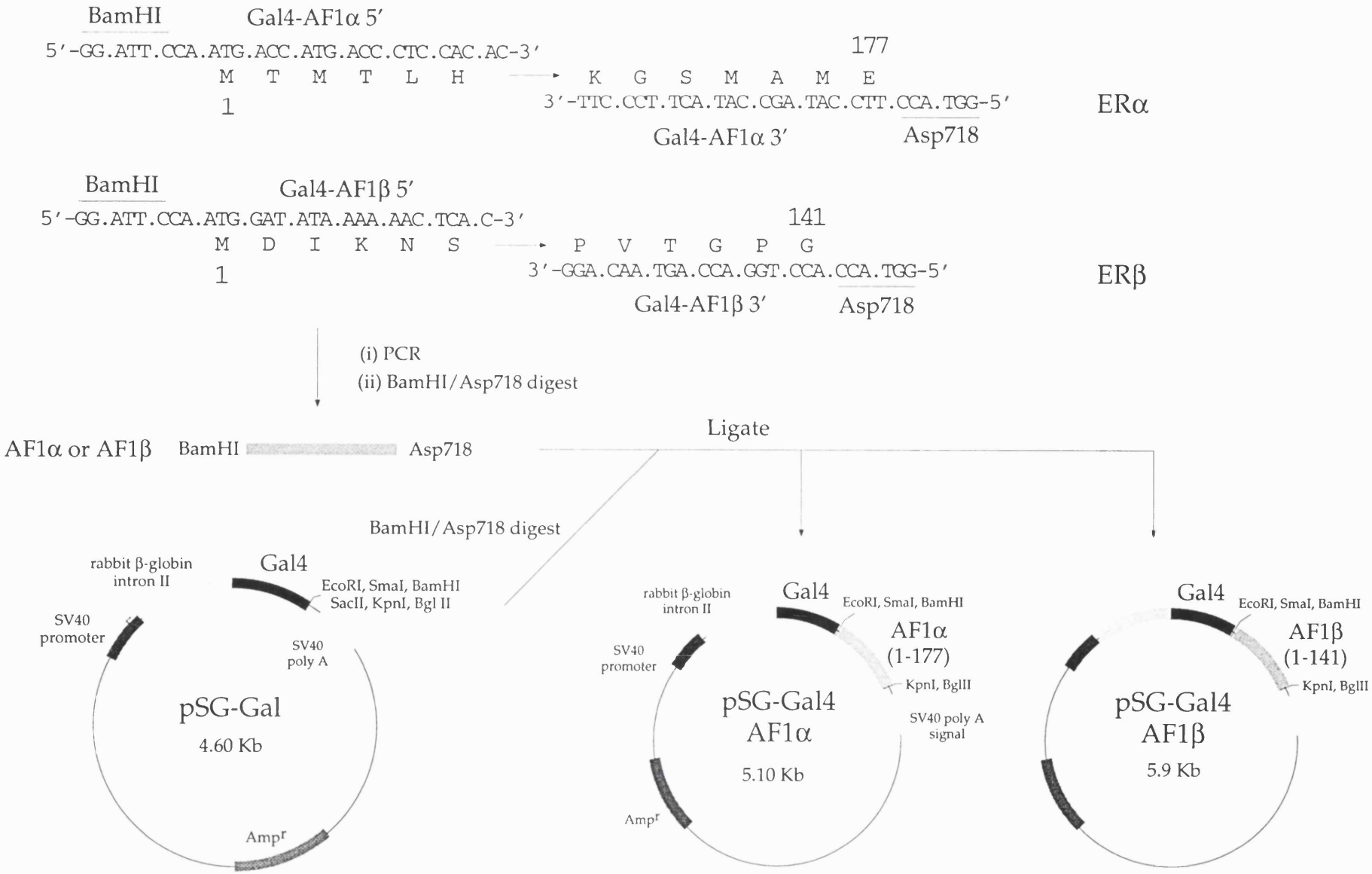
A4 Construction of GST-AF2β

The AF2 domain of ERβ (residues 262-530) was generated by PCR, using primers: GST-AF2.1 and GST-AF2.2. The synthesised fragment was digested with BamHI to produce cohesive ends and then gel purified. This fragment was then subcloned into the vector, pGEX-2TK (3 μg) cut with BamHI and treated with CIP to remove 5' phosphates. The resulting recombinant, GST-AF2β, was identified by digestion with EcoRI and verified by DNA sequencing of the GST-AF2 fusion point.


BamHI
GST-AF2.1
5′-GGA.TCC.GCC.CTG.AGC.CCC.GAG.CAG.CTA-3′
A L S P E Q L
262
530
P Q S Q
3′-G.GTC.TTG.GGT.GTC.GGA.TTC-5′
GST-AF2.2
BamHI
(i) PCR
(ii) BamHI digest
BamHI
BamHI
Ligate
(i) BamHI digest
(ii) removal of 5′ phosphates
GST
lac I
BamHI, SmaI, EcoRI
pGEX-2TK
4.90 Kb
Amp^r
lac I
GST
BamHI
AF2β
(262–530)
GST-AF2β
5.70 Kb
SmaI, EcoRI
Amp^r

A5 Construction of Gal4AF1 -α and -β

The AF1 domains of ERα (residues 1-177) and ERβ (residues 1-141) were generated by PCR, using the appropriate primers (shown in the diagram). The synthesised fragments were digested with BamHI/Asp718 and then gel purified. These fragments were subcloned into the vector, pSG-Gal (3 μg) digested with BamHI/Asp718. The correct recombinants were identified by BamHI/Asp718 digestion and verified by DNA sequencing of the Gal4-AF1 fusion point.

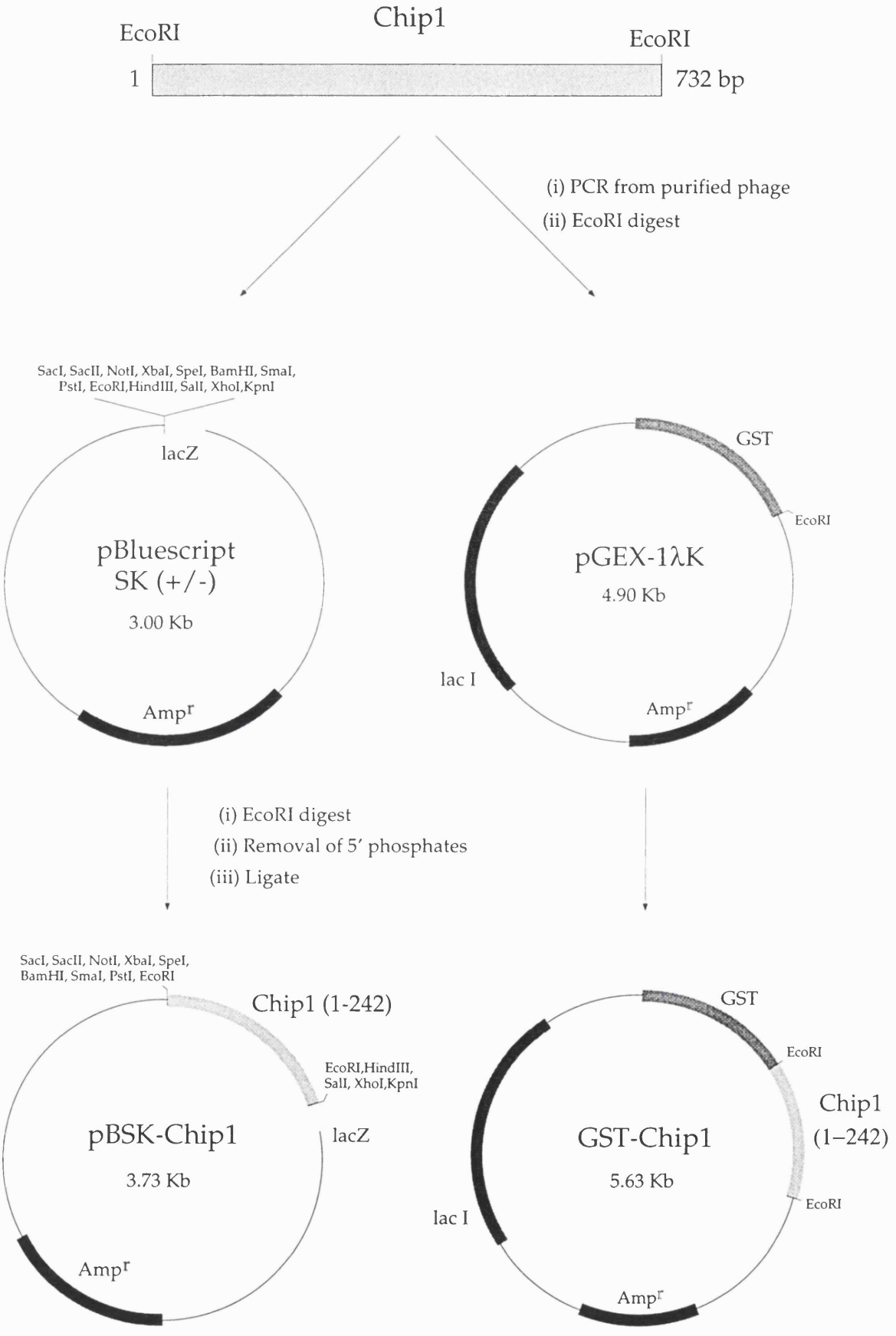

BamHI
Gal4-AF1α 5′
5′-GG.ATT.CCA.ATG.ACC.ATG.ACC.CTC.CAC.AC-3′
M T M T L H
1
177
K G S M A M E
3′-TTC.CCT.TCA.TAC.CGA.TAC.CTT.CCA.TGG-5′
Gal4-AF1α 3′
Asp718
ERα
BamHI
Gal4-AF1β 5′
5′-GG.ATT.CCA.ATG.GAT.ATA.AAA.AAC.TCA.C-3′
M D I K N S
1
141
P V T G P G
3′-GGA.CAA.TGA.CCA.GGT.CCA.CCA.TGG-5′
Gal4-AF1β 3′
Asp718
ERβ
(i) PCR
(ii) BamHI/Asp718 digest
AF1α or AF1β
BamHI
Asp718
Ligate
BamHI/Asp718 digest
rabbit β-globin intron II
Gal4
EcoRI, SmaI, BamHI
SacII, KpnI, Bgl II
SV40 promoter
SV40 poly A
pSG-Gal
4.60 Kb
Ampr
rabbit β-globin intron II
Gal4
EcoRI, SmaI, BamHI
AF1α
(1-177)
KpnI, BglII
SV40 promoter
SV40 poly A signal
pSG-Gal4 AF1α
5.10 Kb
Ampr
Gal4
EcoRI, SmaI, BamHI
AF1β
(1-141)
KpnI, BglII
pSG-Gal4 AF1β
5.9 Kb

A6 Construction of pBSK-Chip1 and GST-Chip1

The Chip1 cDNA was amplified from λgt11 bacteriophage by PCR, using the λgt11 forward and reverse sequencing primers (see below). The Chip1 fragment generated was digested with EcoRI and then gel purified. Chip1 was then subcloned into the vectors, pBSK II (3 μg) and pGEX1λk (3 μg) cut with EcoRI and then treated with CIP to remove 5' phosphates. The resulting recombinants pBSK-Chip1 and GST-Chip1 were identified by digestion with EcoRI and verified by DNA sequencing.

(5'-GGTGGCGACGACTCCTGGAGCCCG-3') : λgt11 Forward

(5'-TTGACACCAGACCAACTGGTAATG-3') : λgt11 Reverse


Chip1
EcoRI
EcoRI
1
732 bp
(i) PCR from purified phage
(ii) EcoRI digest
SacI, SacII, NotI, XbaI, SpeI, BamHI, SmaI,
PstI, EcoRI,HindIII, SalI, XhoI,KpnI
lacZ
pBluescript
SK (+/-)
3.00 Kb
Amp^r
GST
EcoRI
pGEX-1λK
4.90 Kb
lac I
Amp^r
(i) EcoRI digest
(ii) Removal of 5′ phosphates
(iii) Ligate
SacI, SacII, NotI, XbaI, SpeI,
BamHI, SmaI, PstI, EcoRI
Chip1 (1-242)
EcoRI,HindIII,
SalI, XhoI,KpnI
pBSK-Chip1
3.73 Kb
lacZ
Amp^r
GST
EcoRI
Chip1
(1–242)
GST-Chip1
5.63 Kb
EcoRI
lac I
Amp^r

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