Molecular Mechanism of Transcriptional Activation by Oestrogen Receptor Alpha

Ho Yi Mak

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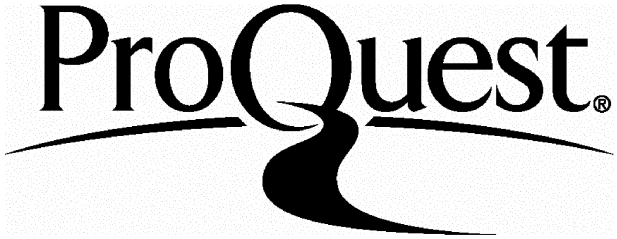
Molecular Endocrinology Laboratory
Imperial Cancer Research Fund
Lincoln's Inn Fields
London

Department of Molecular Pathology
University College London
Gower Street
London



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

Abstract

Oestrogens regulate the transcription of target genes by binding to the oestrogen receptors (ERα and ERβ) which function as ligand inducible transcription factors. Both ERα and ERβ are members of the nuclear receptor superfamily which is characterised by a highly conserved DNA-binding domain. There are two distinct transcriptional activation domains: the ligand independent AF1 at the N-terminus and the ligand dependent AF2 at the C-terminus which is encompassed by the ligand binding domain (LBD).

Following sequence specific binding of oestrogen receptors to the promoter of target genes, additional co-factors are recruited in order to remodel the chromatin structure or to aid the docking of the RNA polymerase II holoenzyme. The AF2 activity of ERα is mediated through interaction between the LBD and coactivator proteins upon ligand binding. In order to define the ERα-coactivator interface at a molecular level, systematic mutagenesis was carried out. This led to the identification of a group of conserved hydrophobic residues in the LBD that are required for binding the p160 family of coactivators. Together with helix 12 and lysine 366 at the C-terminal end of helix 3, they form a hydrophobic groove that accommodates an LXXLL motif, which is essential for coactivator binding to the receptor.

The presence of endogenous coactivators is a major impediment for studying designated receptor-coactivator pairs in mammalian cells. To circumvent this problem, a yeast genetic screen was conducted to identify suppressor mutant coactivators for a transcriptionally defective ERα. The V380H mutant receptor fails to interact with wild-type p160 coactivators such as SRC1e. However, an altered specificity mutant SRC1e recovered from the screen is able to interact with the mutant receptor, and fully rescues its transcriptional activity in transfected mammalian cells. Remarkably, introduction of the analogous mutation into other p160 coactivator family members confers the ability to suppress the V380H mutation. This suggests that at least in the assays employed, recruitment of a p160 coactivator by ERα is sufficient to activate transcription.

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Contents

Chapter 2 Materials and Methods

List of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Appendix I

Abbreviations

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
CBP	CREB binding protein
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CMV	cytomegalovirus
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	deoxyribonucleic acid
DR+X	direct repeat with X base pair spacing
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
ER	oestrogen receptor
ERE	oestrogen response element
ERKO	ER knock-out mice
FBS	foetal bovine 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
HNF4	hepatocyte nuclear factor 4
Hsp	heat shock protein
kb	kilobase
kDa	kilo Dalton
K_d	dissociation constant
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
NP40	nonidet P40
N-terminal	amino terminal
OD_X	optical density at wavelength of x nm
p/CAF	p300/CBP-associated factor
p/CIP	p300/CBP/co-integrator-associated protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
RAC3	receptor associated coactivator 3
RAR	retinoic acid receptor
RID	receptor interacting domain
RIP	receptor interacting protein
RNA	ribonucleic acid
RXR	retinoid X receptor
S	Svedburg units
SDS	sodium dodecyl sulphate
SF1	steroidogenic factor 1

SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
SRC1	steroid receptor coactivator 1
TAF	TBP associated factor
TBP	TATA binding protein
TEMED	N'N'N'N'-tetramethylethylenediamine
TIF	transcription intermediary factor
TK	thymidine kinase
TR	thyroid hormone receptor
TRAM-1	TR activator molecule-1
Tris	tris(hydroxymethyl)aminoethane
UV	ultra violet
VDR	vitamin D receptor

Chapter 1

Introduction

Introduction

The oestrogen receptor alpha (ERα) is a ligand inducible transcription factor. It belongs to the nuclear receptor superfamily whose members are most likely to be present in all metazoans. Activation of ERα is achieved through binding to its natural ligand, 17β-oestradiol. In the classical model, ERα dissociates from heat shock proteins in the presence of ligand and binds to hormone response elements at the promoter of target genes as homodimers. Extensive biochemical and structural analyses have provided information on receptor function such as DNA-binding, ligand-binding and dimerisation. However, the sequence of events which allow activated receptor to stimulate transcription of target genes remain unresolved. It is generally believed that co-factors are recruited to the activated receptor which may modify the local chromatin structure of the promoter or aid the recruitment of the basal transcription machinery. A vast number of candidate co-factors have been cloned, and it is immediately apparent that the receptor must exhibit selectivity since it would not be able to interact simultaneously with all co-factors. Hence, the important question is to discriminate candidate co-factors which are absolutely essential from others which may only be necessary for specific promoters.

The first part of this thesis describes the characterisation of a surface on the ERα ligand binding domain (LBD) which is utilised for interaction with trascriptional coactivator proteins. The determinants that confer high affinity binding by the coactivator to this surface are also identified. Using the knowledge of the ERα-coactivator interface, a directed genetic selection was conducted for altered-specificity receptor-coactivator pairs that may function in the absence of interference from endogenous proteins. This represents a first step in resolving the problem of functional redundancy among candidate co-factors and provides a means to analyse the essential components required for transcriptional activation by ERα.

The nuclear receptor superfamily

In multicellular organisms, two major classes of signalling molecules mediate long range communication between different parts of the body. The

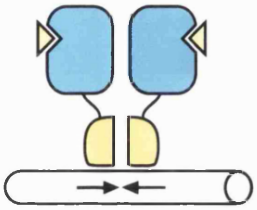
peptide hormones binds to their cell-surface receptors. The signal is then transduced via a number of intracellular secondary messengers and may ultimately influence survival, architecture or gene expression program of the target cell. A second class of signalling molecules are structurally unrelated but have the common properties of being small and lipophilic which allow them to pass through the plasma membrane and bind to their intracellular receptors (Evans, 1988; Jensen, 1991). They include the sex steroids (oestrogen, progesterone and testosterone), adrenal steroids (cortisol and aldosterone), vitamin D, retinoic acids, thyroid hormone and ecdysone. This diverse group of molecules play important roles in the growth, differentiation and homeostasis of the animal body. The sex steroids control the reproductive function of the adult and govern the development of secondary sexual characteristics. The adrenal steroids control the glycogen and mineral metabolism and have widespread effects on the immune system. Vitamin D is critical for calcium metabolism and bone differentiation. Thyroid hormones regulate the development and metabolic behaviour of mammalian cells and are required for metamorphosis in amphibians. Retinoic acids have a major role in the patterning of the embryonic vertebrate body while pulses of ecdysone drives the morphological changes that accompany insect metamorphosis. The importance of small, lipophilic signalling molecules in human physiology are best illustrated by clinical syndromes that arise from insensitivities to these molecules. Individuals with androgen insensitivity syndrome display female sexual phenotype even though they are genotypically male and have an elevated level of serum testosterone (Gottlieb et al., 1998). Hypocalcaemic vitamin D-resistant rickets is a hereditatary disease which is a result of vitamin D insensitivity (Hughes and O'Malley, 1991).

The human glucocorticoid receptor (GR) was the first intracellular receptor to be cloned (Hollenberg et al., 1985). This was soon followed by the cloning of the oestrogen (ER), progesterone (PR) and vitamin D (VDR) receptors (Green et al., 1986; Gronemeyer et al., 1987; McDonnell et al., 1987). Surprisingly, a segment of these receptors displayed similarity to the viral oncogene v-erbA. This was reconciled by the identification of the cellular erbA gene product as the thyroid hormone receptor (TR) (Sap et al., 1986; Weinberger et al., 1986). Comparison of primary amino acid sequences led to the hypothesis that all

intracellular hormone receptors share a common structure in spite of their diverse array of ligands. The recognition of the 'zinc-finger' motifs, indicative of a DNA binding domain, in a highly conserved region suggested that these receptors may constitute a large family of ligand inducible transcription factors. Using the sequence of the putative DNA binding domain, a large number of 'orphan' nuclear receptors had been identified through low stringency hybridisation screening and database searches even though many of their natural ligands remain elusive (for example, Giguére et al., 1988; Blumberg et al., 1998). This approach also led to the cloning of the retinoic acid receptors (RAR) (Giguére et al., 1987; Petkovich et al., 1987). Hence, the original hypothesis that all known lipophilic signalling molecules are bound by a single family of receptor proteins is confirmed.

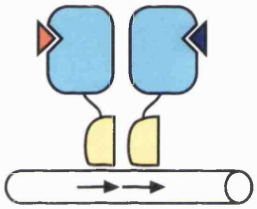
Extensive homology searches place the the intracellular hormone receptors in the nuclear receptor superfamily (Mangelsdorf et al., 1995). The nuclear receptors exhibit a modular structure and loosely conform to the regional definition originally proposed for the ER (Figure 1.1) (Krust et al., 1986). The notion that nuclear receptors are modular in nature actually precedes their molecular cloning. It was first observed that the DNA binding and hormone binding properties of purified GR could be separated by limited proteolysis (Wrange and Gustafsson, 1978). Incidentally, the central DNA binding and the C-terminal ligand binding domains are the most highly conserved regions, not only across species for a given receptor, but also between different nuclear receptors. The DNA binding domain targets the receptor to specific DNA sequences known as hormone response elements (HREs). The ligand binding domain is a multi-functional module: it encompasses the ligand binding pocket, the major dimerisation interface and a ligand dependent transactivation function. It is interesting to note that a large number of orphan receptors including all nuclear receptors identified in the nematode C. elegans have no known ligand. Furthermore, there is no direct relationship between sequence similarity and ligand similarity, for example between RAR and TR. This led to the proposal that the ability to bind ligand may have been acquired independently by nuclear receptors (Escriva et al., 1997; Laudet, 1997). Laudet and co-workers also suggest that the orphan receptors may represent the ancestral form of this class of transcription factors whose activity is originally controlled by post-translational modifications such as phosphorylation. It

Class I
Steroid Receptors



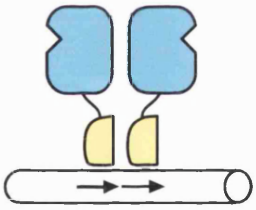
ER *oestrogen*
GR *glucocorticoid*
PR *progesterone*
AR *androgen*
MR *mineralcorticoid*

Class II
RXR Heterodimers



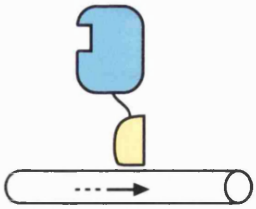
TR *thyroid hormone*
RAR *all-trans RA*
VDR *1,25-$(OH)_2$-VD_3*
PPAR *fatty acid derivatives*
EcR *ecdysone*

Class III
Dimeric Orphan Receptors



COUP ?
HNF4 ?
Tailless ?

Class IV
Monomeric Orphan Receptors



NGFI-B ?
SF-1 (Ftz-F1) ?
Rev-ERB ?

Figure 1.1 Nuclear receptor superfamily. Schematic representation of a typical nuclear receptor which contains a variable N-terminal region (A/B), a conserved DBD (C), a variable hinge region (D), a conserved LBD (E) and a variable C-terminal region (F). Nuclear receptors can be grouped into four classes according to their ligand binding, DNA binding and dimerisation properties: class I steroid receptors which bind as homodimers to palindromic half-sites with a 3 bp spacer, class II RXR heterodimers which bind direct repeats separated by 2 to 5 bp, class III dimeric orphan receptors and class IV monomeric orphan receptors which bind extended half-sites as monomers. Ligands for Class I and II receptors are shown in italics.

is not surprising, therefore, to find that the activity of nuclear receptors with known ligands may also be influenced by the phosphorylation status of the protein (Kato et al., 1995; Rochette-Egly et al., 1997).

The nuclear receptor superfamily can be classified according to the dimerisation and DNA-binding properties of the receptors (Figure 1.1) (Mangelsdorf et al., 1995). Following this scheme, class I receptors include all mammalian steroid receptors which form homodimers and bind to DNA half-sites organised as inverted repeats. Class II receptors are RXR heterodimers which bind to direct repeat of half-sites separated by 2 to 5 base pairs. Class III receptors bind primarily to direct repeats as homodimers while class IV receptors typical binds to DNA as monomers. Based on the relative sequence conservation in the DNA binding and ligand binding domains, an alternative classification has been proposed which divided the nuclear receptor superfamily into six subfamilies (Laudet, 1997). Interestingly, orphan receptors are present in all subfamilies whereas receptors with known ligands are only present in three. This again suggests that the ability of nuclear receptors to bind ligand has been acquired recently in evolution.

The complete genome sequence of C. elegans and Drosophila provide an excellent opportunity to study the evolution of nuclear receptors. In 1995, there were 175 different entries for nuclear receptors from vertebrates, arthropods and nematodes. However, this number was dramatically increased by the discovery that in the C. elegans genome alone, there are more than 200 predicted genes for nuclear receptors (Sluder et al., 1999). Intriguingly, only 20 nuclear receptor genes are found in the complete Drosophila genome (Adams et al., 2000). The large number of predicted nuclear receptors in C. elegans was partly attributable to extensive duplication events on chromosome V which account for half of the nuclear receptor genes. It should also be noted that the gene prediction was based on homology at the DNA binding domain alone and may not represent the number of conventional nuclear receptors. For example, the C. elegans Odr-7 olfactory specific nuclear receptor seems to lack the ligand binding domain (Sengupta et al., 1994). Setting aside the question on the number of *bono fide* nuclear receptors, comparative genome analysis does seem to support the hypothesis that metazoans share a handful of ancestral nuclear receptors which later diversify in each of the

zoological groups (Laudet, 1997). Notably, both the tailless and NGFI-B sub-class of receptors have been found in C. elegans, Drosophila and chordates (Escriva et al., 1997).

In the following sections, a brief account would be given on historical aspects of the identification, molecular cloning and characterisation of the nuclear receptors. This is followed by an account on the physiological roles of oestrogen receptors and its connection with breast cancer. Finally, molecular mechanism of transcriptional regulation by nuclear receptors will be considered.

The identification of intracellular hormone receptors

The idea of intracellular hormone receptor proteins originates from the uptake and concentration of tritium-labelled 17β-oestradiol (E2) by oestrogen responsive tissues such as the rat uterus (Toft and Gorski, 1966; Jensen et al., 1968). When oestrogen target tissues are fractionated in low salt sucrose gradients, a cytosolic steroid-receptor complex which sediments at 8S (~250-300kDa) can be retrieved. In the presence of high salt, this 8S complex is dissociated into a 4S (~65kDa) steroid binding unit. Furthermore, a third complex can be recovered from nuclei of hormone treated target tissues which sediments at 5S (~130kDa) that is capable of DNA binding. These observations suggested the presence of an intracellular oestrogen receptor and led to the proposal that a 'transformation' takes place upon ligand binding by the receptor that converts the 8S form into the 5S form with the concomitant movement from the cytoplasm to the nucleus. A similar 'two-step' model was also proposed for other steroid receptors such as the glucocorticoid and progesterone receptors. It turns out that the 4S steroid binding unit corresponds to an oestrogen receptor monomer. The 8S complex is a heterooligomer which contains in addition to the receptor monomer, a number of heat shock proteins. Hormone binding by the oestrogen receptor leads to the dissociation of heat shock proteins which in turn allows receptor dimerisation to form the 5S complex (Miller et al., 1985b).

The role of heat shock proteins in nuclear receptor function

Heat shock proteins are an evolutionarily conserved group of stress induced proteins. Nevertheless, they are expressed constitutively at significant levels and appear to act as 'chaperones' which ensure the proper folding of other cellular proteins (Jakob and Buchner, 1994). Using a monoclonal antibody, Hsp90 was found to associate with unliganded ER, PR, GR and AR complexes but not with the ligand bound receptors (Joab et al., 1984; Catelli et al., 1985). This led to the proposal that Hsp90 may play a part in keeping steroid hormone receptors in an inactive state. Indeed, isolated ligand binding domains of GR, ER and AR can function as a ligand-inducible switch. They render heterologous fusion proteins inactive which is reversed on hormone binding (Picard et al., 1988; Eilers et al., 1989; Zhu et al., 1998). On the other hand, genetic evidence suggests that the heat shock proteins are required for proper functioning of nuclear receptors in yeast. In a yeast strain which expressed Hsp90 at 5% of the normal level, exogenously expressed GR, ER and RAR failed to activate transcription from reporter genes (Picard et al., 1990; Holley and Yamamoto, 1995). It was found that high affinity hormone binding was abolished in extracts recovered from these cells, and led to the suggestion that Hsp90 may facilitate ligand binding by stabilising the nuclear receptors in a competent state. Furthermore, in the yeast strain carrying a mutant YDJ1 allele, both GR and ER display dramatically increased ligand-independent activity (Kimura et al., 1995). This again points to the involvement of heat shock proteins in nuclear receptor function since the YDJ1 gene product, DnaJ, is a chaperone partner of Hsp70. It can be envisaged that the deregulation of receptor function is a result of mis-folding in the mutant yeast strain. Recently, it was reported that the p23 chaperone acts at yet another step to influence nuclear receptor activity in yeast. In a yeast strain lacking p23, the maximal activity achieved by GR and PR are reduced while that of AR and TR are increased (Freeman et al., 2000). It was argued that p23 may act in a late step of receptor action, for example in the release of ligand or dissociation of ligand bound receptor from its DNA binding site.

It should be noted that no nuclear receptors and their mammalian coactivator partners have been identified in yeast to date. Although exogenously expressed nuclear receptors are capable of activating transcription in yeast, the

mechanism may be fundamentally different (Metzger et al., 1988; Schena and Yamamoto, 1988). Therefore, one must be cautious in interpreting results where transcriptional activity of nuclear receptor in yeast are treated as a reflection of normal receptor function. However, additional evidence for a positive role of heat shock proteins in nuclear receptor function has been reported in Drosophila (Arbeitman and Hogness, 2000). Genetic interaction was observed between mutations in the Drosophila ecdysone receptor (EcR) gene and those in the hsc4 gene which encodes the Hsc70 protein. Animals which are heterozygous for both EcR and hsc4 genes display phenotypes which can be attributed to a reduction in receptor activity arguing that the Hsc70 protein is involved in activating the ecdysone receptor *in vivo*. Notably, no genetic interaction was observed between *usp* and *hsc4* mutations, suggesting that Hsc70 and its associated molecular chaperone-containing heterocomplex does not act on the the EcR hetrodimeric partner, Ultraspiracle (Usp). This corroborates an earlier assumption that only a subset of nuclear receptors require heat shock proteins for proper function, since not all receptors (for example, TR) are associated stably with Hsp90 (Dalman et al., 1990).

Cloning of oestrogen receptors

The human oestrogen receptor (hER) was cloned from cDNA libraries prepared from the MCF-7 breast cancer cell line using a combination of ER monoclonal antibodies and oligonucleotide probes designed from peptide sequences of purified ER (Walter et al., 1985; Green et al., 1986). Northern analysis on a number of breast cancer cell lines indicated that the ER mRNA is approximately 6.2 kilobases in length which encodes a 595 amino acid protein of 66kDa. ER has been found in multiple vertebrate species and cDNA clones were obtained from mouse (White et al., 1987), chicken (Krust et al., 1986), rat (Koike et al., 1987), Xenopus (Weiler et al., 1987), rainbow trout (Pakdel et al., 1990) and salmon (Rogers et al., 2000). The cloning of a second form of the oestrogen receptor (ERβ) came a decade later than the first one (ERα). ERβ has been cloned from rat (Kuiper et al., 1996), human (Mosselman et al., 1996) and mouse (Tremblay et al., 1997). Comparison of primary sequences indicates that the two

receptors share about 95% homology in the DNA binding domain and 55% homology in the ligand binding domain. Although ERα and ERβ are encoded by separate genes and are strictly speaking not isoforms of each other, they have similar affinity and specificity to oestrogens. Nevertheless, there are subtle differences in their affinities towards partial agonists. For example, the phyto-oestrogen Genistein is bound more avidly by ERβ (Kuiper et al., 1997; Barkhem et al., 1998). It was shown that ERα and ERβ can form heterodimers and bind to a consensus oestrogen responsive element (ERE) with an affinity similar to that of ERα and greater than that of ERβ homodimers (Cowley et al., 1997). ERβ also activates ERE-containing reporter genes in an oestradiol dependent manner (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997; Cowley and Parker, 1999). It is unclear whether ERα and ERβ cooperate under physiological conditions to control the expression of oestrogen responsive genes. This is because of their distinct but partially overlapping tissue expression pattern (Kuiper et al., 1997). Furthermore, the two forms of ER can be expressed in different cell types within the same organ, such as the ovary (Sar and Welsch, 1999).

Physiological roles of oestrogen receptors in animal models

The targeted disruption of genes encoding ERα and ERβ in mice have provided invaluable animal models for studying the physiological roles of these receptors, which act as primary mediators of oestrogen action in the body. ERα and ERβ null mice are termed αERKO and βERKO respectively, while the mice homozygous for a targeted disruption of both ER genes are termed αβERKO. It became immediately apparent that disruption of one or both ER genes did not lead to lethality. The animals develop normally and have a life span comparable to their wild-type litter mates. However, these null mice display distinct phenotypes which suggest that both ERα and ERβ are required for the maintenance and normal function of sex accessory tissues.

αERKO

The αERKO female mice are infertile and this can be attributed to their inability to ovulate. Furthermore, enlarged, haemorrhagic and cystic follicles are found in the ovaries of sexually mature animals (Schomberg et al., 1999). It was suggested that the anovulatory phenotype might be secondary to a defect in the hypothalamic-pituitary axis of the αERKO female mice. This was supported by the detection of increased and chronic secretion of lutenising hormone (LH) which clearly deviates from the normal situation where ovulation is triggered by a surge in serum LH (Couse and Korach, 1999).

The uterus of αERKO females possess all three defintive uterine compartments, the myometrium, endometrial stroma and epithelium. However, each of these layers are hypoplastic (Lubahn et al., 1993). The uterine phenotype may be attributed to the loss of upregulation of multiple oestrogen responsive genes which may or may not be the direct transcriptional targets of ERα. The mitogenic effects of oestrogen were thought to be mediated by autocrine and paracrine actions of polypeptide growth factors. This stems from the observations that oestradiol upregulates the uterine levels of trasnforming growth factor-α (TGF-α) (Nelson et al., 1992), insulin-like growth factor-I (IGF-I) (Murphy and Ghahary, 1990), and epidermal growth factor and its receptor (EGF and EGF-R) (Huet-Hudson et al., 1990). The indirect mitogenic effect of oestradiol on uterine epithelium was demonstrated by a series of tissue recombination experiments. By examining tissue recombinants formed by wild-type uterine stroma with αERKO uterine epithelium and vice versa, it was shown that oestrogen-induced proliferation of the uterine epithelium could only be supported by the presence of ERα in the stroma. This implies that epithelial ERα is dispensable for proliferation (Cooke et al., 1997).

The mammary glands of adult αERKO female mice resemble those of newborn female indicating that ERα is necessary for the pre- and postpubertal ductal growth of the murine mammary glands. The deficit in ductal growth is most likely due to insufficient progesterone and prolactin signalling, both of which are downstream targets of ERα action (Day et al., 1990; Kraus et al., 1993). Using the tissue recombination technique, it was shown that the presence of ERα in the stroma is required for the mitogenic actions of oestradiol in the mammary

epithelium arguing once again that peptide growth factors may be involved in a paracrine fashion (Cunha et al., 1997).

The observations that αERKO male mice are infertile provide an important insight into the role of oestrogen in male reproductive function (Eddy et al., 1996). The infertility phenotype was later attributed to the lack of luminal fluid reabsorption by the efferent ductules which connect the testes to the epididymis (Hess et al., 1997). This led to progressive atrophy of the seminiferous epithelium and decrease in sperm counts. Moreover, sperm produced by αERKO male mice fails to fertilise wild-type oocytes *in vitro*. Although the pathology for the αERKO male infertility was extensively studied, a molecular mechanism linking the loss of ERα to the testicular phenotype is still elusive.

βERKO

The defect in reproductive system of βERKO mice is less profound than in αERKO mice. βERKO female mice are fertile but have fewer and smaller litters than wild-type mice. This subfertility phenotype of βERKO female mice may be due to a decrease in the efficiency of oocyte release (Krege et al., 1998). The phenotype of unruptured Graafian follicles in the ovaries of superovulated βERKO female mice resembles that of superovulated Cyclin $D2^{-/-}$ mice (Sicinski et al., 1996). Since the Cyclin D2 gene is induced by oestradiol and FSH treatments in rat granulosa cells and that ERβ is highly expressed in the same cell type (Krege et al., 1998; Robker and Richards, 1998), it was proposed that oestradiol induced proliferation of granulosa cells might be mediated through ERβ activation of the Cyclin D2 gene. Hence the loss of function of ERβ would lead to a deficit in Cyclin D2 expression in granulosa cells and subsequent malfunction of the ovarian follicles (Couse and Korach, 1999).

In contrast to the αERKO female mice, the mammary glands of βERKO female mice appear to undergo normal differentiation and exhibit the lobuloalveolar structures required for lactation which correlates with the low level of expression of ERβ in this tissue. Although ERβ is easily detected in the male reproductive system, the βERKO male mice are fertile, indicating that the effect of oestrogen on male fertility is largely mediated by ERα.

αβERKO

Interpretation of the αERKO and βERKO phenotypes has been complicated by potential functional compensation by the remaining wild-type ER isoform. The role of ERα and ERβ in the physiology of oestrogen target tissues is now clarified through generation of the αβERKO mice (Couse et al., 1999). The αβERKO male mice are infertile and the phenotype closely resembles that of the αERKO mice, reinforcing the importance of ERα in male reproductive function. The hypolplastic uteri observed in αβERKO female mice are similar to that in αERKO mice, suggesting that ERα is the primary mediator in oestradiol induced uterine stromal and epithelial proliferation. One novel phenotype of αβERKO female mice is the apparent sex reversal in the adult ovary which is most likely due to redifferentiation of ovarian components into structures resembling the seminiferous tubules of the testis. Hence, the maintenance of the ovarian identity appears to require the combined action of ERα and ERβ although molecular targets of the receptors remain to be found.

Oestrogen receptor and breast cancer

A link between female sex steroid hormones with breast cancer was first made in 1896 by Beatson when he demonstrated that the removal of ovaries from premenopausal women could, in some cases, impede breast cancer progression (Beatson, 1896). This observation was later extended in a number of mouse models. Notably, production of solid tumours from implanted human breast cancer cells (MCF-7) in athymic, ovariectomised mice, is dependent on oestrogen (Shafie and Grantham, 1981). Patients with ER-positive tumours respond favourably to ovariectomy or to anti-oestrogen (Tamoxifen) therapy whereas the same treatment has little effect on patients with ER-negative tumours (Edwards et al., 1979; Santen et al., 1990). One prevailing hypothesis is that agonist bound ER activates transcription of genes encoding peptide growth factors leading to proliferation of tumour cells in an autocrine or paracrine fashion (Clarke et al., 1991). To this end, it has been domonstrated that TGFα is induced by oestrogen in ER-positive MCF-7 cells and in ER-negative MDA-MB-231 cells stably transfected with ERα (Bates

et al., 1988; Levenson and Jordan, 1998). The latter finding clearly implies that ER is a key transducer of mitogenic signal of oestrogen.

The product of the breast cancer susceptibility gene, *BRCA1*, has been implicated in a number of cellular processes. Recently, it was reported that BRCA1 inhibits the transcriptional activity of ligand-activated ERα in transiently transfected cells (Fan et al., 1999). This led the authors to propose that loss of *BRCA1* may contribute to deregulation of oestrogen-dependent transcriptional pathways and mammary tumourigenesis. However, it is unclear whether this is applicable to clinical situations. This is due to a low frequency of expression of ERα and its transcriptional targets, such as PR and pS2, in breast tumours derived from patients carrying loss of function mutations in the *BRCA1* gene (Osin et al., 1998).

Overexpression of cyclin D1 was frequently observed in breast cancer and it was suggested that such overexpression may promote the growth of oestrogen responsive breast tumours (Bartkova et al., 1994; Musgrove et al., 1994). In addition to its established role in cell cycle progression, it has been reported that cyclin D1 potentiates the transcriptional activity of ERα, independent of complex formation to a CDK partner (Neuman et al., 1997; Zwijsen et al., 1997). Notably, such potentiation occurs in a ligand independent manner and provides a plausible mechanism for oestrogen independent growth of cyclin D1-overexpressing breast tumours. Further reports indicated that cyclin D1 might mediate its action by recruitment of transcriptional coactivators to ERα in the absence of ligand (Zwijsen et al., 1998; McMahon et al., 1999). Although both cyclin D1 and oestrogens are essential for proliferation of breast epithelial cells, their interplay as suggested above is recently questioned by the observations made in a mice strain in which the coding sequences of the cyclin D1 gene have been deleted and replaced by those of cyclin E (Geng et al., 1999). It was shown that cyclin E can substitute cyclin D1 in driving normal mammary epithelial development, even though cyclin E does not bind ERα or serve as its coactivator (Zwijsen et al., 1997; Geng et al., 1999). Moreover, the oestrogen responsive induction of PR proceeds normally in cyclin $D1^{-/-}$ mice, and is unaltered by the ectopic expression of cyclin E. Additional evidence is clearly needed on this topic and one approach may involve overexpressing cyclin D1 in the mammary gland of αERKO mice in the future.

Mutations in the ERα gene are rarely found in breast tumours. In advanced stages of breast cancer, expression of oestrogen receptors are normally lost which correlates with resistance to anti-oestrogen therapy. This is in contrast with numerous mutations in the androgen receptor (AR) gene, found in prostate cancer patients (Gottlieb et al., 1998). In some cases, these mutations appear to confer androgen independent growth of prostatic tumours by altering the ligand binding specificity of the AR. Nevertheless, there are two notable examples of naturally occuring ERα mutant which might contribute to tumour progression. The first one, commonly known as exon 5 variant, is a truncated receptor which lacks the majority of the ligand binding domain (Fuqua et al., 1991). It was reported that this variant is always coexpressed with the wild-type ERα and enhances its transcriptional activity in transfected cells (Zhang et al., 1993; Chaidarun and Alexander, 1998). The second ERα mutant contains a point mutation which replaces Y537 with asparagine (Zhang et al., 1997). This mutant displayed constitutive transcriptional activity and given that it was identified in tumours from metastatic breast cancer patients, it might confer selective advantage for tumour progression.

Crystal structures of nuclear receptors

The success in purifying biologically active DNA binding and ligand binding domains of nuclear receptors allowed the determination of three-dimensional structure of these highly conserved domains (Figure 1.2). The crystal structures of ERα DBD and LBD not only confirm previous findings from biochemical studies, but open new avenues for further investigation into other aspects of receptor function such as co-activator recruitment. The molecular features of nuclear receptor DBD and LBD will be considered in the ensuing sections.

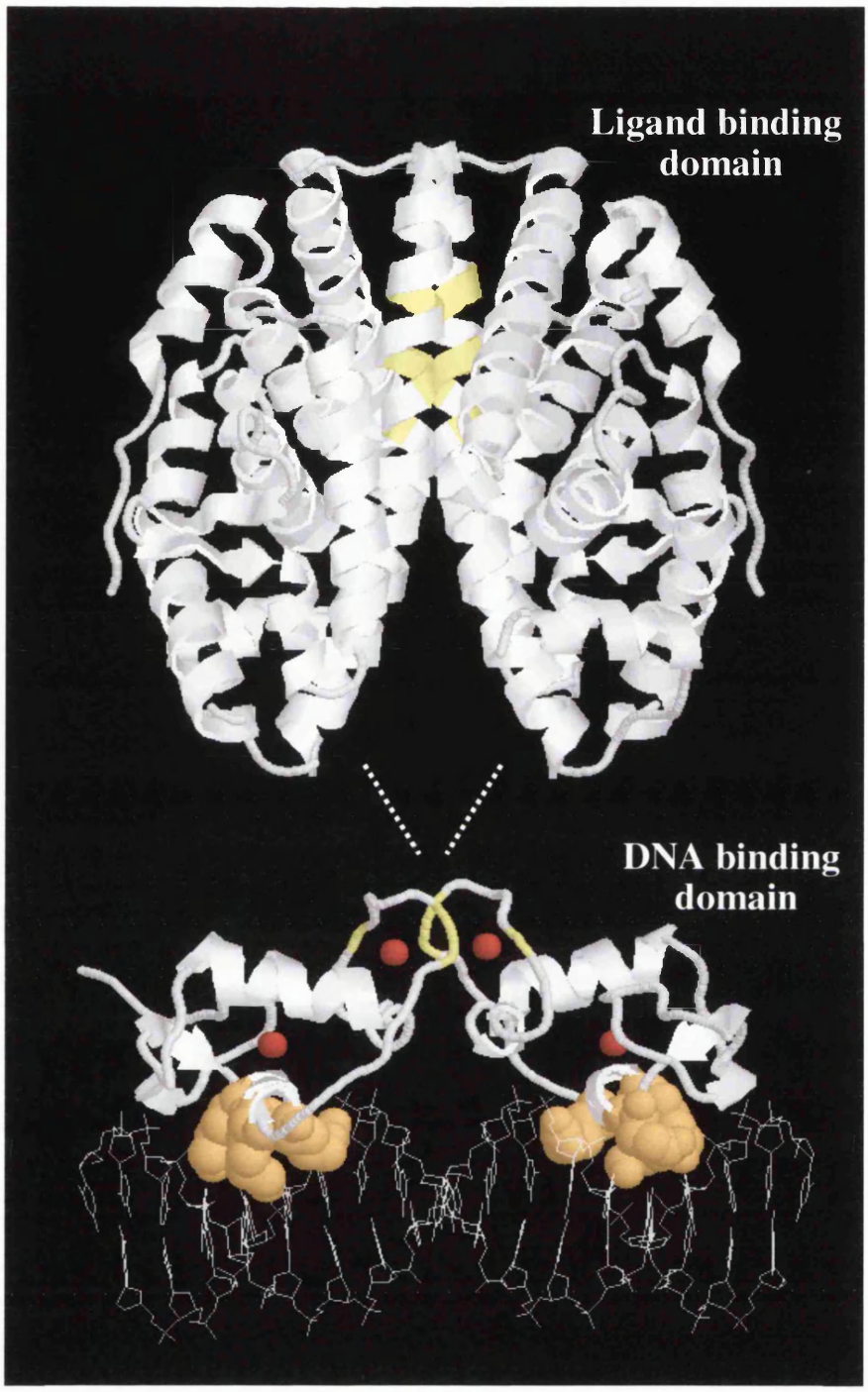


Figure 1.2 The 3-dimensional structures of the ERα DNA binding and ligand binding domain. The ERα DNA binding domain (DBD) crystallised in the presence of its cognate recognition sequence (Schwabe et al., 1993) and its ligand binding domain crystallised in the presence of 17β-oestradiol (Brzozowski et al., 1997) are shown with the dotted lines indicating the hinge region which is unstructured. Residues responsible for DNA recognition are coloured orange and are shown in spacefill mode. The zinc ions are highlighted in red. Residues which contribute to receptor dimerisation are highlighted in yellow.

DNA binding domain

DNA binding is one of the fundamental properties of transcription factors. In nuclear receptors, the DNA binding domain (DBD) consists of approximately 80 to 100 amino acids which are highly conserved throughout evolution. There are eight invariant cysteines in the DBD and the resultant Cys2-Cys2 sequence motifs loosely resemble the 'zinc finger' motifs of the 5S ribosomal RNA transcription factor TFIIIA (Miller et al., 1985a). Two Zn^{2+} ions are coordinated tetrahedrally by the Cys2-Cys2 motifs and are essential for the maintenance of the DBD in its native and active form (Freedman et al., 1988). This was demonstrated by the loss of DNA binding activity and protease sensitivity of the purified DBD upon chelation of metal ions by low pH dialysis and the restoration of its integrity by incubation with Zn^{2+} ions. The importance of the conserved cysteines was in turn shown by genetic selection of DNA-binding defective glucocorticoid receptor in yeast in which mutants with a single mutation at the zinc-coordinating cysteine were recovered at high frequency (Schena et al., 1989).

Nuclear receptors can be classified into two groups according to their 'half-site' recognition sequence. Glucocorticoid receptor (GR), Progesterone receptor (PR), Androgen Receptor (AR) and Mineralocorticoid receptor (MR) recognise the consensus palindromic sequence which consists of the half-site 5'-AGAACA-3' separated by three base pairs. Oestrogen receptors (ER), Thyroid hormone receptors (TR), Retinoic acid receptors (RAR) and Vitamin D receptor (VDR) represent the second group whose half-site recognition sequence is 5'-AGGTCA-3'. The ER consensus is palindromic with the half-site separated by three base pairs, whereas the consensus for VDR, TR and RAR which all form heterodimers with Retinoid X receptor (RXR) are direct repeats with the half-site separated by 3, 4 or 5 base pairs, respectively (Perlmann et al., 1993). Following this scheme, there seems to be a very limited repertoire of DNA binding sites for a large number of nuclear receptors. However, natural hormone response elements (HREs) often deviate from the consensus sequence, which presumably serve to modulate the binding affinity of a given receptor while the flanking sequence would provide an extra level of specificity. Among the natural oestrogen response elements (EREs), only the one found in the Xenopus vitellogenin A2 gene promoter fits the perfect

consensus while others found in the Xenopus vitellogenin B1, human PS2 or human c-fos gene promoters contain one or more changes in one of their half-sites (Klein-Hitpass et al., 1986; Martinez et al., 1987; Berry et al., 1989; Weisz and Rosales, 1990).

The basis of DNA recognition by nuclear receptor DBD was studied extensively a decade ago with the focus on GR and ER. The comparison between these two receptors was particularly informative since they recognise different prototypic half-sites which are arranged in the same way. Hence, difference in binding affinity could be attributed solely to DNA recognition *per se*, independent of the spacing and orientation of the half-sites. Individual zinc fingers of nuclear receptors are encoded by separate exons and it is highly likely that they have evolved to perform distinct functions (Ponglikitmongkol et al., 1988). This notion was reinforced by domain swapping experiments. When the first zinc finger of GR was used to replace its counterpart in ER, the chimeric receptor activated a glucocorticoid response element (GRE)-dependent, but not an ERE-dependent, reporter gene in response to oestradiol (Green et al., 1988). This demonstrated that DNA binding specificity is determined by the first zinc finger of the DBD. Through further mutagenesis, three amino acids, which constitute the so-called 'P-box', were shown to be essential in distinguishing an ERE from a GRE (Mader et al., 1989; Umesono and Evans, 1989). Comparison of amino acid sequences indicate that the P-box is conserved among GR, PR, MR and AR (GS..V) whereas a different consensus is found among ER, RAR, TR and VDR (EG..A/G) which correlate well with their classification based on half-site preference.

The purified ER or GR DBD is monomeric in solution, however DBD monomers bind to each HRE half-site in a cooperative manner and form dimers on DNA (Tsai et al., 1988; Hard et al., 1990a; Hard et al., 1990b; Schwabe et al., 1990). This cooperative mechanism might be pertinent to binding of natural HREs where only one half-site fits the consensus. Hence, binding to the imperfect half-site would be dependent on the initial association of the receptor to the high-affinity half-site. The crystal structures of ER DBD and GR DBD bound to their respective cognate response elements extended previous biochemical characterisation and provided detailed information about the DBD protein structures and the mechanism of DNA recognition (Figure 1.2). The ER and GR

DBD crystal structures together with the solution structures of RAR and RXR DBD reveal a common fold which consists of a pair of amphipathic α-helices oriented at right angles and cross at their mid-points with an extensive hydrophobic core between them (Luisi et al., 1991; Knegtel et al., 1993; Lee et al., 1993; Schwabe et al., 1993). At the N-terminus of each helix are two cysteines which form part of the Cys2-Cys2 zinc-binding motif. A conserved arginine residue (R489 in GR and R234 in ER) appears to stabilise the overall fold of the DBD by making extensive contacts with amino acids in each finger and with phosphate backbone of DNA. This is underscored by the recovery of a R489K mutant GR through genetic selection in yeast for mutations that affect GRE binding (Schena et al., 1989). The R489K mutant exhibited at least 10-fold decrease in its affinity to GRE and this defect is accentuated at cold temperature implying the involvement of R489 in intramolecular protein-protein interaction.

In agreement with the biochemical evidence, two ER DBD monomers dimerise upon binding to adjacent major grooves from one side of the DNA double helix (Schwabe et al., 1993). The dimer interface features direct van der Waals contacts and hydrogen bonds between residues from each monomer, supplemented with bridging contacts involving a number of ordered water molecules. The residues in the dimer interface constitute part of the 'D-box' which were thought to mediate DBD dimerisation previously (Umesono and Evans, 1989). Of the two α-helices in the ER DBD, only the one in the first zinc finger contacts DNA. Four amino acids from this recognition helix (E203, K206, K210 and R211) make hydrogen bonds with the central four base pairs of the 6-bp half-site (Schwabe et al., 1993). Furthermore, seven ordered water molecules complete a network of intermolecular interactions between amino acid side-chains and base pairs together with the phosphate backbone of the DNA. By comparing the structures of ER DBD and GR DBD bound to DNA, it is apparent that the discrimination of half-site sequence is achieved by a combination of specific residues and rearrangement of side-chains of conserved residues (Luisi et al., 1991; Schwabe et al., 1993). E203 of ER makes specific contacts with the divergent base-pairs in ERE and although the GR counterparts of K206, K210 and R211 in ER contact the DNA, their side-chains adopt different conformations. It is interesting to note, of the three residues that constitue the 'P-box', only E203 was found to directly contact DNA.

However, it has been speculated that the other residues in the 'P-box' might play a repulsive role to non-target DNA (Freedman, 1993; Schwabe et al., 1993).

ER and GR form homodimers on palindromic DNA recognition sequence, whereas TR, RAR and VDR all form heterodimers with RXR on direct repeats which implies that a different strategy is employed for the assembly on DNA. This was revealed by the crystal structure of the complex formed by DBD of TR and RXR bound to a thyroid-response element (DR-4: 5'-AGGTCA-3' half sites separated by four base pairs) (Rastinejad et al., 1995). The RXR DBD preferentially occupies the upstream half-site and the TR DBD the downstream one. This polarity is imposed by the stable heterodimer interface formed only in the observed orientation. The mode of DNA recognition by each of the DBDs is similar to that observed in the ER DBD-DNA structure. A unique feature of the TR DBD is a third α-helix at its C-terminus which makes extensive contacts with the minor groove DNA situated between the half-sites. This helix is also thought to play a role in spacer discrimination which prevents binding of TR-RXR to half-sites separated by less than four base pairs. Through computer modelling, it was further suggested that the recognition of cognate DNA binding sites by RXR heterodimers is intimately linked to the use of non-conserved residues by TR, RAR and VDR in the dimer interface which indirectly dictate the spacing permitted between each half-site (Rastinejad et al., 1995).

Ligand binding domain

A number of crystal structures for the ligand binding domain (LBD) of nuclear receptors (NRs) have been reported in the last few years. These crystal structures not only reveal a novel protein fold which is conserved across the nuclear receptor superfamily, but provide important molecular insights into how ligand binding, dimerisation and transcriptional activation are mediated by the LBD. The first reported crystal structure was the dimeric unliganded RXRα (Bourguet et al., 1995). The RARγ, TRβ and PR were crystallised in the presence of their natural agonists (Renaud et al., 1995; Wagner et al., 1995; Williams and Sigler, 1998). The crystal structure of dimeric ERα was solved in the presence of agonists (17β-oestradiol and Diethylstibestrol) and antagonists (Tamoxifen and

Raloxifene) (Brzozowski et al., 1997; Shiau et al., 1998; Tanenbaum et al., 1998). Similarly, the ERβ was crystallised in the presence of a partial agonist (Genistein) and an antagonist (Raloxifene) (Pike et al., 1999). Finally, the crystal structure of PPARγ was solved in the absence or presence of its agonist (Rosiglitazone) (Nolte et al., 1998).

In general, NR LBD structures consist of 12 α-helices and one β-turn which are arranged in a common fold that resembles an antiparallel three-layered α-helical sandwich. By comparing the unliganded RXRα and agonist bound RARγ structures, a mouse-trap model was proposed for ligand binding and the subsequent acquisition of activation function which might be applicable to other NRs (Renaud et al., 1995). By a combination of electrostatic and hydrophobic interactions, the ligand is drawn into the ligand binding cavity which triggers a series of conformational changes. Helix 11 is repositioned to form a continuous helix with helix 10 while helix 12 assumes a position that seals the ligand binding cavity. The movement of helix 12 unleashes the Ω-loop (located between helices 2 and 3), which flips over underneath helix 6 and in the process bends the N-terminal part of helix 3 towards the core of the LBD (Figure 1.3) (Renaud et al., 1995). It should be noted that this model does not seem to apply to PPARγ. It has a potential ligand entry site situated between helix 3 and the β-sheet which is lined by hydrophilic residues that are poorly conserved in the NR superfamily. This is coupled with the observation that helix 12 of unliganded PPARγ assumes a position which closely resembles that of the liganded receptor (Nolte et al., 1998).

The crystal structures of ERα and ERβ provide clear examples of how agonists are accomodated within the ligand binding pocket (LBP) of steroid receptors and how antagonists create disruption to the LBD which lead to its loss of function in transcriptional activation. The LBP volume of oestrogen receptors are significantly larger than the size of their natural ligand, 17β-oestradiol (E2) (Brzozowski et al., 1997). The excess in space of LBP does not allow extensive hydrophobic interactions between the side chains that lined the LBP and the aromatic rings of the steroid molecule. Instead, it is held in place by electrostatic interactions on both ends. The hydroxyl groups at the A- and D-rings of E2 form hydrogen bonds with E353 and R394 at one end and H524 at the other. E353

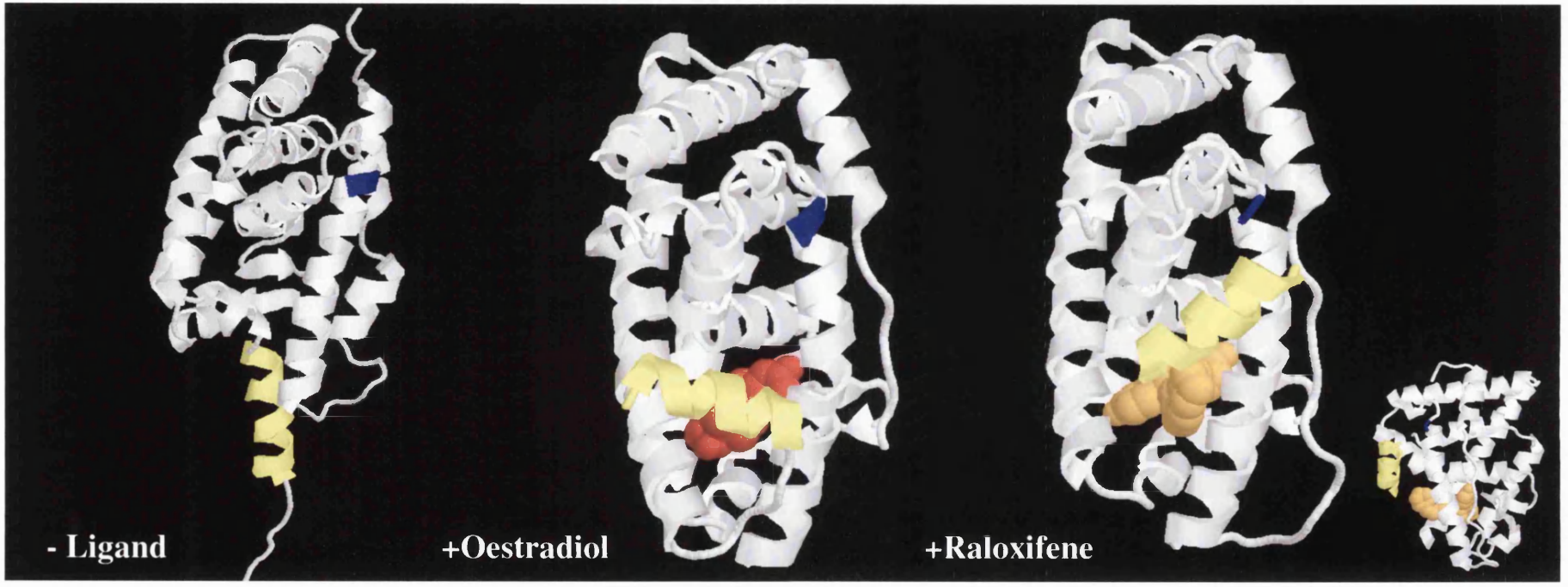


Figure 1.3 Crystal structures of the ERα ligand binding domain. The structures of the ERα ligand binding domain (LBD) crystallised in the presence of the agonist 17β-oestradiol (red) and the antagonist Raloxifene (orange) are compared with the structure of the RXRα LBD crystallised in the absence of ligand (Bourguet et al., 1995, Brzozowski et al., 1997). The helix 12 (highlighted in yellow) adopts three distinctive positions which are thought to correlate with ligand binding. In the absence of ligand, by analogy to the apo-RXRα structure, helix 12 of ERα is likely to protrude from the core of the LBD. Binding of 17β-oestradiol molecule leads to realignment of helix 12 and closure of the ligand binding pocket (LBP). However, the bulky side-chain of the raloxifene molecule projects away from the LBP (as shown in the side-view image), forcing helix 12 to adopt an alternative position. A highly conserved lysine residue in helix 3, which is important for ERα AF2 activity is highlighted in blue. The PDB entry codes for the above structures are 1LBD (apo-RXR), 1ERE (ERα+oestradiol) and 1ERR (ERα+raloxifene).

confers selective binding to E2 since it would not accommodate the hydrogen bond accepting 3-keto group at the A-ring of other steorid hormones, such as progesterone. Although the ER antagonists, Tamoxifen and Raloxifene, are held in place by similar electrostatic interactions as observed for E2 binding, these antagonists induce a distinct conformational change in the ER LBD as a result of their bulky side-chains (Brzozowski et al., 1997; Shiau et al., 1998). The side-chains of these antagonists project from the ligand binding cavity and are stabilised by a salt-bridge with D351. As a result, helix 12 adopts an alternative position and resides in a hydrophobic cleft formed between helices 3, 4 and 5. The complementarity of the hydrophobic cleft with the inner surface of helix 12 indicates that the alternative position is not an artifact produced by the crystal lattice. In fact, the antagonist induced intramolecular interactions provided important clues for the delineation of the ER coactivator docking surface (see Results and Discussion). It is worth noting the critical role of D351 in the action of antagonists. This is supported by the identification of a point mutation, D351Y in ERα, from tumours which are stimulated by Tamoxifen in athymic mice (Catherino et al., 1995). Breast cancer cells stably transfected with this mutant receptor respond to both Tamoxifen and Raloxifene as agonist suggesting that this may be one mechanism for the development of Tamoxifen resistance in breast tumours (Levenson and Jordan, 1998). From a structural point of view, it can be envisaged that the side-chains of antagonists may adopt an alternative position or are simply untethered in the absence of D351 which allow helix 12 to assume its native position.

Dimerisation

The dimerisation properties of ER had been studied extensively by generation of a large panel of deletion and point mutants. Using gel-shift analysis, ER dimer formation on DNA was thought to be largely mediated by an agonist induced dimerisation function in the LBD, supplemented by weak constitutive dimerisation through the DBD (Kumar and Chambon, 1988). Later on, it became apparent that oestrogen binding is not absolutely required for dimerisation and high affinity DNA binding *in vitro* as demonstrated by the mERα mutant G525R

(Fawell et al., 1990). The crystal structures of the hERα LBD confirmed the previous localisation of the dimerisation interface to a group of residues in helix 11 and revealed additional contacts between helix 8 from one monomer and parts of helix 9 and 10 from its dimerisation partner (Brzozowski et al., 1997; Shiau et al., 1998). Dimerisation is driven by interactions between L504, A505, L508, L509 and L511 at the N-terminus of helix 11 which form a tightly packed hydrophobic interface, and is further stabilised by a network of hydrogen-bonding residues.

The symmetrical arrangement of the hERα LBD dimer interface is similar to that of the hRXRα homodimer, hERβ homodimer and the hRARα/mRXRα heterodimer (Bourguet et al., 1995; Pike et al., 1999; Bourguet et al., 2000). Interestingly, asymmetry was observed in the crystal structure of PPARγ/RXRα heterodimer (Gampe et al., 2000). As a result, additional salt bridges between the receptors are proposed to stabilise helix 12 of PPARγ in a position that facilitates the recruitment of coactivators. This may account for the permissiveness of the PPARγ/RXRα heterodimer which can be activated by RXR agonist alone in contrast to what is observed for RAR/RXR heterodimers (Mangelsdorf and Evans, 1995). It should also be noted that the dimerisation interface of ERα homodimer (1700Å^2) is significantly larger than that of PPARγ/RXRα or RARα/RXRα heterodimer (~1000Å^2). The smaller interface in heterodimers involving RXR has been postulated as the basis for its promiscuous binding to a number of receptor partners possibly by compromising the stability of protomer association (Bourguet et al., 2000).

Transcriptional activation by nuclear receptors

Transcriptional activation by the ERα has been studied for more than a decade and its mechanism appears to be applicable to most if not all of the nuclear receptors with known ligand. The ERα functions as a classical transcription enhancer since it stimulates transcription initiation irrespective of the orientation and position of its response element in relation to the transcription start site (Klein-Hitpass et al., 1986; Seiler-Tuyns et al., 1986; Berry et al., 1989). There are two distinct transcriptional activation functions in ERα: the ligand independent activation function 1 (AF1) at the N-terminus and the ligand dependent AF2 at the

C-terminus which is encompassed by the ligand binding domain (LBD) (Webster et al., 1988; Lees et al., 1989; Tora et al., 1989). It appears that AF2 is highly conserved among nuclear receptors because of the sequence and structural conservation of the LBD. However, the AF1 is much more divergent which is partly reflected by the variability in size of the region N-terminal to the DBD, ranging from 23 amino acids in VDR to more than 550 amino acids in PR and AR (Baker et al., 1988; Chang et al., 1988; Kastner et al., 1990). Surprisingly, both ERα and GR had been shown to activate transcription of reporter genes in yeast (Metzger et al., 1988; Schena and Yamamoto, 1988). Despite the original speculation, it is now clear that transactivation by nuclear receptors is unlikely to involve a common mechanism in yeast and in mammalian cells since no yeast homologs of mammalian coactivators have been identified to date.

Transcriptional activation by AF1 of ERα is cell type and promoter specific (Tora et al., 1989). It activates transcription efficiently in CEF cells but not in HeLa cells. In addition, it seems to be more active on the pS2 promoter than on the minimal TATA-based promoter. It is possible that cell-type specific post-translational modification may affect the activation potential of AF1. Furthermore, AF1 may preferentially cooperate with other transcription factors bound on the same promoter while remain silent on others. It has been reported that the AF1 activity of ERα can be modulated by phosphorylation. Serine 118 was found to be a major phosphorylation site within AF1 and replacement of this residue with alanine reduced the transcriptional activity of ERα (Ali et al., 1993). It turns out that S118 can be phosphorylated by at least two distinct mechanisms. On the one hand, S118 is phosphorylated *in vitro* and *in vivo* by the mitogen-activated protein kinase (MAPK) in a ligand-independent manner (Kato et al., 1995; Bunone et al., 1996). By treating cells with epidermal growth factor (EGF), or by expressing MAPK kinase or Ras, both of which activate MAPK, the transcriptional activity of transiently expressed ERα can be enhanced. Besides the transient phosphorylation induced by EGF, a second ligand dependent phosphorylation at S118 was detected which followed a different kinetics (Joel et al., 1998). Recently, it was reported that this ligand dependent event is mediated by cdk7 which is an integral component of the general transcription factor TFIIH (Chen et al., 2000a). It was proposed that the LBD of agonist bound ERα recruits TFIIH, allowing cdk7 to

phosphorylate S118. Phosphorylation of AF1 by cdk7 is not unique to ERα since it has been shown that the AF1 of RARα can be stimulated in a similar manner (Rochette-Egly et al., 1997). It is unclear, however, what is the molecular mechanism that leads to enhancement of AF1 activity following serine phosphorylation in ERα and RARα. It is conceivable that such modification may increase the affinity of AF1 to transcriptional coactivators. Indeed, Giguére and co-workers reported that MAPK mediated phosphorylation of ERβ AF1 may enhance its recruitment of SRC1, a p160 coactivator (see later) (Tremblay et al., 1999).

The ERα ligand dependent AF2 is encompassed by the highly conserved LBD. It is inactive in the absence of ligand and is activated upon agonist binding. On the other hand, binding of antagonists such as ICI 164384 suppresses the AF2 activity (Berry et al., 1990). By deletion and point mutagenesis, an AF2 activation domain (AD) core was localised at the C-terminus of ERα and GR (Danielian et al., 1992). The same region has also been implicated in AF2 activity in RARα and TRα (Barettino et al., 1994; Durand et al., 1994). It turns out that the AF2 AD core is located in the amphipathic α-helix 12 of the LBD. By comparing the crystal structures of the apo-RXRα, agonist bound ERα and antagonist bound ERα, it is apparent that the position of helix 12 in relation to the rest of the LBD is crucial for the AF2 activity (Figure 1.3) (Bourguet et al., 1995; Brzozowski et al., 1997; Shiau et al., 1998). It has been postulated that the position of helix 12 at the agonist bound conformation may be stabilised by additional intramolecular interaction. For example, a salt bridge is formed between a conserved glutamic acid residue in helix 12 and a conserved lysine residue at the C-terminal end of helix 5 in RARγ (Renaud et al., 1995). Mutation of either of these residues completely abrogated the transcriptional activity of the receptor (Durand et al., 1994; Renaud et al., 1995). It is interesting to note that replacement of Y541 in mERα by alanine generates a constitutively active receptor (Weis et al., 1996; White et al., 1997). This conserved tyrosine residue is located just prior to helix 12 and it is tempting to speculate that post-translational modification of this residue may either lock helix 12 in an inactive position or promote helix 12 to adopt the position which is normally induced by agonist binding. Taken together all the evidence presented so far, it is clear that helix 12 which is highly conserved throughout the nuclear receptor superfamily is crucial for AF2 activity. Nevertheless, co-crystal structures

of receptor LBD with fragments of coactivators and biochemical data now indicate that helix 12 may only form part of the coactivator interacting surface (see Results and Discussion). For example, a lysine residue at the C-terminal end of helix 3 has been shown to play an essential role in AF2 function in ERα and TRβ (O'Donnell and Koenig, 1990; Henttu et al., 1997).

The AF1 and AF2 of nuclear receptors has so far been discussed as autonomous entities, however it is conceivable that they may synergise or influence the activity of each other in the context of a full-length protein. This is demonstrated using an F9 embryonal carcinoma (EC) cell differentiation system. RARγ null F9 EC cells are unable to undergo primitive endodermal differentiatiation upon treatment with retinoic acid (RA) (Taneja et al., 1995). This is rescued by stable re-expression of full-length RARγ but not by truncated receptors devoid of AF1, implying that AF1 and AF2 must cooperate to perform its physiological function in this system (Taneja et al., 1997). This report also reinforces the idea that AF1 activity is promoter dependent since the expression of a subset of RA-target genes was restored by re-expression of the RARγ AF1 deletion mutant. Nevertheless, the mutant was clearly not sufficient to elicit the phenotypic rescue. Communication between AF1 and the LBD can also lead to a decrease in the overall activity of the receptor. For instance, phosphorylation of PPARγ AF1 by MAPK reduced its transcriptional activity (Hu et al., 1996). This was later attributed to interdomain communication within the receptor which decreased the affinity of the LBD to its ligand (Shao et al., 1998).

RNA polymerase II and the general transcription factors

RNA polymerase II (pol II) is responsible for the transcription of messenger RNAs and several small nuclear RNA. Understanding of the RNA pol II and the general transciption factors is pertinent to the study of trascriptional activators such as nuclear receptors, since RNA pol II must be recruited to the core promoter of target genes prior to gene activation. This section describes current views on the initiation of RNA pol II transcription.

Eukaryotic core promoters are composed of one or more of the following sequence elements: the TATA element, located ~25-30 base pairs upstream of the

transcription start site; the initiator element (Inr), a sequence that encompasses the start site which can direct initiation of trascription in the absence of a TATA box; and the downstream promoter element (DPE), which was identified in Drosophila TATA-less promoters and is located ~30 base pairs downstream of the start site (Smale and Baltimore, 1989; Burke and Kadonaga, 1997; Smale, 1997). It is the core promoter where the pre-initiation complex (PIC) containing RNA pol II and the general transcription factors (GTFs), TFIIA, B, D, E, F and H are assembled. Except TFIIB, each of the other GTFs is composed of multiple subunits and the complete set of GTFs amounts to about 30 polypeptides. They are remarkably well-conserved through evolution and homologs can be found from yeast to human.

The GTFs could be purified as separate entities and assembled at a promoter in a specific order *in vitro* (Orphanides et al., 1996). TFIID consists of the TATA-binding protein (TBP) and at least eight additional subunits termed TAF_{II}s, for TBP-associated factors. The TATA box is recognised by the TBP whereas the Inr and DPE can be recognised by the $TAF_{II}250$- $TAF_{II}150$ and the $TAF_{II}60$- $TAF_{II}40$ complexes, respectively (Burke and Kadonaga, 1997; Smale, 1997; Chalkley and Verrijzer, 1999). It can be envisaged that the capacity of TFIID to recognise multiple sequence elements of the core promoter ensures that weak promoters where one or more elements are missing would still be recognised. It is interesting to note that a number of TAF_{II}s contain regions that exhibit the classical 'histone-fold' as revealed in crystallographical studies (Burley and Roeder, 1996; Hoffmann et al., 1996; Xie et al., 1996). It is conceivable that exchange of core promoter DNA may occur between the nucleosome and TFIID.

The eight-subunit TFIIH possesses both helicase and kinase activities which are important in promoter melting and promoter clearance, respectively. The ERCC3 subunit of TFIIH is responsible for the ATP-dependent promoter melting and is now thought to act as an unconventional DNA helicase (Kim et al., 2000). It is proposed that ERCC3 functions as a molecular wrench by rotating the DNA relative to fixed upstream DNA-protein interactions made by RNA pol II and other GTFs. The kinase activity of TFIIH originates from three of its subunits: cdk7, cyclin H and MAT-1 which form the cdk-activating kinase (CAK) (Nigg, 1996). It phosphorylates the carboxy-terminal domain (CTD) of the largest subunit of RNA

pol II which consists of multiple tandem repeats of the heptapeptide YSPTSPS. Phosphorylation of the CTD by TFIIH marks the transition from transcription initiation to elongation and facilitates the release of RNA pol II from the GTFs.

The traditional view that PIC formation involves stepwise assembly has been superseded by the proposition that the basal transcription machinery is recruited to the promoter in a single step *in vivo*. This notion was supported by the purification of the RNA pol II holoenzyme in yeast. In search of suppressors of cold-sensitive phenotype of a mutant yeast strain with shortened CTD, Young and co-workers identified nine *srb* genes (for suppressor of RNA polymerase B) which are found to be present in a large multiprotein complex that also contained the RNA pol II, TFIIB, TFIIF and TFIIH (Koleske and Young, 1994). A similar, "mediator complex" which lacks RNA pol II, TFIIH and TFIIB, was isolated by the Kornberg group through a search for protein factors that would enable a purified yeast transcription system to respond to acidic activators (Kim et al., 1994). Since then, multiple mammalian complexes containing homologs of yeast SRB/mediator proteins have been isolated and these are shown to be either positive or negative regulators of transcription (reviewed in Hampsey and Reinberg, 1999). It is noteworthy that the association of the RNA pol II with multiprotein complexes appears to be a common theme throughout the transcription cycle. This is supported by the discovery of the Elongnator complex which preferentially associates with the hyperphospharylated CTD of RNA pol II during transcription elongation (Otero et al., 1999).

Transcriptional control by modification of chromatin structure

DNA in eukaryotic cells are packaged into nucleosomal units around histone octamers which consist of an H3/H4 heterotetramer and two H2A/H2B heterodimer. Nucleosomal units are further compacted into higher order chromatin fibres with the participation of histone H1 (Kornberg and Lorch, 1999). The chromatin structure presents a general obstacle which must be overcome by transcription factors to elicit gene activation. On the other hand, active gene repression may be achieved by modifying the chromatin structure at the promoter region. Chromatin remodelling complexes can be broadly divided into two classes.

One class, represented by the SWI/SNF family and the ISWI family, is characterised by its ATP-dependent chromatin remodelling activities (Varga-Weisz and Becker, 1998; Sudarsanam and Winston, 2000). It has been reported that GR activation of a chromosomally integrated reporter gene, under the control of the mouse mammary tumour virus (MMTV) promoter requires the hSWI/SNF complex (Fryer and Archer, 1998). The second class of chromatin remodelling complexes is characterised by their histone acetyltransferase (HAT) or histone deacetylase activities (Struhl, 1998). They add or remove acetyl groups from the conserved lysine residues at the N-terminal tails of histones. Gcn5 is the first nuclear histone acetylase to be identified and it is present in at least two complexes in yeast, namely the ADA and SAGA complexes (Brownell et al., 1996; Grant et al., 1997). Similarly, a number of histone deacetylase complexes has been identified in yeast which contain distinct catalytic subunits such as Rpd3 and Hda1 (Rundlett et al., 1996). It is interesting to note that Rpd3 and Hda1 are evolutionary conserved and they are also present in mammlian complexes involved in transcriptional repression (Pazin and Kadonaga, 1997). In contrast, HAT activity has been reported for a number mammalian proteins, some of them involve in nuclear receptor transactivation, which do not have yeast homologs (see later). Taken together, the unifying theme is that transcriptional activators and repressors may recruit one or more of the complexes described above and influence the expression of their target genes by remodelling the local chromatin environment.

Histone acetylation has long been associated with transcriptional activity of eukaryotic cells and it was found that hyperacetylation marked 'poised' or actively transcribed regions of the genome (Hebbes et al., 1988; Hebbes et al., 1992). It was proposed that acetylation neutralises the charge of the histone tails and reduces their affinity to DNA, which in turn increases the accessibility of transcription factors to chromatin templates (Hong et al., 1993; Vettese-Dadey et al., 1996). A second model, which is not mutually exclusive to the first, predicts that histone acetylation may create or eliminate sites that is recognised by another factor. For example, interaction between the yeast global transcriptional repressor Tup1 with histones H3 and H4 is abolished upon histone hyperacetylation (Edmondson et al., 1996). It has been reported that the double bromodomain of $hTAF_{II}250$ binds to di-acetylated histone tails (Jacobson et al., 2000). Hence, acetylation of histones by

upstream activators may increase the overall affinity of TFIID to the core promoter. To this end, it was demonstrated that activation of nuclear receptors by hormone treatment induces histone hyperacetylation on chromatin of endogenous target genes (Chen et al., 1999b).

In addition to histone acetylation, histone phosphorylation represents another covalent modification which may affect chromatin structure and gene expression. It has recently been shown that histone H3 phosphorylation at the c-fos promoter in EGF-stimulated cells is closely followed by acetylation of the same molecule (Cheung et al., 2000). Hence, the synergistic coupling of covalent modifications may reinforce the identity of an actively transcribed gene. Nevertheless, it was noted that such coupling may only occur in a subset of genes (Lo et al., 2000). Therefore, the activation status of promoters may be marked by distinct covalent modifications which form the 'histone code' that is recognised by transcriptional regulatory proteins (Strahl and Allis, 2000).

Transcription intermediary factors / Receptor interacting proteins

Upon DNA binding, activated nuclear receptors can stimulate transcription of target genes in a number of ways. They can either act on the repressed chromatin and render the promoter region more accessible to other transcription factors, or on the general transcription machinery. One traditional view is that nuclear receptors may directly contact components of the pol II holoenzyme and recruit it to the core promoter. This is analogous to the mechanism proposed for Sp1 action when it was found to interact with the TBP-associated factor, $TAF_{II}110$ (Chen et al., 1994). It has been reported that the ERα LBD interacts directly with $hTAF_{II}30$ although the functional significance of this interaction is unclear (Jacq et al., 1994). Furthermore, $hTAF_{II}135$ was found to potentiate the AF2 activity of RAR, VDR and TR in mammalian cells (Mengus et al., 1997). Although direct protein-protein interaction between nuclear receptors and the general transcription machinery is feasible, the DNA binding sites for nuclear receptors are not always located in the proximity of the core promoter which impose a physical constraint on such interaction *in vivo*. To this end, it has emerged that a diverse array of transcription intermediary factors may serve as 'molecular bridges'. Some of these

factors may also be recruited by activated nuclear receptors to remodel the chromatin environment.

The hypothesis that nuclear receptors activate transcription by recruiting a common pool of transcription intermediary factors originated from transcription interference experiments (Meyer et al., 1989). It was found that ERα inhibits progestin-induced transcription of reporter gene by PR in a dose- and oestrogen-dependent manner. Notably, the interference required intact activation functions of ERα but not its DNA binding domain. This indicated that ERα and PR were competing for common co-factors which could presumably mediate receptor transactivation. The phenomenon of transcription interference is reminiscent of squelching by Gal4 in yeast (Gill and Ptashne, 1988).

Oestrogen receptor interacting proteins (RIPs) were originally identified by Far-Western blotting technique (Cavaillès et al., 1994; Halachmi et al., 1994). Purified, radio-labelled ERα LBD was used to probe nitrocellulose membranes with immobilised renatured cellular protein extracts. This led to the detection of two protein species of 160kDa and 140kDa which directly interacts with the ERα LBD in an agonist- and AF2- dependent manner. The same two species of proteins were found to interact with DNA-bound RAR/RXR heterodimer (Kurokawa et al., 1995). It is now clear that both the 160kDa and 140kDa signals represent multiple proteins. Furthermore, additional RIPs have been isolated by yeast two-hybrid screening which may interact with nuclear receptors in a ligand dependent or ligand independent manner (Lee et al., 1995). Some of these proteins turn out to be components of multi-protein complexes. Hence, the common theme of transcriptional regulation by nuclear receptors appears to be the recruitment of multi-subunit complexes. A current model is presented in Figure 1.4.

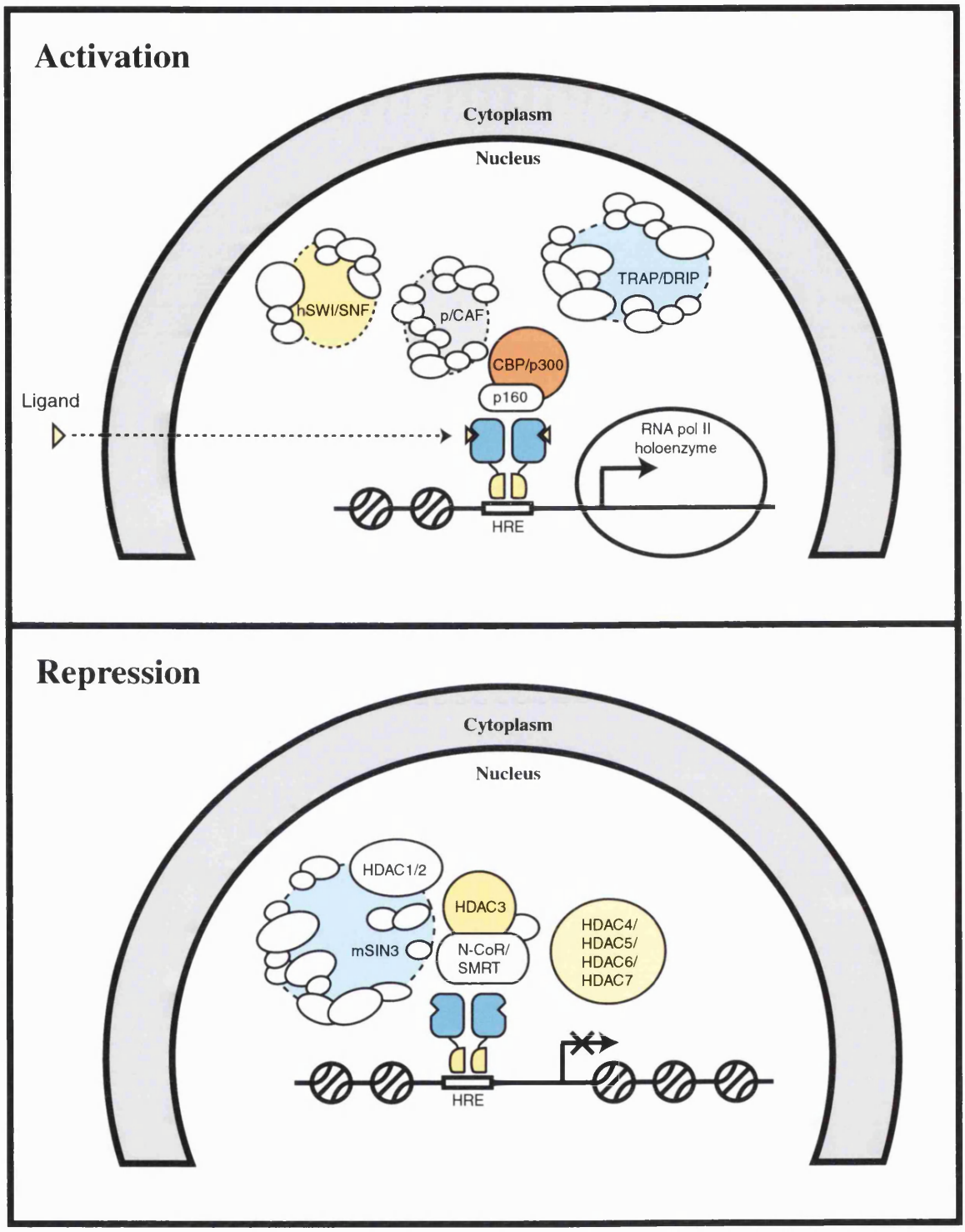


Figure 1.4 Transcriptional regulation by nuclear receptors through recruitment of multi-protein complexes. Trancriptional activation can be achieved by recruitment of acetyltransferases (the p160 proteins, CBP/p300 and the p/CAF complex), ATP-dependent chromatin remodelling complex (hSWI/SNF) or the TRAP/DRIP complex upon ligand binding by the receptor. In the absence of ligand, RAR and TR can actively repress transcription by recruitment of N-CoR/SMRT which in turn interacts with a number of deacetylases. Note the number of ovals reflect the number of known subunits in each complex.

The p160 coactivator family

To date, three mammalian p160 coactivators have been identified which are encoded by distinct genes. They are SRC1 (Onate et al., 1995; Kamei et al., 1996); TIF2/GRIP1 (Hong et al., 1996; Voegel et al., 1996) and RAC3/AIB1/ACTR/TRAM-1/p/CIP (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997a; Takeshita et al., 1997; Torchia et al., 1997). These p160 proteins have a common modular structure (Figure 1.5). At the N-terminus is the highly conserved basic helix-loop-helix (bHLH) PAS domain which is also found in the Per/Arnt/Sim family of transcription factors. Although it has been shown in other factors that the bHLH-PAS domain may mediate DNA-binding and dimerisation, the role of this domain in p160 proteins remains to be clarified (Murre et al., 1989; Huang et al., 1993). The central nuclear receptor interating domain (RID) is followed by two activation domains termed AD1 and AD2 which are capable of activating transcription in mammalian cells when fused to a heterologous DNA binding domain (Kalkhoven et al., 1998; Voegel et al., 1998). Two isoforms of SRC1 have been cloned which differ at their C-termini as a result of alternative splicing (Kalkhoven et al., 1998). Although both of them are co-expressed in a number of cell lines, the SRC1e isoform seems to be a more potent coactivator than the SRC1a isoform.

All three p160 proteins appear to be widely expressed since their corresponding mRNA transcripts are detected in a large number of human or murine tissues and cell lines by Northern analysis (Yao et al., 1996 ; Takeshita et al., 1997; Torchia et al., 1997; Li and Chen, 1998). It is interesting to note that the gene encoding AIB1, at 20q12 was found to be amplified in breast and ovarian cancer (Anzick et al., 1997). This led to the speculation that overexpression of this nuclear receptor coactivator may contribute to tumour progression, perhaps in conjunction with oestrogen receptors.

Several lines of evidence suggest that the p160 proteins are *bona fide* coactivators for nuclear receptors (NRs). Co-expression of p160 proteins potentiate the transcriptional activity of NRs in mammalian cells (Takeshita et al., 1997; Torchia et al., 1997; Kalkhoven et al., 1998). It was shown that TIF2 could

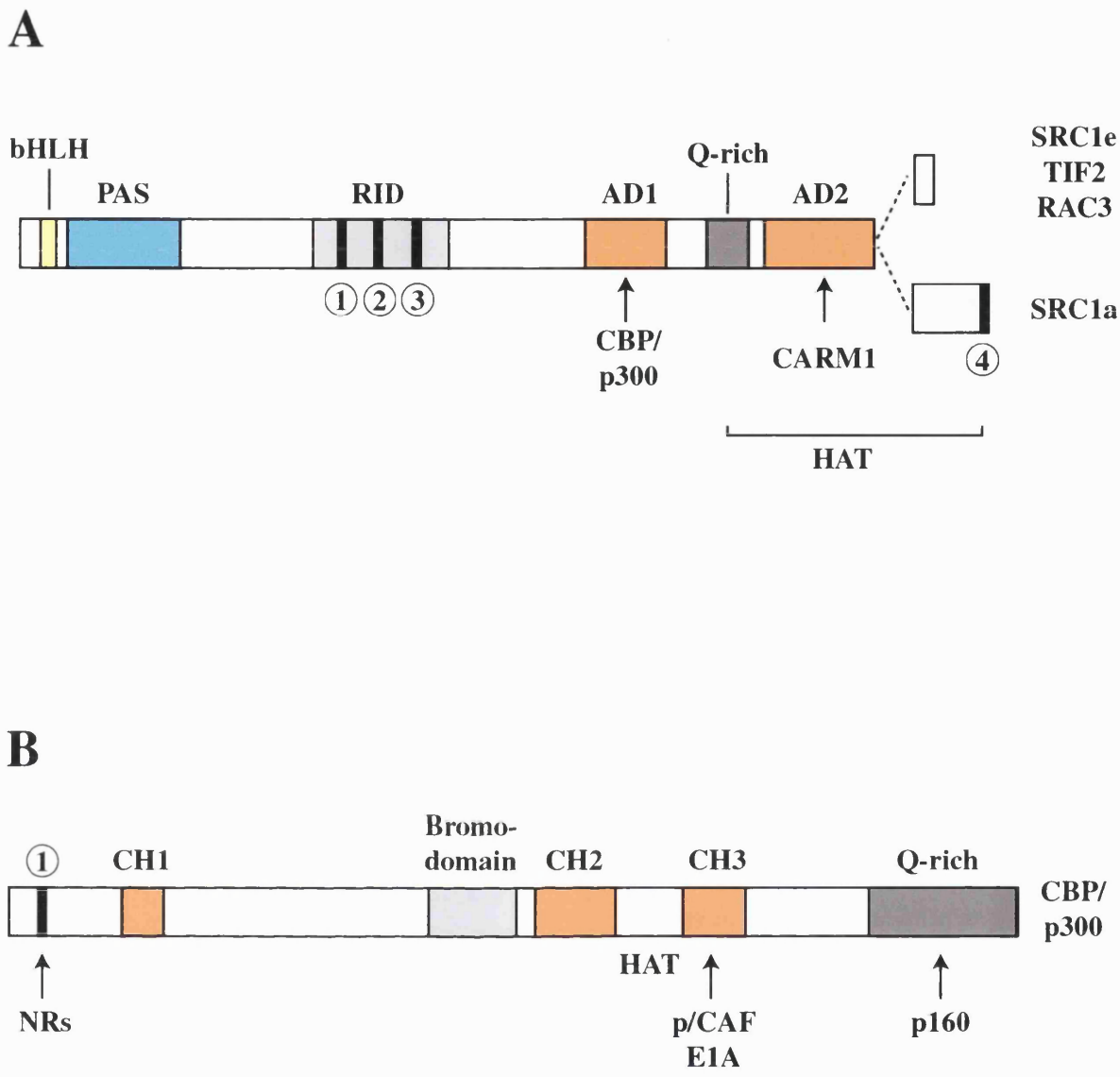


Figure 1.5 Organisation of p160 and CBP/p300. (A) The p160 proteins share a common modular structure. The bHLH-PAS domain at the N-terminus is the most highly conserved region among family members. The receptor interacting domain (RID) contains three LXXLL motifs (denoted by black bars). The activation domain 1 (AD1) interacts with CBP/p300 whereas the activation domain 2 (AD2) interacts with the novel methyltransferase CARM1. The glutamine rich region (Q-rich) mediates binding to the AF1 of NRs. The SRC1a isoform has an alternative C-terminal region which contains an extra LXXLL motif. (B) The CBP/p300 contains three highly conserved cysteine/histidine rich domains, CH1, CH2 and CH3. Notably, p/CAF and E1A bind competitively to the CH3 domain. The bromodomain may involve in interaction with acetylated histones. The N-terminal region of CBP/p300 contains one LXXLL motif (denoted by a black bar) which confers binding to NRs. The C-terminal glutamine rich (Q-rich) region interacts strongly with p160 proteins.

partially relieve the autointerference generated by expressing increasing amount of ERα in transfected cells (Voegel et al., 1996). This lent support to the notion that TIF2 and by inference, other p160 proteins were the common co-factors titrated in transcription interference experiments. Microinjection of antibodies against SRC1 or p/CIP was reported to interfere with transcriptional activation of reporter genes by NRs (Torchia et al., 1997). The p160 coactivators can potentially modulate NR transactivation in a number of ways. It was observed that p160 proteins interact directly with the general coactivator CBP/p300 via their AD1 (Kamei et al., 1996; Kalkhoven et al., 1998; Voegel et al., 1998). Recently, it has also been shown that AD2 of GRIP1 recruits a novel methyltransferase (CARM1) which potentiates the transcriptional activity of NRs only in the presence of p160 coactivators (Chen et al., 1999a). CARM1 can methylate histone H3 *in vitro* but whether this correlates with its role as a secondary coactivator for nuclear receptors is unclear. It has been reported that both SRC1 and RAC3 possess intrinsic histone acetyltransferase (HAT) activity which preferentially acetylates nucleosomal H3 and H4 *in vitro* (Chen et al., 1997; Spencer et al., 1997). Notably, the HAT activity of SRC1 and RAC3 are weaker than that of CBP/p300 and p/CAF (see later). Furthermore, the HAT domain of these p160 proteins does not bear any sequence similarity to the acetyl coA binding site of the p/CAF or GCN5 HAT domains (Clements et al., 1999; Trievel et al., 1999).

The p160 proteins do not only modulate nuclear receptor activity, it has been shown that they may also serve as coactivators of a number of transcription factors such as NFκB and MEF-2C (Na et al., 1998; Sheppard et al., 1999; Chen et al., 2000b). GRIP1 interacts with MEF-2C via its bHLH-PAS domain, which appears to be dispensable for p160 proteins as NR coactivators (Onate et al., 1995; Chen et al., 2000b). Nevertheless, this suggests that the p160 proteins may potentially be limiting when multiple classes of transcription factors are activated concomitantly.

CBP/p300

CBP and p300 are regarded as essential coactivators for a large number of transcription factors including the nuclear receptors (NRs) (Figure 1.5) (Shikama et

al., 1997). CBP was identified through its interaction with the activated form of the CREB transcription factor whereas p300 was identified through its association with the adenoviral-transforming protein E1A (Chrivia et al., 1993; Eckner et al., 1994). It is thought that CBP and p300 function in part as molecular adaptors, physically linking multiple proteins at the promoter and in part as acetyltransferases. Homologs of CBP/p300 have been found in C. elegans and Drosophila but not in yeast, suggesting that this class of proteins may have evolved in metazoans to integrate diverse signals at gene promoters (Akimaru et al., 1997; Shi and Mello, 1998).

The N-terminus of CBP/p300 was shown to directly interact with a number of NRs including RAR, TR, RXR and ER (Chakravarti et al., 1996; Kamei et al., 1996). However, it is now clear that recruitment of CBP/p300 by NRs is most likely to be mediated by the p160 coactivators. The functional importance of CBP/p300 in NR signalling has been demonstrated in a number of ways. They potentiate NR transcriptional activity in transfected mammlian cells and microinjection of blocking antibodies interferes with receptor transactivation (Chakravarti et al., 1996; Hanstein et al., 1996; Kamei et al., 1996). In *in vitro* transcription experiments on chromatin templates, p300 cooperate with ERα to increase the efficiency of productive transcription initiation (Kraus and Kadonaga, 1998). Using the Xenopus oocyte system, it was also shown that p300 requires its histone acetyltransferase (HAT) activity and p160 coactivator interaction domain to facilitate TR transactivation in a chromatin environment (Li et al., 2000). Interestingly, fibroblasts derived from homozygous p300 knockout mice are defective for RAR signalling even though wild-type CBP is present (Yao et al., 1998). Similarly, specific ablation of p300 and CBP using hammerhead ribozymes in F9 embryonal carcinoma cells revealed that they may mediate distinct retinoic acid responses (Kawasaki et al., 1998). Together with the observation that CBP but not p300 haploinsufficiency is the cause of the Rubinstein-Taybi syndrome in human, it has been suggested that there are functional differences between CBP and p300 (Petrij et al., 1995).

As mentioned before, CBP/p300 may function as molecular scaffold that allows communication between the NRs and the RNA pol II holoenzyme. Indeed, the highly conserved CH3 region of CBP has been shown to interact with RNA

helicase A which is a component of the RNA pol II holoenzyme (Nakajima et al., 1997). Deletion of the CH3 region of p300 impairs its ability to potentiate ERα activity in *in vitro* transcription assays (Kraus et al., 1999). Both CBP and p300 have been shown to acetylate histones in mononucleosomes *in vitro* suggesting that nucleosomes may be one of the physiological substrates for the acetyltransferase activity of these proteins *in vivo* (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). In addition, CBP and p300 also modulate the activity of a number of transcription factors through acetylation. It has been reported that the nuclear orphan receptor HNF4 is acetylated by CBP (Soutoglou et al., 2000). Moreover, the acetyltransferase activity of CBP appears to be important for HNF4 dependent gene activation *in vivo*. It seems that acetylation affects both the cellular localisation of HNF4 and its DNA binding activity. p300 has also been shown to acetylate p53 and GATA1 and increase their DNA binding affinity (Gu and Roeder, 1997; Boyes et al., 1998). On the other hand, CBP/p300 can also exert negative effect on gene expression through acetylation. CBP acetylates HMGI(Y), the crucial architectural component of the enhanceosome at the interferon beta gene promoter, and leads to disassembly of the enhanceosome and attenuation of gene expression (Munshi et al., 1998). In Drosophila, dCBP was found to antagonise the wingless signalling pathway by acetylating its downstream transcriptional effector, dTCF (Waltzer and Bienz, 1998). Acetylation of dTCF by dCBP is thought to disrupt the interaction between dTCF and its coactivator, Armadillo. It is interesting to note that the HAT activity of CBP/p300 may itself be regulated in a cell-cycle dependent manner or by other proteins such as E1A and Twist (Ait-Si-Ali et al., 1998; Hamamori et al., 1999).

P/CAF

The p300/CBP-associated factor (P/CAF) was identified on the basis of its sequence similarity with the yeast histone acetyltransferase GCN5 (Yang et al., 1996). In addition to its conserved GCN5 related region, P/CAF contains a unique N-terminal extension which allows it to interact with CBP, p160 proteins and nuclear receptors (Yang et al., 1996; Korzus et al., 1998). P/CAF was shown to potentiate RAR transactivation in transfected cells (Blanco et al., 1998).

Microinjection of blocking antibodies also highlighted the importance of P/CAF HAT activity in mediating RAR signalling (Korzus et al., 1998). Similar to yGCN5, P/CAF exists in a multi-protein complex which contains the human ADA proteins and TAF_{II}s (Ogryzko et al., 1998). In contrast to recombinant P/CAF, the P/CAF complex acetylates nucleosomes avidly (Grant et al., 1997). Interestingly, it has been proposed that a histone octamer-like structure may be formed by selected TAF_{II}s and TAF_{II}s-like polypeptides in the P/CAF complex. This may potentially be important for the association of the P/CAF complex with DNA or for substrate recognition.

The TRAP and DRIP complexes

The TRAP complex was purified by Roeder and co-workers from a cell-line which stably expresses hTRα (Fondell et al., 1996). In the presence of thyroid hormone, at least nine polypeptides, termed TR-associated proteins (TRAPs), were found to bind specifically to the receptor. Furthermore, the TRAP complex was reported to enhance TR transactivation in reconstituted *in vitro* transcription assays on naked DNA templates. Using the VDR ligand binding domain (LBD) as an affinity matrix, a second complex consists of at least nine VDR interacting proteins (DRIPs) was found to associate with the receptor in an agonist dependent manner (Rachez et al., 1998). In contrast to the TRAP complex, the DRIP complex was found to potentiate hormone-dependent transactivation by VDR on chromatin templates (Rachez et al., 1999). Despite their functional differences, which may be due to the purity of components in the *in vitro* transcription systems, the TRAP and DRIP complexes were found to contain a highly similar if not identical set of polypeptides. Some of these polypeptides bear sequence similarity to components of the yeast and human mediator complexes (Ito et al., 1999; Rachez et al., 1999). Remarkably, composition of the TRAP/DRIP complex also resembles that of other mammalian transcription complexes, such as CRSP, ARC and SMCC (Ito et al., 1999; Naar et al., 1999; Ryu et al., 1999). These complexes have been shown to be required for transactivation by Sp1, SREBP, NFκB, p53 and VP16 *in vitro*, although the requirement for a chromatin template is again controversial (Ito et al., 1999; Naar et al., 1999). It is puzzling that no HAT activity has been reported for

the TRAP/DRIP complex (Rachez et al., 1999). In addition, no CBP, p300 or p160 coactivators were found in the complex (Fondell et al., 1999). Therefore, the ability of the TRAP/DRIP complex to activate transcription on chromatin templates is likely to involve alternative chromatin remodelling activities. Another prevailing hypothesis suggests that the TRAP/DRIP complex may link the DNA bound transcription factors to the basal RNA pol II machinery.

The agonist-dependent interaction between the TRAP/DRIP complex with the NR LBD is mediated by the TRAP220/DRIP205 component (Yuan et al., 1998; Rachez et al., 1999). This factor has also been independently indentified as a PPARγ-interacting protein, named PBP (Zhu et al., 1997). Exogenous expression of TRAP220 moderately enhances ligand dependent TR and VDR transactivation in mammalian cells (Yuan et al., 1998; Ren et al., 2000). Notably, it was reported that TRAP220 competes with p160 coactivators for NR binding *in vitro*, suggesting that they may bind to a similar docking surface on the receptor ligand binding domain (Treuter et al., 1999). Recently, another component of the DRIP complex, DRIP150, was found to interact specifically with a functional GR AF1 domain (Hittelman et al., 1999). DRIP150 potentiates GR AF1 activity in mammalian cells. Furthermore, co-expression of DRIP150 and DRIP205 synergistically enhances GR transcriptional activity. This led to the suggestion that the TRAP/DRIP complex may facilitate a functional link between GR AF1 and AF2. It would be interesting to test if a similar mechanism is employed by other NRs.

Candidate modulators of nuclear receptor action

A number of additional proteins have been isolated through ligand dependent interaction with NRs and thus may be considered as candidate modulators of receptor action. The 140kDa signal in the original Far-Western analysis, using the ERα LBD as a probe, represents at least two distinct proteins (Cavaillès et al., 1994; Halachmi et al., 1994). RIP140 interacts with transcriptionally active NRs *in vitro* and *in vivo* (Cavaillès et al., 1995). Intriguingly, RIP140 acts as a transcriptional corepressor for GR and TR2, an orphan nuclear receptor (Lee et al., 1998; Subramaniam et al., 1999). Hence it is

likely to be involved in down-regulation of receptor activity subsequent to activation by hormone binding. TIF1α was identified in a screen designed to isolate proteins which increase the AF2 activity of RXRγ in yeast and is also approximately 140kDa in size (Le Douarin et al., 1995). However, it has been shown that TIF1α interferes with transactivation by NRs in mammalian cells and this was partially attributed to its interaction with heterochromatic proteins (Le Douarin et al., 1996). In addition, it was reported that TIF1α is a protein kinase which undergoes hyperphosphorylation upon association with transcriptionally active NRs although the functional significance of this modification in relation to NR action is unclear (Fraser et al., 1998).

PGC-1 was originally identified as a cold-inducible transcriptional coactivator (Puigserver et al., 1998). Its expression is upregulated in brown fat of cold-exposed animals and it has been proposed that PGC-1 in conjunction with PPARγ and TR may activate genes involved in thermogenesis such as UCP-1. Besides brown fat, PGC-1 expression is restricted to the heart, skeletal muscle, kidney and liver. Although two recent studies suggest that PGC-1 functions as coactivators to other NRs in transfected mammalian cells, it is unlikely that PGC-1 would have a general role in NR signalling (Knutti et al., 2000; Tcherepanova et al., 2000). It was mentioned that the Amplified in Breast Cancer 1 (*AIB1*) gene encodes a p160 coactivator family member, it turns out that the *AIB3* gene may also encode a NR coactivator. The *AIB3* gene, which mapped to 20q11, is amplified in a number of breast cancer cell lines and its gene product has been shown to potentiate the transcriptional activity of ERα and a number of other NRs in transfected cells (Guan et al., 1996; Lee et al., 1999). It has also been independently identified through its ability to interact with NRs in a ligand dependent manner (Caira et al., 2000; Mahajan and Samuels, 2000). It would be interesting to investigate whether overexpression of AIB3 is linked to tumour progression by aberrantly activating the oestrogen receptor signalling pathway.

Active repression by nuclear receptors

In addition to competition for DNA binding sites or common co-factors, TR and RAR are able to actively repress gene expression in the absence of

hormone. A search for proteins which specifically interact with unliganded TR and RAR led to the discovery of two related proteins of approximately 270kDa in size that may mediate gene silencing by these receptors (Figure 1.4) (Chen and Evans, 1995; Horlein et al., 1995; Ordentlich et al., 1999; Park et al., 1999). N-CoR (NR corepressor) and SMRT (silencing mediator for retinoic acid and thyroid hormone receptor) contain multiple separable and non-redundant repressor domains at their N-termini and two C-terminal domains which can independently mediate interaction with NRs (Seol et al., 1996; Li et al., 1997b). N-CoR and SMRT are dissociated from TR and RAR upon hormone addition in both *in vitro* binding assays and in intact cells as determined by chromatin immunoprecipitation technique (Chen and Evans, 1995; Horlein et al., 1995; Perissi et al., 1999). In addition to TR and RAR, transcriptional repression by orphan receptors such as Rev ERB and COUP-TFI have been shown to be mediated by interaction with N-CoR (Shibata et al., 1997; Zamir et al., 1997).

Steroid hormone receptors do not normally associate with corepressors. However, it has been reported that N-CoR avidly associates with ERα in the presence of the partial antagonist Tamoxifen *in vivo* (Lavinsky et al., 1998). Furthermore, tamoxifen resistance of MCF7 cells derived tumours in athymic nude mice correlates with a decrease in N-CoR level. Taken together, these observations suggest that tamoxifen induced ERα/N-CoR interaction may be one way of counteracting the agonistic effect of this drug which is known to be mediated through ERα AF1 in a cell type and promoter specific manner (Berry et al., 1990). Therefore, a decrease in N-CoR level in breast cancer may allow the agonistic effect of Tamoxifen to dominate leading to development of resistance to this compound. Aberrant association of N-CoR/SMRT may also lead to human disease. For example, a number of mutations in the TRβ LBD, identified in thyroid hormone-resistance syndrome patients, confer strong interaction with N-CoR/SMRT which is less sensitive to ligand induced dissociation (Yoh et al., 1997). In addition, it has been shown that the oncoprotein v-erbA interacts constitutively with SMRT, unlike its cellular counterpart TRα (Chen and Evans, 1995). It is tempting to speculate that this may contribute to the oncogenicity of v-erbA.

N-CoR and SMRT mediate transcriptional repression by recruitment of distinct histone deacetylases (HDACs). Histone deacetylation at gene promoters presumably leads to a more compact chromatin environment which is equivalent to a 'silent state' (Pazin and Kadonaga, 1997). Mammalian HDACs can be divided into two classes. Class I includes HDAC1 to HDAC3 which are related to the yeast Rpd3 whereas class II includes HDAC4 to HDAC7 which are homologous to the yeast Hda1 (Taunton et al., 1996; Grozinger et al., 1999). Both HDAC1 and HDAC2 are found in multi-protein complexes which contains the mSin3 protein (Pazin and Kadonaga, 1997). It is thought that N-CoR/SMRT interact with mSin3 and in turn recruit the deacetylase complex to the promoter. Through a second repressor domain, N-CoR/SMRT was also found to interact directly with class II HDACs (Huang et al., 2000; Kao et al., 2000). Recently, purification of a core SMRT complex revealed that HDAC3 can stably associate with SMRT via a third repressor domain (Guenther et al., 2000). Taken together, these data raise the possibility that multiple HDACs may be recruited concomitantly by N-CoR/SMRT. On the other hand, many of the HDACs are expressed in a tissue specific manner, hence the ability of N-CoR/SMRT to recruit HDACs in at least three independent ways may ensure its proper function throughout the animal body.

Binding of receptor interacting proteins to NRs via a common motif

Sequence comparisons reveal little overall similarity between different receptor interacting proteins. However, it has long been suspected that a common mechanism underlies their interaction with NRs which occurs in a characteristic ligand dependent manner. By examining short fragments of RIP140 for interaction with ERα LBD, a common motif was found in all interacting fragments. The consensus sequence of this motif LXXLL, where L is leucine and X is any amino acids, is found nine times in the RIP140 polypeptide (Heery et al., 1997). Isolated LXXLL motif in a 10 amino acid peptide is sufficient to mediate ligand dependent interaction with NRs and that each of the leucines is essential since replacement by alanine renders the motif non-functional. It is now known the LXXLL motif forms an amphipathic α-helix and binds to a hydrophobic surface on the liganded NR

LBD (Darimont et al., 1998; Nolte et al., 1998; Shiau et al., 1998). There are three LXXLL motifs in the receptor interacting domain of the p160 coactivators, which are both necessary and sufficient for interaction with NR LBD (Heery et al., 1997; Torchia et al., 1997; Voegel et al., 1998). Interestingly, a fourth LXXLL motif is present at the C-terminus of SRC1a, one of the isoforms of SRC1 (Figure 1.5) (Kalkhoven et al., 1998). However, the presence of this extra motif does not seem to confer increased binding affinity to NRs by SRC1a (Ding et al., 1998). In conclusion, it is clear that the LXXLL motif is the universal basis for NR recognition and virtually all cloned receptor interacting proteins contain at least one copy of this motif.

More recent studies have attempted to address why are there multiple LXXLL motifs in a single protein. It turns out in the case of p160 coactivators, each of the three LXXLL motifs may mediate high affinity binding only to a subset of receptors (Ding et al., 1998; Kalkhoven et al., 1998; Voegel et al., 1998). For example, the second LXXLL motif of SRC1e and TIF2 appears to be preferentially used for interaction with ERα (Kalkhoven et al., 1998; Voegel et al., 1998). A similar observation is made for TRAP220 where the second of its two LXXLL motifs is preferred by both TR and VDR (Yuan et al., 1998; Rachez et al., 2000; Ren et al., 2000). Further analysis suggests that the determinants of high affinity interaction originate from the flanking sequences of the LXXLL motifs (see Results and Discussion).

It has been reported that the NR co-repressors may use an extended version of leucine rich motif for interaction with unliganded receptors (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). The co-repressor signature motif LXXI/HIXXXI/L is predicted to form an amphipathic α-helix and binds to the unliganded NR LBD at a hydrophobic surface which overlapps the coactivator binding site. The key of discrimination between coactivator and corepressor binding appears to be the ligand induced conformational change in the LBD which leads to the re-positioning of helix 12. It was proposed that in the presence of ligand, helix 12 forms part of a smaller hydrophobic surface which accommodates the coactivator LXXLL motif but is incompatible with the longer α-helical structure that incorporates the co-repressor signature motif (Nagy et al., 1999; Perissi et al., 1999). Given the structural similarity of NR LBD, perhaps the

remaining question is why co-repressor binding is limited to a few NRs whereas coactivator binding appears to be a more general phenomenon. One important factor may be the position helix 12 adopts in the absence of ligand.

Non-genomic functions of nuclear receptors

Nuclear receptors do not only function as classical ligand-inducible activators of transcription, they can also influence gene expression in the absence of DNA binding by modulating the activity of other transcription factors. One classical example of such modulation is the mutual antagonism between AP1 and GR (Jonat et al., 1990). This was attributed in part to inhibition of DNA binding through direct protein-protein interaction (Yang-Yen et al., 1990). Another prevailing hypothesis suggests that transrepression is achieved through competition of a limiting amount of coactivators such as CBP/p300 (Kamei et al., 1996). To this end, overexpression of CBP/p300 in mammalian cells was shown to relieve the transrepression between GR and AP1 (Kamei et al., 1996). The notion that cellular levels of CBP/p300 may be crucial is further supported by the observations that disruption of a single allele of either CBP or p300 led to haploinsufficiency phenotypes (Tanaka et al., 1997; Yao et al., 1998). Competition of coactivators, however, does not seem to apply for AP1 transrepression by TR. Mutations have been identified which selectively ablate transactivation and coactivator recruitment but have no effect on transrepression (Saatcioglu et al., 1997).

Osteoporosis in postmenopausal women correlates with the loss of oestrogen production. This is partly because development of the bone resorbing cells, osteoclasts, is under the control of interleukin-6 (Il-6) whose production is inhibited by oestrogen (Girasole et al., 1992; Jilka et al., 1992). The oestrogen dependent reduction of Il-6 gene expression does not involve binding of ERs to DNA (Stein and Yang, 1995). Instead, ERs inhibit the activity of NFκB and C/EBP which are transcription activators at the Il-6 proximal promoter. Inhibition of NFκB by ERα does not involve competition of coactivator proteins since overexpression of p160 coactivators or CBP/p300 does not alleviate such inhibition (Valentine et al., 2000). This correlates with the observation that an ERα mutant (K366A) which is defective in coactivator binding, is still able to mediate

oestrogen dependent transrepression. In contrast, mutations that disrupt the dimerisation interface of the ERα LBD abolish both transactivation and transrepression (Valentine et al., 2000). It should be noted that an alternative mechanism has been proposed for the GR-mediated inhibition of NFκB activity which involves up-regulation of IκB expression (Auphan et al., 1995; Scheinman et al., 1995). IκB binds to NFκB and retains it in the cytoplasm, thus preventing NFκB from activating its target genes. However, induction of IκB expression by glucocorticoids appears to be cell-type specific (De Bosscher et al., 1997). Furthermore, it has been reported that IκB induction and NFκB inhibition may be two independent events triggered by gluococorticoids (Heck et al., 1997).

Non-genomic function of the GR *in vivo* was most dramatically demonstrated by the generation of the $GR^{dim/dim}$ mice (Reichardt et al., 1998). This mutant mouse strain is homozygous for a point mutation, A458T at the D-loop of the GR DBD. The mutant GR fails to bind cooperatively to palindromic GREs but retains the ability to repress AP1 regulated genes (Heck et al., 1994). Therefore, the $GR^{dim/dim}$ mice would have lost the ability to transactivate genes which are under the control of GREs. Indeed, activation of GRE-dependent reporter genes in embryonic fibroblasts derived from the $GR^{dim/dim}$ mice is severely reduced. Furthermore, the GRE-dependent tyrosine aminotransferase (TAT) gene is no longer induced upon treatment of $GR^{dim/dim}$ mice with dexamethasone (Reichardt et al., 1998). In contrast, the ability of dexamethasone in mediating repression of AP1 target genes such as collagenase-3 is intact in $GR^{dim/dim}$ embryonic fibroblast suggesting that the transrepression function of GR is preserved. Hence, it would be interesting to see which physiological and pharmacological actions of glucocorticoids are indeed mediated by GR transrepression by subjecting the $GR^{dim/dim}$ mice to further analysis. In comparison with the GR-deficient mice ($GR^{-/-}$) which die shortly after birth due to respiratory failure (Cole et al., 1995), it is striking that the $GR^{dim/dim}$ mice undergo normal embryonic and postnatal development. This suggests that GRE-dependent gene activation is not necessary for development and survival.

In addition to inhibitory cross-talk, NRs have also been found to functionally synergise with other transcription factors. For example, ERα has been reported to enhance Sp1 mediated gene activation in an oestrogen dependent

manner (Porter et al., 1997). This is facilitated by direct interaction of ERα with Sp1 which enhances its binding to the promoter DNA. Both ERα and ERβ have been found to activate transcription from an AP1 enhancer element that requires ligand and the AP1 transcription factors Fos and Jun (Webb et al., 1995). Intriguingly, the ligand requirement differs for ERα and ERβ. ERα promotes transcription activation from an AP1 enhancer element in the presence of the natural agoinist 17β-oestradiol, the partial agonists Tamoxifen and Raloxifene and the pure antioestrogen ICI 164384. However, ERβ can only exert the same effect in the presence of Tamoxifen, Raloxifene and ICI 164384 (Paech et al., 1997). This prompted the speculation that the tissue specific oestrogenic effects of partial agonists and antagonists may be dependent on the availability of the two forms of oestrogen receptors.

Steroids hormones invoke a number of rapid, non-transcriptional cellular responses. For example, oestrogen has been shown to induce rapid release of intracellular calcium in granulosa cells (Morley et al., 1992). The unconventional kinetics of such response led to the speculation that there may be cell-surface steroid receptors which do not necessarily resemble the nuclear forms. On the other hand, a subpopulation of 'classical' ER has been found to localise at the cell membrane (Pappas et al., 1995). The downstream events which mediate the transient, non-transcriptional effect of steroids have been investigated in the last few years by Auricchio and co-workers. It was reported that the Src/p21^{ras}/Erk signal transduction pathway could be activated by ER in the presence of 17β-oestradiol but not the pure antioestrogen, ICI 182780 (Migliaccio et al., 1996). Activation was observed within 2 minutes of agonist treatment and was undetectable after 60 minutes. This scheme of signal transduction was eloborated further by the demonstration that binding of progestins to the B isoform of PR induced association of ER with c-Src (Migliaccio et al., 1998). Furthermore, activation of the Src/p21^{ras}/Erk pathway by PR is independent of its transcriptional activity. Most recently, oestradiol and progestin induced activation of the Src/p21^{ras}/Erk pathway has been reported to stimulate S-phase entry (Castoria et al., 1999). Taken together, Auricchio proposed that the mitogenic action of oestradiol and progestin in normal and diseased breast and mammary tissues may be partly mediated through non-transcriptional means. Given the transient nature of

the proposed pathway, it can be envisaged that sustained mitogenic actions should nevertheless involve alteration in gene expression.

Chapter 2

Materials and Methods

MATERIALS

Chemicals and solvents

All chemicals and solvents used were of analytical grade and were obtained from either BDH Chemicals or Sigma Chemicals, except for the following:

Absolute alcohol	Hayman Ltd, UK
Acrylamide	AMRESCO
Agarose	Gibco BRL
Ammonium persulphate	Bio-Rad
Ampicillin	Roche
Amplify	Amersham Pharmacia
Bromophenol blue	Bio-Rad
Coomassie Brilliant Blue R-250	Bio-Rad
Dextran T70	Amersham Pharmacia
Liquid scintillation fluid (Ultima Gold)	Amersham Pharmacia
TEMED	Bio-Rad
Tween 20	Bio-Rad

Radiochemicals

All radiochemicals were supplied by Amersham Pharmacia.

Compound	Specific activity
α-[^{32}P] dCTP	3000Ci/mmol, 10mCi/ml
L-[^{35}S] methionine	>1000Ci/mmol, 15mCi/ml

Enzymes

Restriction enzymes were supplied by New England Biolabs and Roche.

Calf intestinal Alkaline Phosphatase (CIP)	Roche
DNA polymerase I (Klenow fragment)	Roche

eLONGase	Gibco BRL
PfuTurbo DNA polymerase	Stratagene
T4 DNA ligase	New England Biolabs
T4 polynucleotide kinase	New England Biolabs

Miscellaneous

DNA maxiprep kit	Qiagen
DNA miniprep kit	Qiagen
ECL western blotting	Amersham Pharmacia
Detection reagents	
Film: ECL	Amersham Pharmacia
RX	Fuji
Filtration Units	Nalgene or Millipore
Galacto-light β-gal assay kit	TROPIX
Geneclean spin column kit	BIO 101
Gene Pulser cuvettes	Bio-Rad
Glutathione Sepharose 4B	Amersham Pharmacia
Kilobase DNA markers	Gibco BRL
Nitrocellulose membranes	Schleicher and Schuell
Nucleotide triphosphates	Amersham Pharmacia
Oligonucleotides	Synthesised by I. Goldsmith, ICRF
Poly (dI-dC)•(dI-dC)	Amersham Pharmacia
Pre-stained protein markers	New England Biolabs
Protein G sepharose Fast-flow	Amersham Pharmacia
Skimmed milk powder	Marvel
TNT coupled reticulocyte lysate	Promega
in vitro translation kit	

Plasmids

pSG5 TIF2, pSG5 TIF2 m123	Dr. P. Chambon Institute de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France
pCMX RAC3	Dr J. D. Chen Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical Center, USA
pGBDU-C1	Dr P. James Department of Biomolecular Chemistry University of Wisconsin-Madison, USA

Bacterial strain

DH5α

[Ø80d*lacZ*ΔM15, *rec A*1, *end A*1, *gyr A*96, *thi*-1, *hsd R*17 (r_k^-, m_k^+), *sup E*44, *rel A*1, *deo R*, Δ(l*acZYA-arg*F)U169]

HB101

[*thi*-3, *hsd S*20 (r_B^-, m_B^+), *sup E*44, *rec A*13, *ara*-14, *leu B*6, *pro A*2, *lacY*1, *rpsL*20 (str^r), *xyl*-5, *mtl*-1]

Yeast strain

PJ69-4A

[MATa, trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ]

L40

[Matα trp1-901 leu2-3,112 his3-Δ200 ade2 LYS2::(lexAop)4-HIS3 URA3::(LexAop)8-lacZ]

Buffers

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

BBS (2x)	50mM BES adjusted to pH 6.95 with 1N NaOH, 280mM NaCl, 1.5mM Na_2HPO_4 (filter sterilised, stored at –20°C)
CIP buffer (10x)	0.5M Tri-HCl pH 8.5, 1mM EDTA (stored at 4°C)
DCC suspension (Ligand binding assay)	0.025% (w/v) Dextran T70, 0.25% (w/v) charcoal, suspended in TE, pH 7.4 (stored at 4 °C)
DNA loading buffer (10x)	0.2% (w/v) bromophenol blue, 0.25M EDTA pH 8.0, 40% (v/v) glycerol
DNA precipitation mix	1M ammonium acetate, 85% (v/v) ethanol
Gelshift buffer(2x)	100mM KCl, 40mM HEPES pH 7.4 2mM 2-mercaptoethanol, 40% (v/v) glycerol (stored at 4°C)

IP-A	20mM Tris-HCl pH 8.0, 75mM NaCl, 5mM EDTA, 1% Nonidet P-40 (stored at 4°C)
Ligand binding buffer	20mM HEPES pH 7.7, 1.5mM EDTA, 1mM DTT, 0.1% (w/v) BSA, 10% (v/v) glycerol
Ligation buffer (10x)	0.5M Tris-HCl pH 7.8, 0.1M $MgCl_2$, 0.1M DTT 10mM ATP, 250µg/ml BSA (stored at –20°C)
LucLite buffer	0.5M HEPES pH 7.8, 2% (v/v) Triton N101 1mM $CaCl_2$, 1mM $MgCl_2$ (stored at 4°C)
NETN	100mM NaCl, 20mM Tris-HCl pH 8.0 1mM EDTA, 0.5% (v/v) NP40, 1mM DTT 40µg/ml PMSF, 5µg/ml pepstatin 2µg/ml Aprotinin (stored at 4°C without protease inhibitors and DTT)
NTE	0.1M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA
PBSA	140mM NaCl, 2.5mM KCl, 10mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.2
Phenol / Chloroform 1:1	50% (v/v) redistilled phenol, equilibrated in TE pH 8.0, 50% (v/v) chloroform

Poly (dI-dC)•(dI-dC)	Resuspended at 1mg/ml in NTE and heated to 45°C for 10 minutes. (stored at –20°C)
Protein loading buffer (2x)	10% (w/v) SDS, 20% (v/v) glycerol, 250mM Tris-HCl pH6.8, 0.7M 2-mercaptoethanol, 0.02% (w/v) bromophenol blue. (stored at 4°C)
Qiagen buffers:	
P1 (Resuspension buffer)	50mM Tris-HCl pH 8.0, 10mM EDTA 100µg/ml RNase A (stored at 4°C)
P2 (Lysis buffer)	200mM NaOH, 1% (v/v) SDS
P3 (Neutralisation buffer)	3M Potassium acetate pH 5.5 (stored at 4°C)
QBT (equilibration buffer)	750mM NaCl, 50mM MOPS pH 7.0 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100
QC (Wash buffer)	1M NaCl, 50mM MOPS pH 7.0, 15% (v/v) isopropanol
QF (Elution buffer)	1.25M NaCl, 50mM Tris-HCl pH 8.5 15% (v/v) isopropanol
Renilla buffer	0.5M HEPES pH 7.8, 40mM EDTA
Restriction enzyme buffers	As supplied by manufacturers (stored at –20°C)
SDS-PAGE running buffer (10x)	250mM Tris base, 1.9M Glycine 1% (w/v) SDS
TBE (10x)	0.9M Tris-Borate, 20mM EDTA pH 8.0

Western transfer buffer	25mM Tris base, 192mM Glycine 20% (v/v) methanol, pH8.3
Whole cell extract buffer	20mM HEPES pH 7.4, 0.4M KCl 20% (v/v)glycerol, 1mM DTT 40µg/ml PMSF, 5µg/ml pepstatin 2µg/ml Aprotinin (stored at 4°C without protease inhibitors and DTT)

Yeast reagents

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

Drop-out supplement	0.6% (w/v) isoleucine, 0.5% (w/v) phenylalanine 0.4% (w/v) threonine, 0.4% (w/v) lysine 0.4% (w/v) methionine, 1.5% (w/v) valine 0.2% (w/v) arginine (filter sterilised, stored at 4 °C)
Electroporation buffer	10mM Tris-HCl pH 7.5, 1mM $MgCl_2$ 270mM sucrose, (stored at 4°C)
Lysis buffer (plasmid rescue)	50mM Tris-HCl pH 8.0, 62.5mM EDTA 2% (v/v) Triton X-100
Lysis buffer (β-galatosidase assay)	100mM Tris-HCl pH 7.5, 0.5% (v/v) Triton X-100

LacZ buffer	60mM Na_2HPO_4, 40mM NaH_2PO_4,
(β-galatosidase assay)	10mM KCl, 1mM $MgSO_4$,
	38mM 2-mercaptoethanol
Transformation buffer	10mM Tris-HCl pH 7.5, 1mM EDTA
	100mM lithium acetate,
	40% (v/v) polyethylene glycol (MW 3300)

Bacterial media

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
25x M9 salt	1M Na_2HPO_4, 0.5M KH_2PO_4
	0.2M $NaCl_2H$, 0.5M NH_4Cl
M9 agar	1x M9 salt, 1mM $MgSO_4$, 0.1mM $CaCl_2$
	1mM Thiamine, 0.4% (w/v) glucose
	20μg/ml Proline, 20μg/ml Uracil
	50μg/ml Ampicillin, 1.5% (w/v) bactoagar

Yeast media

All media were made with water that was deionised and distilled. Media were sterilised by autoclaving at 121°C for 15 minutes. Organic materials were obtained from Difco.

Minimal media	0.85% (w/v) Yeast Nitrogen Base (without amino acids)
Minimal agar	0.85% (w/v) Yeast Nitrogen Base (without amino acids), 2% (w/v) bactoagar
YP broth	1.1% (w/v) yeast extract, 2.2% (w/v) bacto-peptone 0.055% (w/v) Adenine-HCl
YPD broth	1.1% (w/v) yeast extract, 2.2% (w/v) bacto-peptone 0.055% (w/v) Adenine-HCl, 2% (w/v) glucose
YP agar	1.1% (w/v) yeast extract, 2.2% (w/v) bacto-peptone 0.055% (w/v) Adenine-HCl, 2.2% (w/v) bactoagar
YPD agar	1.1% (w/v) yeast extract, 2.2% (w/v) bacto-peptone 0.055% (w/v) Adenine-HCl, 2.2% (w/v) bactoagar 2% (w/v) glucose

Mammalian cell culture media

Dulbecco's modified Eagle's medium (DMEM)	ICRF media supplies

Foetal bovine serum	Gibco BRL
Trypsin	0.8% (w/v) NaCl, 0.038% (w/v) KCl 0.01% (w/v) Na_2HPO_4, 0.01% (w/v) streptomycin, 100U/ml penicillin, 0.25% (w/v) trypsin, phenol red
Versine	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. All plasmids described in this thesis carry the β-lactamase gene (Amp^r) which confers resistance to ampicillin. Bacterial transformants were grown in L-broth or L-agar containing 50-100μg/ml ampicillin. These bacteria were then stored in L-broth containing 50% (v/v) glycerol at –20°C for up to one year.

Preparation of competent bacteria for transfromation by heat shock

This is an unpublished method by D. M. Heery, derived from Hanahan (Hanahan, 1983). All glassware was pre-washed with L-broth before use. Bacteria were streaked out on L-agar plates and incubated at 37°C overnight. A single colony was then used to innoculate 2ml of L-broth. After overnight incubation with vigorous shaking at 37°C, 100μl of the saturated culture was used to innoculate 100ml of pre-warmed L-broth. This was incubated at 37°C with vigorous shaking until the OD_{600} reached 0.3. The bacteria was then placed on ice for 5 minutes and pelleted by centrifugation at 3000 x *g* for 7 minutes at 4°C. The cell pellet was resuspended in 20ml (20% original volume) of ice cold 100mM $MgCl_2$ and immediately centrifuged at 3000 x *g* for 7 minutes at 4°C. The cell pellet was

finally resuspended in 20ml (20% original volume) of ice cold 100mM $CaCl_2$ / 10% (v/v) glycerol and incubated on ice for 30 minutes. The cell suspension was snap frozen on cardice in aliquots of 0.5ml and then stored at –70°C.

Transformation of competent bacteria by heat shock

Competent cells were thawed on ice and typically, 50µl and 100µl of cells were used for transformation of supercoiled DNA and ligation products respectively (less than 1ng DNA per µl cells). After 10 minutes on ice, the cells were heat shocked at 37°C for 90 seconds and returned to ice for 2 minutes. Following the addition of 5 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 100µg/ml ampicillin, inverted and incubated at 37°C overnight. Competent DH5α cells typically gave 10^6 bacterial colonies per µg of supercoiled DNA.

Preparation of competent bacteria for transfromation by electroporation

All glassware was pre-washed with L-broth before use. Bacteria were streaked out on L-agar plates and incubated at 37°C overnight. A single colony was then used to innoculate 10ml of L-broth and incubated overnight with vigorous shaking at 37°C. This was then subcultured into 1 litre of pre-warmed L-broth and incubated with vigorous shaking at 37°C until the OD_{600} reached 0.5 to 0.8. The cells were then chilled on ice for 15 minutes and pelleted by centrifugation at 4000 x *g* for 15 minutes at 4°C. Subsequently, the cells were resuspended in 1 litre of ice-cold water. This cell suspension was then concentrated by a stepwise decrease in resuspension volume after repeated centrifugation as above; starting from 500ml ice cold water to 20ml ice cold water / 10% (v/v) glycerol to 2ml ice cold water / 10% (v/v) glycerol. Finally, the cells were snap frozen on cardice in aliquots and stored at –70°C.

Transformation of competent bacteria by electroporation

Competent cells were thawed on ice. DNA solution of low ionic strength (less than 1ng DNA/μl cells) at a maximum volume of 2μl was added to 50μl of cells. The mixture was transferred to pre-chilled 1mm electroporation cuvette and electroporated using a Bio-Rad gene pulser, at 1.67kV, 25μF and 200 ohms, giving a time constant of approximately 4 milliseconds. Following electroporation, 0.5ml L-broth was added and the cells were incubated at 37°C for 30-50 minutes. The cells were then spread on L-agar plates containing 100μg/ml ampicillin, inverted and incubated at 37°C overnight. Competent DH5α and HB101 cells typically gave 10^8 bacterial colonies per μg of supercoiled DNA.

Preparation of plasmid DNA

Small scale preparation of plasmid DNA (miniprep)

The Qiagen miniprep kit was routinely used to prepare up to 15μg of high-copy plasmid DNA. Individual bacterial colonies were used to inoculate 3ml of L-broth containing 50μg/ml ampicillin and incubated overnight at 37°C with vigorous shaking. Typically, 1.5ml of overnight culture was pelleted using a microcentrifuge and the plasmid DNA prepared according to the manufacturer's protocol. The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (Vogelstein and Gillespie, 1979). The alkaline lysis procedures were modified from the method by Birnboim and Doly (Birnboim and Doly, 1979). Bacteria were lysed under alkaline conditions in the presence of RNaseA, and the lysate was subsequently neutralised and adjusted to high-salt binding conditions, ready for purification on a silica-gel membrane. The membrane allowed selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer. RNA, cellular proteins and metabolites were not retained on the membrane but found in the flowthorough. Endonucleases from *endA*$^+$ strains such as HB101 were removed by a wash step with a buffer containing chaotropes, to ensure that plasmid DNA was not degraded. After removing the salts, plasmid DNA was eluted with either water or 10mM Tris-HCl pH 8.5. Typically, the recovery efficiency was higher than 85%.

Large scale preparation of plasmid DNA (Maxiprep)

The Qiagen maxiprep kit was routinely used to prepare up to 500μg of high-copy plasmid DNA. Plasmid DNA prepared by this method was used for sub-cloning, transient transfection, *in vitro* transcription/translation and sequencing. Individual colonies or 10μl of saturated bacterial culture were used to inoculate 100ml of L-broth containing 50μg/ml ampicillin and incubated overnight at 37°C with vigorous shaking. The bacteria were havested by centrifugation at 6000 x *g* for 15 minutes at 4°C. The pellet was then processed according to the manufacturer's protocol. The protocol was based on a modified alkaline lysis procedures; the bacterial cells were lysed in NaOH-SDS in the presence of RNaseA. SDS solubilises the phospholipid and protein components of cell membrane, leading to lysis and release of cell contents. NaOH denatures the chromosomal and plasmid DNA, as well as proteins while RNaseA digests the liberated RNA during lysis. The lysate was neutralised by the addition of acidic postassium acetate. The high salt condition caused SDS to precipitate with the denatured proteins, chromosomal DNA and cellular debris trapped in salt-detergent complexes. Plasmid DNA, being smaller and covalently closed, renatured and remained in solution. Bacterial lysates were cleared by high speed centrifugation with the plasmid DNA remaining in the cleared supernatant. This was then loaded onto a pre-quilibrated anion-exchange column which was operated by gravity flow under low-salt and pH conditions. RNA, proteins, dyes and low molecular weight impurities were removed by a wash buffer of medium ionic strength. Plasmid DNA was eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. The purified DNA was air-dried and redissolved in 10mM Tris-HCl pH 8.5. The DNA concentration and purity were determined by measurement of the OD_{260} and OD_{280} (Sambrook et al., 1989).

DNA manipulation and subcloning

Restriction endonuclease digestion

Restriction enzyme digests were performed at 37°C in buffers supplied by the menufacturer unless other conditions were recommeded. DNA was digested with 10-fold excess of enzyme with the final volume of glycerol not exceeding 5% (v/v). For analysis of digestion products by gel electrophoresis, reactions were stopped by the addition of DNA loading buffer to 10% (v/v) of the total volume of digestion. Alternatively, digestions were stopped by loading the entire reactions to Geneclean Spin columns for purification of the digested DNA directly (see purification of restriction fragments by Geneclean method).

Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose at 0.8–2% (w/v) in 1xTBE by heating the suspension in a microwave oven. The solution was allowed to cool, ethidium bromide added to 0.2μg/ml and the solution poured into a gel mould. Once set, the gel was submerged in 1xTBE buffer in a gel tank and the DNA samples, containing 10% (v/v) DNA loading buffer, loaded into the wells. Electrophoresis was carried out at 8V/cm until the DNA fragments were well resolved. DNA was visualised by illumination on a long wave UV light box and photographed. The size of DNA fragments was estimated by comparing their mobility with that of restriction fragments of known size in 1kb marker (Gibco BRL).

Purification of restriction fragments by Geneclean method

Restriction fragments (>200bp) were purified from TBE agarose gels using Geneclean Spin kit (BIO 101) according to maufacturer's protocol. For each spin column, up to 5μg of DNA was purified from a gel slice (<300 μl in volume) which contained the fragment of interest. The method is based on the principle that DNA binds to silica in high concentrations of chaotripic salt and elutes in low salt. The mechanism of DNA binding to silica may involve chaotropic salt disruption of

the water structure around negatively charged silica, allowing it to form cation bridge with the negatively charged phosphate backbone of DNA. Elution of DNA with water is facilitated by rehydration of the silica matrix that breaks the attraction between the matrix and DNA.

Purification of restriction fragments by glass wool method

Restriction fragments (<200bp) were purified from TAE agarose gels using the glass wool method. An extraction module was assembled by placing a 0.5ml eppendorf tube punctured at the bottom with a 23G needle into a 1.5ml eppendorf tube. After filling the conical part of the 0.5ml tube with glass wool, the fragment of interest was excised from the gel and placed on top of the glass wool. The whole extraction module was spun at 5000rpm for 5 minutes at room temperature in a microcentrifuge. The DNA in the eluate collected in the 1.5ml tube was precipitated by addition of the DNA precipitation mix. After incubation at –20°C for at least 30 minutes, the DNA was pelleted by centrifugation at 13000rpm for 15 minutes. The DNA pellet was then washed with 70% ethanol and spun for a further 5 minutes. After removal of the supernatant and air-drying, the pellet was resuspended in distilled water.

Preparation of vectors

In order to prepare vectors for subcloning, 2μg of plasmid DNA was typically digested with the appropriate restriction enzyme(s) and was then subjected to agarose gel electrophoresis. After purification by Geneclean method, the digested DNA was treated with 20 units of calf intestinal phosphatase (CIP). CIP removes the 5' terminal phosphates from the cut end of the vector so that self ligation is prevented. After incubation at 37°C for 20 minutes, the DNA was re-purified by Geneclean and eluted in water at a final concentration of 30-50ng/μl.

Oligonucleotide phosphorylation 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 phosphorylated prior to annealing. 100ng of oligonucleotide was incubated with 1x T4 DNA ligase buffer (containing 1mM ATP) and approximately 10 units of T4 polynucleotide kinase in a final volume of 20µl at 37°C for 30 minutes. After the addition of 80µl NTE buffer, the complementary oligonucleotides were mixed and the solution heated to 90°C for 5 minutes. The solution was allowed to cool slowly to room temperature to facilitate the annealing of the oligonucleotides.

Ligations

Ligations were performed with 30-50ng 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 20µl which contained 1µl T4 DNA ligase (6 Weiss units). All reactions were incubated overnight at 15°C.

Polymerase chain reaction

Polymerase chain reactions (PCR) were performed using eLONGase DNA polymerase (Gibco BRL) or PfuTurbo DNA polymerase (Stratagene). A 50µl reaction included 0.5mM of each dNTP, 400nM of each primer, 20ng termplate DNA, buffer containing 1–2mM Mg^{2+} as supplied by the manufacturer and 2.5 units of enzyme. Mineral oil was used to cover the reaction mixture to prevent evaporation during thermo-cycling. A hot-start protocol was adopted where the enzyme was added to the reaction mixture pre-heated to 82°C. The DNA template was denatured completely by heating at 94°C for 2 minutes before the reaction mixture was subjected to 3 thermo-cycles of: 94°C for 30 seconds, (T_m – 5°C) of the primers for 45 seconds and 72°C for 2 minutes. This was followed by another 30 thermo-cycles of: 94°C for 30 seconds, T_m of the primers for 30 seconds and 72°C for 2 minutes. A final extension of 72°C for 5 minutes was carried out after the last cycle.

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 0.5–1μg plasmid DNA, 8μl of Terminator Ready Mix (containing Taq polymerase and dye-labelled terminators) and 30ng of primer in a 20μl reaction. PCR reactions typically consisted of 25 thermo-cycles of: 94°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Following the PCR, the DNA was precipitated by addition of 80μl of distilled water, 5μl of 3M sodium acetate pH5.2 and 300μl of 95% ethanol, followed by incubation at room temperature for 15 minutes. The DNA was pelleted by centrifugation at 13000rpm for 20 minutes and washed again with 70% ethanol. The air-dried pellet was then processed by the ICRF equipment park.

***In vitro* protein analysis**

***In vitro* protein synthesis**

A coupled rabbit reticulocyte lysate system was used to synthesise proteins *in vitro* (Promega). In a typical 25μl reaction, 1μg of circular DNA template and 22.5μCi of [^{35}S]-methinoine were used and the reaction assembled according to the manufacturer's protocol. The translation reactions were incubated at 30°C for 90 minutes and then stored at −70°C. Typically, 1/25 of the reaction was subjected to SDS polyacrylamide gel electrophoresis in order to assess the size and yield of the radiolabelled product.

SDS polyacrylamide gel electrophoresis

Proteins were analysed on discontinuous polyacrylamide gels using the ATTO Corporation dual slab chamber. Gels were prepared from two solutions to form the stacking and resolving gels. The resolving gel typically contained 10% acrylamide (30% acrylamide, 0.8% bis-acrylamide stock), 375mM Tris-HCl pH8.8

and 1% SDS. The stacking gel contained 5% acrylamide, 125mM Tris-HCl pH6.8 and 1% SDS. Polymerisation of the resolving gel was initiated by the addition of TEMED and ammonium persulphate. The solution was then poured between the gel plates to within 1cm of the base of the prospective wells and overlaid with distilled water. Once polymerisation was complete, the distilled water was poured off. Polymerisation of the stacking gel was initiated by the addition of TEMED and ammonium persulphate. The solution was then poured on top of the resolving gel and a comb inserted. Once the stacking gel had set, the comb was removed and the wells were rinsed with distilled water to remove unpolymerised acrylamide. Samples in protein loading buffer were boiled for 3 minutes before loading as were prestained molecular weight markers. The gel was run in 1xSDS PAGE buffer at 150V until the dye front had migrated to the bottom of the gel. The plates were then separated and the gel fixed in 10% acetic acid, 30% methanol for 5 minutes and then incubated in Amplify for 15 minutes. The gel was dried under vacuum at 80°C for 60 minutes and the radiolabelled bands visualised by fluorography. For western blotting, the gels were not fixed but processed as described below.

Western blotting

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

The blotting apparatus was disassembled and the nitrocellulose membrane rinsed with distilled water, incubated briefly with 1x Ponceau Red stain for visualisation of the transferred proteins on the membrane. The dye was washed off by rinsing with PBSA. The membrane was incubated, with gentle shaking, in

blocking solution (0.05% (v/v) Tween-20, 3% (w/v) non-fat milk in PBSA) for 1 hour at room temperature. The membrane was then washed three times (5 minutes each wash) with washing solution (0.05% (v/v) Tween-20, PBSA) and then incubated for 1 hour at room temperature with the primary antibody in a sealed bag. Typically, the primary antibody was diluted to 1:1000 in incubation buffer (0.05% (v/v) Tween-20, 0.3% (w/v) BSA and 0.1% (w/v) Thimerosal in PBSA). The anti-fungal agent Thimerosal allowed the storage of diluted antibody at 4°C for subsequent experiments. After incubation with the primary antibody, the membrane was washed three times (5 minutes each wash) with washing solution and then incubated for 1 hour at room temperature with horse radish peroxidase (HRP) conjugated antibody against the primary antibody at 1:2000 dilution in washing solution. The membrane was finally washed three times (10 minutes each wash) with washing solution and was then processed for enhanced chemilluminescence (ECL) according to manufacturer's protocol (Amersham Pharmacia).

If the membrane was to be reprobed with a different primary antibody and background signals were not desired, the membrane was stripped of the first set of antibodies by treatment with stripping buffer (100mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5mM Tris-HCl pH6.7) and incubated at 60°C for 30 minutes with gentle shaking. The membrane was then washed three times (10 minutes each wash) with washing solution before being blocked and incubated with antibodies again as described above.

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 assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blur G-250 shifts from 465nm to 595nm when binding to protein occurs. The dye concentrate was used according to the manufacturer's instructions (Bio-Rad). Typically, 2-5µl of cell extract was added to 1ml of Bradford reagent diluted 1:5 with distilled water. After mixing, the samples were transferred to polystyrene cuvettes and incubated for 5 minutes prior to reading the absorbance at OD_{595} with

a spectrophotometer. The protein concentrations were determined by extrapolation from a standard curve prepared from the absorbance at OD_{595} of a series of dilutions of a BSA standard. In this assay, BSA binds twice as much dye compared to other proteins and this was taken into account when calculating the concentration of protein extracts.

Purification of GST fusion protein

The fusion protein GST-SRC1 (570-780) was expressed and purified from the *Escherichia coli* strain DH5α. Bacteria transformed with the plasmid encoding the fusion protein were grown overnight at 37°C in L-broth (50μg/ml ampicillin) with vigorous shaking. The saturated culture was diluted 1:10 with fresh medium and grown until the OD_{600} reached 0.6 (in approximately 1 hour). IPTG was then added to a final concentration of 0.1mM and the culture grown for a further 3 hours with vigorous shaking. The cells were harvested by centrifugation at 5000 x *g* for 5 minutes at 4°C. The cell pellet was resuspended in 1/10 volume of ice cold NETN (containing protease inhibitors) and the cells lysed by sonication for three periods of ten seconds (power level 22) at 4°C using a Soniprep 150 ultrasonic disintegrator with a 3mm probe. The bacterial lysate was cleared by centrifugation at 10000 x *g* for 5 minutes at 4°C. The supernatant was incubated with Glutathione sepharose beads (equilibrated in NETN) for 1 hour at 4°C with constant mixing. The beads were then washed three times with NETN to remove unbound proteins. Finally, the GST fusion protein was eluted by incubation with elution buffer (20mM glutathione, 100mM Tris-HCl pH8.0 and 120mM NaCl) for 1 hour at 4°C with constant mixing. The beads were pelleted by centrifugation and glycerol was added to the supernatant to 10% (v/v) and stored at −70°C.

Pull down assay

GST fusion proteins were expressed and purified as described above and left attached to the Glutathione sepharose. The beads were resuspended in NETN and incubated with 20μl of diluted *in vitro* synthesised protein at 4°C with constant mixing. The beads were washed three times with ice cold NETN to remove

unbound proteins, dried under vacuum in a speedivac, and resuspended in 30µl of protein loading buffer. The sample was boiled for 3 minutes and analysed by SDS-PAGE.

Co-immunoprecipitation

293-T cells transfected with mammalian expression plasmids were washed twice with room temperature PBSA and lysed using ice cold IP-A buffer which contained a cocktail of protease inhibitors (Roche). For each 10cm culture dish, 1ml of IP-A buffer was used. The cell suspension was passed through a 21 gauge needle ten times and incubated on ice for 20 minutes. The crude lysate was centrifuged at 20000 x *g* for 20 minutes at 4°C and the supernatant was precleared by incubating with Protein G Sepharose (equilibrated in IP-A) for 30 minutes at 4°C. The cleared lysate was divided into 400µl aliquots and subjected to immunoprecipitation with the addition of 25µg of Anti-FLAG M2 agarose (Kodak), plus or minus 17β-oestradiol (1µM final) in a total volume of 1ml. After incubation at 4°C for 5 hours, agarose beads were washed four times with IP-A and once with PBS. The beads were then resuspended in protein loading buffer, boiled for 3 minutes and then subjected to SDS-PAGE.

Gel shift assay

For each reaction, 2µg of whole cell extract was preincubated with or without antisera in 1x gel shift buffer, 0.5% (w/v) BSA and 1µg of poly (dI-dC)•(dI-dC) for 5 minutes at room termperature. 1ng of the [^{32}P]-labelled oligonucleotide probe containing a consensus oestrogen response element from the vitellogenin A2 gene promoter was added and the reaction was allowed to proceed for a further 25 minutes. A 6% polyacrylamide (30% acrylamide, 0.8% bis-acrylamide stock) 0.5x TBE non-denaturing gel was pre-run for 30 minutes at 100V. The reactions were loaded on the gel and run in 0.5x TBE at 220V for 60-90 minutes to allow separation of protein-DNA complexes from unbound DNA. Gels were fixed for 15 minutes (10% acetic acid and 30% methanl), dried under vacuum at 80°C for 50 minutes and visualised by autoradiography.

Radiolabelled probes were prepared by annealing complementary pairs of oligonucleotides to form the binding site and 5' overhanging ends. The oligonucleotides were labelled by filling in the overhanging ends in the presence of α-[^{32}P]-dCTP using the Klenow fragment of *E. coli.* DNA polymerase I. Typically, 500ng of annealed oligonucleotides were incubated in a final volume of 50μl containing 50μCi α-[^{32}P]-dCTP and 2 units of Klenow enzyme at 37°C for 1 hour. The probe was then purified by elution through a G-50 Sepharose column with TE buffer.

Ligand binding assay

Ligand binding assay was performed essentially as described in Fawell *et al* with 17β-[^{3}H]oestradiol (Fawell et al., 1990). The tritiated ligand was supplied in solution in toluene containing solvent which interfered with the ligand binding. Hence, appropriate volume of 17β-[^{3}H]oestradiol was dried under vacuum in a speedivac and resuspended in absolute ethanol to give a 1μM stock solution. The assay was performed over a range of tritiated ligand concentration (0.125–8nM). The 1μM stock solution was diluted with ligand binding buffer to give an 80nM solution which was then subjected to two-fold serial dilution to give 40, 20, 10, 5, 2.5, 1.25nM working stock solutions. Whole cell extract from COS-1 cells transiently transfected with wild type or mutant mERα was diluted using ligand binding buffer to 0.1–0.5μg/μl. The ligand binding reaction (50μl final volume) was set up in 1.5ml 'safe-seal' polyproprylene tubes (Sorensen) with the reagents added in the following order: 35μl ligand binding buffer, 10μl diluted cell extract (1–5μg), 5μl diluted tritiated ligand. Control reactions for non-specific binding of ligand by cell extract were performed in the presence of 4.2μM unlabelled 17β-oestradiol. The reactions were allowed to proceed overnight at 4°C.

The input radioactivity at each concentration of 17β-[^{3}H]oestradiol was measured by transferring 5μl diluted tritiated ligand to a scintillation vial containing 2ml of scintillation fluid (Ultima Gold) immediately after the set up of reactions. The vials were loaded into a scintillation counter and the number of counts per minute recorded for each sample.

The ligand binding reactions were stopped by addition of 50µl DCC suspension, mixed thoroughly, and incubated at 4°C for 10 minutes. The samples were centrifuged at 13000rpm in a microcentrifuge at room temperature for 5 minutes to pellet the charcoal in DCC suspension. 80µl of the supernatant (80% of reaction) was transferred to a scintillation vial containing 2ml of scintillation fluid (Ultima Gold) and the radioactivity measured as above.

Yeast culture and manipulation

Storage of yeast

The *Saccharomyces cerevisiae* strain PJ69-4A was maintained on YPD agar at 4°C for up to 3 months. Yeast transformed with episomal expression plasmid was maintained on minimal agar plate supplemented with drop out supplement and appropriate amino acids (selective plate) and stored at 4°C for up to 2 weeks. For long term storage at −70°C, glycerol was added (50% (v/v) final) to a saturated culture in YPD broth and snap frozen on cardice in cryovials.

Preparation of carrier DNA

This is a method derived from Schiestl and Gietz (Schiestl and Gietz, 1989). Salmon sperm DNA (Sigma) was dissolved in TE at 5mg/ml by incubation overnight at 4°C with constant mixing on a rotating wheel to give a homogeneous viscous solution. It was then sonicated twice for 30 seconds at 4°C using a Soniprep 150 ultrasonic disintegrator with a 3mm probe at power level 3–4. The DNA solution was extracted once with phenol (TE equilibrated), once with phenol (TE equilibrated):choloroform (50:50) and once with choloroform. The DNA was then precipitated by adding 1/10 volume of 3M sodium acetate pH5.4 and 2.5 volume of ice cold absolute ethanol and incubated at −20°C for 30 minutes. The DNA was pelleted by centrifugation at 12000 x *g* at 4°C for 15 minutes and washed with 70% ethanol. After air-drying, the DNA pellet was re-dissolved in TE at 5mg/ml before being denatured in a boiling water bath for 20 minutes. The DNA

solution was then immediately cooled in an ice water bath, divided into aliquots and stored at –20°C.

Transformation by lithium acetate

This protocol is by Stan Hollenberg which is modified from a published method (Schiestl and Gietz, 1989). Yeast grown on YPD agar or selective plates was used to inoculate 50ml YPD broth and grown overnight at 30°C with moderate shaking. The culture in log phase (OD_{600}~1.0) was harvested by centrifugation at 3000 x *g* for 5 minutes at room temperature. The cells were resuspended in 30ml distilled water and re-spun as above. The cell pellet was then resuspended in 2ml 0.1M lithium acetate and incubated at room temperature for 10 minutes. Plasmid DNA was dispensed to 1.5ml eppendorf tube together with carrier DNA (50µg per 1µg plasmid DNA). This was followed by the addition of 100µl of yeast suspension which was mixed thoroughly with the DNA. After the addition of 700µl transformation buffer, the mixture was incubated at 30°C for 30 minutes. Finally, 88µl DMSO was added to the yeast suspension which was then heat shocked at 42°C for 7 minutes. The cells were pelleted by centrifugation at 6000rpm in a microcentrifuge for 10 seconds, washed with 1ml distilled water and then re-spun. The cells were spread on selective plates, inverted and incubated at 30°C for up to 3 days.

Transformation by electroporation

Yeast grown on YPD agar or selective plates was used to inoculate 100ml YPD broth and grown overnight at 30°C with moderate shaking. The culture in log phase (OD_{600}~0.7) was harvested by centrifugation at 3000 x *g* for 5 minutes at 4°C. The cell pellet was washed once with ice cold distilled water and once with electroporation buffer. The cells were pelleted by centrifugation as above after each washing. The cells were finally resuspended in 2ml (2% original volume) electroporation buffer. 50µl of cell suspension was added to a pre-chilled eppendorf tube containing up to 5µl of DNA solution. The mixture was transferred to pre-chilled 2mm electroporation cuvette and electroporated using a Bio-Rad

gene pulser, at 450V, 250μF and 400 ohms, giving a time constant of approximately 70 milliseconds. Following electroporation, 1ml of ice cold distilled water was added and the cells retrieved and pelleted by centrifugation at 6000rpm in a microcentrifuge at room temperature for 3 minutes. The cells were then spread on selective plates, inverted and incubated at 30°C for up to 3 days.

Rescue of plasmid DNA from yeast

Yeast grown on selective plates was used to inoculate 10ml minimal medium supplemented with drop out supplement and appropriate amino acids (selective medium) and grown overnight at 30°C with moderate shaking. The cells were harvested by centrifugation at 3000 x *g* for 5 minutes and the cell pellet resuspended in 1ml distilled water. The cell suspension was transferred into 1.5ml eppendorf tube and spun at 4000rpm in a microcentrifuge for 15 seconds. The cell pellet was then resuspended in 250μl lysis buffer before the addition of equal volume (250μl) of glass beads (0.5mm diameter). After the yeast:glass bead mixture was vortexed for 2 minutes, an equal volume (500μl) of phenol (TE equilibrated) was added and the mixture was vortexed for further 1 minute. The solid phase was pelleted by centrifugation at 13000rpm in a microcentrifuge for 5 minutes. The aqueous phase was then transferred to a new tube, mixed with 1 volume of phenol:choloroform, and re-extracted as before. The plasmid DNA in the aqueous phase was precipitated by addition of 3.5 volume of DNA precipitation mix, incubated at −20°C for 30 minutes, and pelleted by centrifugation at 13000rpm in a microcentrifuge for 15 minutes. The DNA pellet was washed twice with 70% ethanol, air-dried and resuspended in 100μl distilled water. 1μl of the yeast miniprep DNA was then used to transform *E. coli.* HB101 by electroporation. For selection of LEU2 containing plasmids, HB101 transformants were plated onto M9 plates where HB101 was normally unable to grow because of a defect in the leuB gene.

β-galactosidase assay

This method is derived from a published version by Dodou and Treisman (Dodou and Treisman, 1997). Yeast grown on selective plates was used to inoculate 10ml selective medium (minus or plus ligand) and grown overnight at 30°C with moderate shaking. The cells were harvested by centrifugation at 3000 x *g* for 5 minutes and the cell pellet resuspended in 1ml distilled water. The cell suspension was transferred into 1.5ml eppendorf tube and spun at 6000rpm in a microcentrifuge for 3 minutes. The cell pellet was then resuspended in 200μl lysis buffer, snap frozen on cardice, and allowed to thaw slowly at room temperature. This procedure permeabilised the yeast cell without breaking it completely open. The assay was carried out in 1.5ml eppendorf tube at room temperature by adding 20–50μl of yeast suspension to 500μl lacZ buffer. At timed interval (usually 15 seconds), 100μl ONPG (4mg/ml) was added to start the reaction. When the colour of the reaction mixture turned visibly yellow, the reaction was stopped by the addition of 250μl 1M Na_2CO_3 and the time noted. The cells were then pelleted by centrifugation at 6000rpm in a microcentrifuge for 5 minutes and the supernatant transferred into cuvettes for measurement at OD_{420}. The cell pellet was resuspended in 1ml distilled water and transferred into cuvettes for measurement at OD_{600} in order to estimate the cell density. The β-galactosidase activity was calculated as Miller units, i.e. $1000 \times OD_{420} / (OD_{600} \times$ reaction time in minutes).

Growth assay on selective medium (Drop test)

Individual yeast transformants were maintained on selective plates and were re-streaked one day before the Drop test. Freshly grown yeast was used to inoculate 1.5ml distilled water. The density of different cell suspensions was normalised to give OD_{600}~0.5. 10μl of yeast cell suspension was then dropped onto selective agar plates with or without ligand. The plates were incubated at 30°C for 1 to 3 days and monitored everyday for growth of yeast.

Cell culture

Maintenance of cell stocks

Mammalian cells were grown as a monolayer on 175cm^2 tissue culture flasks at 37°C in a humidified atmosphere maintained at 10% (v/v) CO_2. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS). Cell cultures were subcultured once or twice a week depending on growth rate. The medium was removed and the cells were washed twice with PBSA before prewarmed trypsin / versine mix (1:10) was applied. The trypsin / versine mix was withdrawn once it was allowed to disperse throughout the monolayer and the cells were incubated with minimal volume of trypsin / versine mix at 37°C for 3 minutes. The flask was gently tapped until all the cells were dislodged, which was followed by the addition of 10ml DMEM (10% (v/v) FBS) that neutralised the trypsin. The cell suspension was transferred to a sterile bottle and then spun at 1100rpm in an MSE bench top centrifuge for 5 minutes. The pellet was gently resuspended in fresh growth medium and subcultured at a suitable dilution.

Storage of cell stocks

Subconfluent monolayer cultures were trysinised and pelleted as described above. The cell pellet was then resuspended in 3.5ml of DMEM containing 10% (v/v) FBS and 10% (v/v) DMSO. 1.5ml aliquots were made in cryovials which were wrapped in tissue and frozen at −20°C for 2 hours, −70°C overnight and then transferred to liquid nitrogen for long term storage. Cells recovered from liquid nitrogen were thawed rapidly at 37°C. These were pelleted by centrifugation at 1100rpm in an MSE bench top centrifuge for 5 minutes to allow the removal of DMSO containing medium. The cell pellet was then resuspended in growth medium and plated out in tissue culture flask.

Charcoal treatment of serum

Foetal bovine serum (FBS) contains endogenous steroid hormones that might mask the effect of exogenously added ligand in transfection experiments. Serum use for transfections was therefore pre-treated with dextran coated charcoal (DCC) which removes all small molecules from the serum, including steroid hormones. 500ml of dextran coated charcoal suspension (1% (w/v) activated charcoal, 0.1% (w/v) dextran T70 and 10mM Tris-HCl pH7.4) was divided equally among four 250ml disposable centrifuge bottles and spun at 2000 x *g* for 5 minutes at 4°C. The supernatant was removed and the pellet was resuspended with 250ml of FBS. The suspension was shaken vigorously at 55°C for 30 minutes and then re-spun. The serum was decanted into bottles containing a fresh dextran coated charcoal pellet, re-incubated and re-spun as above. The treated serum was filter sterilised twice using 0.45μm and 0.22μm Nalgne filter units respectively, aliquoted and stored at –20°C.

Transient transfection

Calcium phosphate precipitation - BBS method

HeLa and COS-1 cells were routinely transfected by a calcium phosphate / DNA co-precipitation method modified from the method of Chen and Okayama (Chen and Okayama, 1987). For transfection in 96-well microtitre plates, cells were plated at 50-70% confluency by diluting the required number of cells in DMEM containing 5% (v/v) DCC treated FBS and adding 100μl of the cell suspension per well. For each precipitate, supercoiled DNA enough for 25 wells were prepared (30ng of DNA per well). It was first mixed with 12.5μl $CaCl_2$, before 125μl of 2x BBS was added. After through mixing, the solution was left at room temperature for 20 minutes. The DNA solution was mixed again before 10μl was added slowly to each well. The cells were then incubated for 16 hours at 37°C, 10% (v/v) CO_2. The medium was then removed and the cells gently washed twice with DMEM containing 2% (v/v) DCC treated FBS to remove residual precipitate. The cells were re-fed with fresh DMEM containing 5% (v/v) DCC treated FBS and

ligand (dissolved in ethanol and stored at –20°C) or ethanol carrier and incubated at 37°C, 10% (v/v) CO_2. Cells were routinely harvested 24 hours after transfection.

For transfection in 24-well microtitre plates, procedures were essentially the same as above except for the following. The cells were plated at 50-70% confluency by diluting the required number of cells in DMEM containing 5% (v/v) DCC treated FBS and adding 1ml of the cell suspension per well. For each precipitate, supercoiled DNA enough for 5 wells were prepared (300ng of DNA per well) and 100µl precipitate was added to each well.

Calcium phosphate precipitation - HBS method

293-T cells were transient transfected by the HBS – calcium phosphate / DNA co-precipitation method after Graham and van der Eb (Graham and Van der Eb, 1973) using the Profection mammalian transfection system (Promega). Cells were plated at 60% confluency in 10cm tissue culture dish in 10ml of DMEM containing 10% (v/v) FBS. The DNA / calcium phosphate precipitate was prepared by addition of 62µl 2M $CaCl_2$ to a diluted DNA solution (432µl) containing 20µg of plasmid DNA. This mixure was then added dropwise with occasionally agitation to 500µl of 2x HBS solution. After incubation at room temperature for 30 minutes, the precipitate was added dropwise to the dish. After 16 hours incubation at 37°C, 10% (v/v) CO_2, the medium was removed and the cells re-fed with DMEM containing 10% (v/v) FBS. Cells were rountinely harvested 24 hours post transfection.

Electroporation

COS-1 cells were transfected by electroporation. Cells were grown to 80% confluency in 175cm^2 tissue culture flasks, trypsinised (as described for Maintenance of cell stocks), recovered by centrifugation and the pellet resuspended in 2ml PBSA. 1ml of the cell suspension was added to 18µg of plasmid DNA in a 0.4cm electroporation cuvette, mixed and incubated on ice for 10 minutes. The cells were then electroporated using a Bio-Rad gene pulser, at 450V and 250µF giving a time constant of approximately 5 milliseconds. The cuvettes were then

returned to ice for 10 minutes, before the cell suspension was removed and diluted in DMEM containing 10% (v/v) FBS and plated on 15cm tissue culture dish. After 16 hours incubation at 37°C, 10% (v/v) CO_2, the medium containing dead cells was replaced and the cells harvested after incubation for further 24 hours.

Reporter assays for 24-well plate transfection

Transiently transfected cells were harvested 24 hours after the addition of ligand, using a method based on the LucLite luciferase reporter gene assay (Packard). Luciferase catalyses the reaction between luciferin and ATP which results in light emission and the production of adenyl-oxyluciferin. The system employed here prevents the binding of adenyl-oxyluciferin to luciferase, allowing the enzymatic reaction to produce a stable and linear glow-type signal instead of a flash-type signal. The cells were washed twice with PBSA and then lysed with 60µl LucLite buffer on ice for 10 minutes. 20µl of the cell lysate was then added to 20µl 2x LucLite reagent in a 96-well MICROLITE plate (Dynex). After incubation at room temperature in the dark for 15 minutes, the signal was measured using the MLX microplate luminometer (Dynex).

For β-galactosidase assay, a Galacto-Light Plus assay system (TROPIX) was employed. The Galacton chemiluminescent substrate (1,2-dioxetane) emits light which persists at a near constant level with a half-life of approximately 180 minutes. The reaction buffer was made by diluting the Galacton substrate 100-fold with the diluent buffer. 5µl of the cell lysate from above was added to 50µl of reaction buffer in a 96-well MICROLITE plate (Dynex). After incubation at room temperature for 45-60 minutes, 75µl of Accelerator II was added to each sample. After incubation in the dark at room termperature for further 15 minutes, the signal was measured using the MLX microplate luminometer (Dynex).

Reporter assays for 96-well plate transfection

Transiently transfected cells were harvested 24 hours after the addition of ligand, using a method based on the LucLite luciferase reporter gene assay (Packard). 50µl of culture medium was removed from each well which was

followed by the addition of 50µl of 1x LucLite reagent. After incubation at room temperature for 5 minutes, the assay mix (100µl) containing the LucLite reagent, medium, and lysed cell contents were transferred to a 96-well MICROLITE plate (Dynex). After incubation in the dark at room termperature for 10 minutes, the signal was measured using the MLX microplate luminometer (Dynex).

The Renilla luciferase assay was carried out in the same 96-well MICROLITE plate containing the firefly luciferase reaction. The Renilla luciferase reagent was prepared by diluting the substrate, Coelenterazine (dissolved in DMSO at 1mg/ml and stored at –20°C), in Renilla buffer to a final concnetration of 10ng/µl. 25µl per well of the Renilla luciferase reagent was then added to the firefly luciferase reaction. After incubation in the dark at room termperature for 10 minutes, the signal was measured using the MLX microplate luminometer (Dynex). The EDTA in the Renilla buffer chelates the divalent cations required for firefly luciferase reaction and quenches the light emission, allowing the Renilla luciferase signal to be detected.

Preparation of whole cell extracts

Cells on 10 or 15 cm plates were washed three times with ice cold PBSA and scraped off the dish with a rubber policeman in 10ml ice cold PBSA. After centrifugation at 1200rpm for 5 minutes in an MSE bench top centrifuge, the cell pellet was snap frozen at –70°C. Cell pellets were thawed on ice in approximately 5 volumes of whole cell extract buffer and passed through a 21 gauge needle ten times. Insoluble material was pelleted by centrifugation at 20000 x *g* for 20 minutes at 4°C. Aliquots of the supernatant were stored at –70°C.

Chapter 3

Molecular determinants of the mERα-coactivator interface

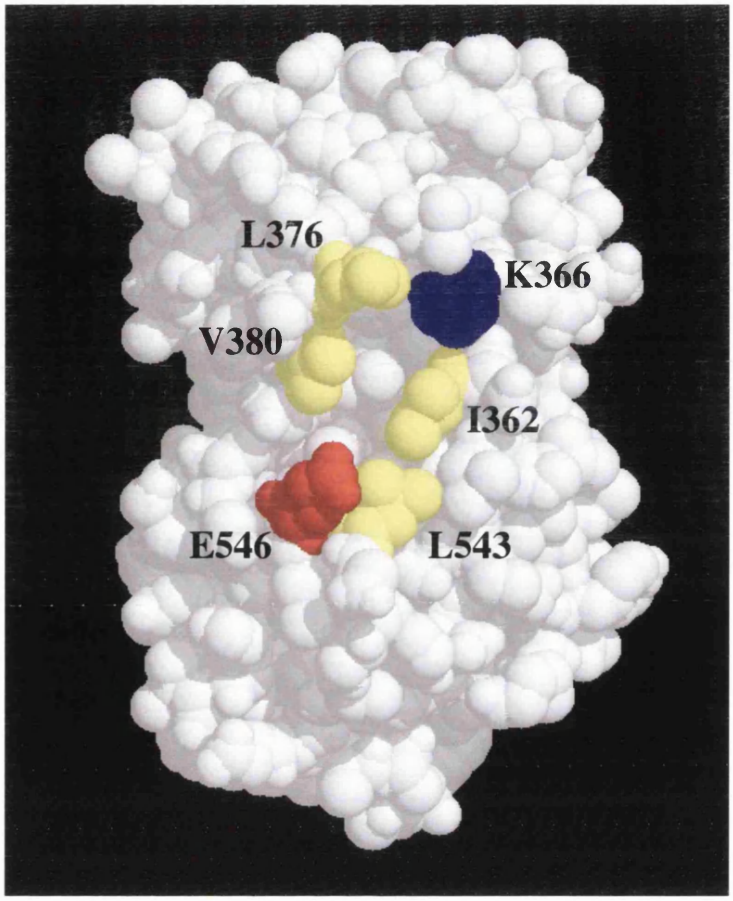
Introduction

The ligand dependent interaction of the p160 coactivators with mERα is mediated by LXXLL motifs, three of which are conserved in both sequence and spacing in all family members (Heery et al., 1997; Torchia et al., 1997). The hydrophobic nature of the LXXLL motifs led to the prediction that a complementary hydrophobic docking surface is likely to be located on the surface of the ligand binding domain (LBD) of the receptor. The ligand dependent transcriptional activity (AF2) of ERα could be attributed to the recruitment of coactivators by the LBD. Therefore, mutation of residues which compromised AF2 activity might imply direct participation in coactivator binding by these residues. It was shown that the integrity of helix 12 at the C-terminus of the LBD and a lysine residue in helix 3 are critical for ERα AF2 activity (Danielian et al., 1992; Henttu et al., 1997). Structural analysis of the LBDs of a number of nuclear receptors suggests that agonist binding results in the realignment of helix 12 (Renaud et al., 1995; Wagner et al., 1995; Wurtz et al., 1996; Brzozowski et al., 1997). Its importance in ERα function is underscored by the observation that it is misaligned in the presence of an antiestrogen, raloxifene, which blocks AF2 activity (Brzozowski et al., 1997).

Inspection of the crystal structure of hERα ligand binding domain in the presence of 17β-oestradiol indicated that, helix 12 and lysine 366 at the C-terminal end of helix 3, are located on the surface of the LBD (Figure 3.1A) (Brzozowski et al., 1997). The observation that these two elements are not in close proximity to each other prompted us to speculate that they are only part of the surface responsible for the docking of p160 coactivator proteins. Because of the hydrophobic nature of the LXXLL motif, we focused on a hydrophobic patch on the surface of the mERα LBD whose boundary seemed to be defined by helix 12 and lysine 366. This hydrophobic patch appears to consist mainly of three residues from helices 3 and 5, namely I362, L376 and V380. Sequence analysis of the nuclear receptor superfamily revealed that the corresponding residues in other receptors are almost always hydrophobic (Figure 3.1B).

Figure 3.1 Structure of hERα ligand binding domain in the presence of 17β-oestradiol. (A) Residues implicated for participation in p160 coactivator binding are highlighted yellow (hydrophobic), red (acidic) and blue (basic). The residues are numbered as in mERα. The space-filled model is generated using RasMol and is based on the co-ordinates under the Protein Data Bank entry code 1ERE. (B) Sequence alignment of mERα LBD helices 3, 4, 5 and 12 with corresponding regions of members of the nuclear receptor superfamily whose agonist-bound crystal structures are solved. Note the absolute conservation of residues (marked with asterisks) in mERα and hERα which are involved in coactivator binding. The boundaries for helices 3, 4, 5 and 12 are assigned according to the hERα LBD structure. The alignment was generated by ClustalX and formatted using MacBoxshade.

A



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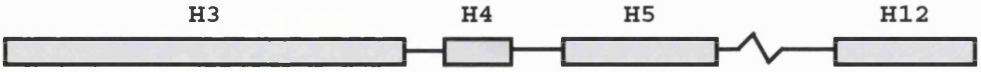


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                                  *   *       *   *           *
mERα    344 ASMMGLLTNLADRELVHMINWAKRVPGFGDLNLHDQVHLLE.....LYDLLLEMLDA 550
hERα    340 ASMMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLE.....LYDLLLEMLDA 546
hPPARγ  277 IRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLK.....LHPLLQEIYKD 473
hRARγ   224 LGLWDKFSELATKCIIKIVEFAKRLPGFTGLSIADQITLLK.....MPPLIREMLEN 418
hTRβ    261 LEAFSHFTKIITPAITRVVDFAKKLPMFCELPCEDQIILLK.....FPPLFLEVFED 461
hPR     712 SSLLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQ.....FPEMMSEVIAA 915
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A hydrophobic surface of the mERα LBD is required for coactivator binding

To assess the contribution of I362, L376 and V380 to the transcriptional activity of mERα and its binding to coactivators, each of them was replaced by alanine. Full length wild-type or mutant receptors were transiently transfected into COS-1 cells and tested for their ability to activate an ERE-tk-luciferase reporter gene. The transcriptional activity of the L376A mutant mERα was impaired, but nevertheless was stimulated by overexpressed SRC1e, a member of the p160 coactivator family (Figure 3.2). Mutant receptors bearing the mutation I362A or V380A activated the reporter gene to the same extent as the wild-type receptor. Their transcriptional activity could also be further enhanced by cotransfecting SRC1e. Consistent with the transient transfection assays, binding of *in vitro* translated mutant receptors to GST-SRC1 (aa 570 to 780), which encompasses its receptor interacting region, is comparable to that of wild-type mERα (Figure 3.3).

Next, I362, L376 and V380 were replaced with aspartic acid, a charged residue which might actively interfere with packing of hydrophobic side chains. All three mutant receptors had dramatically reduced transcriptional activity. There was no detectable *in vitro* binding of the I362D and V380D mutants to GST-SRC1 (aa 570 to 780), and markedly reduced binding was observed for the L376D mutant. Our results implied that I362, L376 and V380 of mERα are in close proximity to the bound p160 coactivator. However, since removal of individual hydrophobic side-chain from any of the three positions was insufficient to abolish coactivator binding by the receptor, these residues might be redundant in the formation of the coactivator interaction surface.

Of the four highly conserved hydrophobic residues in helix 12 of the mERα LBD, only L543 is exposed on the surface in the crystal structure. It was previously shown that alanine substitution of both L543 and L544 abrogated transcriptional activity of the mutant receptor (Danielian et al., 1992). In the light of the crystal structure, we tested whether L543 alone is required for coactivator binding and AF2 activity. The L543A mutant displayed negligible transcriptional activity when transiently transfected into COS-1 cells as full length receptor (Figure 3.5), in contrast to the phenotype observed for the single alanine substitution of I362, L376 or V380. To verify that the mutation is affecting AF2 alone and not interfering with possible cooperation between AF1 and AF2, a

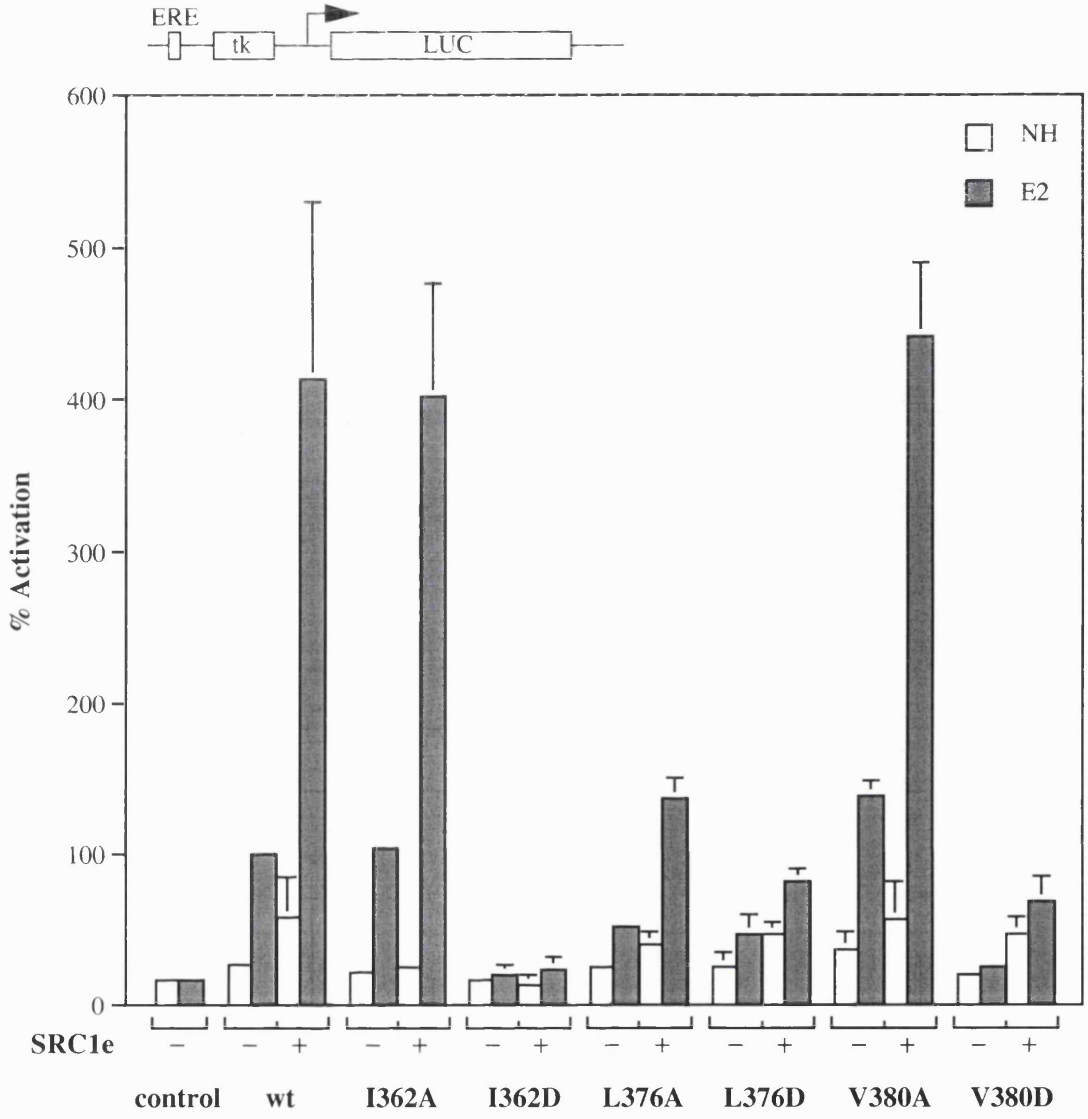


Figure 3.2 Surface hydrophobic residues in helices 3 and 5 of mERα are involved in coactivator binding. COS-1 cells were transiently transfected with expression vector for wild-type (wt) or mutant receptors and pERE-tk-GL3 reporter in the absence (-) or presence (+) of 100ng full length SRC1e. A cytomegalovirus promoter driven pJ7-lacZ plasmid was co-transfected as the internal control. After transfection, cells were treated with ethanol vehicle alone (NH) or oestradiol (E2) at 10nM for 24 h. Subsequently, cells were assayed for luciferase and β-galactosidase activity. Normalised values are expressed as percentage activity compared with wild-type mERα alone in the presence of E2 (100%). The results shown represent the average of at least two independent experiments assayed in duplicate + standard errors. ERE, oestrogen response element.

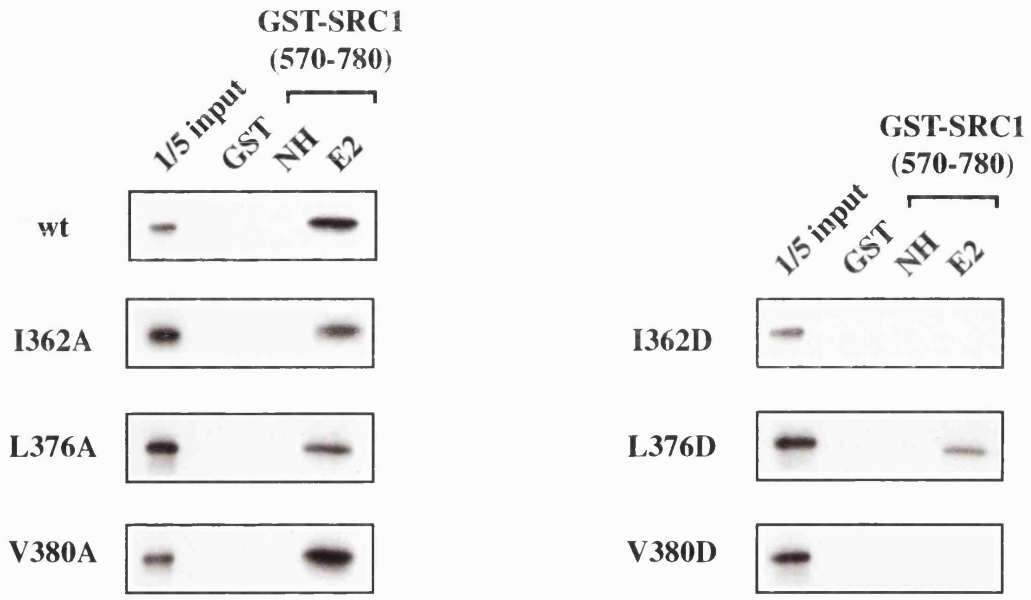


Figure 3.3 Binding activity of wild-type or mutant receptors to SRC1 in GST-pull down assays. In vitro translated, [^{35}S]methionine-labelled wild-type or mutant receptors were incubated with GST-SRC1 (570-780) coupled to Sepharose beads either in the absence (NH) or presence (E2) of 1μM oestradiol. Bound proteins were eluted and separated on 10% SDS-polyacrylamide gels. Labelled proteins were detected by fluorography. The input lane represents 20% of the total volume of the lysate used in each reaction.

chimeric receptor consisting of the LBD with the L543A mutation fused to Gal4 DNA-binding domain was made. The chimeric receptor was unable to activate a Gal4 reporter gene in COS-1 cells and very weak activity was observed when SRC1e was overexpressed concomitantly (Figure 3.5). In GST pull-down experiments, no detectable binding was observed between the L543A mutant with GST-SRC1 (aa 570 to 780) (Figure 3.6). Hence, L543 seems to be essential for AF2 activity, at least in part due to its participation in coactivator binding.

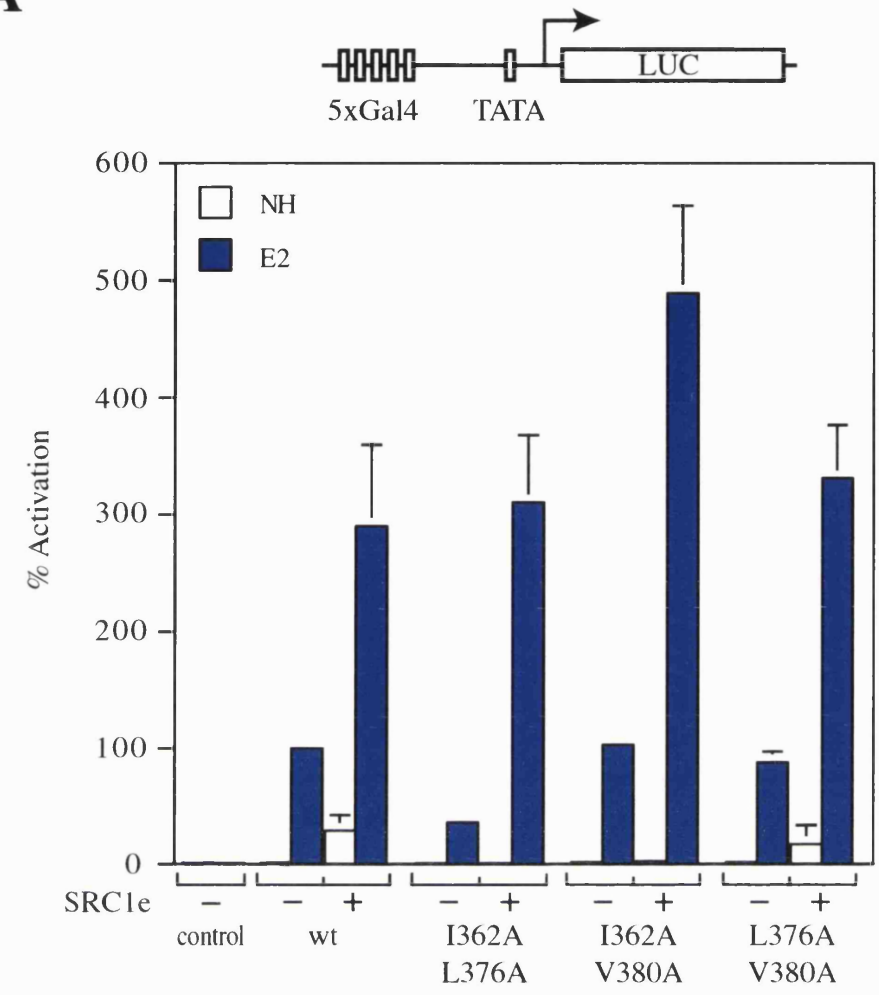
Differential contribution of hydrophobic residues in AF2 activity

It is apparent from the phenotypes of the mutants with single alanine substitution that hydrophobic residues which form the putative coactivator interaction surface might not contribute equally to the AF2 activity of mERα. To extend this observation, we generated mutants with double or triple point mutations in which I362, L376 and V380 were replaced by alanine in all possible combinations. Alanine substitution of any two of the three residues failed to reduce the transcriptional activity of Gal4 DBD-chimeric mutant receptors in transiently transfected COS-1 cells (Figure 3.4). Furthermore, all of the double mutants displayed wild-type binding activity to GST-SRC1 (aa 570 to 780) in GST pull-down experiments (Figure 3.4). A dramatic decrease in transcriptional activity was only observed when all three residues were substituted with alanine, both as full-length or as Gal4-chimeric receptor in COS-1 cells (Figure 3.5). Nevertheless, the triple mutant could be partially rescued by overexpressed SRC1e and was more active than the L543A mutant (Figure 3.5).

Next, we tested whether the difference in transcriptional activity was correlated with the ability of these mutants to bind coactivator. In GST pull-down experiments, weak binding between the triple I362A-L376A-V380A mutant and GST-SRC1 (aa 570 to 780) was observed. There was no detectable binding between the L543A mutant with the same SRC1 construct (Figure 3.6). To obtain a quantitative comparison *in vivo*, mammalian two-hybrid interaction assays were conducted. SRC1 (aa 570 to 780) was fused to the Gal4 DBD and the LBD of the wild-type or mutant receptors to the VP16 acidic activation domain. Upon transient

Figure 3.4 Analysis of double hydrophobic mutant receptors. (A) Wild-type or mutant chimeric receptors consisting of the LBD of mERα fused to the DNA-binding domain of Gal4 were transiently transfected into COS-1 cells. The p5Gal-E1B-luciferase reporter gene was co-transfected in the presence (+) or absence (-) of 100ng full length SRC1e and pJ7-lacZ was used as an internal control. Data are presented as described for Figure 3.2. The results shown represent the average of at least two independent experiments assayed in duplicate + standard errors. (B) Binding of mutant recpetors to GST-SRC1 (aa 570-780) *in vitro* was examined under the same conditions as described for Figure 3.3.

A


LUC
5xGal4
TATA
600
500
400
300
200
100
0
% Activation
NH
E2
SRC1e
–
–
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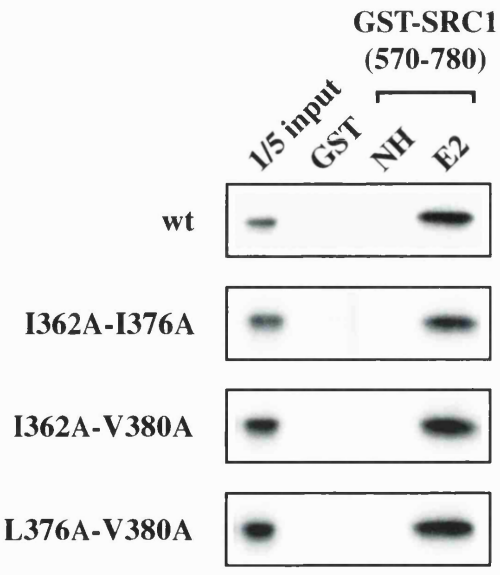
V380A
L376A
V380A

B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1/5 input
GST
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E2
wt
I362A-I376A
I362A-V380A
L376A-V380A

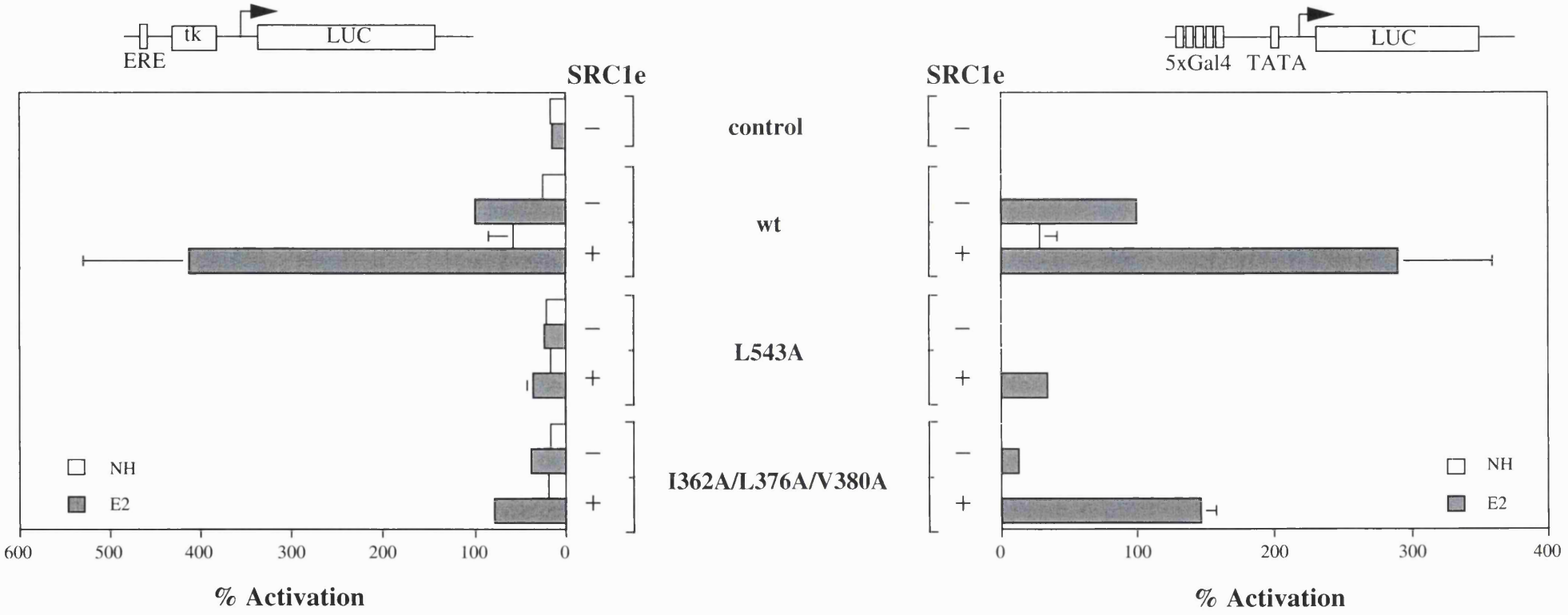

Figure 3.5 Functional analysis of L543A and I362A-L376A-V380A mutant receptors. Wild-type or mutant full length (left) or chimeric receptors consisting of the LBD of mERα fused to the DNA-binding domain of Gal4 (right) were transiently transfected into COS-1 cells. Luciferase reporter genes as indicated were co-transfected in the presence (+) or absence (-) of 100ng full length SRC1e and pJ7-lacZ was used as an internal control. Data are presented as described for Figure 3.2. The results shown represent the average of at least two independent experiments assayed in duplicate + standard errors.

Figure 3.6 Binding of L543A and I362A-L376A-V380A mutant receptors to SRC1. (A) Binding of mutant receptors to GST-SRC1 (aa 570 to 780) *in vitro* were examined using GST pull-down assays under the same conditions as described for Figure 3.3. (B) *In vivo* interaction of mutant mERα LBDs with SRC1 (aa 570 to 780). The expression vectors used are schematically represented with the numbers indicating the amino acid position in the full length protein. The darkly shaded box represents the Gal4 DNA binding domain (aa 1 to 147) and the lightly shaded box represents the activation domain of VP16 (aa 410 to 490). HeLa cells were transiently transfected with the indicated expression vectors together with a p5Gal-E1B-GL3 reporter gene and the pJ7-lacZ internal control plasmid. Following transfection, cells were treated with ethanol vehicle alone (NH) or oestradiol at 10nM (E2). After 24 h, cell extracts were prepared and assayed for luciferase and β-galactosidase activities. Normalised values are expressed as fold induction compared with that of the Gal4 DNA binding domain alone (set as 1). The results shown represent the average of at least two independent experiments assayed in duplicate + standard errors. nd, not determined.

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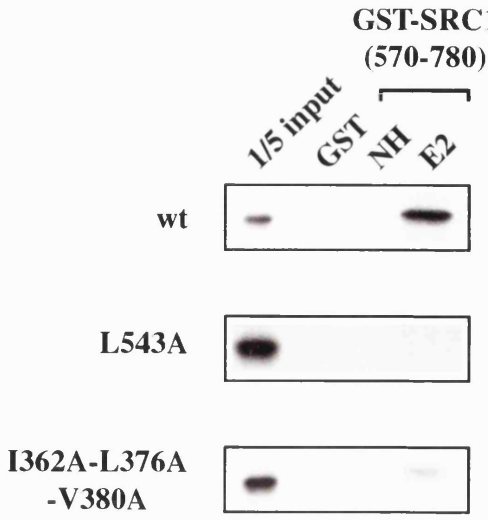

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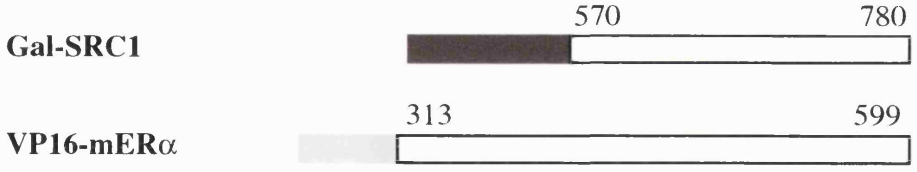


	Fold induction of 5Gal-E1B-GL3	
	NH	E2
Gal4 DBD	1.0	nd
Gal-SRC1/VP16	1.6±0.9	nd
Gal4 DBD/VP16-wt	0.7±0.2	1.1±0.3
Gal4-SRC1/VP16-wt	30.9±17.6	3858.0±95.7
Gal4-SRC1/VP16-L543A	0.9±0.2	22.7±2.6
Gal4-SRC1/VP16-I362A-L376A-V380A	0.7±0.1	64.6±9.7

transfection into HeLa cells, interaction between receptor and SRC1 leads to activation of a Gal4 reporter. In these assays, interaction of the triple I362A-L376A-V380A and single L543A mutants with SRC1 was 60-fold and 168-fold lower than that of the wild-type receptor respectively (Figure 3.6). Together with the observation that L358A, F371A and L383A mutants retain wild-type transcriptional activity (P. M. A. Henttu and M. G. Parker, unpublished observations), these results suggest a hierarchy of conserved hydrophobic residues which form the coactivator interacting surface by virtue of their differential contribution to the AF2 activity of the receptor.

Dual property of lysine 366 in mediating AF2 activity of mERα

Lysine 366 is the only positively charged residue in the predominantly hydrophobic coactivator interacting surface of mERα. It was shown previously that the K366A mutant exhibited negligible transcriptional activity and minimal binding to the coactivator SRC1 *in vitro* (Henttu et al., 1997). However it was unclear whether this effect was due to the lack of charge or the long aliphatic stem of the lysine side chain since alanine is lacking both. To address this question, a mutant receptor was generated in which K366 was replaced by leucine, whose side chain mimics the aliphatic stem of lysine but is devoid of the terminal positive charge. In transiently transfected COS-1 cells, the transcriptional activity of the K366L mutant was intermediate to that of the wild-type receptor but exceeded that of the K366A mutant when tested as full length or Gal4-chimeric receptor (Figure 3.7). This intermediate activity was paralleled by the interaction of K366L mutant with SRC1, which was reduced by 10-fold compared with wild-type mERα but was 20-fold greater than that of the K366A mutant in mammalian two-hybrid interaction assays (Figure 3.8B). This suggests that the terminal charge of K366 is required for optimal transcriptional activity and coactivator binding, but the aliphatic stem of its side chain is sufficient for the partial activity observed.

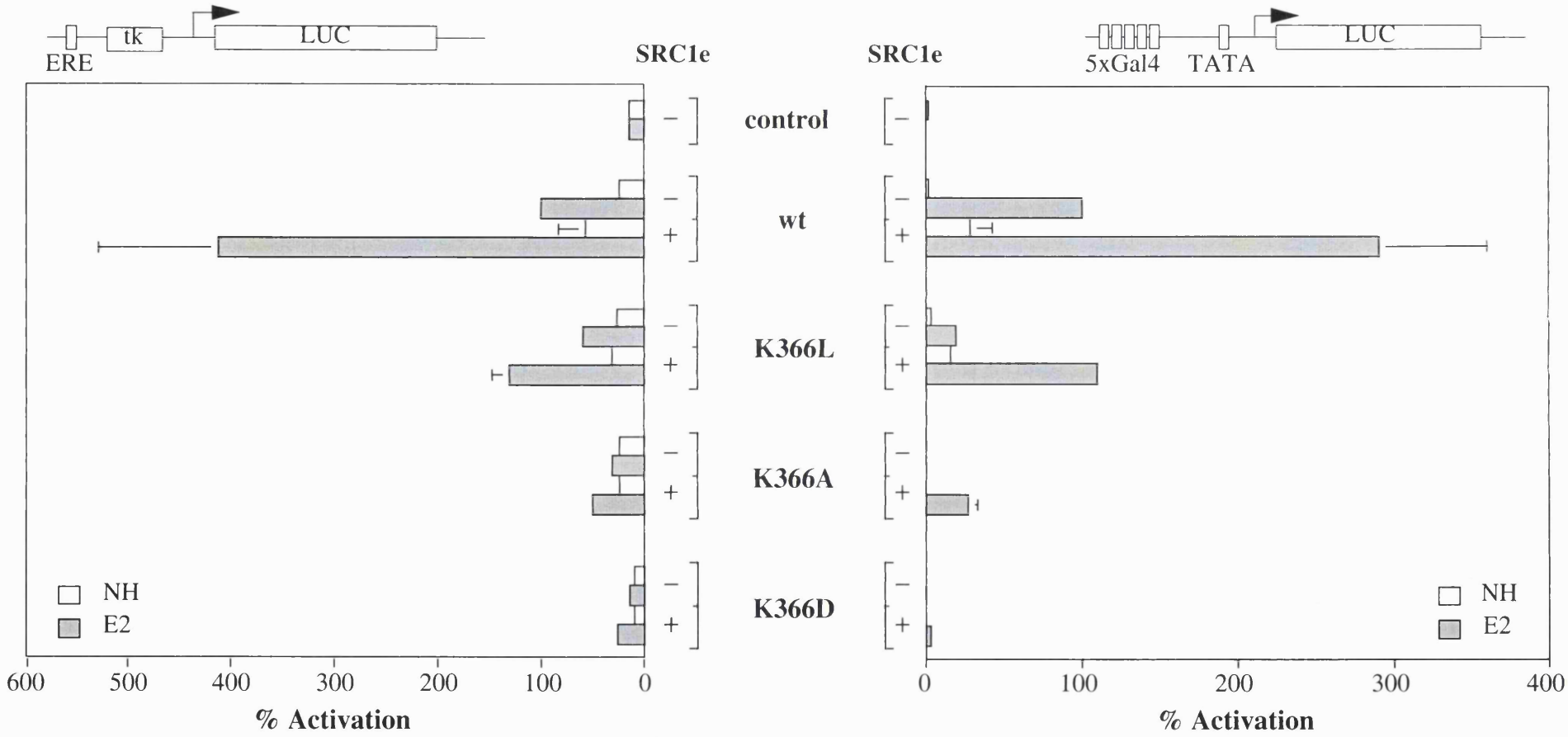
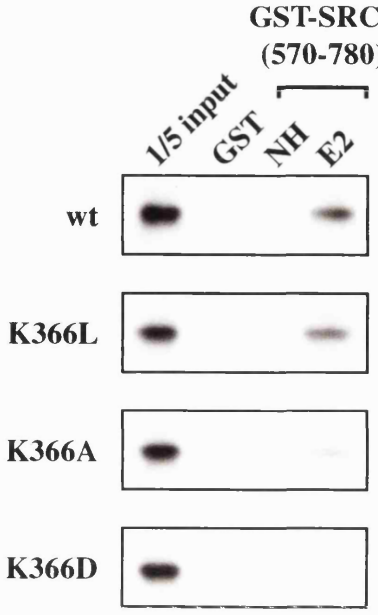


Figure 3.7 Mutation of K366 reveals its dual property in AF2 activity. (A) COS-1 cells were transiently transfected with expression vector for wild-type or mutant receptors (left) or Gal4-chimeric receptors (right), the pJ7-lacZ internal control plasmid and the luciferase reporter plasmid as indicated. Data are presented as described in Figure 3.2. The results shown represent the average of at least two independent experiments assayed in duplicate + standard errors.

Figure 3.8 Binding of K366 mutant receptors to SRC1. (A) Binding of mutant recpetors to GST-SRC1 (aa 570 to 780) were examined under the same conditions as described in Figure 3.3. (B) *In vivo* interaction of mutant mERα LBDs with SRC1 (aa 570 to 780) in transiently transfected HeLa cells. Data are presented as described in Figure 3.6. The results shown represent the average of at least two independent experiments assayed in duplicate + standard errors.

A



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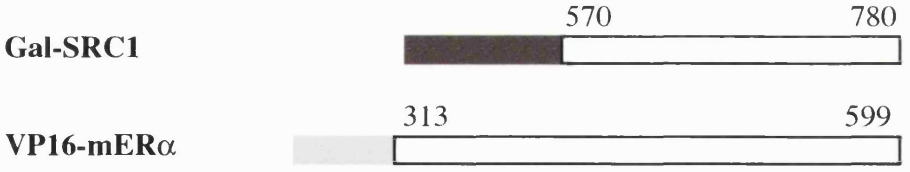


	Fold induction of 5Gal-E1B-GL3	
	NH	E2
Gal4 DBD	1.0	nd
Gal-SRC1/VP16	1.6±0.9	nd
Gal4 DBD/VP16-wt	0.7±0.2	1.1±0.3
Gal4-SRC1/VP16-wt	30.9±17.6	3858.0±95.7
Gal4-SRC1/VP16-K366L	1.0±0.1	350.0±45.6
Gal4-SRC1/VP16-K366A	1.0±0.3	16.0±1.4
Gal4-SRC1/VP16-K366D	0.9±0.1	1.3±0.2

Next, K366 was replaced by aspartic acid and arginine. The K366D mutant had negligible transcriptional activity (Figure 3.7) and displayed no binding to SRC1 both *in vitro* (Figure 3.8A) and *in vivo* (Figure 3.8B), a phenotype more severe than that of the K366A mutant. However, the K366R mutation had no effect on the transcriptional activity of the receptor (data not shown). The first result confirms the requirement for a positive charge at the C-terminal end of helix 3 while the latter suggests that the exact positioning of the positive charge is not crucial. Taken together, it can be concluded that both the terminal positive charge and the aliphatic stem of the K366 side-chain are involved in mediating the AF2 activity of the receptor.

Mutation of residues which constitute the coactivator interaction surface does not affect ligand binding or DNA binding

Expression of the wild-type and mutant receptors was verified by Western blotting (Figure 3.9). To ensure that mutations at the coactivator interaction surface had no effect on the integrity of the LBD structure, ligand binding assays were performed. All receptor proteins bound 17β-oestradiol with similar affinities (Table 1). It was concluded that the slight variation of the dissociation constants could not account for the dramatic decrease of transcriptional activity of the mutant receptors since a saturating concentration of hormone (10nM) was used in transfection experiments. In addition, all mutant receptors bound to DNA as dimers in a gel retardation assay (Figure 3.10). The identity of the receptor-DNA complex was confirmed by the supershift observed in the presence of the ERα-specific antibody MP16. Therefore, alteration of transcriptional activity of the mutant receptors were attributed to defects in coactivator interactions rather than ligand binding or DNA binding.

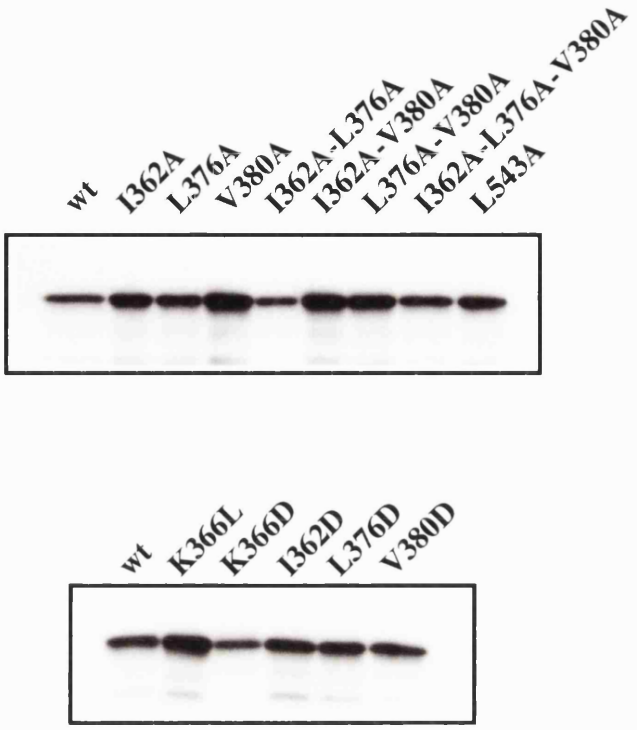


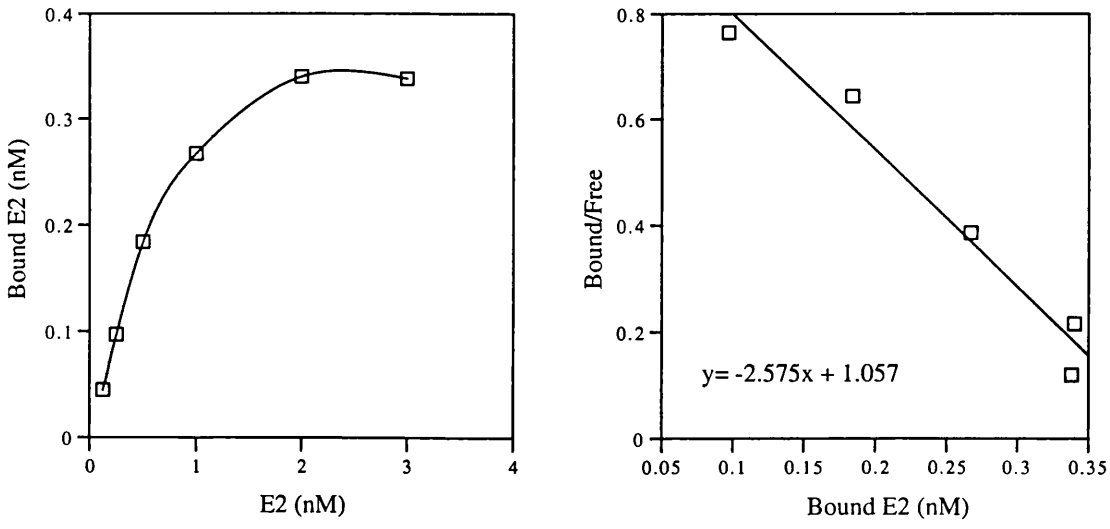
Figure 3.9 Expression of mutant receptors. Full-length wild-type (wt) or mutant receptors were transiently expressed in COS-1 cells. Whole cell extracts containing 10μg of proteins were separated on 8% polyacrylamide gels and transferred onto nitrocellulose membranes. Western blotting was carried out using the ERα specific monoclonal antibody H222 (1:2000) and the HRP-conjugated rabbit anti-rat antibody (1:3000). The signals were detected by enhanced chemiliminescence (ECL).

Table 1

Oestrogen binding activity of wild-type and mutant receptors

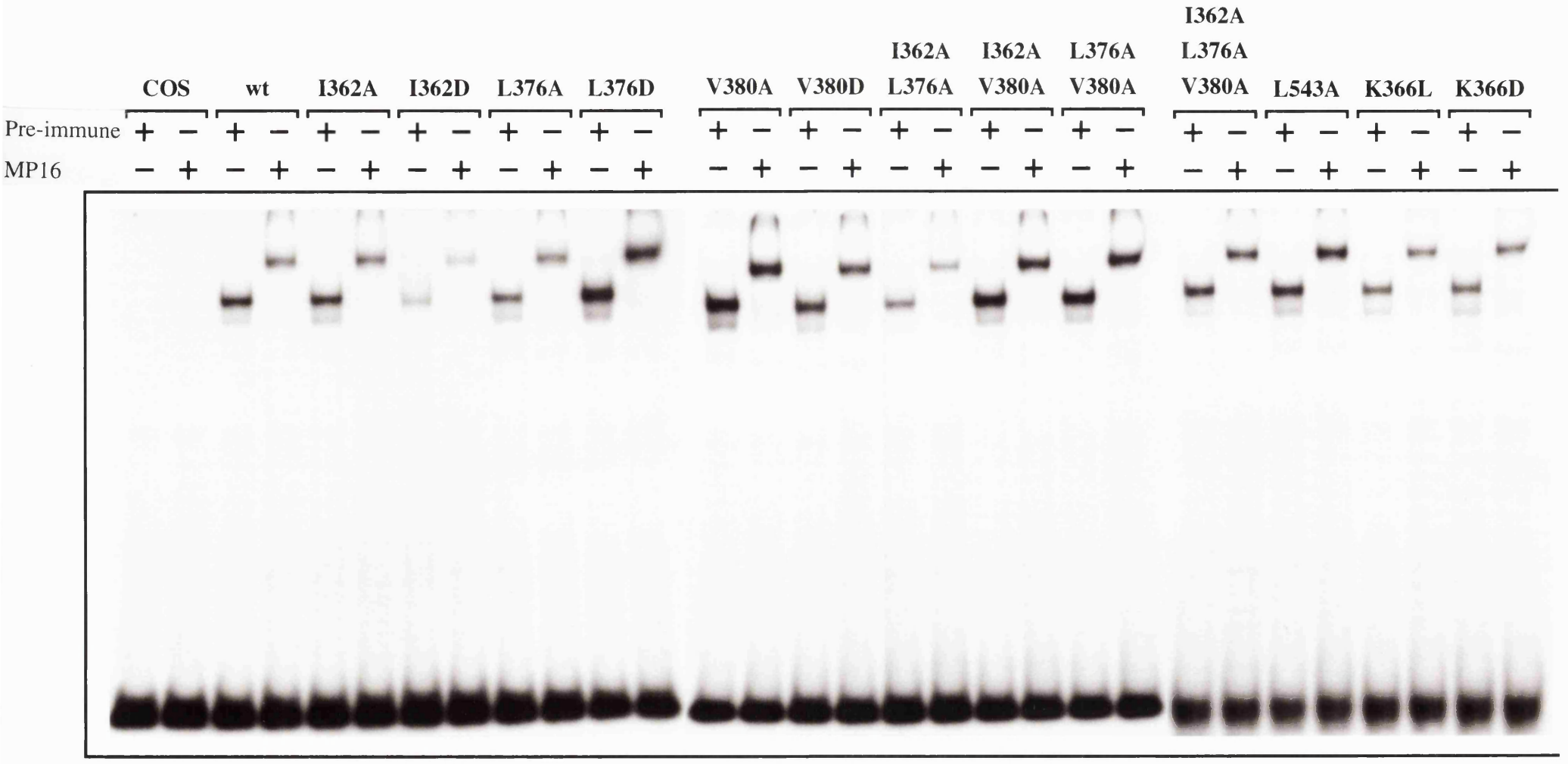
Receptor	K_d (nM)
Wild-type mERα	0.33
I362A-L376A-V380A	0.28
I362D	0.49
L376D	0.86
V380D	0.90
K366L	0.31
K366D	0.33

Extracts prepared from transiently transfected COS-1 cells were analysed for ligand binding activity. The dissociation constant (K_d) for ligand binding for each mutant was determined by Scatchard analysis. The K_d for mutant receptors I362A, L376A and V380A were not determined. However, they showed similar specific binding as wild type mERα to 17β-oestradiol at a concentration of 8nM.



Graphical representation of results from a ligand binding assay performed on wild-type mERα transiently expressed in COS-1 cells. Saturated specific binding of 17β-[^{3}H]oestradiol is shown on the left and the Scatchard plot is shown on the right.

Figure 3.10 Mutant receptors bind to DNA with affinity similar to the wild-type receptor. Full length wild-type (wt) or mutant receptors were transiently expressed in COS-1 cells. Equal amounts of receptor were analysed for DNA binding in an electrophoretic mobility shift assay using a [^{32}P]-labelled oligonucleotide containing a single consensus oestrogen response element from the vitellogenin A2 gene promoter. Binding reactions were performed either in the presence of ERα-specific antibody MP16 or pre-immune serum. Protein-DNA complexes were separated on 6% native polyacrylamide gels and detected by autoradiography.


COS
wt
I362A
I362D
L376A
L376D
V380A
V380D
I362A L376A
I362A V380A
L376A V380A
I362A L376A V380A
L543A
K366L
K366D
Pre-immune
MP16

Specificity of LXXLL motifs to the mERα coactivator interaction surface

Having probed the coactivator interaction surface of the mERα LBD, attempts were made to identify potential determinants for high affinity binding of p160 coactivators to the surface, in collaboration with Sue Hoare in the lab. There are three LXXLL motifs in the receptor interaction domain of each of the p160 coactivator family members (Figure 3.11). Previous work indicated that motif 2 in SRC1 is preferentially used in mediating interaction between mERα and SRC1 *in vitro* (Kalkhoven et al., 1998). To test the affinity of different SRC1 motifs towards the docking site in greater detail, increasing concentrations of 14-mer peptides, M1, M2 or M3, encompassing either SRC1 motif 1, 2 or 3 respectively were used to compete for the *in vitro* binding of GST-mERα LBD with SRC1e. Inhibition of SRC1 binding by the M2 peptide was approximately 8-fold better than that by the M1 or M3 peptides (Figure 3.12A and B), implying that SRC1 motif 2 has a higher affinity to the mERα LBD. Next, a panel of M2 peptides ranging from 8- to 22-mers (designated M2(8) to M2(22)) were used to investigate whether the length of the peptide would affect its ability to inhibit receptor-coactivator interaction. Inhibition by the M2(12) peptide was about 100-fold stronger than that by the M2(8) peptide; however, further extension of the peptide at the N- or C- termini did not increase the degree of inhibition (Figure 3.13). This suggests that the determinants of SRC1 motif 2 for its high affinity binding to the mERα docking site is N-terminal to the minimal LXXLL motif. In particular, we noted a cluster of three basic residues at positions -2 to -4 of motif 2 which are conserved across all p160 coactivator family members. To determine whether the three basic residues are sufficient to confer specificity, we synthesised an M3 peptide with residues at positions -2 to -4 substituted for by the corresponding basic residues of SRC1 motif 2. The resultant peptide, M3(M2), had an inhibition profile similar to that of the native M2 peptide (Figure 3.14). Conversely, we replaced the three basic residues in M2 with the corresponding residues from SRC1 motif 3, M2(M3) behaved in a manner similar to that of the native M3 peptide (Figure 3.14). Finally, we tested the effect of replacing the individual basic residues with alanine, but found that all three mutant peptides were capable of inhibiting the binding of SRC1 to a similar extent as the wild-type peptide (data not shown). Thus, our results suggest that the preference for motif 2 when ERα

and SRC1 interact is conferred, at least in part, by basic residues which are N-terminal to the minimal LXXLL motif. Moreover, these three residues seem to be sufficient for transforming a low-affinity motif into a high-affinity motif for the docking site on mERα.

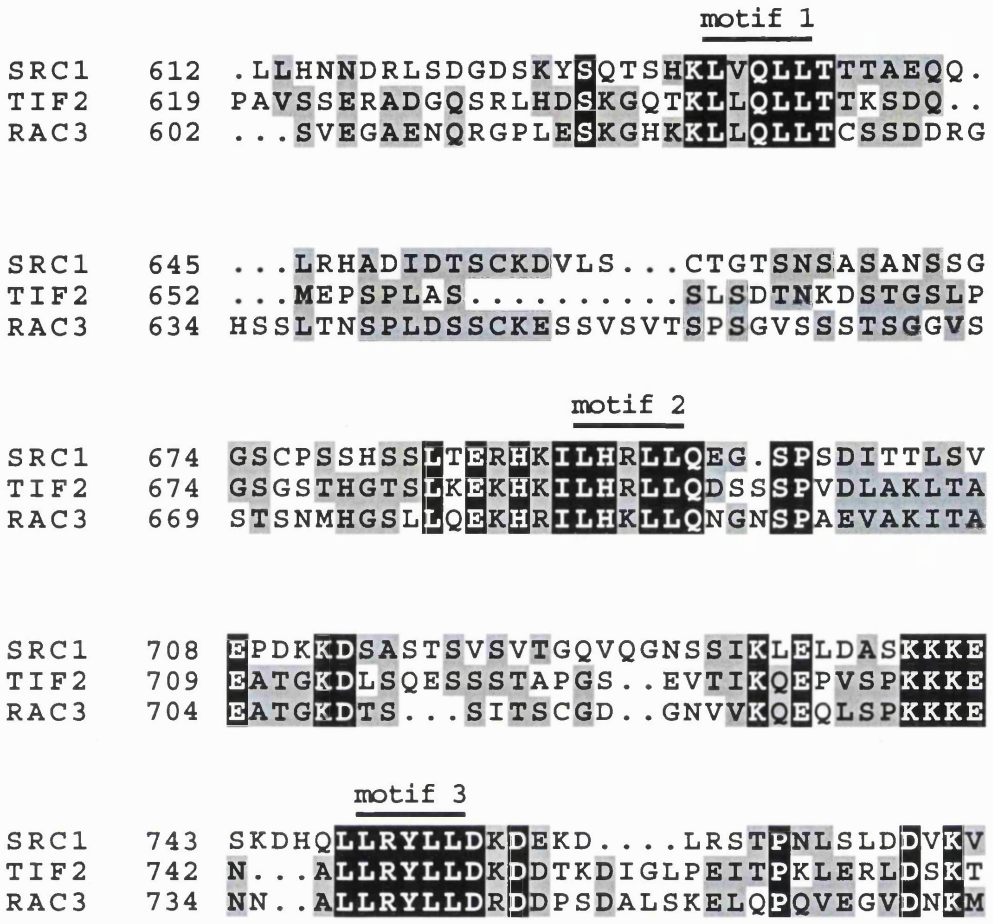
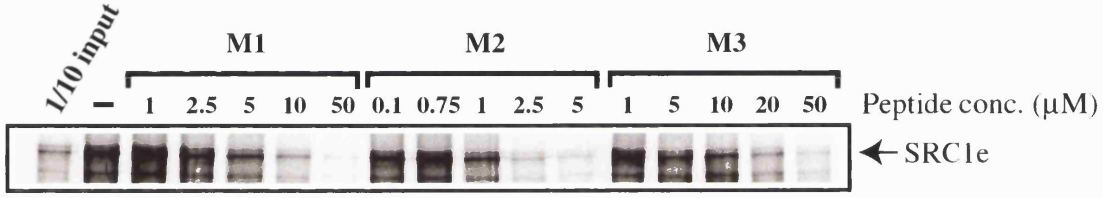


Figure 3.11 Sequence alignment of receptor interaction domain of p160 coactivators. Identical residues are highlighted in black and conserved residues are shaded in grey. The alignment was generated by ClustalX and formatted with MacBoxshade.

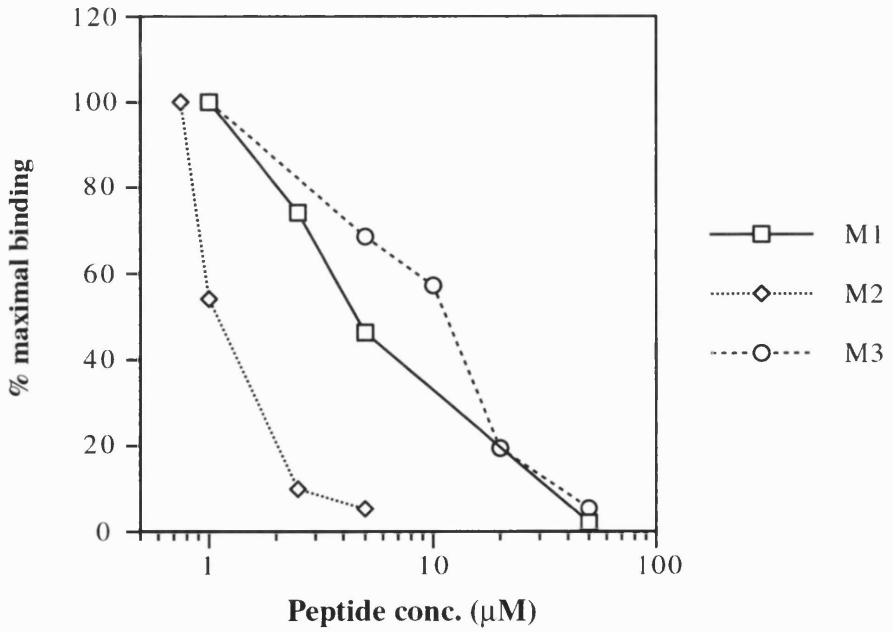
Figure 3.12 Inhibition of mERα-SRC1 interaction in vitro by LXXLL motif containing peptides. (A) Comparison of peptides encompassing either SRC1 LXXLL motif 1, 2 or 3. A GST fusion protein of mERα LBD which had been coupled to Sepharose beads was incubated with *in vitro* translated [^{35}S]methionine-labelled SRC1e protein and increasing amount of LXXLL motif containing peptide, in the presence of 1μM oestradiol. Bound labelled proteins were eluted, separated on 10% SDS-polyacrylamide gels and detected by fluorography. The imput lane represents 10% of the total volume of the lysate used in each reaction. (B) Graphical representation of results from Figure 6A. The amount of bound SRC1e protein were quantified using a Phosphoimager and expressed as percentage maximal binding relative to the amount of bound proteins in the absence of any LXXLL motif containing peptide (100%). At least two independent experiments were performed and the data shown are from one representative experiment, performed by Sue Hoare.

A

		-7 -6 -5 -4 -3 -2 -1 +1 +2 +3 +4 +5 +6 +7
SRC1 M1	(626-639)	Y S Q T S H K L L Q L L T T
SRC1 M2	(683-696)	L T E R H K I L H R L L Q E
SRC1 M3	(742-755)	E S K D H Q L L R Y L L D K



B



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M2 (8)                    I L H R L L Q E
M2 (12)               A R H K I L H R L L Q E
M2 (14)           L T A R H K I L H R L L Q E
M2 (17)           L T A R H K I L H R L L Q E G S P
M2 (22)           L T A R H K I L H R L L Q E G S P S D I T T
```

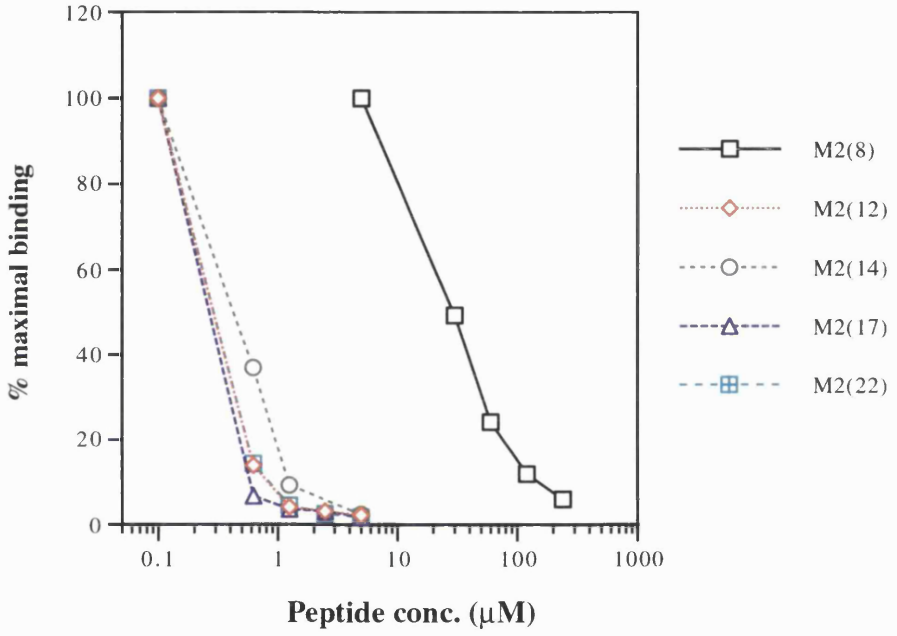


Figure 3.13 Differential inhibition of mERα-SRC1 interaction *in vitro* by LXXLL motif 2 containing peptides. Effect of flanking residues on inhibition of mERα-SRC1 interaction by motif 2-containing peptide. Increasing amounts of M2 peptide whose length varied from 8 to 22 residues were used to inhibit interaction between GST-mERα LBD and [^{35}S]methionine-labelled SRC1e protein in an assay described for Figure 3.12A. Data are presented as described for Figure 3.12B and are from one representative experiment. At least two independent experiments were performed.

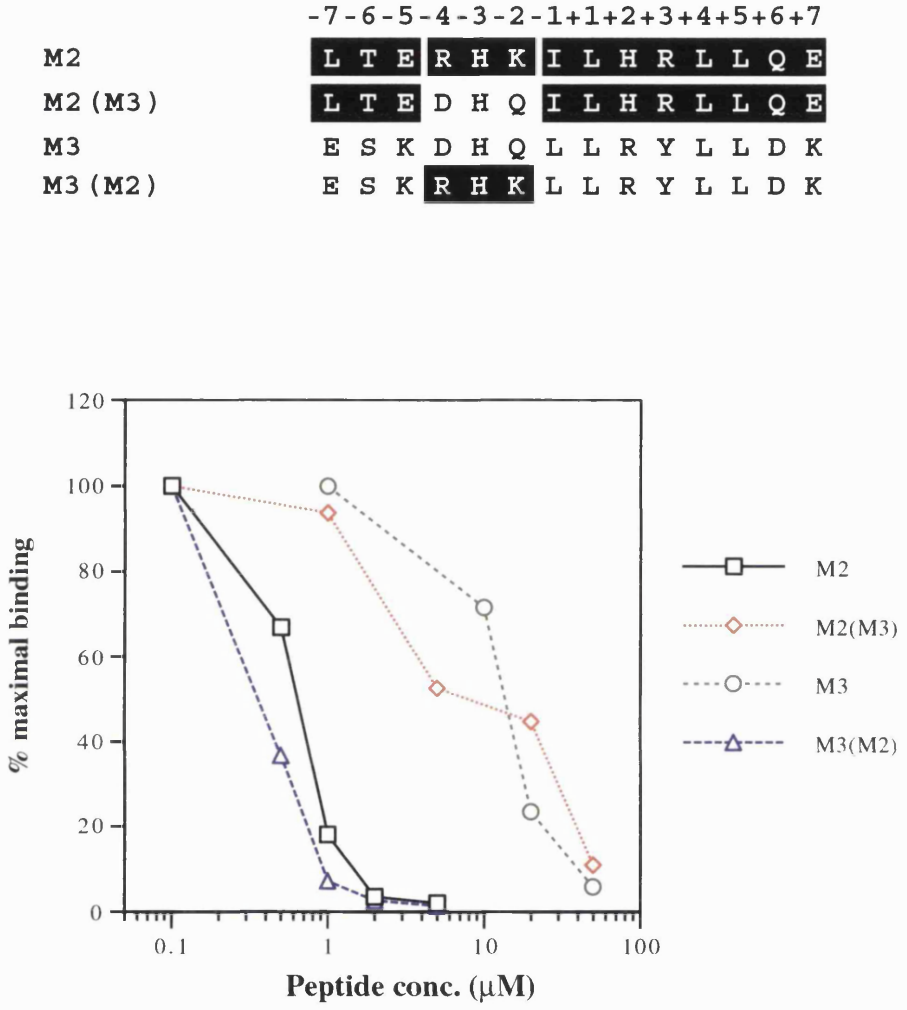


Figure 3.14 Three basic residues N-terminal to LXXLL core motif 2 confers high affinity binding to mERα. Residues $^{-4}$DHQ^{-2} of SRC1 motif 3 was substituted by residues $^{-4}$RHK^{-2} from the corresponding positions of motif 2 in a 14-mer peptide and vice versa. Increasing amount of wild-type or mutant peptides were incubated with GST-mERα LBD and [^{35}S]methionine-labelled SRC1e protein in an assay described for Figure 3.12A. Data from one representative experiment are presented as described for Figure 3.12B. At least two independent experiments were performed.

Summary

Transcriptional activation by ERα is achieved through its interaction with coactivator proteins upon ligand binding. It has been shown that the recruitment of p160 family of coactivators is dependent on a short hydrophobic motif, LXXLL, three of which are conserved in individual family members (Heery et al., 1997; Torchia et al., 1997). In this chapter, a cluster of residues in the LBD of mERα were identified which comprise an interaction surface to allow docking of the motif. Because of the structural similarity of the NR LBD, it is likely that the same surface is also utilised by other receptors for coactivator binding. In addition, three basic residues N-terminal to the core LXXLL motif 2 in SRC1 were identified as the determinants which confer high affinity binding to the mERα coactivator interaction surface.

Chapter 4

Identification of altered specificity coactivator for mERα

Introduction

In order to establish the functional role of p160 coactivators in mammalian cells in the absence of interference from endogenous proteins, a yeast two-hybrid screen was conducted to identify mutant coactivators which would interact with designated mERα. This approach was necessary because endogenous coactivators were in most cases sufficient to support oestrogen dependent transcriptional activation of reporter genes (Figure 4.1A). As a result, potentiation of ERα transcriptional activity by exogenously expressed coactivators was modest and it was not feasible to pinpoint the coactivator which is in direct contact with ERα during transcriptional activation. In view of the large number of proteins which are capable of interacting with ERα in a ligand dependent manner, it is especially important to distinguish between the 'core' coactivator components and others which may play indirect or modulatory roles.

To investigate the functional consequence of direct interaction between p160 coactivators and ERα in mammalian cells, mutant receptors that were incapable of binding endogenous coactivators were generated. These mutant receptors were then used as 'baits' in a genetic screen for altered specificity mutant SRC1. The ERα mutants alone were unable to activate reporter genes in mammalian cells (Figure 4.1B). Transcriptional activation would then be conditional upon exogenous expression of altered specificity SRC1 if and only if SRC1 was sufficient to anchor the 'core' coactivator components to the receptor (Figure 4.1C). In contrast, failure to activate transcription should imply that direct ERα–SRC1e interaction was insufficient in eliciting oestrogen dependent gene activation under physiological conditions.

Mutant mERα impaired for interaction with transcriptional coactivators

The molecular determinants of the mERα–coactivator interface have been established in biochemical and crystallographical studies and selected residues which mediate the protein-protein interactions are highlighted in Figure 4.2A (Brzozowski et al., 1997; Shiau et al., 1998; Mak et al., 1999). The role of V380, a

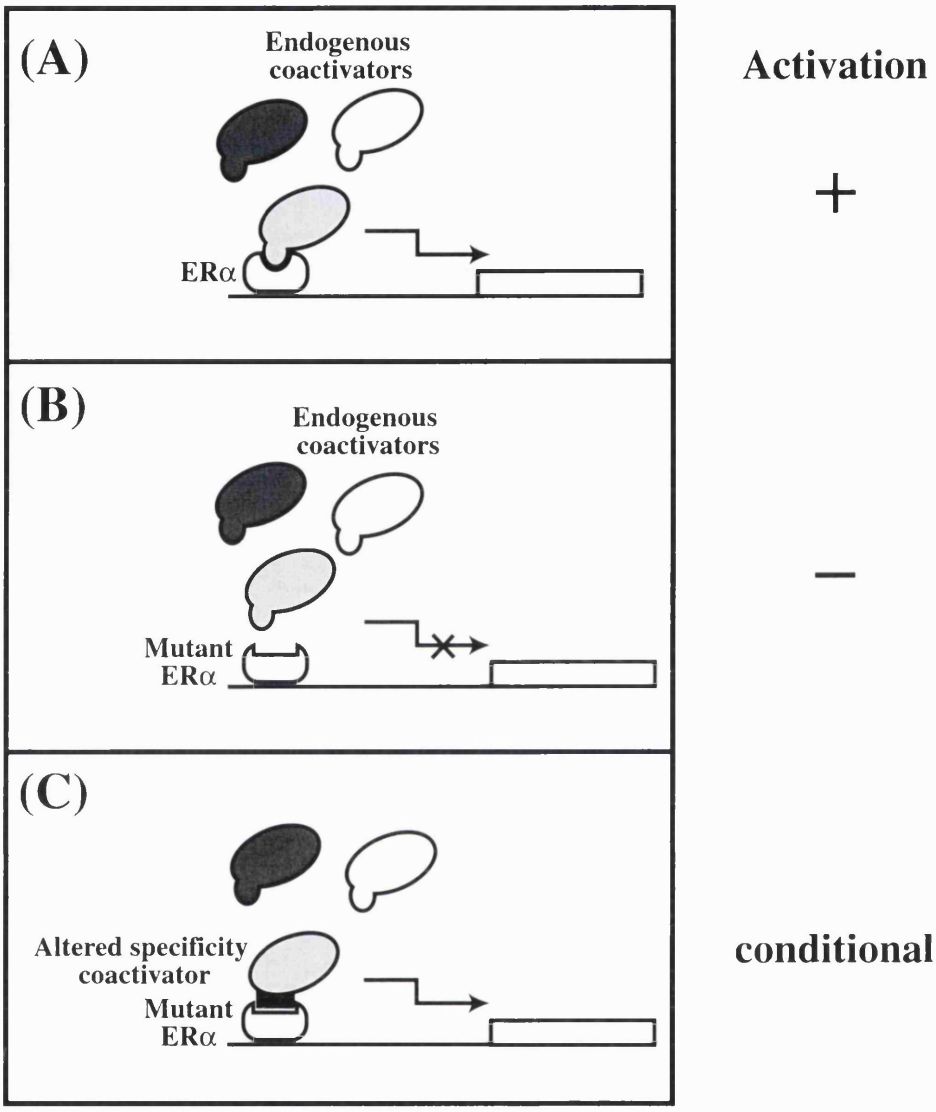


Figure 4.1 Oestrogen dependent gene activation through ERα-coactivator interaction. Model for gene activation by wild-type and mutant ERα. (A) Under physiological conditions, wild-type ERα interacts with endogenous coactivators in a ligand dependent manner to activate transcription of a reporter gene. Different shading represents distinct species of proteins which are capable of interacting with ligand bound ERα whose relative functional importance is unresolved. (B) Disruption of coactivator interaction surface of ERα prevents its binding to any endogenous coactivators. The mutant ERα is therefore unable to activate transcription. (C) Conditional gene activation may be achieved by co-expression of the mutant ERα with its altered specificity coactivator partner on the assumption that the coactivator is directly recruited by ERα under physiological conditions.

conserved residue on the surface of mERα LBD in coactivator binding has been analysed in the previous chapter. While the V380D mutant receptor failed to bind SRC1e, the V380A mutant receptor was normal. In contrast, replacement of L543 by alanine was sufficient to abolish coactivator interaction (Mak et al., 1999). This prompted us to speculate that there is a hierarchy of conserved hydrophobic residues which form the mERα coactivator interaction surface and mutations at V380 which abolish coactivator binding might be more amenable to complementation.

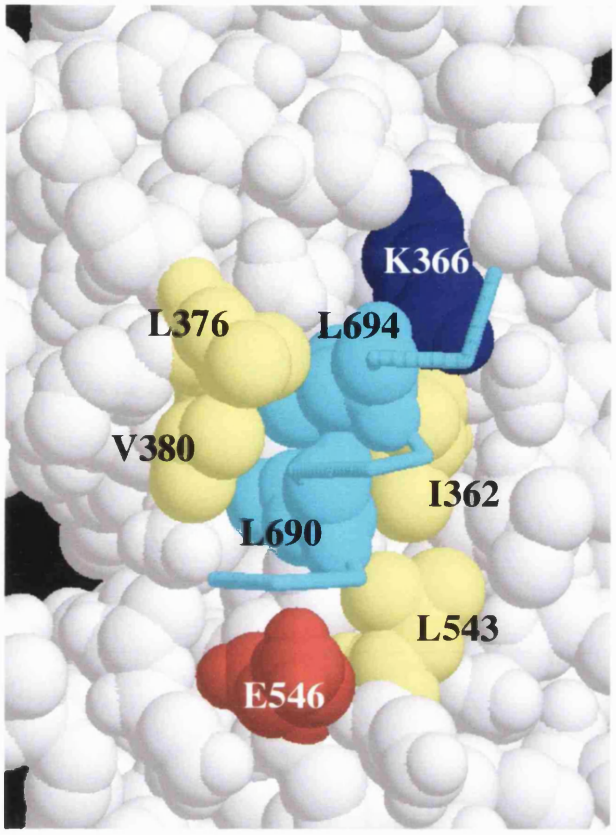
Two additional mutant receptors, V380H and V380R, were generated which satisfied the criteria for potential complementation by altered specificity SRC1e. Histidine and arginine were chosen in the hope that their bulky and charged side chains would sufficiently disrupt the original protein-protein interactions and at the same time provide interesting molecular features for complementaion. Both mutant receptors failed to interact with SRC1e m13, *in vitro* (Figure 4.2B) and *in vivo* (see below). In SRC1e m13, the first and the third LXXLL motifs were rendered non-functional by mutation into LXXAA. However, the intact second LXXLL motif was shown to mediate mERα–SRC1e interaction at an affinity similar to wild-type SRC1e (Kalkhoven et al., 1998). The structural integrity of V380H was demonstrated by its binding affinity to 17β-oestradiol (K_d=0.87nM for V380H and K_d=0.33nM for wild-type receptor). The ligand binding affinity of V380R was not determined. However, when the analogous mutation was introduced into hTRβ, the ligand binding affinity of the corresponding mutant (I302R) was reported to be wild-type (Feng et al., 1998). It is resonable to extrapolate that the ligand binding affinity of V380R is comparable to that of wild-type receptor.

Design of a yeast two-hybrid screen for altered specificity SRC1

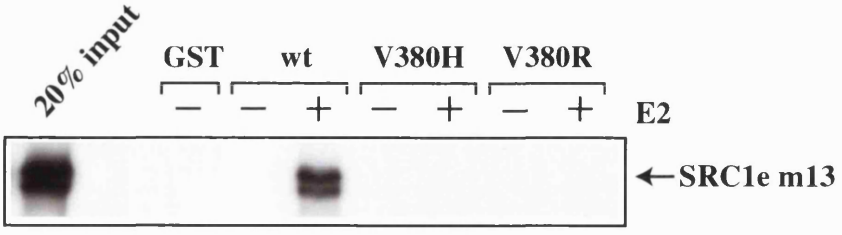
The crystal structure of the agonist bound human ERα (hERα) LBD complexed with GRIP1 NR box II peptide indicated that V376 of hERα interdigitated with L690 and L694 of GRIP1 (Shiau et al., 1998). We proposed that similar van der Waals contacts exist between V380 of mERα and L690 and L694 of SRC1e since the residues which consitute the receptor-coactivator interface are

Figure 4.2 Structure and function of mERα coactivator interaction surface. (A) A close-up view of the agonist bound hERα-GRIP1 NR box II peptide co-crystal structure showing the receptor-peptide interface. The residues that form the coactivator interacting surface in the receptor moiety are coloured in yellow (hydrophobic), red (acidic) and blue (basic) and are numbered as in mERα. The peptide is coloured in cyan. The two leucine residues, in close contact with V380 of mERα, are shown in space-filled mode to highlight the interaction. The model was generated by RasMol and was based on the coordinates under the Protein Data Bank entry code 3ERD. (B) Binding activity of wild-type and mutant receptors to SRC1e m13 in GST pull-down assay. *In vitro* translated, [^{35}S]methionine-labelled SRC1e m13 was incubated with GST-fusion of wild-type or mutant mERα LBD coupled to Sepharose beads in either the absence or presence of 1μM 17β-oestradiol (E2). Bound proteins were eluted and separated on SDS-8% polyacrylamide gels. Labelled proteins were detected by fluorography. The imput lane represents 20% of the total volume of the lysate used in each reaction.

A


K366
L376
L694
V380
I362
L690
L543
E546

B


20% input
GST
wt
V380H
V380R
– – + – + – +
E2
←SRC1e m13

highly conserved (Figure 4.2A). Furthermore, the failure of V380H, V380R and V380D to bind wild-type SRC1e was most likely due to disruption of these contacts.

In order to isolate altered specificity mutant SRC1e capable of interacting with mutant receptors, a yeast two-hybrid library was constructed in which L690 and L694, which form part of the second LXXLL motif in SRC1e were randomly mutatated. The second LXXLL motif was chosen because it was shown to preferentially interact with mERα (Kalkhoven et al., 1998; Mak et al., 1999). The mutant library was based on a construct encompassing the entire receptor interacting domain (aa 570 to 782) of SRC1e fused to the Gal4 DNA binding domain (pGBDU-SRC1 m13). The first and the third LXXLL motifs were rendered non-functional by mutation into LXXAA and this was denoted by the suffix m13 (Figure 4.3). This ensured that interaction with the mutant receptors would be restored solely by mutations based on the second LXXLL motif and not by cooperation with a wild-type motif. It also justified the use of SRC1 m13 as a wild-type reference for receptor-coactivator interaction and function in subsequent experiments.

The ligand binding domain (aa 313 to 599) of mERα which contained point mutations at V380 was fused to the Gal4 activation domain (AD). This is because chimeric receptor consisting of heterologous DBD and mutant mERα LBD may possess significant transcriptional activity in yeast even though they displayed negligible activity in mammalian cells. When the LBD of a series of mutant receptors: L543A, K366L, K366A or K366D, was fused to the LexA DBD and expressed in the *S. cerevisiae* strain L40, they all activated a lacZ reporter gene at a higher level than the wild-type receptor in an agonist dependent manner (Figure 4.4). In the most extreme case, K366D was 45-fold more active than the wild-type receptor. Correlation between transcriptional activity in yeast and mammalian cells was only observed for the triple point mutant I362A-L376A-V380A. It should be noted that these mutant receptors were all characterised to have reduced or negligible affinity to mammalian transcriptional coactivators (Chapter 3). These results suggest that, upon ligand binding, a subset of mutant receptors are capable of displaying structural features which permit fortuitous interactions with

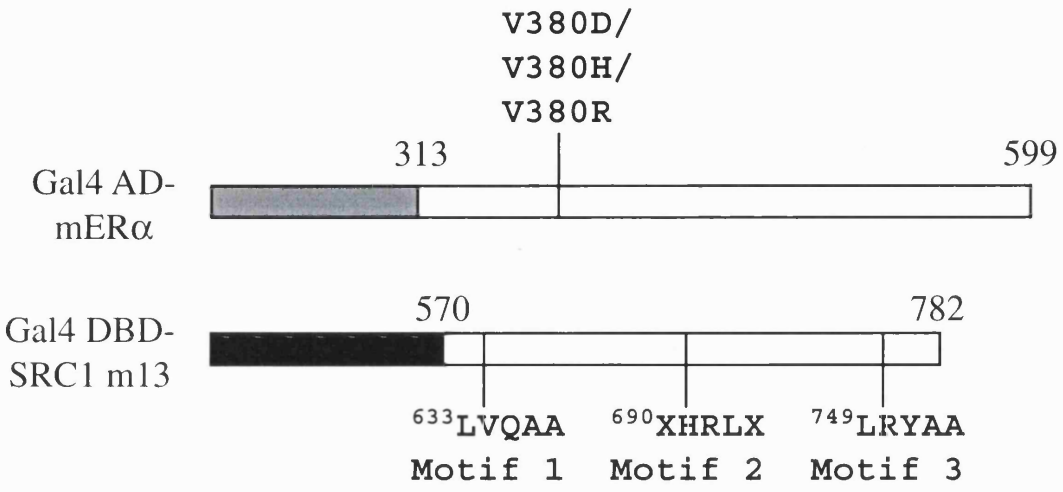


Figure 4.3 Schematic representation of the constructs used in the yeast two-hybrid screen for SRC1 mutants which suppress mutations in V380 of mERα. The numbers indicate the amino acid position in the full-length protein. The letter X represents any amino acids and signifies the two randomised positions at LXXLL motif 2 of SRC1. In addition, LXXLL motifs 1 and 3 of SRC1 were rendered non-functional by mutation into LXXAA and the construct was denoted by the suffix 'm13'. The lightly shaded box represents the activation domain (AD) of Gal4 (aa 768 to 881) and the darkly shaded box represents the DNA binding domain (DBD) of Gal4 (aa 1 to 147).

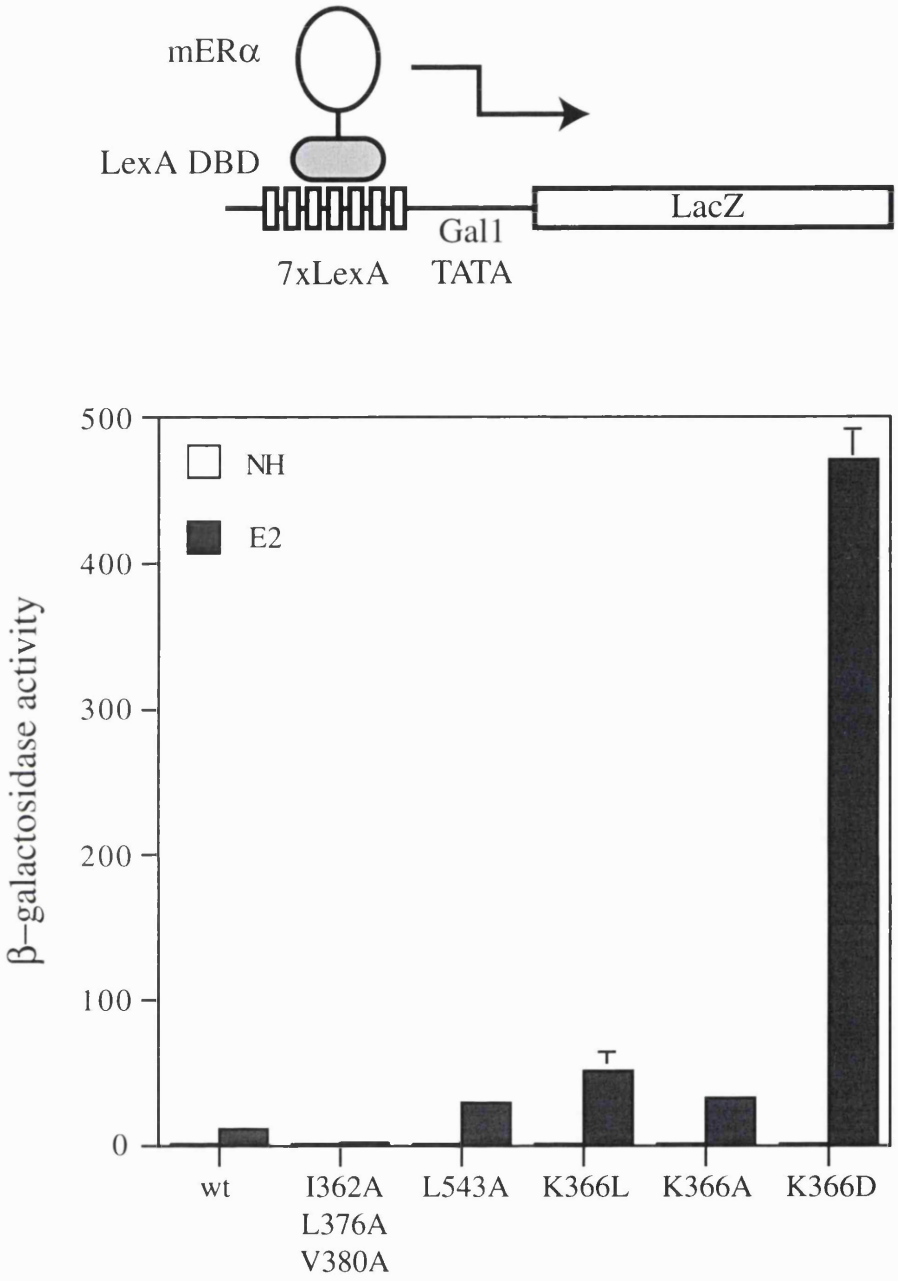


Figure 4.4 Transcriptional activity of LexA DBD-mERα LBD fusion proteins in *S. cerevisiae* strain L40. Wild-type and mutant mERα LBD (aa 313 to 599) was expressed as LexA DBD fusions from the episomal vector pBTM116. L40 tranformants were grown overnight in selective medium in the absence (NH) or presence of 1μM 17β-oestradiol (E2). The β-galactosidase activity was measured using ONPG as substrate and was expressed as Miller units. The results shown represent the average activity of two independent transformants.

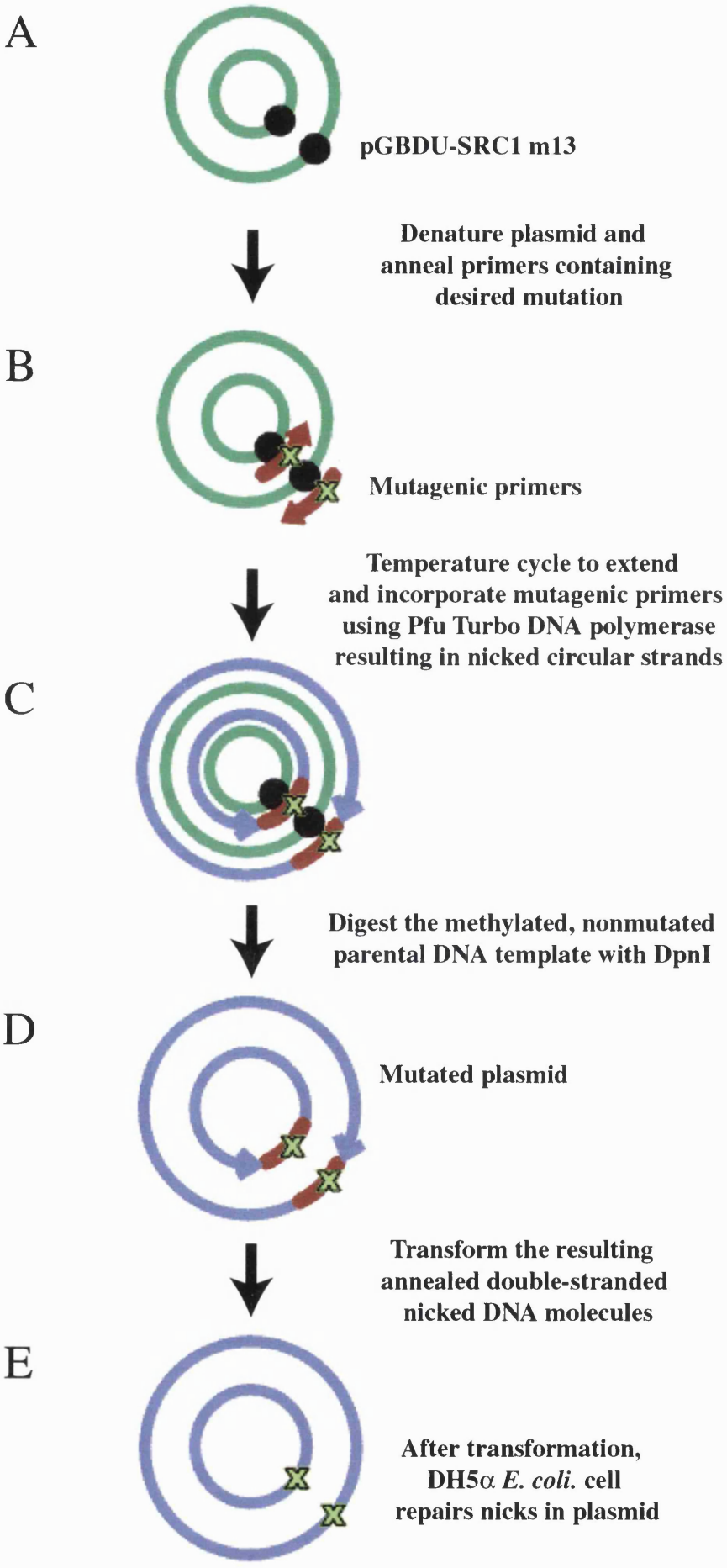
endogenous yeast transcriptional coactivators or general transcription machinery. Therefore, the receptor LBD was expressed as activation domain fusions in the subsequent genetic screen in order to minimise artificial activation of reporter genes.

Construction of mutant SRC1 library

The two leucine residues, L690 and L694, in SRC1 were randomly mutated by a method based on limited rounds of extension from mutagenic primers using a high fidelity DNA polymerase, PfuTurbo (Figure 4.5, Stratagene-QuickChange Mutagenesis Kit). The yeast expression plasmid, pGBDU-SRC1 m13, described in the previous section was used as the starting material. It was first denatured and then allowed to anneal with complementary primers which encompassed the nucleotide sequence for amino acids E685 to S700. The codons for L690 and L694 were substituted with the sequence NNS (where N=G, A, T or C and S=G or C) and hence the theoretical complexity of the library should be 1024. Extension and incorporation of mutagenic primers was facilitated by temperature cycling (22 cycles) which resulted in nicked circular strands. The parental, methylated DNA template was then digested with restriction endonuclease *DpnI* (18 recognition sites in pGBDU-SRC1 m13) which only cleaved at methylated sites and hence left the *in vitro* synthesised, mutated DNA intact. The mutated DNA was then transformed into the *E. coli.* strain DH5α by electroporation where the nicks of the plasmid were repaired.

A total of 2800 independent bacterial colonies were harvested from which the library DNA was prepared. Sequencing of randomly selected clones indicated that 80% of the library contained the targeted mutations. The remaining 20% of the library contained plasmids where deletions or insertions were found in the region of interest. Nevertheless, the number of 'correctly' mutated sequences in the library were still more than two times the theoretical complexity.

Figure 4.5 Mutagenesis scheme for generating SRC1 yeast two-hybrid library. (A) The parental pGBDU-SRC1 m13 plasmid (green circles) with the target site for mutation marked by black dots. (B) Annealing of mutagenic primers (brown arrows) containing the desired mutation (green crosses). (C) Extension from mutagenic primers using the high fidelity PfuTurbo DNA polymerase. The newly generated nicked circular strands were coloured blue. (D) Digestion of methylated parental plasmid with *DpnI*. (E) Transformation of mutated DNA molecules into *E. coli.* followed by repair of the nicks in the plasmids.

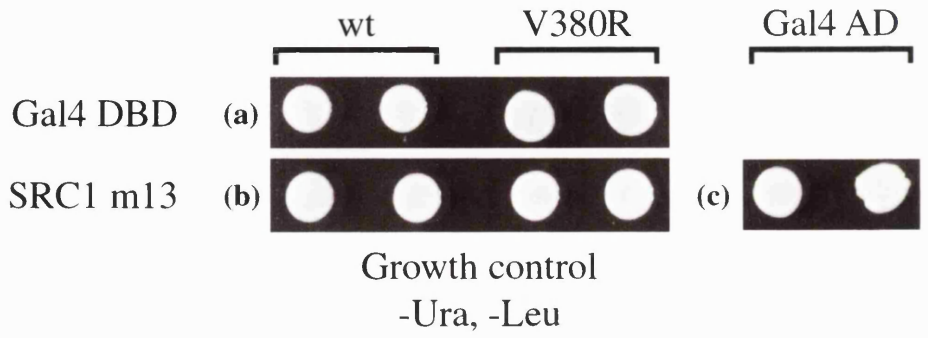

A
pGBDU-SRC1 m13
Denature plasmid and
anneal primers containing
desired mutation
B
Mutagenic primers
Temperature cycle to extend
and incorporate mutagenic primers
using Pfu Turbo DNA polymerase
resulting in nicked circular strands
C
Digest the methylated, nonmutated
parental DNA template with DpnI
D
Mutated plasmid
Transform the resulting
annealed double-stranded
nicked DNA molecules
E
After transformation,
DH5α *E. coli.* cell
repairs nicks in plasmid

Analysis of mERα–SRC1 interaction in yeast

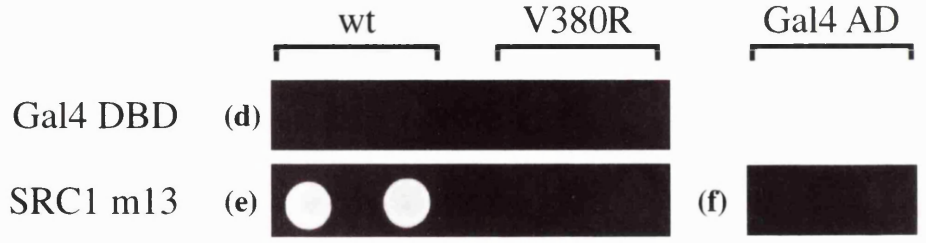
A series of control experiments were performed to assess the interaction between SRC1 m13 and wild-type or mutant versions of mERα in the yeast strain PJ69-4A which was to be used for the yeast two-hybrid screen. Growth assays were employed such that positive interaction between mERα and SRC1 m13, which were expressed as two hybrid fusion proteins (Figure 4.3), would confer growth of yeast tranformants on synthetic media lacking either histidine or adenine in the presence of agonist. This was due to activation of the HIS3 or ADE2 reporter gene which was under the control of the Gal1 or Gal2 promoter, respectively (Figure 4.6). The Gal1 promoter contained four and the Gal2 promoter two Gal4 DNA binding sites which allowed the docking of Gal4 DBD-SRC1 fusion protein (Bram et al., 1986). The Gal4 DBD-SRC1 fusion did not possess intrinsic transcriptional activity since co-expression of Gal4 AD alone did not enable the yeast strain to grow on synthetic media lacking either histidine or adenine (Figure 4.6 compare panels (c), (f) and (i)). In addition, co-expression of Gal4 AD fusions of wild-type or V380R mutant receptors with Gal4 DBD alone did not confer growth under the same conditions (Figure 4.6 compare panels (a), (d) and (g)). Our results indicated that mERα LBD could not interact with the Gal4 DBD in yeast.

The yeast strain which co-expressed SRC1 m13 and wild type mERα as two-hybrid fusion proteins grew on Ade^- or His^- medium in the presence of 17β-oestradiol. In contrast, co-expression of SRC1 m13 and V380R was unable to support growth (Figure 4.6 compare panels (b), (e) and (h)). These data provided evidence that V380R was unable to interact with SRC1 m13 in intact cells and extended the earlier observations made *in vitro* (Figure 4.2B). The interaction between SRC1 m13 and V380H or V380D was assessed in similar settings. Expression of Gal4 AD fusion of either V380H and V380D failed to activate the the HIS3 reporter gene in the presence of Gal4 DBD-SRC1 m13, indicating that neither of the mutant receptors was able to interact with SRC1 *in vivo* (Figure 4.7). Taken together, these results verified the lack of interaction between SRC1 and the three mutant receptors, V380H, V380R and V380D in yeast and qualified them as candidates for complementation by suppressor mutations in SRC1.

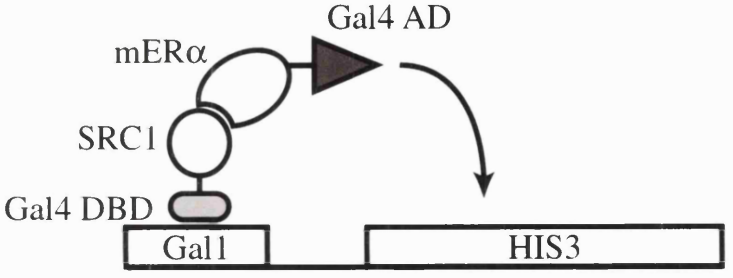
Figure 4.6 Analysis of mERα–SRC1 interaction in yeast. Yeast two-hybrid interaction assay (Drop test) using the Gal1-HIS3 and Gal2-ADE2 reporters in the strain PJ69-4A. 10µl of yeast cell suspension (OD_{600}~0.5) transformed with the indicated constructs was dropped onto Ura^-, Leu^- plate (growth control, panels (a to c)), Ura^-, Leu^-, His^- plate in the presence of 5mM 3-aminotriazole (3-AT) and 100nM 17β-oestradiol (E2) (panels (d to f)), and Ura^-, Leu^-, Ade^- plate in the presence of 100nM 17β-oestradiol (E2) (panels (g to i)). Growth of transformants indicated positive interaction between the two-hybrid protein partners as a result of reporter gene activation. The Gal4 DBD vector contained the URA3 marker and the Gal4 AD vector contained the LEU2 marker and both plasmids were selected for by omitting uracil and leucine in the medium. 3-aminotriazole was used to suppress the basal activity of the Gal1-HIS3 reporter. Plates were incubated at 30°C for 2 days. Two independent transformants are shown for each two-hybrid pair.

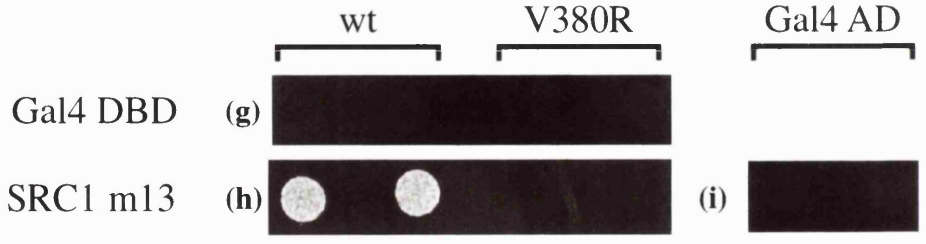


Growth control
-Ura, -Leu

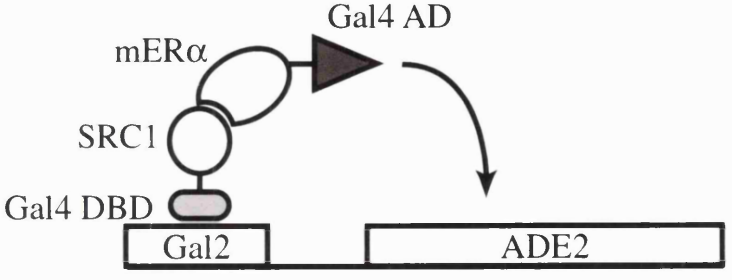


-Ura, -Leu, -His
+5mM 3-AT
+100nM E2





-Ura, -Leu, -Ade
+100nM E2



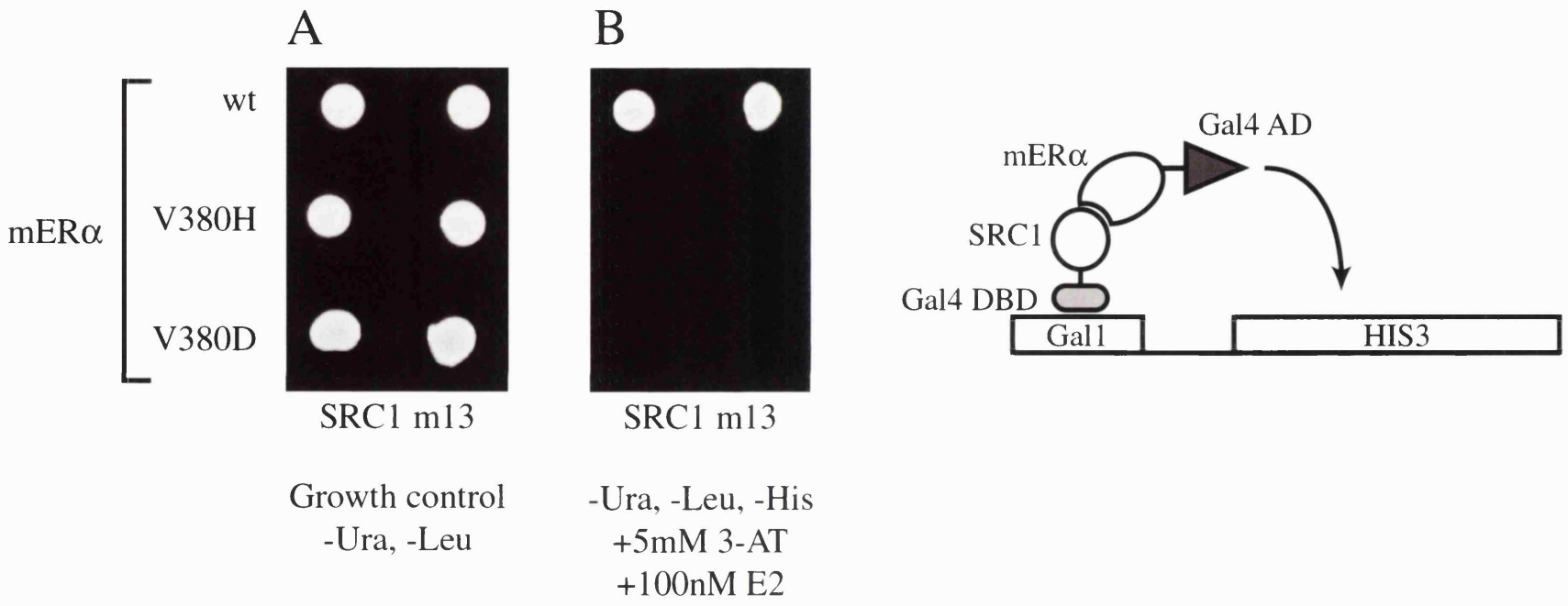


Figure 4.7 Analysis of mERα-SRC1 interaction in yeast. Yeast two-hybrid interaction assay (Drop test) using the Gal1-HIS3 reporter in the strain PJ69-4A. 10µl of yeast cell suspension (OD600~0.5) transformed with the indicated constructs was dropped onto (A) Ura^-, Leu^- plate (growth control) and (B) Ura^-, Leu^-, His^- plate in the presence of 5mM 3-aminotriazole (3-AT) and 100nM 17β-oestradiol (E2). Growth of transformants indicated positive interaction between the two-hybrid protein partners as a result of reporter gene activation. The Gal4 DBD vector contained the URA3 marker and the Gal4 AD vector contained the LEU2 marker and both plasmids were selected for by omitting uracil and leucine in the medium. 3-aminotriazole was used to suppress the basal activity of the Gal1-HIS3 reporter. Plates were incubated at 30°C for 2 days. Two independent transformants are shown for each two-hybrid pair.

A yeast two-hybrid screen for altered specificity SRC1

The HIS3 reporter in the two-hybrid host strain PJ69-4A was chosen for the primary selection of altered specificity mutant SRC1 because of its sensitivity over the ADE2 reporter. It was noticed that the growth rate of PJ69-4A coexpressing two-hybrid fusions of wild-type mERα and SRC1 was slower when plated on Ade^- medium than on His^- medium (Figure 4.6 compare panels (e) and (h)). This indicated that the ADE2 reporter was more stringent than the HIS3 reporter such that weak interactions between two-hybrid proteins might not be detected. Hence the HIS3 reporter was favoured in anticipation that the interaction between mutant receptors and altered specificity SRC1 might not be as strong as the wild-type receptor-coactivator pair.

The host strain PJ69-4A was first transformed with plasmids encoding Gal4 AD fusions of V380H, V380R or V380D before the mutant SRC1 library was introduced. A total of 59000 (V380H), 51750 (V380R) and 64000 (V380D) transformants were screened for suppressor mutation in SRC1 which would confer growth of the transformants on His^- medium in the presence of 5mM 3-aminotriazole and 100nM 17β-oestradiol. 3-aminotriazole was used to suppress the basal activity of the HIS3 reporter. The number of transformants screened was 20-fold in excess of the complexity of the library indicating that a saturation screen was conducted. After incubation for 5 days, one transformant was recovered from the V380H screen and another one from the V380R screen. No transformant was recovered from the V380D screen. The suppressor allele in the V380H screen was designated SRC1 VHC for V380H complement and that in the V380R screen was designated SRC1 VRC for V380R complement.

The interaction between V380H and V380R with their respective suppressor mutants was verified using the ADE2 reporter in PJ69-4A. On Ade^- medium, co-expression of V380H with SRC1 VHC conferred growth in a ligand dependent manner (Figure 4.8). Similar observations were made when V380R and SRC1 VRC were co-expressed. It was significant that the ADE2 reporter was only activated in the presence of ligand. It implied that mutations in the SRC1 suppressor alleles indeed rescued the binding between the mutant receptors and SRC1 and did not transform the Gal4 DBD-SRC1 fusions into constitutive

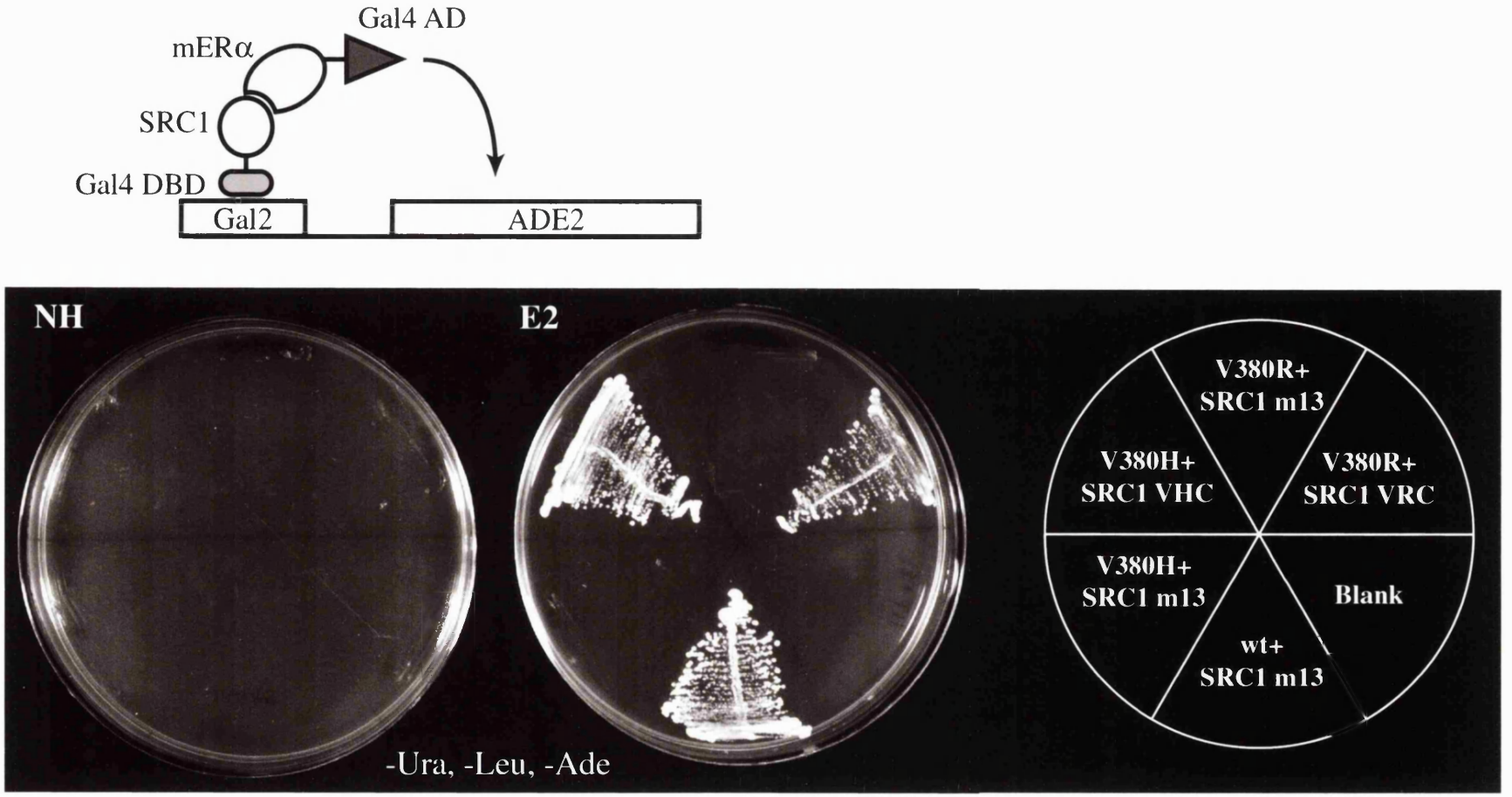


Figure 4.8 Ligand dependent interaction of mutant mERα and altered specificity SRC1 *in vivo*. Yeast two-hybrid interaction assay using the Gal2-ADE2 reporter in the strain PJ69-4A. Transformants with the indicated constructs were streaked onto Ura^-, Leu^-, Ade^- plate in the absence (NH) or presence of 100nM 17β-oestradiol (E2). E2 dependent ADE2 gene activation by co-expression of Gal4 AD-wild type mERα and Gal4 DBD-SRC1 m13 acted as a positive control. Plates were incubated at 30°C for 2 days.

transcriptional activators, a possibility which we could not exclude in the primary screen.

Analysis of the SRC1 suppressor alleles

To gain insights into the molecular basis of complementation, the DNA sequence of the mutant SRC1 alleles were determined. Surprisingly, both SRC1 VHC and SRC1 VRC alleles contained insertional mutations at the position where the second LXXLL motif of SRC1 is normally found. The VHC mutant allele consisted of a wild-type LXXLL motif followed immediately by a C-terminal insertion of 15 amino acids containing a variant motif YXXLK (Figure 4.9). In the VRC mutant allele, an insertion of 48 amino acids was found and as a result, one wild-type and three variant LXXLL motifs were positioned in tandem (Figure 4.9). It was plausible that these alleles arose from incorporation of multiple mutagenic primers rather than mispriming *per se* during the library construction. This was supported by the observation that codons which encoded L690 and L694 in the mutant alleles differed from that of the wild-type. The mutant alleles represented rare species in the library and provided an explanation for the recovery of a single allele for each mutant receptor from an apparent saturation screen.

Given the presence of a wild-type LXXLL motif in both VHC and VRC mutant SRC1 alleles, the ability of SRC1 VHC and SRC1 VRC to interact with wild-type mERα was tested in yeast. Co-expression of mERα and SRC1 VHC or SRC1 VRC in the yeast host strain PJ69-4A led to activation of a lacZ reporter gene in an agonist dependent manner (Figure 4.10). Using the same reporter, the strength of interaction between the mutant receptor-coactivator pairs (V380H+SRC1 VHC and V380R+SRC1 VRC) was found to be approximately 50% when compared with their wild-type counterparts. These results demonstrated that SRC1 VHC and SRC1 VRC could mediate robust *in vivo* interaction with V380H and V380R, respectively.

Figure 4.9 Sequence alignment of wild-type and altered specificity SRC1 alleles. The black boxes encompass the 15 amino acid and 48 amino acid insertions found in the VHC and VRC alleles respectively. The wild-type LXXLL motifs are indicated with (*) and the variant motifs are indicated with (^).

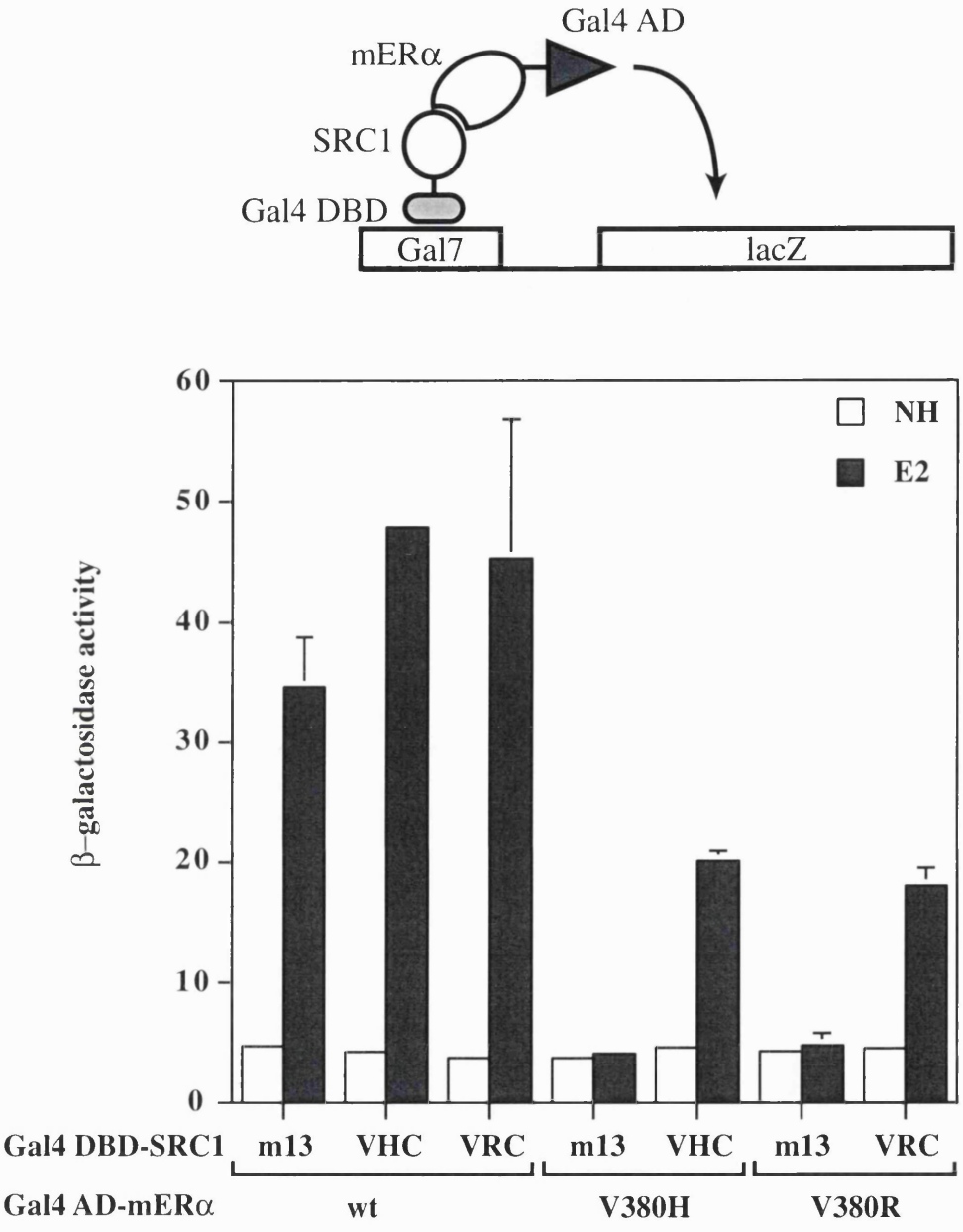


Figure 4.10 Interaction of altered specificity SRC1 with wild-type and mutant mERα. Yeast two-hybrid interaction assay using the Gal7-lacZ reporter in the strain PJ69-4A. Transformants with the indicated constructs were grown overnight in selective medium in the absence (NH) or presence of 1μM 17β-oestradiol (E2). The β-galactosidase activity was measured using ONPG as substrate and was expressed as Miller units. The results shown represent the average activity of two independent transformants.

Summary

By combining crystallographic and biochemical knowledge of the mERα–SRC1 interface, a genetic screen was conducted to identify SRC1 altered specificity mutants which could interact with the remodelled docking surface on the mERα LBD. This approach relied on the assumption that mutations which hindered wild-type receptor-coactivator interaction would not disrupt the three dimensional structure of the mERα LBD, but at the same time generate molecular features which were amenable to complementation. One suppressor allele was recovered for each of the mutant receptors, V380H and V380R. Both SRC1 VHC and SRC1 VRC were shown to interact with their respective mutant receptor partners in a ligand dependent manner *in vivo*. It was surprising that a wild-type LXXLL motif was featured in both altered specificity mutant SRC1 together with sequences which contain variant motifs in its vicinity. The role of the wild-type motif in altered specificity receptor-coactivator interaction is a subject for further investigation.

Chapter 5

Functional analysis of altered specificity p160 coactivators

Introduction

In order to determine the functional consequence of allowing ERα to interact solely with a specific p160 coactivator in mammalian cells, two altered specificity SRC1 mutants were generated. SRC1 VHC and SRC1 VRC, could interact with the modified coactivator docking surface of the V380H and V380R mutant receptors, respectively. Since the mutant receptors are unable to interact with wild-type coactivators, activation of reporter genes can be studied in a null background, equivalent to the situation where most if not all endogenous coactivators are eliminated from mammalian cells. In view of the complexity of the SRC1 VRC mutant allele, functional analysis was confined to SRC1 VHC.

In this chapter, the ability of SRC1 VHC to restore the transcriptional activity of V380H in transfected mammalian cells was examined in the hope of addressing whether a direct ERα-SRC1 interaction is normally employed to trigger gene activation under physiological conditions. Furthermore, the ability of the suppressor mutation in SRC1 VHC to function as a protein-protein interaction module was explored by introducing analogous mutations to the other p160 coactivators, TIF2 and RAC3.

Interaction of mERα V380H and SRC1e VHC *in vitro*

It was established in the previous chapter that mERα V380H and SRC1 VHC interacted in a yeast two-hybrid setting (Figure 4.8 and 4.10). To test whether the full-length mutant receptor and its coactivator partner could interact with each other *in vitro*, mERα V380H and SRC1e VHC were transiently expressed in 293-T cells and the lysate was subjected to immunoprecipitation reactions. As shown in Figure 5.1B, V380H co-immunoprecipitated with SRC1e VHC in a ligand dependent manner *in vitro*. The strength of interaction was approximately 50% when compared with the wild-type receptor-coactivator pair (Figure 5.1B compare lanes 8 and 10). In addition, a ligand dependent interaction between SRC1e VHC and wild-type mERα was detected because of the presence of an intact LXXLL motif (Figure 5.1B, lanes 5 and 6). Taken together, these results verified the yeast two-hybrid analysis and demonstrated that SRC1e VHC could interact with V380H *in vivo* and *in vitro*.

A

Wild-type ^{685}ERHKILHRLLQEGSPSDITT

SRC1 VHC ^{685}ERHKILHRLLQEGSPSERHKIYHRLKQESPSDITT

B

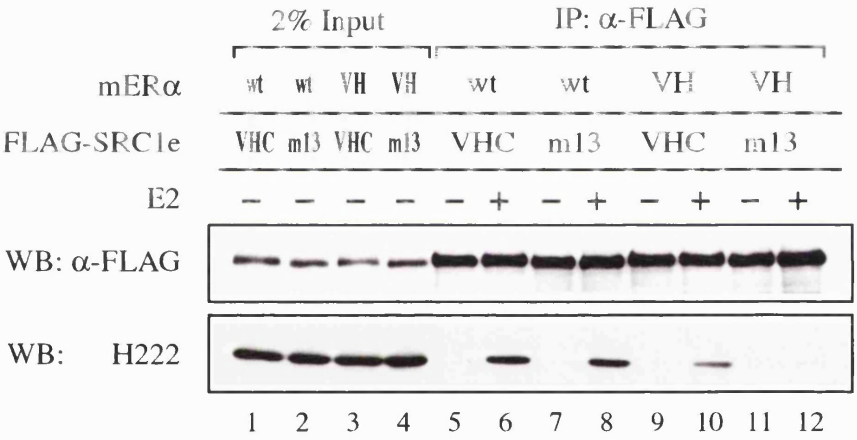


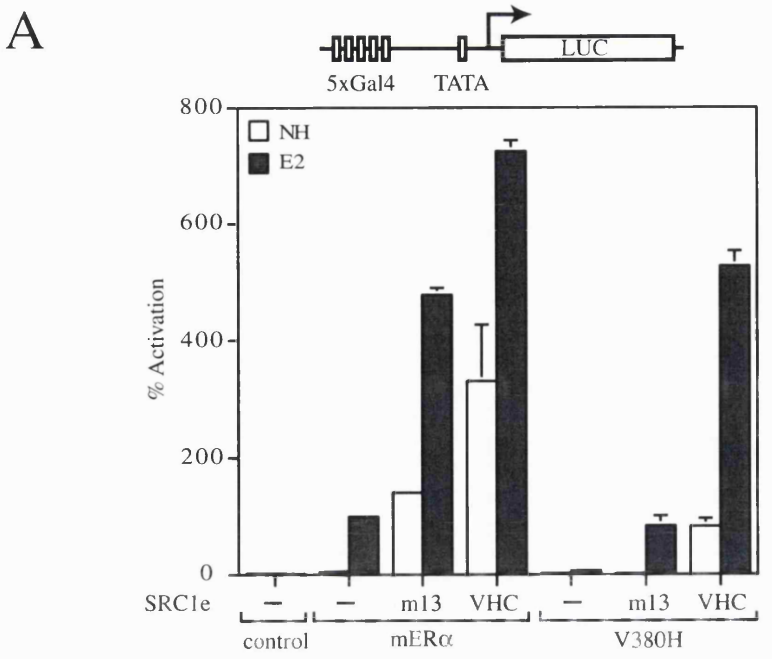
Figure 5.1 Ligand dependent interaction of mERα V380H with SRC1e VHC *in vitro*. (A) Sequence comparison of wild-type SRC1 and SRC1 VHC in the vicinity of the second LXXLL motif. The 15-amino acid insertion in the mutant is encompassed by a black box. (B) Full length mERα and FLAG-epitope tagged wild-type or mutant SRC1e were transiently expressed in 293-T cells and the whole cell lysate was subjected to immnuoprecipitation with an anti-FLAG antibody immobilised on agarose beads in the absence or presence of 1μM 17β-oestradiol (E2). SRC1e was detected by Western blotting (WB) using an anti-FLAG antibody. The co-immunoprecipitated mERα was detected using the monoclonal antibody H222. VH stands for V380H in the panel.

Functional rescue of mERα V380H by altered specificity SRC1e

Having established that SRC1e VHC interacts with mERα V380H, its ability to restore the transcriptional activity of the mutant receptor was next examined. The ability of Gal4 DBD–ERα LBD chimeric receptors to activate a Gal4 reporter gene was examined in HeLa cells. Exogenously expressed SRC1e m13 and SRC1e VHC potentiated the transcriptional activity of wild-type chimeric receptor by five- and seven-fold, respectively (Figure 5.2A). The V380H mutant chimeric receptor had negligible transcriptional activity indicating that it was unresponsive to endogenous coactivators (Figure 5.2A). Exogenously expressed SRC1e m13 partially rescued this defect. Remarkably, co-expression of SRC1e VHC with the mutant chimeric receptor led to more than 80-fold induction of reporter activity. The level of activation was comparable to that achieved by the wild-type receptor-coactivator pair. Similar observations were made in COS-1 cells (Figure 5.5C) and in 293-T cells (data not shown). Taken together, our results clearly establish that the ligand dependent transcriptional activity of mERα V380H could be restored by co-expressing SRC1e VHC. Moreover, recruitment of SRC1e by the mutant receptor alone appears to be sufficient to instigate transcriptional activation in mammalian cells.

To demonstrate the functional rescue in another setting, full length wild-type or V380H mutant receptors were transiently transfected into HeLa cells and tested for their ability to activate a 2×ERE-PS2-luciferase reporter in the presence of SRC1e. Exogenously expressed SRC1e m13 and SRC1e VHC potentiated the transcriptional activity of wild-type receptor by two- and four-fold, respectively (Figure 5.2B). Co-expression of SRC1e VHC led to a complete rescue of transcriptional activity of V380H and activated the reporter gene to a level higher than that achieved by the wild-type receptor-coactivator pair (Figure 5.2B). This represented a more than 10-fold potentiation of transcriptional activity of V380H. We noted that V380H alone was able to activate the reporter gene to a detectable level. Such activation could be attributed to AF1 of the receptor since it was shown that AF1 contributed significantly to receptor activity on this reporter when assayed under similar conditions (Cowley and Parker, 1999). It should be added that V380H displayed negligible transcriptional activity and was functionally

Figure 5.2 Functional rescue of mERα V380H by SRC1e VHC. (A) Wild-type or mutant chimeric receptors consisting of the LBD of mERα fused to Gal4 DBD were transiently transfected into HeLa cells together with the p5Gal-E1B-GL3 reporter in the absence (–) or presence of full-length SRC1e m13 or SRC1e VHC as indicated. The pRL-CMV plasmid which encoded the Renilla luciferase gene driven by a cytomegalovirus (CMV) promoter was cotransfected as the internal control. After transfection, cells were treated with ethanol vehicle alone (NH) or 17β-oestradiol (E2) at 10nM for 24h. Subsequently, cells were assayed for fire-fly luciferase (LUC) and Renilla luciferase activity. Normalised values are expressed as percentage of activity compared with that of wild-type mERα alone in the presence of E2 (100%). The results shown represent the average of at least two independent experiments assayed in quadruplicate + standard errors. (B) Full length wild-type or mutant mERα were transiently transfected into HeLa cells together with the p2×ERE-PS2-GL3 reporter. Experimental procedures and data presentation are as described for (A). (C) Full length wild-type or mutant mERα were transiently transfected into HeLa cells together with the p2×ERE-TATA-GL3 reporter. Experimental procedures and data presentation are as described for (A).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LUC
5xGal4
TATA
800
600
400
200
0
NH
E2
% Activation
SRC1e
–
–
m13
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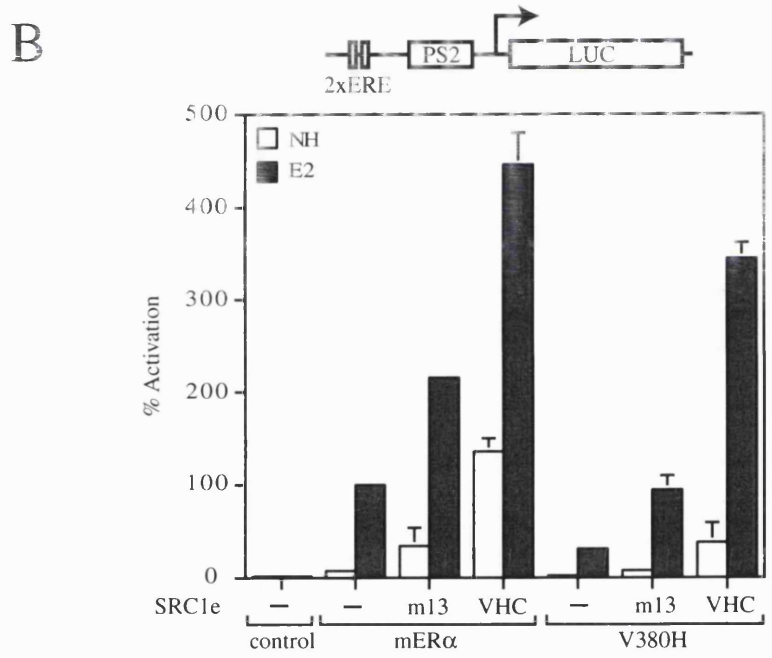
–
m13
VHC
control
mERα
V380H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LUC
2xERE
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400
300
200
100
0
NH
E2
% Activation
SRC1e
–
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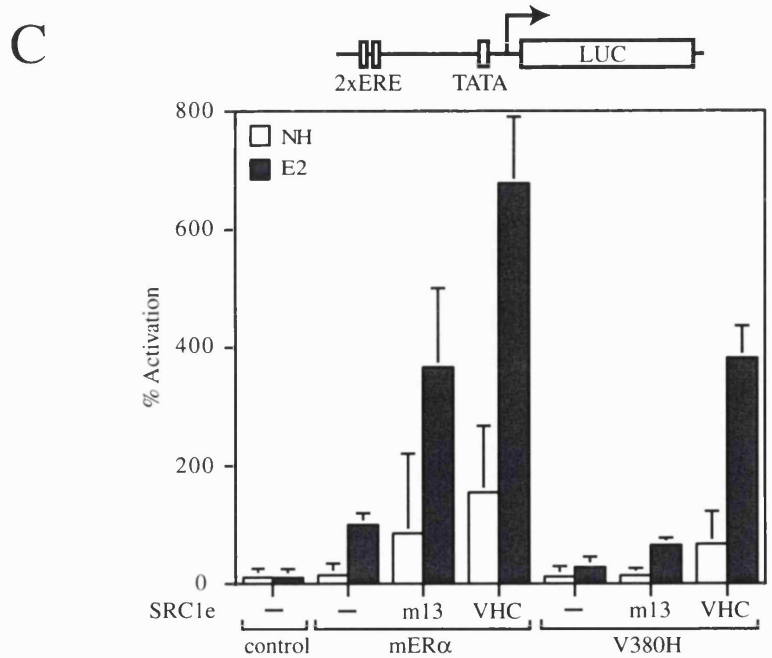
mERα
V380H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mERα
V380H

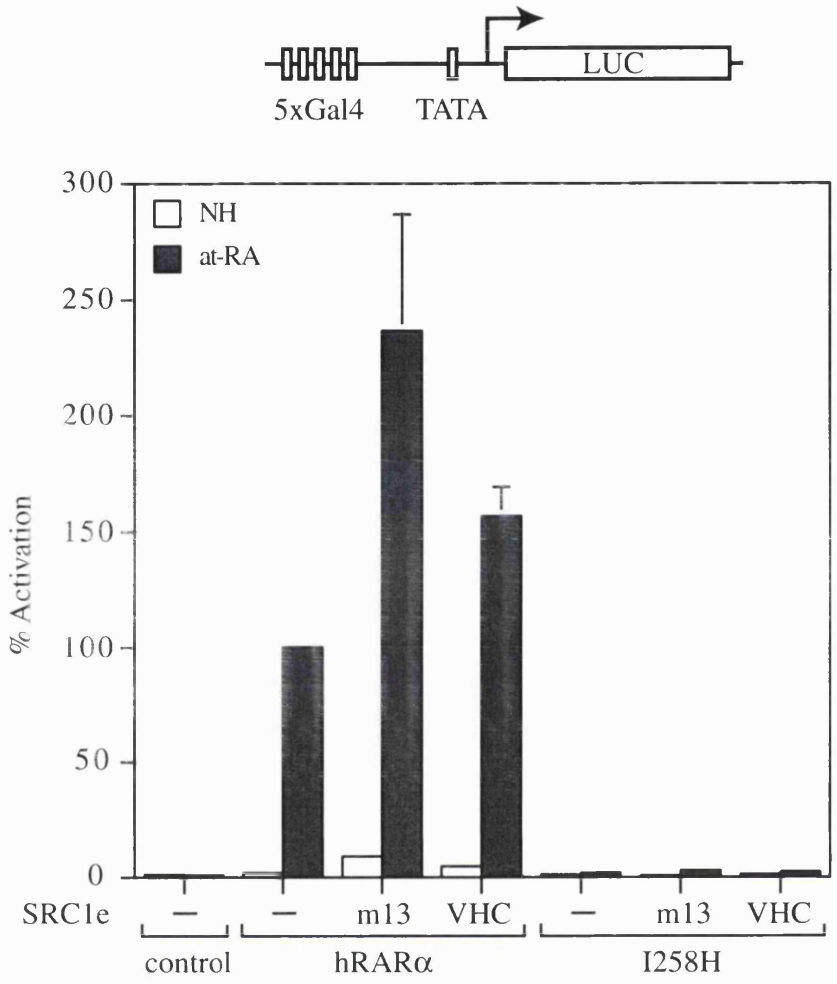


Figure 5.3 SRC1e VHC does not rescue a transcriptionally inactive hRARα. Wild-type or mutant (I258H) chimeric receptors consisting of the LBD of hRARα fused to Gal4 DBD were transiently transfected into HeLa cells together with the p5Gal-E1B-GL3 reporter, the internal control pRL-CMV and SRC1e expression plasmids as indicated. After transfection, cells were treated with ethanol vehicle alone (NH) or all-trans retinoic acid (at-RA) at 100nM for 24h. Subsequently, cells were assayed for fire-fly luciferase (LUC) and Renilla luciferase activity. Normalised values are expressed as percentage of activity compared with that of wild-type hRARα alone in the presence of at-RA (100%). The results shown represent the average of at least two independent experiments assayed in quadruplicate + standard errors.

rescued by SRC1e VHC when assayed on a 2×ERE-TATA-luciferase reporter (Figure 5.2C). These results indicate that functional rescue by SRC1e VHC is applicable to full length mutant receptor and further suggest that it should be possible to test whether recruitment of V380H and SRC1e VHC to the promoter is sufficient to activate endogenous oestrogen responsive genes in the future.

Next, we asked whether SRC1e VHC was able to rescue the transcriptional activity of other nuclear receptors which bear mutations analogous to V380H in mERα. As predicted by sequence analysis and by inspection of the hRARγ LBD crystal structure, I258 in human retinoic acid receptor (hRARα) occupies a similar position in helix 5 of the LBD as V380 in mERα. When I258 was replaced with histidine (I258H), a chimeric receptor containing the LBD of the mutant receptor fused to Gal4 DNA binding domain was unable to activate a reporter gene when transiently transfected in HeLa cells (Figure 5.3). In addition, co-expression of SRC1e VHC had no effect on the transcriptional activity of the I258H mutant. It is noteworthy that SRC1e VHC appears to be less potent than SRC1e m13 as a coactivator for the wild-type hRARγ, in contrast to the observations made for mERα (Figures 5.2 and 5.3). This suggests that the altered specificity mutation in SRC1e VHC does not mediate promiscuous protein-protein interaction. Rather, the functional rescue of mERα V380H by SRC1e VHC appears to be specific and is most likely due to recognition of features of the mERα LBD that are not present in other nuclear receptor.

Molecular determinants of the mERα V380H–SRC1e VHC interaction

The molecular basis of complementation was examined. In particular, we were interested in the contribution of the wild-type LXXLL motif and its flanking residues in mERα V380H–SRC1e VHC interaction. First, the wild-type LXXLL motif in SRC1 VHC was rendered non-functional by replacing L693 and L694 with alanine. The alanine substitutions in SRC1 VHC eliminated its binding to both wild-type and V380H mutant receptors in a yeast two-hybrid interaction assay (Figure 5.4B). Furthermore, the SRC1 L690Y-L694K mutant did not interact with V380H in similar assays (Figure 5.4B). This indicates that the variant motif

YXXLK, found in the 15-amino acid insertion of SRC1 VHC, was unable to mediate protein-protein interaction with V380H independently.

It was shown that I689 made extensive van der Waals contacts with the ERα coactivator docking surface (Shiau et al., 1998) and this –1 position of LXXLL motif is frequently occupied by hydrophobic residues indicating its functional importance. When I689 of SRC1e VHC was replaced with alanine, the mutant coactivator was unable to bind wild-type or V380H mutant receptors *in vitro* (Figure 5.5A and B). In keeping with the loss of interaction, the I689A mutant neither potentiated the transcriptional activity of wild-type mERα nor functionally rescued V380H in transient transfection assays (Figure 5.5C). Taken together, these results suggest that the LXXLL motif together with its flanking residues are likely to function in conjunction with the 15 amino acid insertion in SRC1e VHC as an integral module, and are indispensible for its interaction with mERα V380H.

To investigate whether the modified coactivator docking surface of V380H is the target of SRC1e VHC, we tested the ability of SRC1e VHC to potentiate V380H transcriptional activity in the presence of antiooestrogens. It has been shown that protrusion of the bulky side-chain of Tamoxifen from the ERα ligand binding pocket leads to repositioning of helix 12. As a result, the coactivator docking surface is blocked (Shiau et al., 1998). The 'pure' antioestrogen, ICI 182780, possesses a similar bulky side-chain and is expected to exert the same effect on the positioning of helix 12. Since the alternative position of helix 12 is driven by steric hindrance actively imposed by antioestrogens, it is postulated that the modified V380H coactivator docking surface would also be obscured as in the wild-type receptor. In transiently transfected HeLa cells, SRC1e VHC failed to restore the transcriptional activity of V380H in the presence of Tamoxifen or ICI 182780 (Figure 5.6). This implied that SRC1e VHC was unable to interact with the docking surface of antioestrogen bound mutant receptor. Therefore, the V380H–SRC1e VHC interaction is most likely to involve a surface which overlaps the coactivator docking surface of the wild-type mERα.

Figure 5.4 Interaction of mutant SRC1 with mERα. (A) Sequence comparison of mutant SRC1 tested in the interaction assay. Mutations are marked with asterisks. (B) Yeast two-hybrid interaction assay using the Gal7-lacZ reporter in the strain PJ69-4A. Transformants with the indicated constructs were grown overnight in selective medium in the absence (NH) or presence of 1μM 17β-oestradiol (E2). The β-galactosidase activity was measured using ONPG as substrate and was expressed as Miller units. The results shown represent the average activity of two independent transformants.

m13 refers to SRC1 m13
L693A-L694A refers to SRC1 VHC L693A-L694A
L690Y-L694K refers to SRC1 L690Y-L694K

A

Wild-type	685	ERHKILHRLLQEGSPSDITT
SRC1 VHC	685	ERHKILHRLLQEGSPS**ERHKIYHRLKQESPS**DITT
SRC1 VHC L693A-L694A	685	ERHKILHR**AA**QEGSPS**ERHKIYHRLKQESPS**DITT
SRC1 L690Y-L694K	685	ERHKI**Y**HRL**K**QEGSPSDITT

B

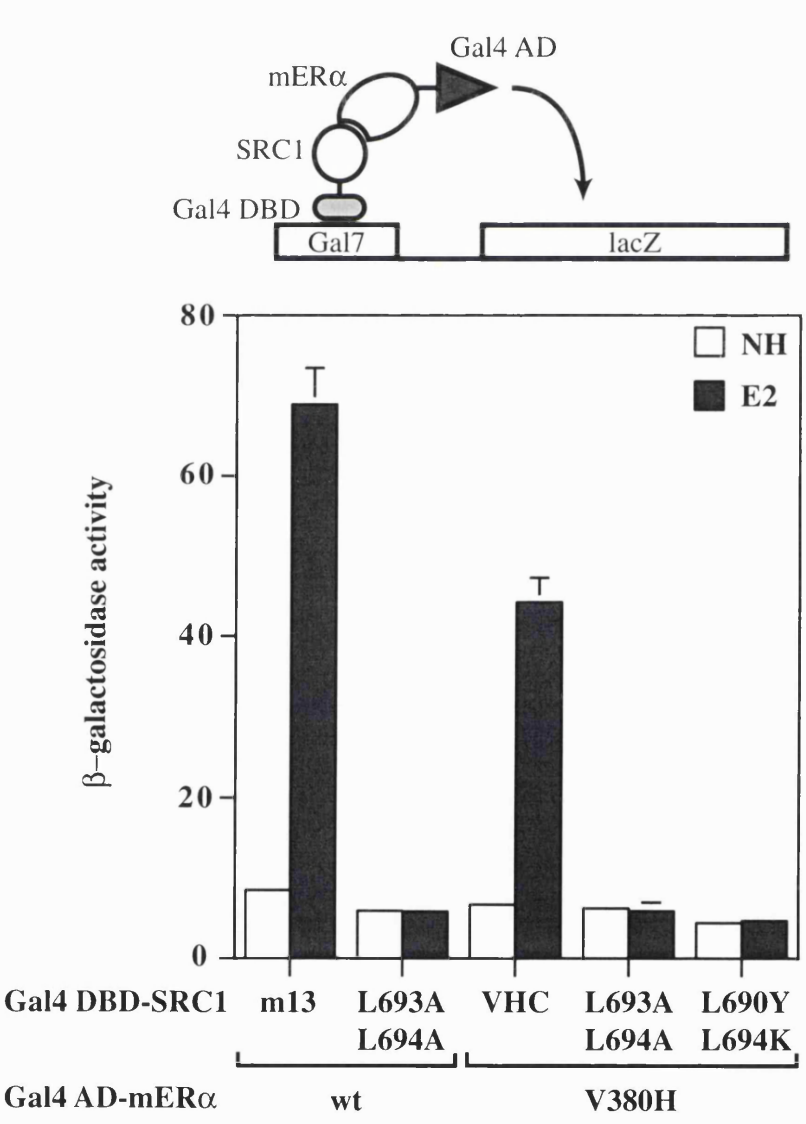


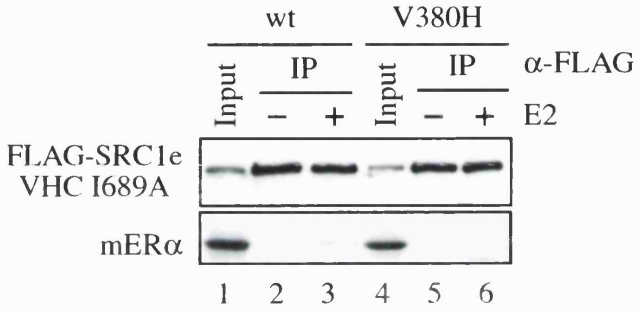
Figure 5.5 Molecular determinants of the mERα V380H–SRC1e VHC interaction. (A) The point mutation I689A (marked with an asterisk) was introduced into full length SRC1e VHC. The sequence in the vicinity of the I689A mutation is shown to hightlight the spacial relationship between I689, the LXXLL motif and the 15-amino acid insertion found in SRC1e VHC. (B) The I689A mutation in SRC1e VHC abolished its *in vitro* binding to wild-type and V380H mutant mERα. Co-immunoprecipitation was carried out as described for Figure 5.1B. The input control represents 2% of the whole cell extract employed in the immunoprecipitation (IP) reaction. (C) Wild-type or mutant chimeric receptors consisting of the LBD of mERα fused to Gal4 DBD were transiently transfected into COS-1 cells together with the p5Gal-E1B-GL3 reporter in the absence (–) or presence of full-length SRC1e m13, SRC1e VHC or SRC1e VHC I689A as indicated. The pRL-CMV plasmid was co-transfected as an internal control. Data are presented as described for Figure 5.2A.

m13 refers to SRC1e m13
VHC refers to SRC1e VHC
I689A refers to SRC1e VHC I689A

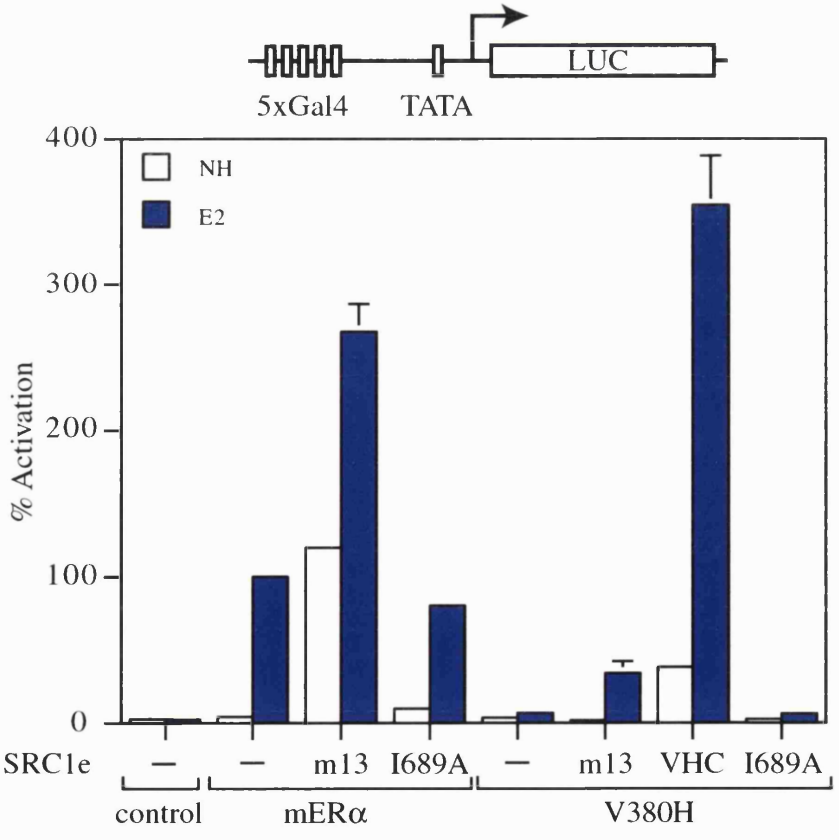
A


SRC1 VHC
685 ERHKILHRLLQEGSPS
ERHKIYHRLKQESPSDITT
SRC1 VHC
I689A
685 ERHKALHRLLQEGSPS
ERHKIYHRLKQESPSDITT

B


wt
V380H
Input
IP
Input
IP
α-FLAG
– + – +
E2
FLAG-SRC1e
VHC I689A
mERα
1 2 3 4 5 6

C


LUC
5xGal4
TATA
400
300
200
100
0
NH
E2
% Activation
SRC1e
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m13
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I689A
control
mERα
V380H

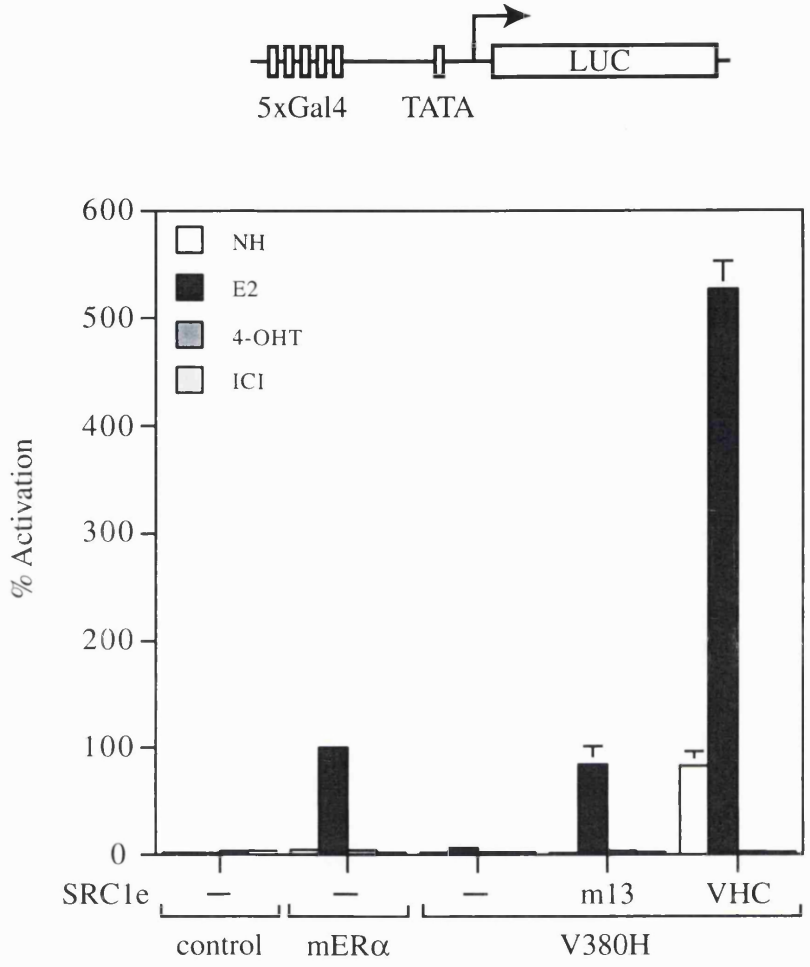


Figure 5.6 Antioestrogens prevent functional rescue of V380H by SRC1e VHC. Wild-type or mutant chimeric receptors consisting of the LBD of mERα fused to Gal4 DBD were transiently transfected into HeLa cells together with the p5Gal-E1B-GL3 reporter in the absence (-) or presence of full-length SRC1e m13 or SRC1e VHC as indicated. The pRL-CMV plasmid was co-transfected as an internal control. After transfection, cells were treated with ethanol vehicle alone (NH), 17β-estradiol (E2) at 10nM, 4-hydroxytamoxifen (4-OHT) at 1μM or ICI 182780 (ICI) at 100nM for 24h. Subsequently, cells were assayed for fire-fly luciferase (LUC) and Renilla luciferase activity. Normalised values are expressed as percentage of activity compared with that of wild-type mERα alone in the presence of E2 (100%). The results shown represent the average of at least two independent experiments assayed in quadruplicate + standard errors.

Functional rescue of mERαV380H by TIF2 and RAC3 altered specificity mutants

To explore the possibility that the suppressor mutation in SRC1e VHC functions as a protein-protein interaction module, analogous mutations were introduced into other p160 coactivator family members. The sequence conservation between the three p160 coactivators in the vicinity of the second LXXLL motif allowed us to place the 15 amino acids insertion found in SRC1e VHC at precisely the same position C-terminal to the motif in TIF2 and RAC3 (Figures 5.7A and 5.8A). The mutants, designated TIF2 VHC and RAC3 VHC were then tested for their ability to interact with mERα V380H. Both TIF2 VHC and RAC3 VHC co-immunoprecipitated with mERα V380H in a ligand dependent manner *in vitro* (Figure 5.7B, lanes 9 and 10; Figure 5.8B, lanes 9 and 10). In common with SRC1e VHC, they also bound to wild-type mERα because of the presence of an intact LXXLL motif (Figure 5.7B, lanes 5 and 6; Figure 5.8B, lanes 5 and 6). These data suggest that, when placed in a similar context, the suppressor mutation originally recovered in SRC1e can function in other p160 coactivators and confers the ability to interact with mERα V380H.

Next, we tested whether TIF2 VHC and RAC3 VHC can rescue the transcriptional activity of mERα V380H in transient transfection assays. In HeLa cells, co-expression of the V380H mutant receptor with TIF2 VHC led to an 89-fold induction of transcriptional activity on a Gal4 reporter gene (Figure 5.7C). The level of activity achieved was comparable to that of the wild-type receptor–coactivator pair indicating a complete functional rescue. When RAC3 VHC was tested in the same setting, the transcriptional activity of mERα V380H was induced by 22-fold (Figure 5.9A), approximately 60% of that achieved by the wild-type counterparts. We therefore conclude that TIF2 VHC and RAC3 VHC are capable of rescuing the transcriptional activity of mERα V380H, albeit to a varying degree. Furthermore, these results imply that recruitment of any p160 coactivator is sufficient to mediate mERα transactivation.

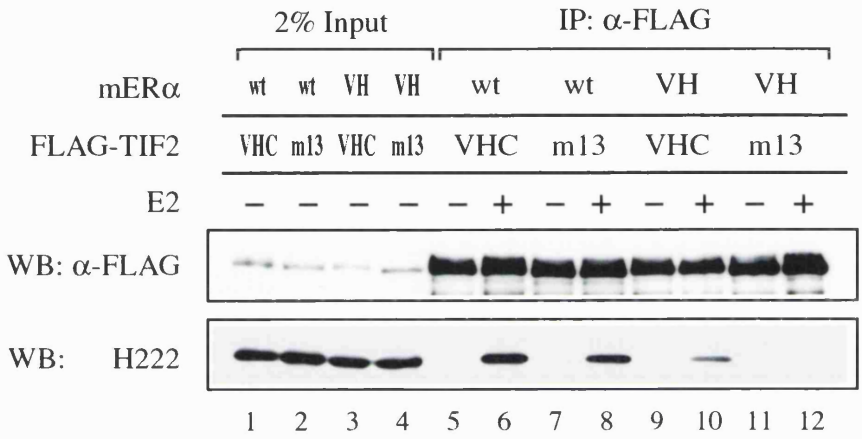
Figure 5.7 Analysis of the altered specificity TIF2. (A) Sequence comparison of wild-type TIF2 and TIF2 VHC. The 15 amino acid insertion found in SRC1e VHC (encompassed by the black box) was placed immediately C-terminal to the TIF2 wild-type LXXLL motif 2 as indicated. (B) Ligand dependent interaction of mERα V380H with TIF2 VHC *in vitro.* Full length mERα and FLAG-epitope tagged wild-type or mutant TIF2 were transiently expressed in 293-T cells and the whole cell lysate was subjected to immnuoprecipitation with an anti-FLAG antibody immobilised on agarose beads in the absence or presence of 1μM E2. TIF2 was detected by Western blotting (WB) using an anti-FLAG antibody. The co-immunoprecipitated mERα was detected using the monoclonal antibody H222. VH stands for V380H in the panel. (C) Wild-type or mutant chimeric receptors consisting of the LBD of mERα fused to Gal4 DBD were transiently transfected into HeLa cells together with the p5Gal-E1B-GL3 reporter in the absence (–) or presence of full-length TIF2 m13 or TIF2 VHC as indicated. The pRL-CMV plasmid was co-transfected as an internal control. Data are presented as described for Figure 5.2A.

A

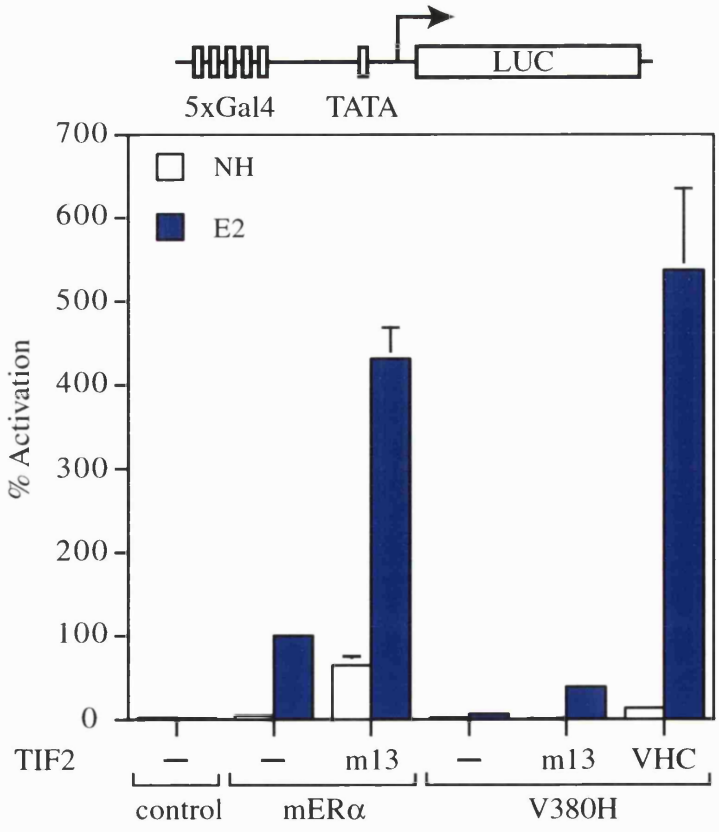
TIF2 ^{685}EKHKILHRLLQDSSSPVDLA

TIF2 VHC ^{685}EKHKILHRLLQEGSPSERHKIYHRLK
QDSSSPVDLA

B



C



A

RAC3 ^{680}EKHRILHKLLQNGNSPAEVA

RAC3 VHC ^{680}EKHRILHKLLQEGSPSERHKIYHRLKQE
GNSPAEVA

B

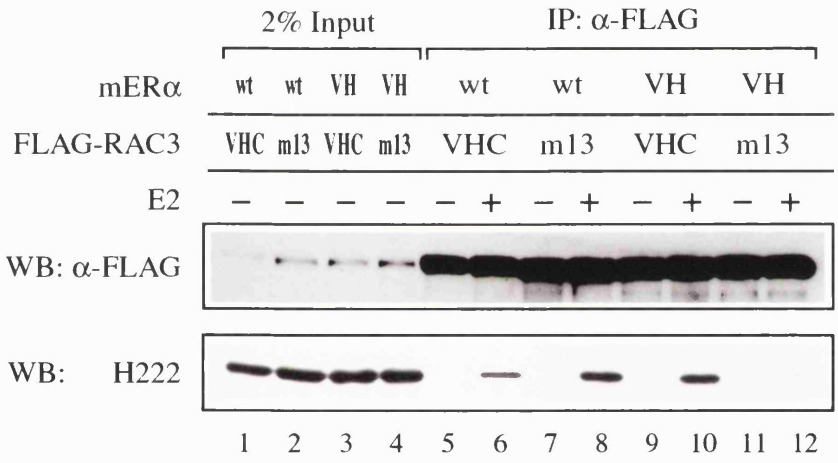


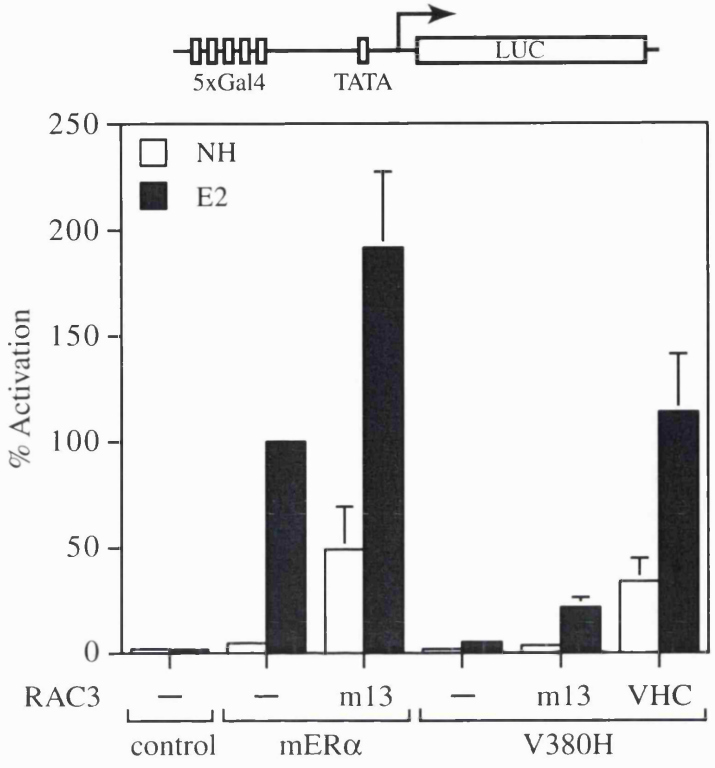
Figure 5.8 Analysis of the altered specificity RAC3 *in vitro*. (A) Sequence comparison of wild-type RAC3 and RAC3 VHC. The 15 amino acid insertion found in SRC1e VHC (encompassed by the black box) was placed immediately C-terminal to the RAC3 wild-type LXXLL motif 2 as indicated. (B) Ligand dependent interaction of mERα V380H with RAC3 VHC in vitro. Full length mERα and FLAG-epitope tagged wild-type or mutant RAC3 was transiently expressed in 293-T cells and the whole cell lysate was subjected to immnuoprecipitation with an anti-FLAG antibody immobilised on agarose beads in the absence or presence of 1μM E2. RAC3 was detected by Western blotting (WB) using an anti-FLAG antibody. The co-immunoprecipitated mERα was detected using the monoclonal antibody H222. VH stands for V380H in the panel.

One possibility for the incomplete functional rescue of V380H by RAC3 VHC was that the second LXXLL motif was not preferentially used for ERα-RAC3 interaction. If this was the case, the suppressor mutation might not be presented in an optimal conformation which would hinder the rescue. In SRC1e and TIF2, the second LXXLL motif was clearly preferred for interaction with oestrogen receptor and that retention of this motif alone allowed SRC1e and TIF2 to function almost as efficiently as their wild-type counterparts in stimulating ERα transcriptional activity (Kalkhoven et al., 1998; Voegel et al., 1998). To gain insights into the preference of LXXLL motifs in RAC3 by mERα, a complete series of RAC3 mutants were generated where the LXXLL motifs were rendered non-functional by mutation into LXXAA either individually or in all possible combinations. We then tested the ability of these mutants to potentiate the transcriptional activity of a Gal4–chimeric oestrogen receptor in HeLa cells. Unlike SRC1e and TIF2, mutation of a single LXXLL motif impaired the ability of RAC3 as a coactivator with the effects most pronounced when motif 1 was mutated (Figure 5.9B). When only one LXXLL motif was retained, none of the mutants was able to recapitulate the full activity of the wild-type RAC3. Mutation of all three motifs eliminate the ability of RAC3 to potentiate ERα activity. Our functional data correlates well with other studies where ERα-RAC3 interaction was examined (Chen et al., 1999b; Leo et al., 2000) and led us to postulate that cooperation of multiple LXXLL motifs might be necessary to foster ERα-RAC3 interaction. Hence, the incomplete functional rescue of mERα V380H by RAC3 VHC could be attributed to the absence of cooperating motifs for the 'functional' motif in RAC3 VHC which resulted in suboptimal interaction with the receptor.

We showed that the altered specificity mutation from SRC1e VHC could be transposed to TIF2 and RAC3 and confers the ability to suppress the V380H mutation in mERα. This provides evidence that our novel approach should be applicable in defining the functional role of other candidate coactivators which had been shown to interact with nuclear receptors. More importantly, our results demonstrated that SRC1e, TIF2 and RAC3 are functionally redundant and that directed recruitment of a single species of p160 coactivator is sufficient to mediate transcriptional activation by ERα.

Figure 5.9 Functional analysis of mutant RAC3. (A) Wild-type or mutant chimeric receptors consisting of the LBD of mERα fused to Gal4 DBD were transiently transfected into HeLa cells together with the p5Gal-E1B-GL3 reporter in the absence (–) or presence of full-length RAC3 m13 or RAC3 VHC as indicated. The pRL-CMV plasmid which encoded the Renilla luciferase gene driven by a cytomegalovirus (CMV) promoter was cotransfected as an internal control. After transfection, cells were treated with ethanol vehicle alone (NH) or 17β-oestradiol (E2) at 10nM for 24h. Subsequently, cells were assayed for fire-fly luciferase (LUC) and Renilla luciferase activity. Normalised values are expressed as percentage of activity compared with that of wild-type mERα alone in the presence of E2 (100%). The results shown represent the average of at least two independent experiments assayed in quadruplicate + standard errors. (B) Potentiation of mERα transcriptional activity by RAC3 mutants. Wild-type chimeric receptor consisting of the LBD of mERα fused to Gal4 DBD were transiently transfected into HeLa cells together with the p5Gal-E1B-GL3 reporter in the absence (–) or presence of full-length RAC3 mutants as indicated. m1 denotes a non-functional LXXLL motif 1 and the same nomenclature applies to all other mutants. The pRL-CMV plasmid was cotransfected as the internal control. Experimental procedures and data presentations are as described for (A).

A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m13
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mERα
V380H

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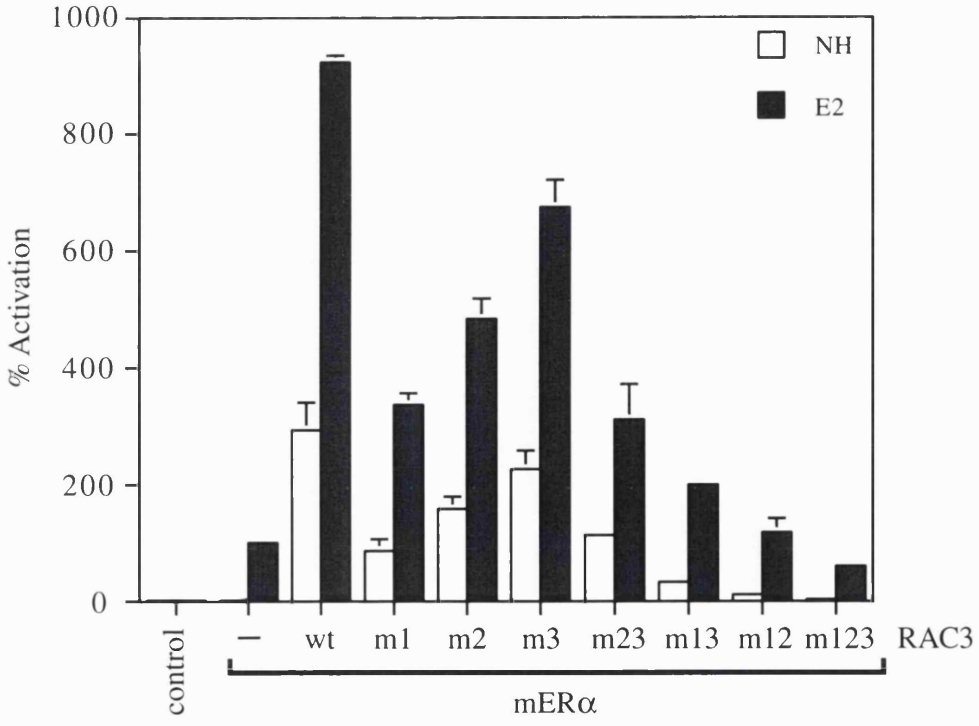
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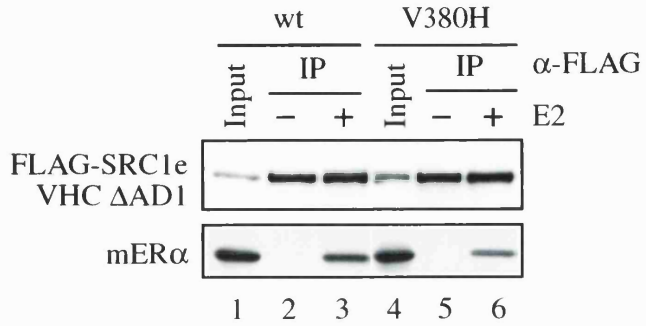
RAC3
mERα

Role of CBP/p300 in functional rescue of mERα V380H by SRC1e VHC

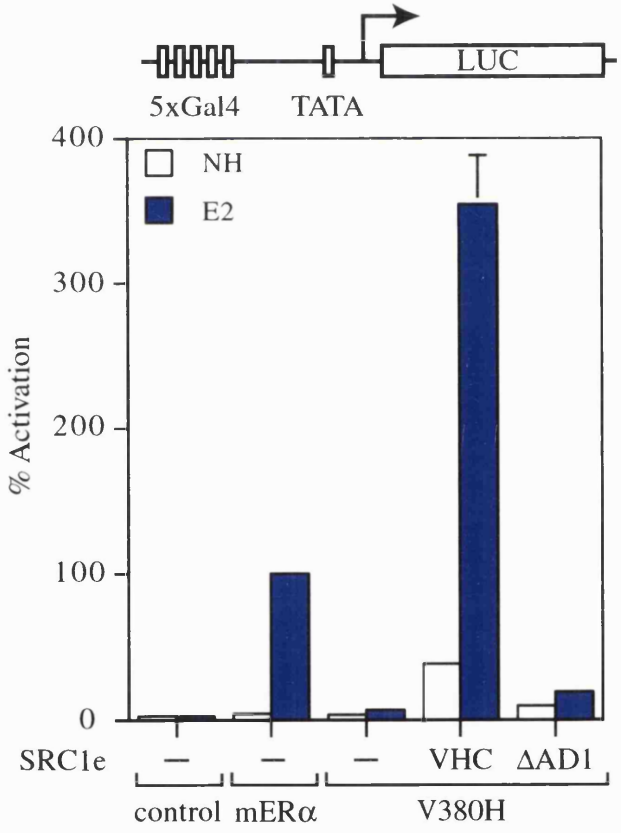
Activation domain 1 (AD1) of SRC1e and other p160 coactivator family members was shown to physically interact with CBP and p300 (Chen et al., 1997; Kalkhoven et al., 1998; Voegel et al., 1998). To directly address whether recruitment of CBP/p300 is the primary role of p160 coactivators, we utilised a SRC1e VHC construct which lacks AD1 and tested its ability to mediate transactivation by the V380H mutant receptor. The deletion mutant was expressed at a comparable level to the wild-type control (data not shown) and the deletion did not grossly affect the structure of the coactivator as examplified by its binding to both wild-type and V380H mutant receptors *in vitro* (Figure 5.10A). However, expression of SRC1e VHC ΔAD1 failed to rescue the transcriptional activity of a chimeric receptor consisting of the mERα V380H LBD fused to Gal4 DNA binding domain in transiently transfected COS-1 cells (Figure 5.10B). Similar results were also obtained in HeLa cells (data not shown). This demonstrates that recruitment of CBP/p300 by SRC1e VHC was essential for the functional rescue of the transcriptionally defective V380H mutant. Our data further suggests that p160 coactivators serve as ligand dependent adaptor proteins whose primary function is to recruit the general coactivator CBP/p300 to the promoter where ERα is bound. Hence, disruption of p160 coactivator function *in vivo* would significantly compromise transcriptional activity of ERα. This provides a plausible molecular mechanism for the block in the development and function of oestrogen responsive tissues as reported in SRC1 and RAC3 null mice (Xu et al., 1998; Xu et al., 2000).

Figure 5.10 Functional analysis of SRC1e VHC ΔAD1. (A) Ligand dependent interaction of mERα V380H with SRC1e VHC ΔAD1 *in vitro*. Full length mERα and the FLAG-epitope tagged SRC1e was transiently expressed in 293-T cells. The whole cell lysate was subjected to immnuoprecipitation with an anti-FLAG antibody immobilised on agarose beads in the absence or presence of 1μM E2. SRC1e was detected by Western blotting using an anti-FLAG antibody. The co-immunoprecipitated mERα was detected using the monoclonal antibody H222. The input control represents 2% of the whole cell extract employed in the immunoprecipitation (IP) reaction. (B) Wild-type or mutant chimeric receptors consisting of the LBD of mERα fused to Gal4 DBD were transiently transfected into HeLa cells together with the p5Gal-E1B-GL3 reporter in the absence (–) or presence of full-length SRC1e VHC or SRC1e VHC ΔAD1 as indicated. The pRL-CMV plasmid was cotransfected as the internal control. After transfection, cells were treated with ethanol vehicle alone (NH) or 17β-oestradiol (E2) at 10nM for 24h. Subsequently, cells were assayed for fire-fly luciferase (LUC) and Renilla luciferase activity. Normalised values are expressed as percentage of activity compared with that of wild-type mERα alone in the presence of E2 (100%). The results shown represent the average of at least two independent experiments assayed in quadruplicate + standard errors.

A


wt
V380H
Input
IP
Input
IP
α-FLAG
– + – +
E2
FLAG-SRC1e
VHC ΔAD1
mERα
1 2 3 4 5 6

B


LUC
5xGal4
TATA
400
300
200
100
0
NH
E2
% Activation
SRC1e
–
–
–
VHC
ΔAD1
control
mERα
V380H

Summary

In this chapter, the functional consequence of direct interaction between mERα and p160 coactivators was determined by utilising the V380H mutant receptor and its altered specificity coactivator partner, SRC1e VHC. Since V380H is unresponsive to endogenous coactivators, its functional rescue by SRC1e VHC in mammalian cells provides evidence that SRC1e alone is sufficient for mERα transactivation. Interestingly, TIF2 and RAC3 carrying analogous mutations can also rescue the mutant receptor, suggesting that there is functional redundancy between the three p160 coactivators. The success of transposing the altered specificity mutations into TIF2 and RAC3 implies that it may be applicable as a universal adaptor module which allows other receptor interacting proteins to bind V380H. If this is feasible, it would provide a powerful means for determining the role of other putative coactivators in mERα transactivation in the future.

Chapter 6

Discussion

The ERα coactivator interaction surface

Transcriptional activation by the oestrogen receptor α (ERα) is achieved through its interaction with coactivator proteins upon ligand binding. It has been shown that recruitment of the p160 family of coactivators is dependent upon the integrity of a short hydrophobic motif, LXXLL, three of which are conserved in individual family members (Heery et al., 1997; Torchia et al., 1997). Here, a cluster of residues is identified in the LBD of mERα which comprise an interaction surface to allow docking of the motif.

The coactivator interaction surface of mERα LBD is composed mainly of hydrophobic residues from helices 3, 5 and 12 which closely resembles a similar surface described for human thyroid hormone receptor β (hTRβ) (Darimont et al., 1998; Feng et al., 1998). More importantly, side chains of residues characterised here, namely I362, L376, V380 and L543, were shown to make van der Waals contacts with side chains of the three LXXLL motif leucines and of the isoleucine immediately N-terminal to the motif in the crystal structure of the agonist bound hERα LBD complexed with GRIP1 NR box II peptide (Shiau et al., 1998). While the aspartic acid substitutions demonstrated that these residues are in close contact with the motif in functional assays, the alanine substitutions led to the notion that they could be divided into two classes (Figures 3.2 to 3.6). One class, including L358, I362, F371, L376, V380 and L383 are likely to contribute to the optimal binding of coactivators but are dispensible, since removal of one or two side chains of these residues had little effect on receptor function. In contrast, L543 is essential for ligand dependent coactivator binding and AF2 activity of mERα. This residue was shown to make intramolecular van der Waals contacts with residues in helix 3 in the crystal structure of oestradiol bound ERα (Brzozowski et al., 1997). Therefore, it can be postulated that L543 plays a pivotal role not only in coactivator binding *per se*, but in the completion and stabilisation of the coactivator interaction surface upon ligand binding. Hence, the L543A mutation might destabilise the position of helix 12 in addition to obliterating an essential contact with the LXXLL motif. Our biochemical analysis complements and extends crystallographic studies in one important aspect. It was found that a hierarchy exists among ERα surface residues that constitute the coactivator

docking surface, which is not apparent from the crystal structures. This provides critical information for further targeted modulation of oestrogen receptor function.

In the structure of the agonist bound ERα LBD complexed with NR box II peptide, K366 and E546 were shown to form hydrogen bonds with the main chain of the peptide (Shiau et al., 1998), similar to the observation made in the holo-PPARγ-SRC1 co-crystal structure (Nolte et al., 1998). This led to the suggestion that these oppositely charged residues which are situated at opposite ends of the coactivator interaction surface might serve as a 'charge clamp' and stabilise the helical structure of the peptide. Although the phenotypes of the K366L and E546A mutations implied that the charges of these residues might be involved in p160 coactivator binding by mERα (Figures 3.7 and 3.8) (Danielian et al., 1992; Mak et al., 1999), our assays did not allow us to distinguish between their role in recognition or equilibrium binding. However, SRC1 binding to mERα (Kalkhoven et al., 1998) and the ability of a peptide containing SRC1 motif 2 to inhibit such binding occurs at high salt concentration *in vitro* (Sue Hoare, unpublished data) suggesting that ionic interaction between mERα and SRC1 is dispensible for equilibrium binding. Given that the GRIP1 NR box II peptide used in crystallisation studies is not structured on its own (Darimont et al., 1998), we imagine the initial recognition of the peptide to the docking surface of the receptor could not be due to the complementarity between the surface of the LBD and the LXXLL motif. Two possibilities arise, recognition might be achieved by the polarity of the surface imposed by K366 and E546, favouring the formation of helical structure of the peptide in one orientation. On the other hand, K366 and E546 could be recognised directly by flanking residues of the LXXLL motif which do not appear to participate in equilibrium binding. Since the SRC1 moiety in the PPARγ-SRC1 complex appeared to be largely unstructured except for the a short helix containing the LXXLL motif (Nolte et al., 1998), we speculate that the mechanism of recognition postulated for the NR box II peptide might also be applicable for native p160 coactivator proteins.

Specificity determinants of the LXXLL motifs to the ERα coactivator interaction surface

The ERα has been demonstrated to interact preferentially with LXXLL motif 2 in SRC1 and TIF2 (Kalkhoven et al., 1998; Voegel et al., 1998). This motif was also shown to bind with highest affinity to TRβ (Darimont et al., 1998), while alternative motifs are preferentially utilised by other receptors (Ding et al., 1998; McInerney et al., 1998; Needham et al., 2000). Sequence alignment indicates that conservation of a particular LXXLL motif in the three p160 coactivators is greater than the conservation between individual motifs (Figure 3.11). Since such conservation sometimes extends beyond the minimal LXXLL sequence, it is conceivable that residues flanking the LXXLL motif could confer preferential binding of particular motifs to different receptors. Using a peptide inhibition assay, we confirmed our previous observation that SRC1 motif 2 has a higher affinity towards the mERα coactivator binding site (Kalkhoven et al., 1998). Moreover, we identified three basic residues, N-terminal to the core LXXLL motif, as the determinants for such high affinity binding (Figures 3.12 to 3.14). Since this cluster of basic residues is highly conserved among all three p160 coactivator family members, we speculate that they are common features shared by motif 2 of p160 coactivators for mediating high affinity binding to the ERα. Residues flanking the GRIP1 LXXLL motif 2 have also been shown to modulate its affinity with TRβ, although the relative contributions of the N- and C-terminal residues were not assessed. Taken together, the three basic residues identified in SRC1 appear to function as a unit in conferring specificity to an LXXLL motif. This seems to be independent of other flanking residues of the motif, since replacement of $^{-4}$DHQ^{-2} of SRC1 motif 3 with $^{-4}$RHK^{-2} from motif 2 was sufficient to confer high affinity binding to the ERα coactivator interaction surface.

The specificity determinants N-terminal to the LXXLL motif are disordered in the structure of the agoinst bound ERα complexed with NR box II peptide (Shiau et al., 1998). Therefore, it is unlikely that they form stable interactions with residues of the ERα in equilibrium binding. It was proposed that these basic residues might be accomodated by a shallow groove between helix 5 and helix 12 in TRβ (Darimont et al., 1998). Alternatively, we envisage that the three basic residues could be involved in long range recognition of surface features of ERα

which are not necessarily in the proximity of the coactivator docking site. Although not detected in our peptide inhibition assays, microinjection studies also implicated residues C-terminal to SRC1 motif 2 in selective ERα binding (McInerney et al., 1998). It is tempting to speculate that the same principle of long range recognition might be applicable. Recently, it was shown that ERβ preferentially binds to LXXLL motifs which are preceded by a proline at the –2 position (Chang et al., 1999). This distinction in the preference for residues flanking the LXXLL motif suggests that there are subtle differences between the coactivator interaction surface of ERα and ERβ. Evans and co-workers reported that two lysine residues immediately N-terminal to the RAC3 LXXLL motif 1 were acetylated *in vitro* by p300 (Chen et al., 1999b). It was suggested that acetylation might eliminate two potential electrostatic interactions between ERα and RAC3 and lead to their dissociation. Although we do not favour the notion of stable interactions between $^{-4}$RHK^{-2} of SRC1 motif 2 with ERα, it is plausible that acetylation may play a part in the release of SRC1 from the ERα docking surface. To this end, acetylation at the ε-NH_3^+ group of the lysine at the –2 position might create steric hindrance that is sufficient to destabilise the ERα–SRC1 interaction.

Taken together, a stepwise model can be proposed for p160 coactivator binding to ERα. The first step involves the flanking residues of the LXXLL motif whose primary function is to direct the core motif to a broad area of the receptor which encompasses the coactivator interaction surface. Once the LXXLL motif is in the vicinity of the surface, specific hydrophobic and ionic interactions between the motif and the receptor ensue, resulting in stable interaction of the coactivator with ERα.

Crucial role of helix 12 positioning in nuclear receptor transcriptional activity

It is now clear that the conserved residues from helices 3, 5 and 12 of NR LBD form the coactivator interaction surface, which formally defines the ligand dependent AF2 activity (Darimont et al., 1998; Feng et al., 1998; Nolte et al., 1998; Mak et al., 1999). In conjunction with the observations that helix 12 occupies distinct positions on the LBD surface in the presence of agonist and antagonist (Brzozowski et al., 1997; Shiau et al., 1998), the molecular basis of

antagonist action has become apparent. Upon agonist binding, helix 12 of ERα adopts a position that seals the ligand binding cavity and concomitantly completes the coactivator docking surface. However, the steric hindrance generated by the bulky side-chain of antioestrogens (Raloxifene or Tamoxifen) prevents helix 12 from adopting this position. Instead, it is docked at the hydrophobic groove between helices 3 and 5 (Brzozowski et al., 1997; Shiau et al., 1998). This is clearly incompatible with coactivator recruitment since the LXXML motif in helix 12 has occupied the space where the LXXLL motif of the coactivators would normally dock. It seems that this antagonist induced alternative position of helix 12 is not unique to ERα since it is also observed in the Raloxifene bound ERβ (Pike et al., 1999), the selective antagonist BMS614 bound RARα and the constitutively active RXRαF318A mutant receptor (Bourguet et al., 2000). The last observation can be explained by the complementarity observed at the interface between helix 12 and the hydrophobic groove, suggesting that this 'alternative positioning' of helix 12 may yield an energetically favourable conformation that is selectively preserved in crystallisation. Nevertheless, in the absence of steric hindrance imposed by antagonist binding, it is conceivable that the helix 12 of RXRαF318A can also freely adopt the 'agonist-induced' position which may be reinforced by the presence of transcriptional coactivators.

The principle outlined above may be applicable to the strucuture of ERβ LBD crystallised in the presence of Genistein (Pike et al., 1999). Genistein (GEN) is a phyto-oestrogen that is found at significant levels in soya beans and binds to ERβ with ~30 fold higher affinity than ERα (Kuiper et al., 1997). However, GEN is a full-agonist to ERα but a partial agonist to ERβ (Barkhem et al., 1998). With the ERβ-GEN LBD structure, it is now possible to speculate on the basis of such partial agonism. Although GEN is buried inside the ligand binding pocket of ERβ in a manner similar to that observed for E2 in ERα, one clear deviation from the agonist bound ERα LBD structures is the position of helix 12 (Brzozowski et al., 1997; Pike et al., 1999). Similar to the Raloxifene bound structure, helix 12 in the ERβ-GEN LBD structure lies between helices 3 and 5 although it projects away from the core of the LBD instead of running along the hydrophobic cleft (Pike et al., 1999). Nevertheless, this suggests that helix 12 in the ERβ-GEN LBD may compete directly with transcriptional coactivators for access to their docking site.

As GEN is buried inside the ligand binding pocket, it presents no steric hindrance to helix 12 in adopting the agonist-induced position which may account for the partial agonistic activity of this ligand. The effect of GEN binding on ERα and ERβ transactivation adds an important consideration in the design of synthetic ligands. It raises the possibility that high affinity, isoform specific ligand binding does not necessarily lead to preferential activation of the same receptor isoform.

The importance of helix 12 in nuclear receptor function is further underscored by the discovery of mutations which are proposed to affect its agonist-induced position in human diseases. Two mutations in PPARγ were identified in patients with severe insulin resistance, diabetes mellitus and hypertension (Barroso et al., 1999). The mutated residues, one located in helix 3 (V290) and the other at the N-terminus of helix 12 (P467), are proposed to mediate important intramolecular interactions within the receptor LBD. As a result, receptors bearing the V290M or P467L mutation were found to have severely impaired transcriptional activity which is attributed to compromised coactivator and ligand binding. Incidentally, a T277A mutation has been found in hTRβ from a patient with thyroid hormone resistance syndrome (Collingwood et al., 1998). T277 occupies the analogous position as V290 in PPARγ and it is conceivable that the T277A mutation would destabilise helix 12 positioning in a similar manner. Indeed, the mutant hTRβ displays reduced transcriptional activity which is attributed to impaired coactivator binding.

Probing the functional role of protein-protein interaction by altered specificity mutants

Genetic selection for second-site suppressors of mutations, which affect specific function of a protein is a powerful means of identifying its interaction partners. It also provides useful information on the protein-protein interface. Furthermore, altered specificity protein pairs can be studied at a functional level in the absence of interference from other potential interaction partners, which are only capable of binding to the wild-type parental protein. This strategy has been used to study protein functions in both prokaryotic and eukaryotic systems and was employed in this thesis to probe the functional roles of p160 coactivators in nuclear

receptor action. In bacteria, the specificity determinants of dimerisation in the bacteriophage λcI transcription factor was shown to involve two pairs of oppositely charged residues (Whipple et al., 1998). Second, the transcriptional activation function of λcI was found to rely on its direct interaction with the σ^{70} subunit of bacterial RNA polymerase, through identification of a mutation in the σ^{70} gene which specifically suppress the transcriptional defect of a λcI mutant (Li et al., 1994).

In yeast, genetic selection of TATA-binding protein (TBP) mutant with altered DNA-binding specificity led to the identification of a TBP mutant which binds specifically to a mutated sequence TGTAAA (Strubin and Struhl, 1992). This altered specificity protein-DNA pair was later used in tandem with an altered specificity TBP-TFIIB pair to show that TBP-TFIIB are targeted by a subset of mammalian transcriptional activators to instigate transcription initiation (Tansey and Herr, 1997). It is interesting to note that the TBP-TFIIB altered specificity pair was designed based on their co-crystal structure. The success was largely due to the nature of interaction which relied on a single pair of charged amino acids. The examples presented so far involve protein-protein or protein-DNA pairs which are known to interact with each other and the random mutation of specific regions that are critical for interaction. A 'pure' genetic appraoch was taken in the identification of dominant suppressors for an UNC-4 homeodomain protein mutant in C. elegans, which is defective in the wiring of motor neuron circuit (Winnier et al., 1999). The UNC-4 protein contains an eh1 repressor domain which in other proteins, is responsible for interaction with transcriptional co-repressors such as Groucho. Misenese mutation of highly conserved residues in the eh1 domain led to loss of function in UNC-4 which was suppressed by a single mutation in the eh1-interacting region of C. elegans Groucho, UNC-37. The phenotypic rescue clearly demonstrates the functional significance of the UNC-4/UNC-37 interaction and indicates that UNC-37 is the obligate partner for transcriptional repression by UNC-4.

In mammalian systems, multiple proteins may possess an identical protein-protein interaction module which allow them to perform similar functions in biochemical assays. However, it is often difficult to decipher the significance of such interaction under physiological conditions. One way to probe the role of a

specific protein-protein interaction is the use of altered specificity mutants. This was successfully employed to confirm the essential role of FOG, a transcriptional coactivator for GATA-1, in erythroid differentiation (Crispino et al., 1999). Orkin and co-workers first identified mutations in GATA-1 which disrupted GATA-1/FOG interaction in a 'split two-hybrid' screen. A GATA-1 mutant was then used as bait in a second yeast two-hybrid screen for FOG suppressor mutants which specifically rescue their interaction. Our strategy for the identification of SRC1 altered specificity mutants is similar, although the scale of our genetic screen was smaller. This was facilitated by the clear indication from the co-crystal structure that V380 of mERα interdigitated with L690 and L694 of SRC1. As a result, targeted random mutation were made at only two residues in SRC1 which ensured a low complexity of the library, and in turn allowed parallel screening of three mutant receptors. One disadvantage of this strategy was that no altered specificity SRC1 would be recovered, if suppression of the receptor mutations actually required alteration of other side chains in addition to those of L690 and L694. There was a precedent in which a single mutation in the growth hormone receptor necessitated simultaneous mutation of five residues in the growth hormone molecule to reconstitute a functional protein-protein interface (Atwell et al., 1997). To this end, random mutagenesis of a broader region which encompassed SRC1 LXXLL motif 2 would represent an alternative strategy.

A model for the mERα V380H–SRC1 VHC interaction

It is surprising that both SRC1 VHC and SRC1 VRC, which are capable of interacting with mERα V380H and V380R respectively, retain a wild-type LXXLL motif in the vicinity of additional variant motifs. Furthermore, the exclusive recovery of insertional mutant SRC1 in the screen argued that mutations in L690 and L694 alone were not sufficient to rescue binding to the mutant receptors. Several lines of evidence suggested that both the wild-type LXXLL motif and the 15 amino acid insertion found in SRC1 VHC are necessary for mutant receptor-coactivator interaction (Figures 5.4 to 5.6). First, mutant SRC1 with a single copy of the variant motif YXXLK found in SRC1 VHC was unable to interact with V380H. Second, disruption of the wild-type LXXLL motif in SRC1 VHC

abolished its ability to bind V380H. Finally, SRC1 VHC was unable to rescue the transcriptional activity of V380H in the presence of antioestrogens such as Tamoxifen and ICI 182780, implying that SRC1 VHC could not interact with antagonist bound V380H. Tamoxifen binding forces helix 12 to adopt a position which occludes the docking site for the wild-type LXXLL motif, without altering the rest of the ERα LBD structure (Shiau et al., 1998). The last observation, therefore, suggests that V380H-SRC1 VHC interaction employs a variant interface which is most likely to be based on the one utilised by their wild-type counterparts.

A model can be proposed which may be applicable for both SRC1 VHC and SRC1 VRC (Figure 6.1). Although the wild-type LXXLL motif alone is not sufficient to stably interact with the remodelled coactivator docking surface of V380H or V380R, it is tempting to speculate that it remained as a recognition or anchoring module for the mutant mERα-SRC1 interaction. Nevertheless, stable equilibrium binding requires the sequence insertions in the mutant alleles which might interact directly with the histidine or arginine side chain where V380 is normally found. Alternatively, subtle structural changes associated with V380H or V380R might be specifically recognised by the additional sequences. A third possibility is that the sequence insertions may contact a second site on the receptor surface which is only available in the presence of ligand. In the case of SRC1 VHC, this second site has to be in close proximity to the modified coactivator interaction surface owing to the length of the sequence insertion. It is important to note that the sequence insertion in SRC1 VHC is unlikely to alter the structural integrity of the protein. This is because the SRC1 moiety in the holo-PPARγ-SRC1 complex appears to be largely unstructured except for the short helices which contain the LXXLL motifs (Nolte et al., 1998). Therefore, the 15 amino acid insertion is likely to be accomodated in the random coil region without major disruption to the tertiary structure. Setting aside the question concerning the precise nature of the mutant receptor-coactivator interaction, it is clear that the altered specificity mutation in SRC1 VHC does not constitute a promiscuous protein binding motif. This is supported by the observation that SRC1 VHC was

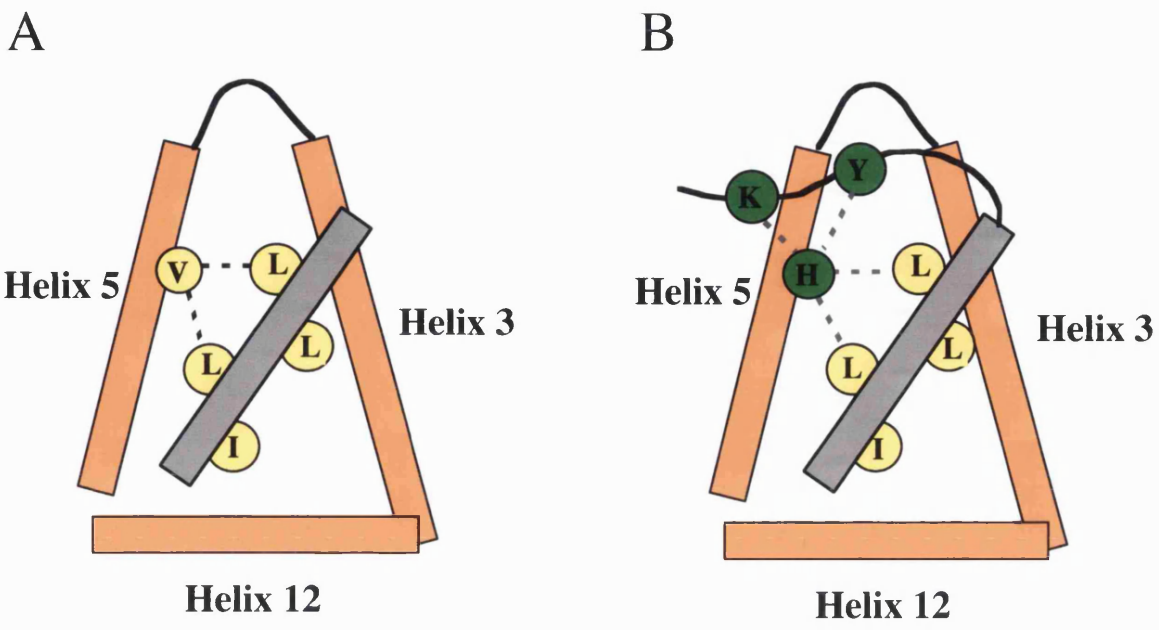


Figure 6.1 Model for mERα-SRC1 VHC interaction. The orange rectangles represents helices 3, 5 and 12 of mERα LBD while the grey rectangle represents the amphipathic α-helix in SRC1 which contains the LXXLL motif 2. (A) V380 of mERα makes strong van der Waals contacts (denoted by dotted black lines) with L690 and L694 of SRC1 in the wild-type receptor-coactivator interface. (B) In one model, side-chain of the histidine residue which replaces V380 in the modified mERα coactivator interaction surface, forms multiple weak interactions (denoted by dotted grey lines) with L690 and L694 together with residues present in the variant motif YXXLK of SRC1 VHC. Note the α-helix which contains the wild-type LXXLL motif is proposed to remain as a recognition or anchoring module for the altered specificity receptor-coactivator interaction.

unable to rescue an hRARα mutant which bears a mutation analogous to V380H in mERα. Future efforts should be directed to deciphering the molecular determinants of the mERα V380H–SRC1 VHC by computer modelling or crystallographical studies.

Transcriptional activation by ERα through direct recruitment of p160 coactivator

The co-expression of SRC1 VHC fully restored the transcriptional activity of ERα V380H (Figure 5.2). Although a large number of proteins have been reported to interact with ERα and modulate its activity, our results suggested that direct recruitment of the p160 coactivator alone is sufficient to mediate ERα transactivation. Using a version of SRC1 VHC which lacked its CBP/p300 binding domain (ΔAD1), we demonstrated that the recruitment of CBP/p300 by SRC1 was an obligatory second step for SRC1 mediated gene activation. Although this sequence of events has been postulated previously, our data extend the existing model and suggest that no other transcriptional co-factors are necessary to support ERα transactivation in mammalian cells, at least in a subset of promoters. How does this compare with other existing models for NR transcriptional activation?

A model proposed by Roeder and co-workers suggested that transcriptional activation by NR might require the sequential recruitment of coactivators with HAT activity (p160 proteins, CBP/p300, p/CAF) and the TRAP/DRIP complex (Fondell et al., 1999). The major assumption of this model is that the two sets of co-factors should have non-overlapping functions and are responsible for discreet steps in gene activation. Accordingly, coactivators with HAT activity may involve histone modifications, releasing the promoter from a repressed state. Recruitment of the TRAP/DRIP complex would then follow and stimulate transcription initiation by promoting the assembly of the basal transcription machinery. However, several lines of evidence suggest that such sequential recruitment of co-factors might not occur *in vivo*. The TRAP complex was reported to stimulate transcription from a naked DNA template *in vitro* (Fondell et al., 1996; Fondell et al., 1999). In contrast, two other groups which independently isolated a related DRIP/ARC complex, observed robust stimulation of *in vitro* transcription from a

chromatin template with their purified complex (Naar et al., 1999; Rachez et al., 1999). Although no HAT activity was detectable, the ability to facilitate transcription from a chromatin template clearly indicates that the TRAP/DRIP complex is able to alter the local chromatin structure directly or indirectly (Rachez et al., 1999). This dual function of TRAP/DRIP complex is mirrored by the p160-CBP-p/CAF ensemble. In addition to its HAT activity, CBP can also interact with RNA helicase A which is a component of the RNA pol II holoenzyme (Nakajima et al., 1997). Hence, NR binding to a p160 protein, which in turn recruits CBP-p/CAF, can trigger both chromatin remodelling and assembly of basal transcription machinery at the core promoter. Taken together, it is conceivable that recruitment of either the p160 coactivators or the TRAP/DRIP complex is sufficient for NR transactivation in the absence of co-regulator exchange.

Recent reports concerning the activation of the oestrogen responsive pS2 gene support our hypothesis that the p160 coactivators are sufficient to mediate ERα transactivation. In chromatin immunoprecipitation (ChIP) experiments, RAC3 was found to associate with the endogenous pS2 promoter for up to one hour after hormone treatment (Chen et al., 1999b). The kinetics of RAC3 association coincided with that of histone H4 hyperacetylation at the same promoter. Furthermore, cessation of hormone-induced pS2 gene activation also correlated with the dissociation of RAC3 and CBP with the same promoter. It was shown that the pS2 promoter is complexed with two strongly positioned nucleosomes which are not displaced upon hormone induced transcriptional activation (Sewack and Hansen, 1997). This implies that histone acetylation, not nucleosome displacement or remodelling, is the major form of chromatin modification pertinent to the pS2 gene activation. Since the TRAP/DRIP complex possess no HAT activity (Rachez et al., 1999), it is unlikely that this complex would be recruited by ERα at the pS2 promoter, at least for the initial phase of gene activation. It would be interesting to learn whether the selective use of p160 coactivators by ERα applies to activation of other oestrogen responsive genes. Targeted disruption of the TRAP220 gene in mice provided additional evidence that TRAP/DRIP complex may be differentially utilised by different NRs. TRAP220 serves as an adaptor unit for the recruitment of the TRAP/DRIP complex to activated NRs (Yuan et al., 1998; Rachez et al., 1999). In embryonic

fibroblasts derived from TRAP220 null mice, TR-driven transcriptional activation of a reporter gene is defective (Ito et al., 2000). On the other hand, transactivation by RAR is unaffected. To this end, it is tempting to speculate that transactivation of ERα, which appears to be primarily dependent on p160 coactivators, might also be normal in the TRAP220$^{-/-}$ fibroblasts. Examination of endogenous target gene activation is clearly necessary to verify and extend these observations made in cell lines.

The current discussion has not considered the recuritment of the SWI/SNF complex by NRs. The SWI/SNF complex was proposed to influence the chromatin environment in a number of ways. In biochemical assays, it was shown to render nucleosome accessible for transcription factor binding. Furthermore, it can mediate cis- or trans- displacement of nucleosomes (Sudarsanam and Winston, 2000). It has emerged that the SWI/SNF complex may be recruited to specific promoters by transcription factors. In yeast, the sequential recruitment of transcriptional activator and chromatin remodelling complexes has been demonstrated in the control of the *HO* gene activation (Cosma et al., 1999). Upon DNA binding, the transcriptional activator Swi5p first recruits the SWI/SNF complex which is followed by the SAGA complex. This prompted the hypothesis that the SWI/SNF complex may lower the energy barrier between different nucleosomal states, a prerequisite for histone modification by the HAT complexes (Kingston and Narlikar, 1999). In mammalian systems, it has also been reported that GR binding to the MMTV promoter led to the recruitment of the hSWI/SNF complex. Nucleosome disruption by the complex then allowed the binding of other transcription factors to the same promoter (Belikov et al., 2000). Furthermore, GR activation of a chromosomally integrated, MMTV-driven reporter gene, was shown to require the hSWI/SNF complex (Fryer and Archer, 1998). Nevertheless, it is unclear whether the recruitment of HAT complexes by GR represents a second step prior to gene activation at the MMTV promoter. It should be noted that, through whole genome expression analysis, only 6% of genes in S. cerevisiae were found to be affected by a loss of function mutation in the Swi2p catalytic subunit of the SWI/SNF complex (Holstege et al., 1998). This suggests that the SWI/SNF complex is dispensible for activation of a large proportion of genes in yeast and perhaps in higher eukaryotes. Indeed, activation of the oestrogen responsive pS2 gene does not involve

nucleosome remodelling attributable to the SWI/SNF complex, implying that it may not be recruited by ERα at this promoter (Sewack and Hansen, 1997).

In conclusion, we favour a model in which NR transactivation is mediated via recruitment of either the p160 coactivators or the TRAP/DRIP complex (Figure 6.2). Differential use of the p160 coactivators or the TRAP/DRIP complex by NRs may be governed by criteria such as their relative abundance, their binding affinity to the NR in question and the promoter architecture. However, we can not exclude the possibility that both coactivator complexes may be used in parallel in a subset of promoters. To gain further support of our hypothesis, it would be of interest to test the transcriptional activity of the altered specificity ERα-SRC1 pair on a chromatin based template. We are currently investigating whether expression of ERα V380H and SRC1 VHC in mammalian cells could support activation of endogenous oestrogen responsive target genes such as PR, pS2 and cathepsin D. A second approach would involve *in vitro* transcription assays with chromatin templates which had been successfully employed to study the role of p300 in ERα transactivation (Kraus and Kadonaga, 1998).

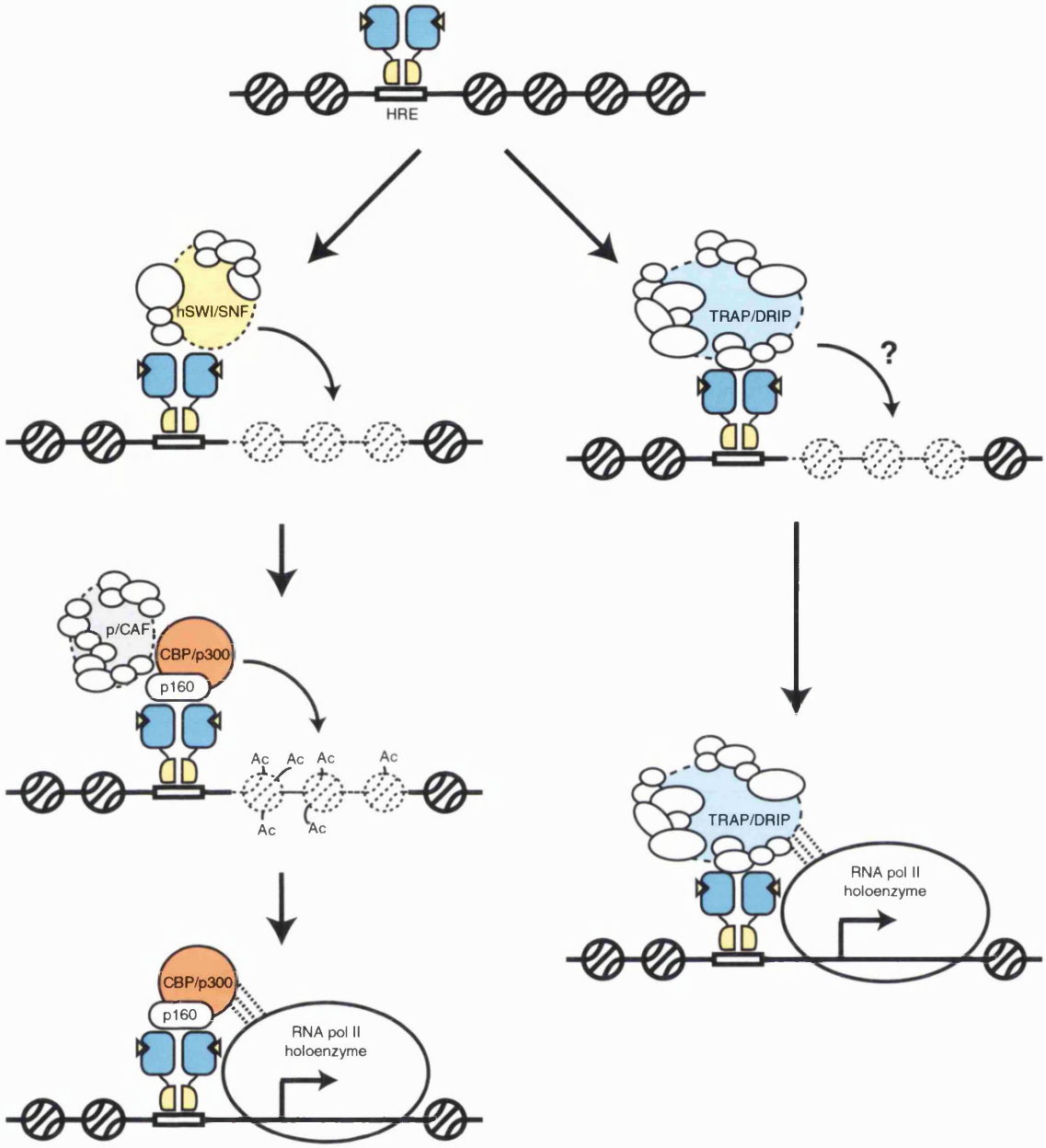


Figure 6.2 Model for transcriptional activation by nuclear receptors. Upon binding to hormone response element (HRE) at target gene promoters, NR may recruit the SWI/SNF complex to increase the fluidity of the local chromatin environment. This allows additional transcription factors to bind to the same promoter and / or facilitate histone modifications by HAT activity containing coactivators such as p160 proteins, CBP/p300 and p/CAF. The p160 coactivators form a crucial platform for the association of CBP/p300 and p/CAF with NR. CBP/p300 in turn recruits the RNA pol II holoenzyme via direct protein-protein interaction. Alternatively, HRE binding by NR may be followed by recruitment of the TRAP/DRIP complex which is able to overcome the repressive effect of chromatin by an unidentified mechanism. In addition, the TRAP/DRIP complex may directly recruit the RNA pol II holoenzyme to the core promoter. Ac represents acetylated histone tail.

The SRC1 VHC suppressor mutation is a transposable protein-protein interaction module

Introduction of the SRC1 VHC suppressor mutation into the other p160 coactivators allowed us to generate mutant versions of TIF2 and RAC3 which could interact with ERα V380H (Figures 5.7 and 5.8). TIF2 VHC and RAC3 VHC possess similar binding characteristics as SRC1 VHC. This suggests that the suppressor mutation which consists of a wild-type LXXLL motif followed by the sequence ERHKIYHRLKQESPS may function as a transposable protein-protein interaction module, enabling heterologous proteins to interact with ERα V380H. Theoretically, this module could be introduced into other LXXLL motif containing NR interacting proteins. Of particular interest is the generation of an altered specificity mutant TRAP220 which is capable of binding ERα V380H. The functional analysis of TRAP220 in mammalian cells had been hampered by the fact that exogenous expression of TRAP220 did not give rise to significant (< 3-fold) potentiation of NR transcriptional activity (Yuan et al., 1998; Ren et al., 2000). Notably, potentiation of ERα activity by TRAP220 has not been reported. This raises the speculation that TRAP220 and by inference, the TRAP/DRIP complex, is not required for ERα transactivation. To this end, the use of an altered specificity mutant should allow us to probe the functional consequence of direct recruitment of TRAP220 by ERα, and address the above issue unequivocally.

Functional redundancy of p160 coactivators

In mammalian cells, the transcriptional activity of ERα V380H could be rescued by SRC1 VHC, TIF2 VHC or RAC3 VHC. Hence, the recruitment of any one of the p160 coactivators appears to be sufficient to instigate ERα transactivation. This clearly suggests that the three p160 proteins are functionally redundant and that expression of one family member could potentially compensate for the absence of others. Indeed, such functional compensation may explain the relatively mild phenotype of the SRC1 null mice (Xu et al., 1998). In these mice, an increase in the level of TIF2 mRNA transcripts was observed in selected tissues. The increase in TIF2 expression might therefore limit the range and severity of defects originated from the SRC1 deficiency.

There had been speculations that RAC3 might be functionally distinct from SRC1 and TIF2. First, it was reported that microinjection of anti-SRC1 antibody inhibited the activity of a retinoic acid responsive reporter gene. This inhibition was overcome by exogenous expression of SRC1 or TIF2 but not the mouse homologue of RAC3, p/CIP (Torchia et al., 1997). However, the p/CIP clone used in the report was defective in potentiating transcription from either ERE or RARE tethered reporter genes (Torchia et al., 1997). This is in clear contrast to RAC3 which consistently potentiates ERα transactivation to the same extent as SRC1 and TIF2 under our experimental conditions. It was later noted that the p/CIP clone contained a frameshift at its C-terminus which gave rise to a form that lacked the activation domain 2 (AD2) (Takeshita et al., 1997). The AD2 is present in all p160 family members and its absence may affect the overall integrity of p/CIP as a transcriptional coactivator. This issue could be resolved by repeating the microinjection experiments using RAC3 in the future. It was noted that the gene encoding RAC3, but not SRC1 or TIF2, is amplified in breast and ovarian cancers (Anzick et al., 1997). This prompted the speculation that overexpression of RAC3 may play a unique role in promoting mammary and ovarian tumour progression. However, the selective amplification of the RAC3 gene may be attributed to its genomic locus rather than any potential functional difference between the p160 coactivators. The RAC3 gene is adjacent to another two genes which are also amplified in breast cancer at 20q11-12 (Guan et al., 1996). Notably, one of these genes has been found to encode another putative NR coactivator, AIB3/ASC-2 (Lee et al., 1999; Caira et al., 2000; Mahajan and Samuels, 2000). The amplification of 20q11-12 may, therefore, lead to simultaneous overexpression of two NR coactivators and increase the likelihood of aberrant transcriptional activation by NRs such as ERα. This is reminiscent to the synergistic potentiation of NR activity by overpressing two coactivators in mammalian cells (Chen et al., 1999a). Taken together, we postulate that the RAC3 gene amplification in breat cancer is unlikely to reflect a unique functional role. Its selection over the SRC1 or TIF2 gene may be due to its proximity to the AIB3 gene which also encodes a putative NR coactivator. Therefore, it can be envisaged that the concomitant overexpression of RAC3 and AIB3, but not RAC3 alone, may account for the proposed selective advantage in mammary tumour progression.

The RAC3 null mice was reported to display distinct phenotypes when compared with the SRC1 null mice. This led O'Malley and co-workers to suggest that the physiological role of RAC3 is different from that of SRC1 *in vivo* (Xu et al., 1998; Xu et al., 2000). The SRC1 null mice display partial steroid hormone and thyroid hormone resistance (Xu et al., 1998; Weiss et al., 1999). On the other hand, the RAC3 null mice exhibited retarded growth, delayed puberty and impaired female reproductive function which were not observed in the SRC1 null mice (Xu et al., 2000). By in situ hybridisation analysis, it was reported that SRC1 is highly expressed in the mammary gland, pituitary gland, olfactory epithelium and cardiac muscles (Jain et al., 1998; Misiti et al., 1999; Xu et al., 2000). On the other hand, RAC3 expression was detected in the mammary gland, oocyte, olfactory bulb and vascular smooth muscle by X-gal staining of the RAC3 heterozygous mice which contain a lacZ gene inserted into the RAC3 locus (Xu et al., 2000). It was postulated, therefore, that the distinct phenotypes of SRC1 and RAC3 null mice were due to the differential expression of these two coactivators (Xu et al., 2000). However, such conclusion must be treated with caution since two methods with different sensitivity were used for detecting the SRC1 and RAC3 transcripts. Furthermore, the lacZ gene insertion removed multiple introns which may contain sequences that govern RAC3 expression. As a result, signals from the X-gal staining may only partially indicate the endogenous RAC3 expression pattern. It should be noted that both mutant mice strains exhibit a common deficiency in mammary gland development in response to oestrogen and progesterone (Xu et al., 1998; Xu et al., 2000). Although SRC1 and RAC3 are highly expressed in this tissue, they do not seem to functionally compensate for each other. This may imply that the absolute level of p160 proteins is critical in mammary gland development. Alternatively, SRC1 and RAC3 may be expressed in distinct cell types within the mammary gland.

Our hypothesis on the functional redundancy of p160 coactivators addresses their role in ERα transactivation at a cellular level, and is compatible with the available animal models. We predict that co-expression of ERα and a single species of p160 coactivator is sufficient to stimulate transcription initiation for at least a subset of oestrogen responsive genes. This does not exclude the possibility that different p160 family members may have distinct expression

patterns and in some cell types, only one member is present. Removal of p160 proteins (for example, by targeted gene disruption) from these latter cell types would therefore lead to impaired transactivation by ERα and a deficiency in steroid hormone response. The complex phenotypes of the SRC1 and RAC3 null mice can be attributed to both cell autonomous and cell non-autonomous effects. For example, the RAC3 null mice have a lower level of systemic oestrogen which predictably affects multiple aspects of sexual maturation and reproductive function of the female mice (Xu et al., 2000). This again highlights the value of using altered specificity mutants, which allow the study of cell autonomous function of a receptor-coactivator pair independent of endogenous proteins.

Evolution of p160 coactivators

Identification of functional homologs of p160 proteins in other species provides a powerful means in understanding the evolution of this family of transcriptional coactivators. High level of sequence identity should indicate regions of functional importance. Furthermore, identification of homologs in model organsims such as C. elegans and Drosophila, should allow genetic dissection of protein functions and verification of priniciples established in mammalian cell culture systems. A single homolog in more primitive species would also circumvent the problem of functional redundancy which has hindered the intepretation of results in mice models. In collaboration with Shaun Cowley, attempts were made to identify p160 homologs in both chicken and Drosophila. These two organisms were chosen because Far Western blot analysis indicated that proteins of ~160kDa in size, among other signals, could be detected in cell extracts prepared from chicken embryonic fibroblasts and Drosophila embryo. Nevertheless, we were aware that functional homologs do not necessarily share similar sizes and that the 160kDa signal may not represent our candidate proteins. In these preliminary experiments, we used the ligand binding domain of ERα as a probe, making the assumption that the coactivator interaction surface on the activated ERα LBD is an evolutionarily conserved feature of NRs which is recognised by coactivators from diverse animal species.

By expression screening, cDNA encoding the receptor interaction domain of the chicken RAC3 homolog (cRAC3) was cloned. In parallel, cDNA encoding the PAS domain of cRAC3 was amplified using a pair of degenerate oligonucleotides (Appendix I, Figure AI.1). Assembly of multiple additional cRAC3 cDNA clones and conceptual translation provided the primary amino acid sequence of cRAC3 which lacked ~100 residues at the N-terminus. cRAC3 is 65% identical to its human counterpart and sequence alignment of human (Li et al., 1997a), chicken and Xenopus (Kim et al., 1998) RAC3 reveals extensive sequence homolgy throughout the entire protein (Appendix I, Figure AI.1). This suggests that RAC3 is most likely to be conserved in vertebrates. Expression pattern of a gene sometimes gives important insights into its roles in the development or maintenance of specific tissues. Preliminary in situ hybridisation was performed on 2.5 to 3 days old chicken embryos (Appendix I, Figure AI.2). It is apparent that cRAC3 is expressed in multiple embryonic tissues, however the precise identification of mesenchymal structures awaits further analysis of serial sections. Nevertheless, it was readily noted that cRAC3 is expressed in the apical ectodermal ridge (AER) of the chicken limb buds. Since the AER is a crucial signalling centre which governs limb outgrowth (Schwabe et al., 1998), it is tempting to speculate that cRAC3 or other p160 coactivators may facilitate NR function in the AER and contribute to its activity. Notably, differential expression of RAR isoforms have been reported in the limb bud of mouse and chicken embryos (Dolle et al., 1989; Smith and Eichele, 1991). Although there is no clear indication of RAR expression at the AER, a retinoic acid (RA) responsive reporter gene in transgenic mice could be activated in the AER upon RA treatment, indicating the presence of RAR signalling components in this structure (Mendelsohn et al., 1991; Rossant et al., 1991). No defect in limb development has been reported in the RAC3 null mice (Xu et al., 2000), however it is conceivable that RAC3 function may be compensated by the other p160 proteins.

In order to identify chicken homologs of SRC1 and TIF2, a pair of degenerate oligonucleotides were used to amplify cDNA encoding part of the activation domain 2 (AD2) which is bordered by residues conserved in both proteins. Conceptual translation of the cDNA clones indicated that the chicken SRC1 (cSRC1) and TIF2 (cTIF2) are 76% and 90% identical to their human

counterparts, respectively (Appendix I, Figure AI.3). The precise nature of AD2 in p160 coactivators has thus far been ill-defined, however it is apparent from the sequence comparison that this region may be conserved in vertebrates. Unfortunately, the high level of sequence conservation also prevented us from identifying residues which might be critical in AD2 function. Functionally similar proteins may play distinct roles in the context of a whole animal through differential expression. Therefore, it would be interesting to compare the expression patterns of cSRC1, cTIF2 and cRAC3 in the future.

Repeated attempts to isolate Drosophila p160 homolog by expression screening or by amplification of cDNA with degenerate oligonucleotides were unsuccessful. Indeed, sequence homology searches were unable to identify p160-like proteins in the Drosophila genome (Adams et al., 2000). However, the notion that p160 coactivators may be unique to vertebrates has recently been challenged. A novel Drosophila gene, named taiman (tai), was identified in a genetic screen for mutations that cause migration defects to border cells (Bai et al., 2000). The Drosophila ovary consists of egg chambers in which border cells are found. During oogenesis, the border cells migrate from the anterior tip of the egg chamber to the border of the oocyte through the interior of the chamber (Spradling, 1993). Loss of function mutation in the tai gene causes the border cells to migrate at a slower rate. Surprisingly, molecular cloning of the tai gene revealed that it may be a functional homologue to the mammalian p160 coactivators (Bai et al., 2000). The predicted TAI protein contains a PAS domain at its N-terminus, three NR interacting motifs (two LXXLL and one LXXML motif) in the central region and a glutamine rich region at the C-terminus which may serve as transcriptional activation domain. The PAS domain is the only region which can be aligned with confidence owing to extremely low sequence homology between the human p160 proteins with TAI (Appendix I, Figure AI.4). Even for the PAS domain, the human p160 coactivators appear to share a higher degree of similarity with Drosophila ARNT, SIM and aryl hydrocarbon receptor than TAI. It is therefore not surprising that TAI has eluded from sequence homology searches.

The most significant feature of TAI is perhaps the NR interacting motifs whose spacing closely matchs that of the LXXLL motifs in their human counterparts. It has been noted that the spacing between the three LXXLL motifs

in vertebrate p160 coactivators is highly conserved, which may underlie the optimal presentation of these motifs to the NRs. It is tempting to speculate that the conserved mechanism for NR interaction together with the putative transcriptional activation domain may be sufficient to qualify TAI as a prototype of NR coactivator. In addition, TAI is a nuclear protein and may potentiate the activity of the ecdysone receptor in cell culture assays (Vogel, 2000). Since sequence homology seraches using TAI failed to recover additional Drosophila or C. elegans proteins with significant similarity, TAI may represent the sole p160 coactivator homolog in Drosophila. In addition, any C. elegans homolog is likely to have very low sequence homology with its counterpart in other species. An alternative approach to identify p160 homologs would be by yeast two-hybrid screening using Drosophila or C. elegans NRs as 'baits'. Indeed, the Drosophila NR co-repressor homolog, SMRTER, was identified in a yeast two-hybrid screen for ecdysone receptor interacting proteins (Tsai et al., 1999). Interestingly, SMRTER also has very limited sequence homology with the vertebrate NR co-repressors N-CoR and SMRT, although a putative NR interacting motif IXXIIXXXI can be found.

Taken together, it is difficult to judge whether TAI represents an ancestral form of the vertebrate p160 coactivators. Alternatively, C. elegans, Drosophila and vertebrate NR coactivators may be derived from a common ancester found in more primitive metazoan. Evolution in individual branches may then allow diversification, accounting for the low sequence homology between the Drosophila and vertebrate proteins. Furthermore, it can be postulated that the presence of three p160 coactivators are a result of recent gene duplication, unique to vertebrates. Hence there may not be enough time to acquire highly specialised functions, which in part explain the overlapping biological roles of the vertebrate p160 proteins.

Concluding remarks

The complete genome sequences of an increasing number of organisms including humans have provided the foundation for functional genomics. Previously uncharacterised gene products can be assigned to particular cellular processes through analysis of their expression pattern, sub-cellular localisation and protein interaction partners. In addition, methods have been devised for large scale

protein interaction mapping which allow the delineation of protein networks responsible for a given developmental process (Walhout et al., 2000). Nevertheless, the functional significance of individual interactions in such networks is not always established.

In this thesis, an altered specificity SRC1 was used to demonstrate that direct interaction between ERα and p160 coactivator is sufficient to elicit hormone induced gene activation even though ERα has been shown to bind to many other putative transcriptional co-regulators. This suggests that generation of altered specificity mutants may represent a generally applicable solution in dissecting the functional roles of individual components in a complex network of protein-protein interaction.

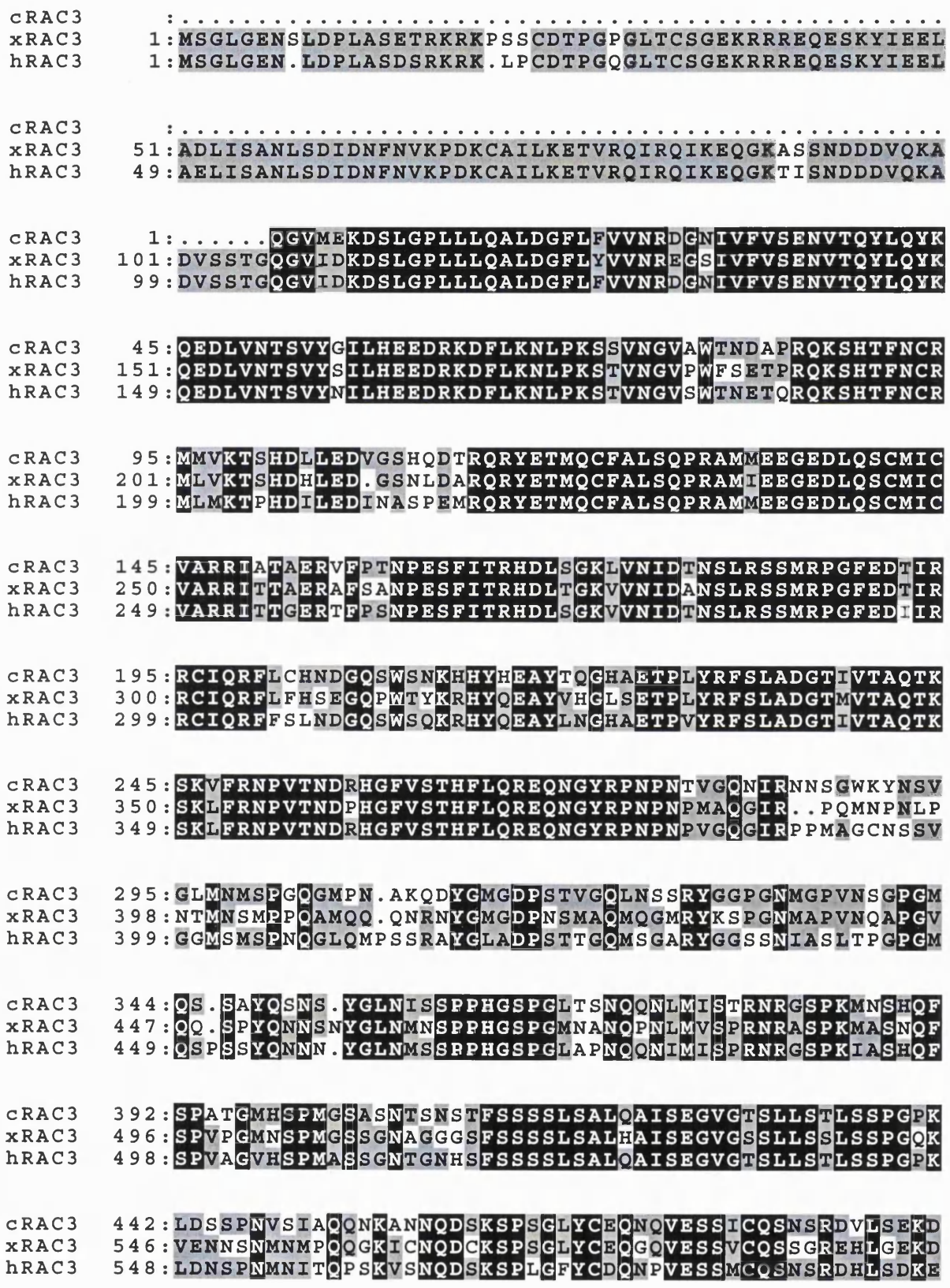
Appendix I

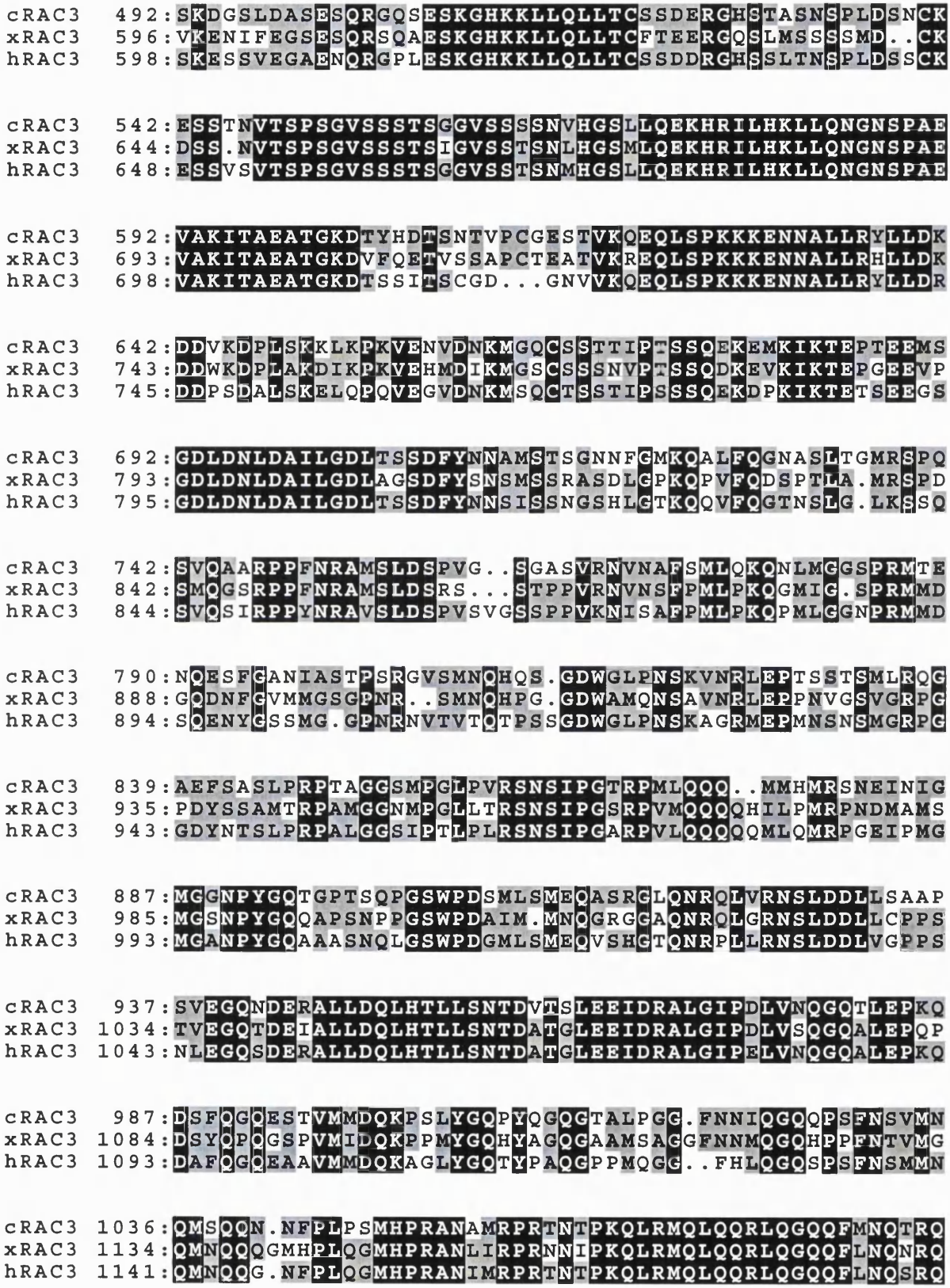
p160 coactivator homologs

Figure AI.1 Sequence comparison of chicken, Xenopus and human RAC3. The primary amino acid sequence of chicken RAC3 (cRAC3) is deduced from conceptual translation of the available cDNA clones. Thus far, we have been unable to obtain cDNA clones encoding the N-terminus of cRAC3. Black boxes indicate identical residues, and grey boxes similar residues in all three sequences.

Conserved residues (numbering as in human RAC3)	Sequence of degenerate oligonucleotides
^{105} QGVIDKD	5'-CARGGIGTIATIGAIAARGA-3'
^{221} YETMQCF	5'-GCRAARCAYTGCATIGTYTCRTA-3'
^{1070} EEIDRAL	5'-CCIARIGCICKRTCIATYTCYTC-3'
^{1410} MGPDQKYC	5'-RCARTAYTTYTGRTCIGGICCCAT-3'

R=A or G Y=C or T K=G or T I=inosine


cRAC3 :
xRAC3 1:MSGLGENSLDPLASETRKRKPSSCDTPGPGLTCSGEKRRREQESKYIEEL
hRAC3 1:MSGLGEN.LDPLASDSRKRK.LPCDTPGQGLTCSGEKRRREQESKYIEEL
cRAC3 :
xRAC3 51:ADLISANLSDIDNFNVKPDKCAILKETVRQIRQIKEQGKASSNDDDVQKA
hRAC3 49:AELISANLSDIDNFNVKPDKCAILKETVRQIRQIKEQGKTISNDDDVQKA
cRAC3 1:.....QGVMEKDSLGPLLLQALDGFLFVVNRDGNIVFVSENVTQYLQYK
xRAC3 101:DVSSTGQGVIDKDSLGPLLLQALDGFLYVVNREGSIVFVSENVTQYLQYK
hRAC3 99:DVSSTGQGVIDKDSLGPLLLQALDGFLFVVNRDGNIVFVSENVTQYLQYK
cRAC3 45:QEDLVNTSVYGILHEEDRKDFLKNLPKSSVNGVAWTNDAPRQKSHTFNCR
xRAC3 151:QEDLVNTSVYSILHEEDRKDFLKNLPKSTVNGVPWFSETPRQKSHTFNCR
hRAC3 149:QEDLVNTSVYNILHEEDRKDFLKNLPKSTVNGVSWTNETQRQKSHTFNCR
cRAC3 95:MMVKTSHDLLEDVGSHQDTRQRYETMQCFALSQPRAMMEEGEDLQSCMIC
xRAC3 201:MLVKTSHDHLED.GSNLDARQRYETMQCFALSQPRAMIEEGEDLQSCMIC
hRAC3 199:MLMKTPHDILEDINASPEMRQRYETMQCFALSQPRAMMEEGEDLQSCMIC
cRAC3 145:VARRIATAERVFPTNPESFITRHDLSGKLVNIDTNSLRSSMRPGFEDTIR
xRAC3 250:VARRITTAERAFSANPESFITRHDLTGKVVNIDANSLRSSMRPGFEDTIR
hRAC3 249:VARRITTGERTFPSNPESFITRHDLSGKVVNIDTNSLRSSMRPGFEDIIR
cRAC3 195:RCIQRFLCHNDGQSWSNKHHYHEAYTQGHAETPLYRFSLADGTIVTAQTK
xRAC3 300:RCIQRFLFHSEGQPWTYKRHYQEAYVHGLSETPLYRFSLADGTMVTAQTK
hRAC3 299:RCIQRFFSLNDGQSWSQKRHYQEAYLNGHAETPVYRFSLADGTIVTAQTK
cRAC3 245:SKVFRNPVTNDRHGFVSTHFLQREQNGYRPNPNTVGQNIRNNSGWKYNSV
xRAC3 350:SKLFRNPVTNDPHGFVSTHFLQREQNGYRPNPNPMAQGIR..PQMNPNLP
hRAC3 349:SKLFRNPVTNDRHGFVSTHFLQREQNGYRPNPNPVGQGIRPPMAGCNSSV
cRAC3 295:GLMNMSPGQGMPN.AKQDYGMGDPSTVGQLNSSRYGGPGNMGPVNSGPGM
xRAC3 398:NTMNSMPPQAMQQ.QNRNYGMGDPNSMAQMQGMRYKSPGNMAPVNQAPGV
hRAC3 399:GGMSMSPNQGLQMPSSRAYGLADPSTTGQMSGARYGGSSNIASLTPGPGM
cRAC3 344:QS.SAYQSNS.YGLNISSPPHGSPGLTSNQQNLMISTRNRGSPKMNSHQF
xRAC3 447:QQ.SPYQNNSNYGLNMNSPPHGSPGMNANQPNLMVSPRNRASPKMASNQF
hRAC3 449:QSPSSYQNNN.YGLNMSSRPHGSPGLAPNQQNIMISPRNRGSPKIASHQF
cRAC3 392:SPATGMHSPMGSASNTSNSTFSSSSLSALQAISEGVGTSLLSTLSSPGPK
xRAC3 496:SPVPGMNSPMGSSGNAGGGSFSSSSLSALHAISEGVGSSLLSSLSSPGQK
hRAC3 498:SPVAGVHSPMASSGNTGNHSFSSSSLSALQAISEGVGTSLLSTLSSPGPK
cRAC3 442:LDSSPNVSIAQQNKANNQDSKSPSGLYCEQNQVESSICQSNSRDVLSEKD
xRAC3 546:VENNSNMNMPQQGKICNQDCKSPSGLYCEQGQVESSVCQSSGREHLGEKD
hRAC3 548:LDNSPNMNITQPSKVSNQDSKSPLGFYCDQNPVESSMCQSNSRDHLSDKE


cRAC3 492:SKDGSLDASESQRGQSESKGHKKLLQLLTCSSDERGHSTASNSPLDSNCK
xRAC3 596:VKENIFEGSESQRSQAESKGHKKLLQLLTCFTEERGQSLMSSSSMD..CK
hRAC3 598:SKESSVEGAENQRGPLESKGHKKLLQLLTCSSDDRGHSSLTNSPLDSSCK
cRAC3 542:ESSTNVTSPSGVSSSTSGGVSSSSNVHGSLLQEKHRILHKLLQNGNSPAE
xRAC3 644:DSS.NVTSPSGVSSSTSIGVSSTSNLHGSMLQEKHRILHKLLQNGNSPAE
hRAC3 648:ESSVSVTSPSGVSSSTSGGVSSTSNMHGSLLQEKHRILHKLLQNGNSPAE
cRAC3 592:VAKITAEATGKDTYHDTSNTVPCGESTVKQEQLSPKKKENNALLRYLLDK
xRAC3 693:VAKITAEATGKDVFQETVSSAPCTEATVKREQLSPKKKENNALLRHLLDK
hRAC3 698:VAKITAEATGKDTSSITSCGD...GNVVKQEQLSPKKKENNALLRYLLDR
cRAC3 642:DDVKDPLSKKLKPKVENVDNKMGQCSSTTIPTSSQEKEMKIKTEPTEEMS
xRAC3 743:DDWKDPLAKDIKPKVEHMDIKMGSCSSSNVPTSSQDKEVKIKTEPGEEVP
hRAC3 745:DDPSDALSKELQPQVEGVDNKMSQCTSSTIPSSSQEKDPKIKTETSEEGS
cRAC3 692:GDLDNLDAILGDLTSSDFYNNAMSTSGNNFGMKQALFQGNASLTGMRSPQ
xRAC3 793:GDLDNLDAILGDLAGSDFYSNSMSSRASDLGPKQPVFQDSPTLA.MRSPD
hRAC3 795:GDLDNLDAILGDLTSSDFYNNSISSNGSHLGTKQQVFQGTNSLG.LKSSQ
cRAC3 742:SVQAARPPFNRAMSLDSPVG..SGASVRNVNAFSMLQKQNLMGGSPRMTE
xRAC3 842:SMQGSRPPFNRAMSLDSRS...STPPVRNVNSFPMLPKQGMIG.SPRMMD
hRAC3 844:SVQSIRPPYNRAVSLDSPVSVGSSPPVKNISAFPMLPKQPMLGGNPRMMD
cRAC3 790:NQESFGANIASTPSRGVSMNQHQS.GDWGLPNSKVNRLEPTSSTSMLRQG
xRAC3 888:GQDNFGVMMGSGPNR..SMNQHPG.GDWAMQNSAVNPLEPPNVGSVGRPG
hRAC3 894:SQENYGSSMG.GPNRNVTVTQTPSSGDWGLPNSKAGRMEPMNSNSMGRPG
cRAC3 839:AEFSASLPRPTAGGSMPGLPVRSNSIPGTRPMLQQQ..MMHMRSNEINIG
xRAC3 935:PDYSSAMTRPAMGGNMPGLLTRSNSIPGSRPVMQQQQHILPMRPNDMAMS
hRAC3 943:GDYNTSLPRPALGGSIPTLPLRSNSIPGARPVLQQQQQMLQMRPGEIPMG
cRAC3 887:MGGNPYGQTGPTSQPGSWPDSMLSMEQASRGLQNRQLVRNSLDDLLSAAP
xRAC3 985:MGSNPYGQQAPSNPPGSWPDAIM.MNQGRGGAQNRQLGRNSLDDLLCPPS
hRAC3 993:MGANPYGQAAASNQLGSWPDGMLSMEQVSHGTQNRPLLRNSLDDLVGPPS
cRAC3 937:SVEGQNDERALLDQLHTLLSNTDVTSLEEIDRALGIPDLVNQGQTLEPKQ
xRAC3 1034:TVEGQTDEIALLDQLHTLLSNTDATGLEEIDRALGIPDLVSQGQALEPQP
hRAC3 1043:NLEGQSDERALLDQLHTLLSNTDATGLEEIDRALGIPELVNQGQALEPKQ
cRAC3 987:DSFQGQESTVMMDQKPSLYGQPYQGQGTALPGG.FNNIQGQQPSFNSVMN
xRAC3 1084:DSYQPQGSPVMIDQKPPMYGQHYAGQGAAMSAGGFNNMQGQHPPFNTVMG
hRAC3 1093:DAFQGQEAAVMMDQKAGLYGQTYPAQGPPMQGG..FHLQGQSPSFNSMMN
cRAC3 1036:QMSQQN.NFPLPSMHPRANAMRPRTNTPKQLRMQLQQRLQGQQFMNQTRQ
xRAC3 1134:QMNQQQGMHPLQGMHPRANLIRPRNNIPKQLRMQLQQRLQGQQFLNQNRQ
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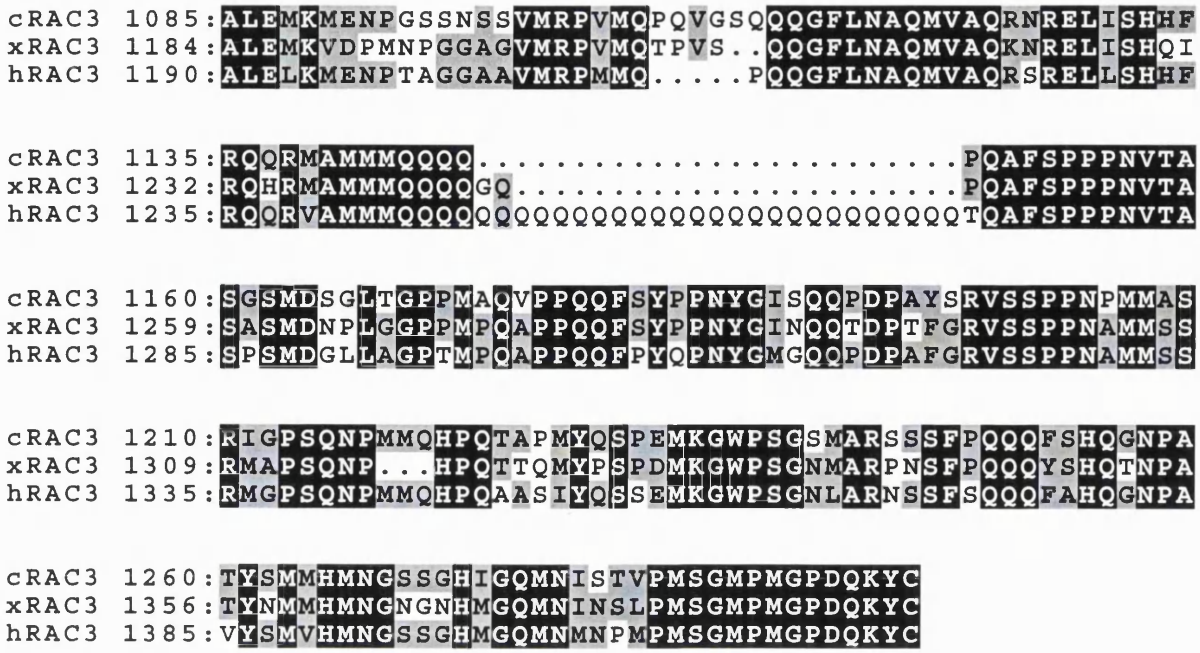

cRAC3 1085:ALEMKMENPGSSNSSVMRPVMQPQVGSQQQGFLNAQMVAQRNRELISHHF
xRAC3 1184:ALEMKVDPMNPGGAGVMRPVMQTPVS..QQGFLNAQMVAQKNRELISHQI
hRAC3 1190:ALELKMENPTAGGAAVMRPMMQ.....PQQGFLNAQMVAQRSRELLSHHF
cRAC3 1135:RQQRMAMMMQQQQ.....PQAFSPPPNVTA
xRAC3 1232:RQHRMAMMMQQQQGQ.....PQAFSPPPNVTA
hRAC3 1235:RQQRVAMMMQQQQQQQQQQQQQQQQQQQQQQQQQQTQAFSPPPNVTA
cRAC3 1160:SGSMDSGLTGPPMAQVPPQQFSYPPNYGISQQPDPAYSRVSSPPNPMMAS
xRAC3 1259:SASMDNPLGGPPMPQAPPQQFSYPPNYGINQQTDPTFGRVSSPPNAMMSS
hRAC3 1285:SPSMDGLLAGPTMPQAPPQQFPYQPNYGMGQQPDPAFGRVSSPPNAMMSS
cRAC3 1210:RIGPSQNPMMQHPQTAPMYQSPEMKGWPSGSMARSSSFPQQQFSHQGNPA
xRAC3 1309:RMAPSQNP...HPQTTQMYPSPDMKGWPSGNMARPNSFPQQQYSHQTNPA
hRAC3 1335:RMGPSQNPMMQHPQAASIYQSSEMKGWPSGNLARNSSFSQQQFAHQGNPA
cRAC3 1260:TYSMMHMNGSSGHIGQMNISTVPMSGMPMGPDQKYC
xRAC3 1356:TYNMMHMNGNGNHMGQMNINSLPMSGMPMGPDQKYC
hRAC3 1385:VYSMVHMNGSSGHMGQMNMNPMPMSGMPMGPDQKYC

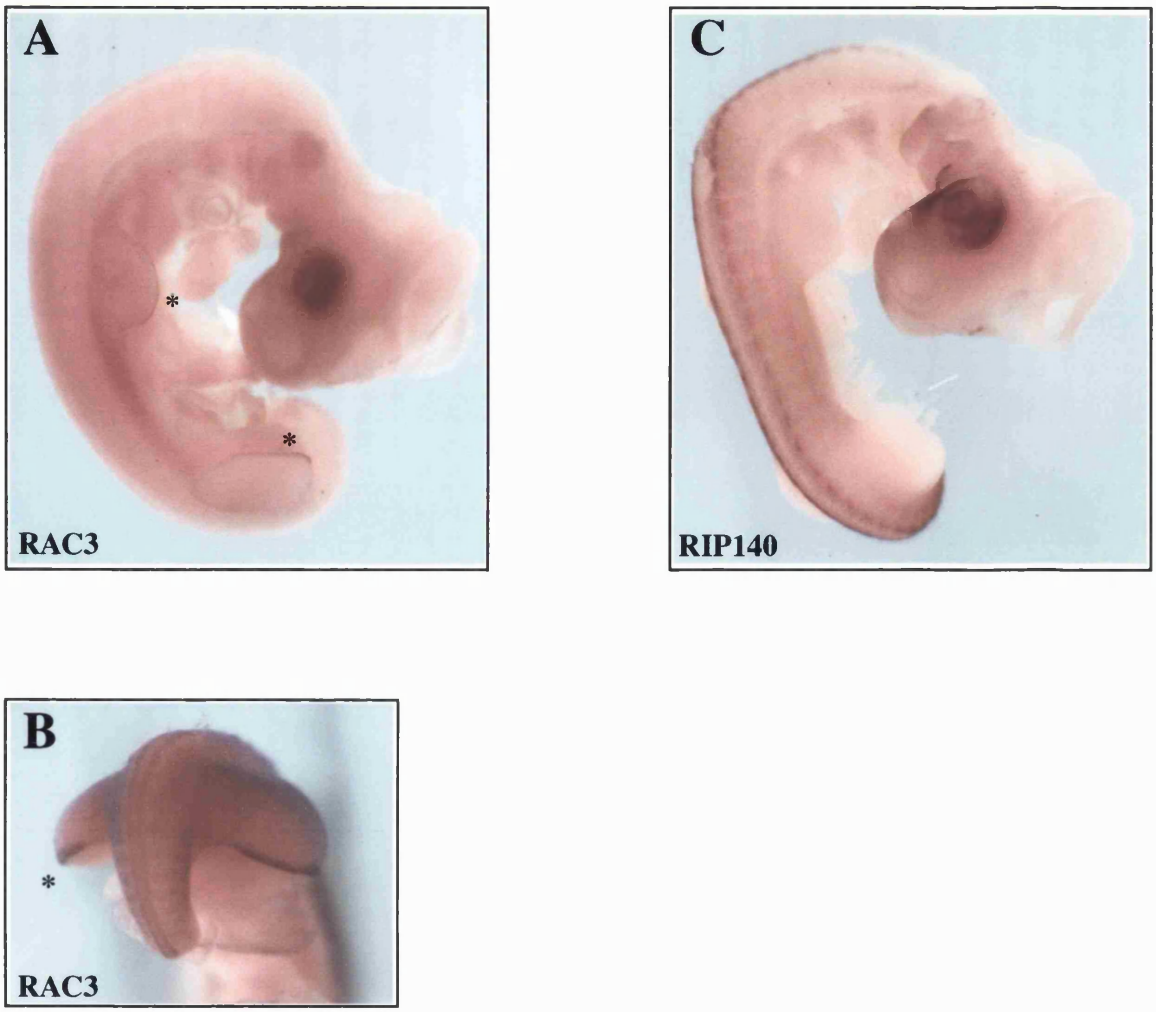


Figure AI.2 Localisation of RAC3 and RIP140 mRNA transcripts in chicken embryos. In situ hybridisation was carried out using digoxigenin (DIG) labelled anti-sense RNA probes on 2.5 to 3 days old chicken embryos. The signals were detected with an alkaline phosphatase conjugated anti-DIG antibody and the chromogenic substrate mix of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) plus 4-nitroblue tetrazolium chloride (NBT). The same patterns of expression were detected using probes derived from multiple regions of the cDNA clones. Expression of cRAC3 is shown in panel (A) with the distinctive signal at the apical ectodermal ridge (AER) of the limb buds (marked with asterisks). A close-up view of the hindlimb buds is presented in panel (B) which shows strong signals at the AER. Notably, the expression pattern of cRAC3 is clearly different from that of cRIP140, another NR interacting protein, as shown in panel (C).

Figure AI.3 Sequence comparison of chicken and human SRC1 and TIF2. Alignment of the primary amino acid sequences of chicken and human SRC1 (A) and TIF2 (B). The numbering for the chicken sequences is arbitrary. Black boxes indicate identical residues, and grey boxes similar residues in both chicken and human sequences.

Conserved residues in both SRC1 and TIF2 (numbering as in human SRC1)	Sequence of degenerate oligonucleotides
[1105] NAQMLAQ	5'-AAYGCICARATGYTIGCNCA-3'
[1353] PEQVNDPA	5'-GCIGGRTCRTTIACYTGYTCNGG-3'

R=A or G Y=C or T N=A,G,T or C I=inosine

A

```
cSRC1     1:NAQMLAQRQRELYSQQHRQRQLMQQ.RAILMRQQSFGNNLPPSAGLPVQM
hSRC1  1105:NAQMLAQRQRELYSQQHRQRQLIQQQRAMLMRQQSFGNNLPPSSGLPVQM

cSRC1    50:GAARLPQAPPQQFPYPPNYGTSPGNPPTSTSPFSQLASSPEAALANRGGM
hSRC1  1155:GNPRLPQGAPQQFPYPPNYGTNPGTPPASTSPFSQLAANPEASLANRNSM

cSRC1   100:VTRGMMGSVGGQFGTGMTPQTQQNIFQYSGSGMGQQNEPTFAPSLSPSSP
hSRC1  1205:VSRGMTGNIGGQFGTGINPQMQQNVFQYPGAGMVPQGEANFAPSLSPGSS

cSRC1   150:LMSPQLPPSQSPMLQPAPPAPGYQSPDMKTWQQGAMGNSNVFSQTGQTQP
hSRC1  1255:MVPMPIPPPQSSLLQQTPPASGYQSPDMKAWQQGAIGNNNVFSQAVQNQP

cSRC1   200:APAQQGMYNNMSITVSMAGGNTNVQNMNPMAGQMQMNSLQMPGMNSMCPE
hSRC1  1305:TPAQPGVYNNMSITVSMAGGNTNVQNMNPMMAQMQMSSLQMPGMNTVCPE

cSRC1   250:QVNDP
hSRC1  1355:QINDP
```

B

```
cTIF2     1:NAQMLAQRQREILSQHLRQRQMQQQQQQVQQRTLMMRGQGLSMTPSMVAT
hTIF2  1231:NAQMLAQRQREILNQHLRQRQMHQQQQ.VQQRTLMMRGQGLNMTPSMVAP

cTIF2    51:GGIPATMSNPRIPQANAQQFPSPPNYGISQQPDPGFTGATTPQSPLMSPR
hTIF2  1280:SGMPATMSNPRIPQANAQQFPFPPNYGISQQPDPGFTGATTPQSPLMSPR

cTIF2   101:LAHTQSPMIQQTQANPAYQSSTEMNGWAQGNMGGNSMFSQQSPPHFGQQA
hTIF2  1330:MAHTQSPMMQQSQANPAYQAPSDINGWAQGNMGGNSMFSQQSPPHFGQQA

cTIF2   151:STSMYNNNMNINVSMATNTSGMNNMNQMTGQISMTSATSVPTSGLSSMGP
hTIF2  1380:NTSMYSNNMNINVSMATNTGGMSSMNQMTGQISMTSVTSVPTSGLSSMGP

cTIF2   201:EQVNDP
hTIF2  1430:EQVNDP
```

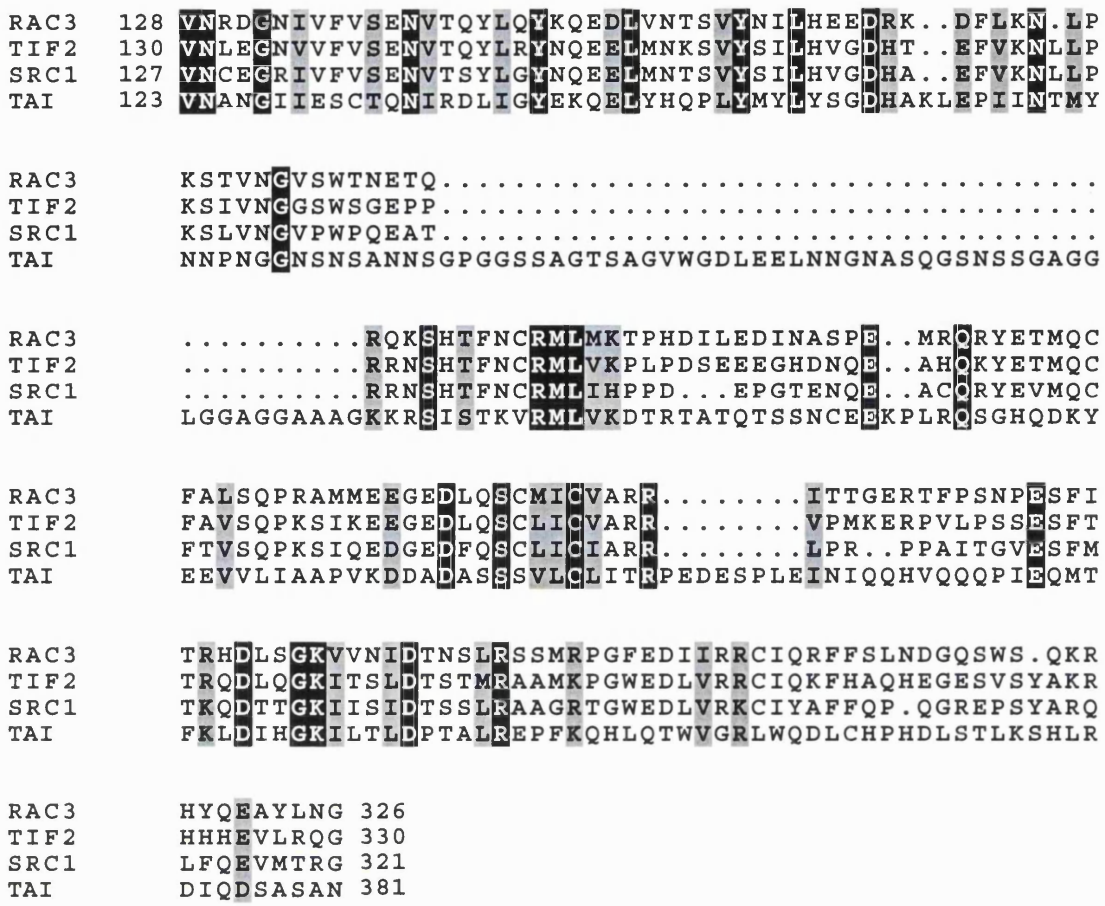
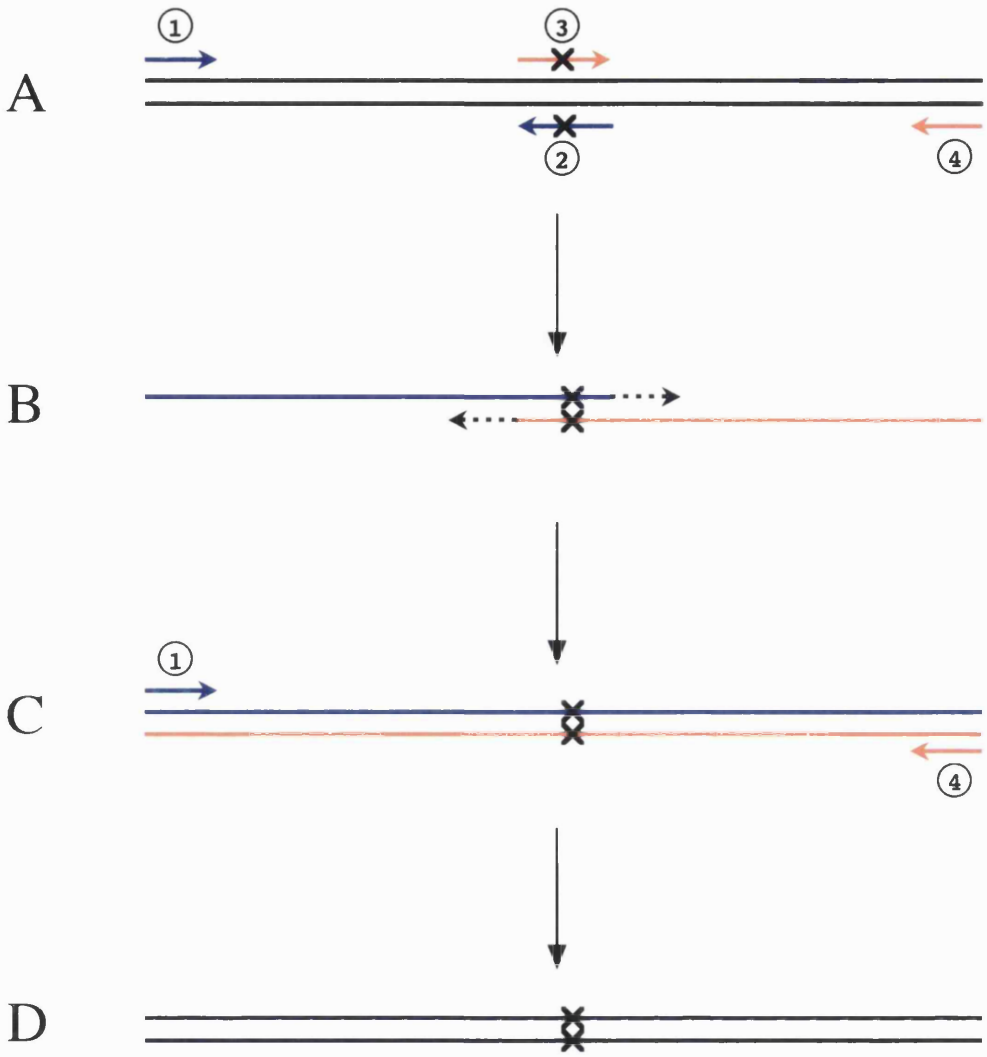


Figure AI.4 Sequence comparison of human p160 coactivators with their putative Drosophila homolog (TAI). Sequences of the PAS domain are aligned. Black boxes indicate identical residues, and grey boxes similar residues in all four sequences.

Appendix II

Plasmids construction



AII.1 Scheme for recombinant PCR. (A) Two independent PCR reactions were set up using primers 1+2 and primers 3+4. Primers 2 and 3 contained the targeted mutation (marked with crosses). (B) Purified products of the first PCR reaction were allowed to anneal. Addition of dNTPs and DNA polymerase facilitated extension from the 3' end of the first PCR products. Three temperature cycles of (94°C 1 min; 40°C 2 mins; 72°C 2 mins) with a gradual decrease in temperature from 94°C to 40°C in 3 mins were performed. (C) Extended DNA fragments from (B) were subjected to PCR reactions using primers 1+4. (D) Amplifed double-stranded DNA fragment containing the targeted mutation.

Figure AII.2 Construction of pSG-Gal

The cDNA encoding Gal4 DNA binding domain (aa 1 to 147) was amplified by PCR using primers Gal4N and Gal4C and the vector pSG424 as template. The PCR product was then digested with EcoRV and BglII. The vector pSG5 was first digested with EcoRI and the 3' recessed ends subsequently filled in using the Klenow enzyme. The treated vector was further digested with BglII and dephosphorylated using the calf intestinal alkaline phosphatase (CIP). This was then used for subcloning the digested Gal4 DBD PCR product. The recombinants were verified by DNA sequencing.

Gal4N (5' primer)	5'-CACGATATCAAGCTTCCTGAGATGAAGCTACTGTCTTCT-3'
Gal4C (3' primer)	5'-ATAGATCTGGTACCGTCCGCGGATCCCCG-3'

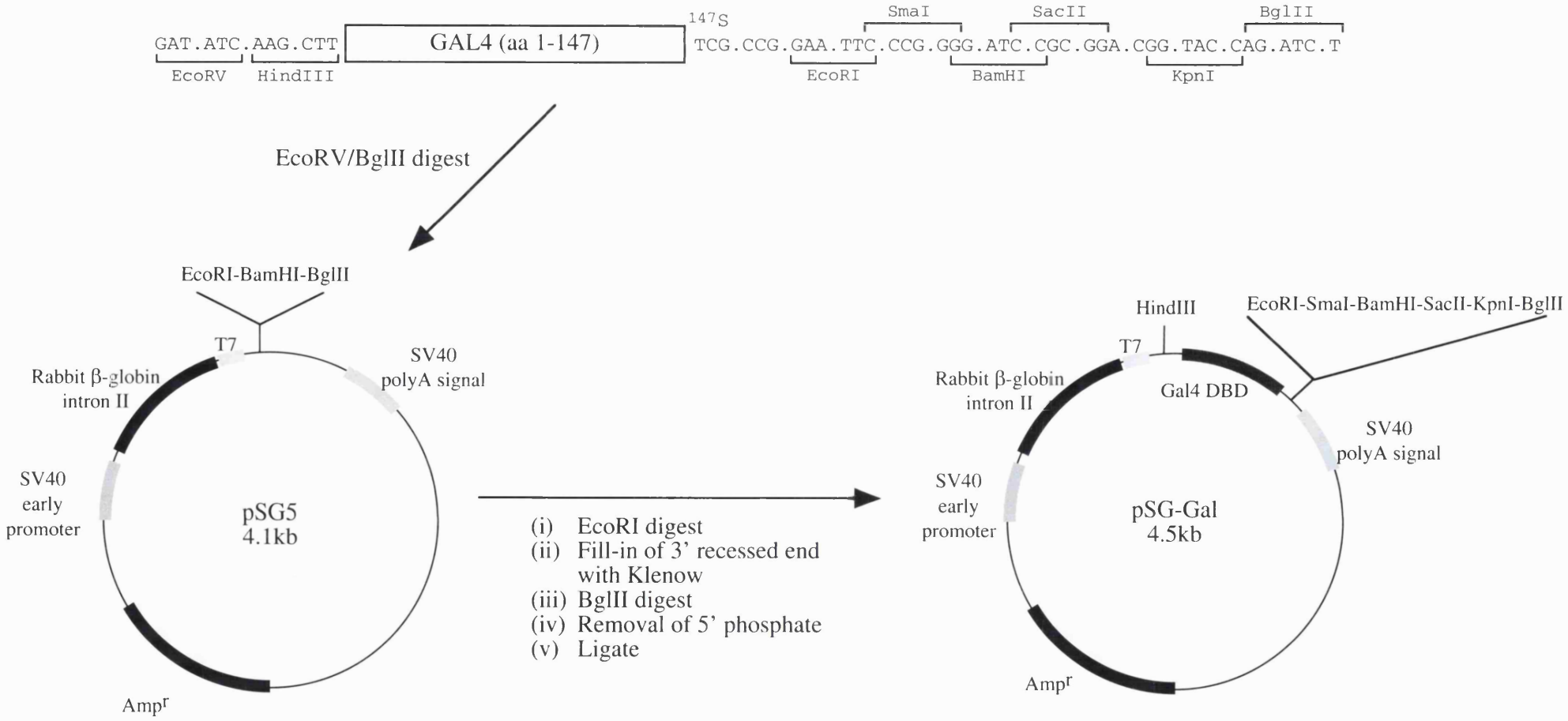

GAT.ATC.AAG.CTT
EcoRV
HindIII
GAL4 (aa 1-147)
147S
TCG.CCG.GAA.TTC.CCG.GGG.ATC.CGC.GGA.CGG.TAC.CAG.ATC.T
EcoRI
SmaI
SacII
BamHI
KpnI
BglII
EcoRV/BglII digest
EcoRI-BamHI-BglII
T7
Rabbit β-globin intron II
SV40 polyA signal
SV40 early promoter
pSG5 4.1kb
Amp^r
(i) EcoRI digest
(ii) Fill-in of 3' recessed end with Klenow
(iii) BglII digest
(iv) Removal of 5' phosphate
(v) Ligate
HindIII
EcoRI-SmaI-BamHI-SacII-KpnI-BglII
T7
Rabbit β-globin intron II
Gal4 DBD
SV40 polyA signal
SV40 early promoter
pSG-Gal 4.5kb
Amp^r

Figure AII.3 Construction of pSG5 MORK

pSG5 was digested with EcoRI and treated with CIP. The cDNA encoding full length mERα was obtained by EcoRI digestion of pSP65 MORK (Fawell *et al.*, 1990). This cDNA contains silent mutations (^{V537}GTT.GTG.CCC→ GT*G*.*GTA*.*CCC*;) which introduce a unique KpnI site (mutated bases are underlined and the KpnI recognition site is in italics). MORK stands for mouse oestrogen receptor KpnI. The pSG5 MORK recombinants were verified by DNA sequencing.

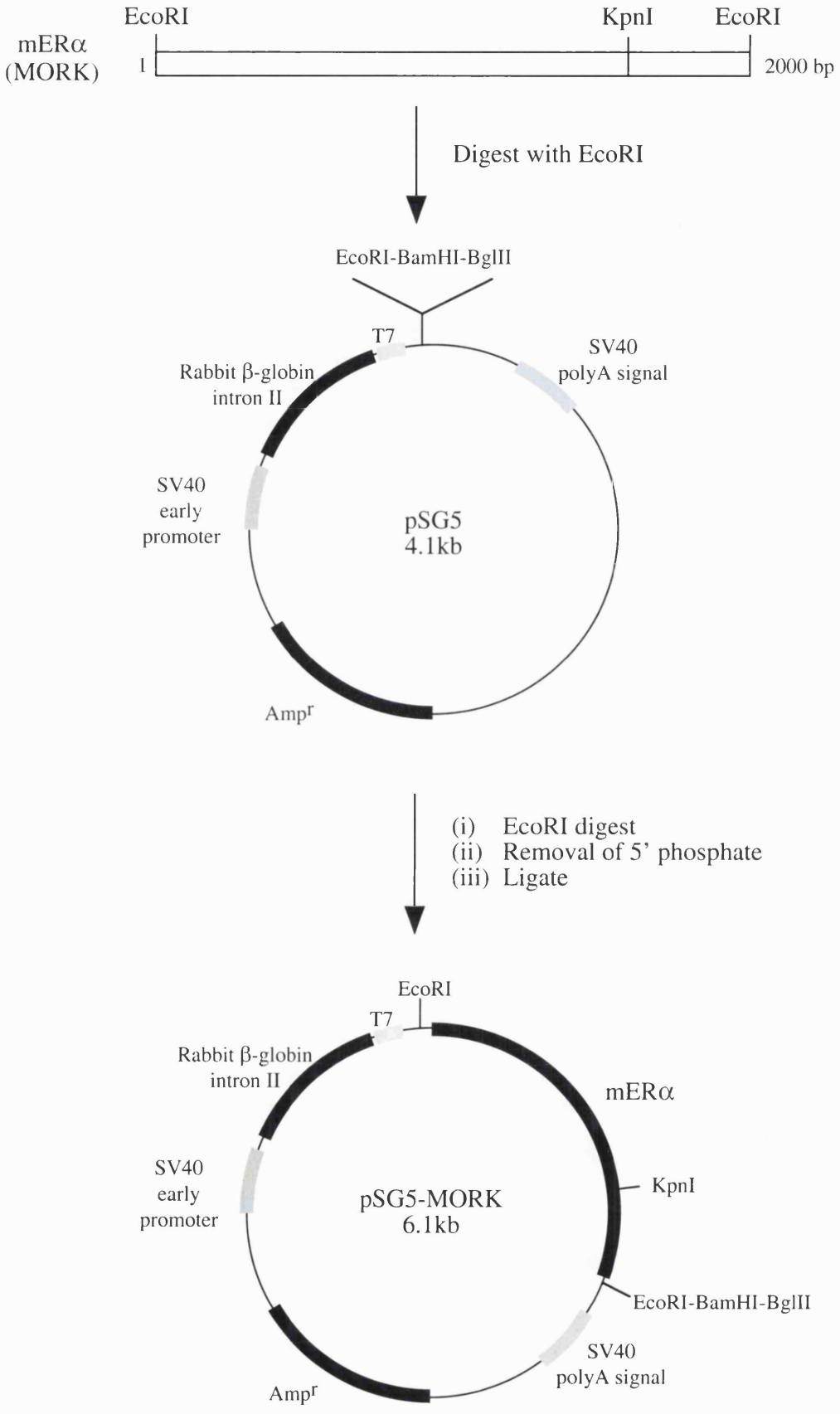

EcoRI
KpnI
EcoRI
mERα
(MORK)
1
2000 bp
Digest with EcoRI
EcoRI-BamHI-BglII
T7
SV40
polyA signal
Rabbit β-globin
intron II
SV40
early
promoter
pSG5
4.1kb
Amp^r
(i) EcoRI digest
(ii) Removal of 5' phosphate
(iii) Ligate
EcoRI
T7
Rabbit β-globin
intron II
mERα
SV40
early
promoter
pSG5-MORK
6.1kb
KpnI
EcoRI-BamHI-BglII
SV40
polyA signal
Amp^r

Figure AII.4 Construction of pSG5 MORK mutants

Point mutations in helices 3 and 5 of mERα were introduced by recombinant PCR. The PCR products were digested with NdeI and BglII and subcloned into pSP65 MORK digested with the same enzymes. cDNA encoding the full length mutant receptors were subcloned into the mammalian expression vector pSG5 as an EcoRI fragment. All recombinants were verified by DNA sequencing.

Outer primers	
MOR3 (5' primer)	5'-CGACGCCAGAATGGCCGAGAGAGACTG-3'
MOR4 (3' primer)	5'-TAGAGGGGCACAACGTTCTTGCATTTC-3'
Mutagenic primers	(only the sense strand is shown below, mutated codons are underlined)
I362+	5'-GAGCTGGTTCATATGVVTAACTGGGCAAAGAG-3'
V380+	5'-AATCTCCATGATCAGVVTCACCTTCTCGAGTG-3'
L376A+	5'-CTTGGGGACTTGAATGCCCATGATCAGGTCC-3'
L376D+	5'-TTGGGGACTTGAATGACCATGATCAGGTCC-3'
K366LR+	5'-ATGATCAACTGGGCACKGAGAGTGCCAGGCT-3'

V=A, C or G K=G or T

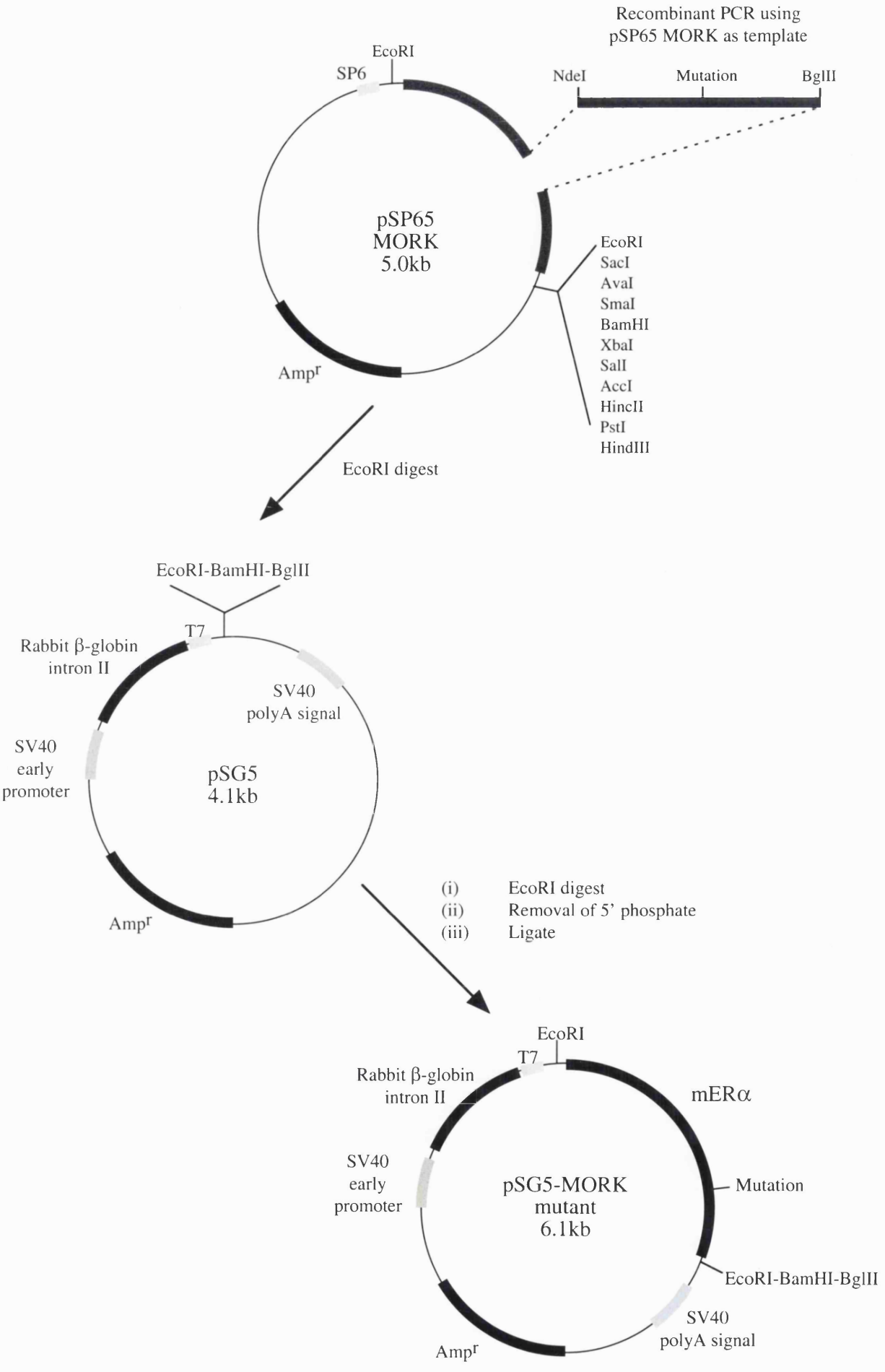

Recombinant PCR using
pSP65 MORK as template
EcoRI
SP6
NdeI
Mutation
BglII
pSP65
MORK
5.0kb
Amp^r
EcoRI
SacI
AvaI
SmaI
BamHI
XbaI
SalI
AccI
HincII
PstI
HindIII
EcoRI digest
EcoRI-BamHI-BglII
T7
Rabbit β-globin
intron II
SV40
polyA signal
SV40
early
promoter
pSG5
4.1kb
Amp^r
(i) EcoRI digest
(ii) Removal of 5' phosphate
(iii) Ligate
EcoRI
T7
Rabbit β-globin
intron II
mERα
SV40
early
promoter
pSG5-MORK
mutant
6.1kb
Mutation
EcoRI-BamHI-BglII
SV40
polyA signal
Amp^r

Figure AII.5 Construction of pSG-Gal MORK LBD and pSG-VP16 MORK LBD

The cDNA encoding mERα LBD (aa 313 to 599) was amplified by PCR using primers MOR2 and MOR6 and pSG5 MORK as template. The PCR products were digested with EcoRI and BamHI and subcloned into pSG-Gal or pSG-VP16 (Butler and Parker, 1995) digested with EcoRI and BglII. Hence the BglII site of the vector was destroyed upon ligation. All recombinants were verified by DNA sequencing.

MOR6 (5' primer)	5'-TATGAATTCTCCTTGACAGCTGACC-3'
MOR2 (3' primer)	5'-TAGGATCCTCAGATCGTGTTGGGGAA-3'

Restriction sites in the primers are underlined.

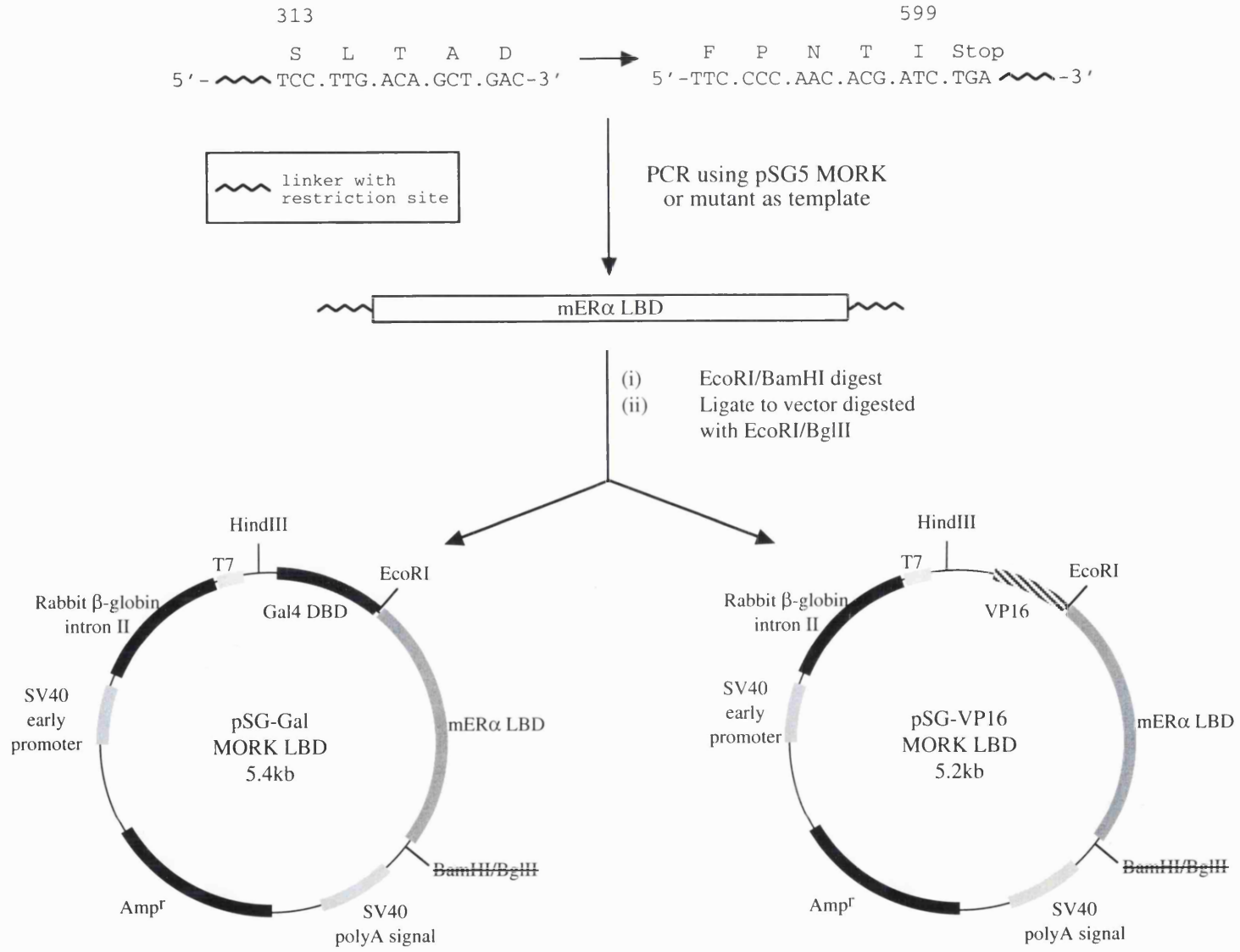

313
599
S L T A D
F P N T I Stop
5'-TCC.TTG.ACA.GCT.GAC-3'
5'-TTC.CCC.AAC.ACG.ATC.TGA-3'
linker with restriction site
PCR using pSG5 MORK or mutant as template
mERα LBD
(i) EcoRI/BamHI digest
(ii) Ligate to vector digested with EcoRI/BglII
HindIII
T7
EcoRI
Rabbit β-globin intron II
Gal4 DBD
SV40 early promoter
pSG-Gal MORK LBD 5.4kb
mERα LBD
BamHI/BglII
Ampr
SV40 polyA signal
HindIII
T7
EcoRI
Rabbit β-globin intron II
VP16
SV40 early promoter
pSG-VP16 MORK LBD 5.2kb
mERα LBD
BamHI/BglII
Ampr
SV40 polyA signal

Figure AII.6 Construction of pBTM116 MORK LBD and pGAD424 MORK LBD

The cDNA encoding mERα LBD (aa 313 to 599) was amplified by PCR using pSG5 MORK as template. For pBTM116 MORK LBD, primers MOR1 and MOR2 were used. The PCR products were digested with SacII/BamHI and subcloned into pBTM116 (Vojtek *et al.*, 1993) digested with the same enzymes. For pGAD424 MORK LBD, primers MOR2 and MOR6 were used. The PCR products were digested with EcoRI/BamHI and subcloned into pGAD424 (Clontech) digested with the same enzymes. All recombinants were verified by DNA sequencing.

MOR1 (5' primer)	5'-AATACCGCGGTCCTTGACAGCTGAC-3'
MOR6 (5' primer)	5'-TATGAATTCTCCTTGACAGCTGACC-3'
MOR2 (3' primer)	5'-TAGGATCCTCAGATCGTGTTGGGGAA-3'

Restriction sites in the primers are underlined.

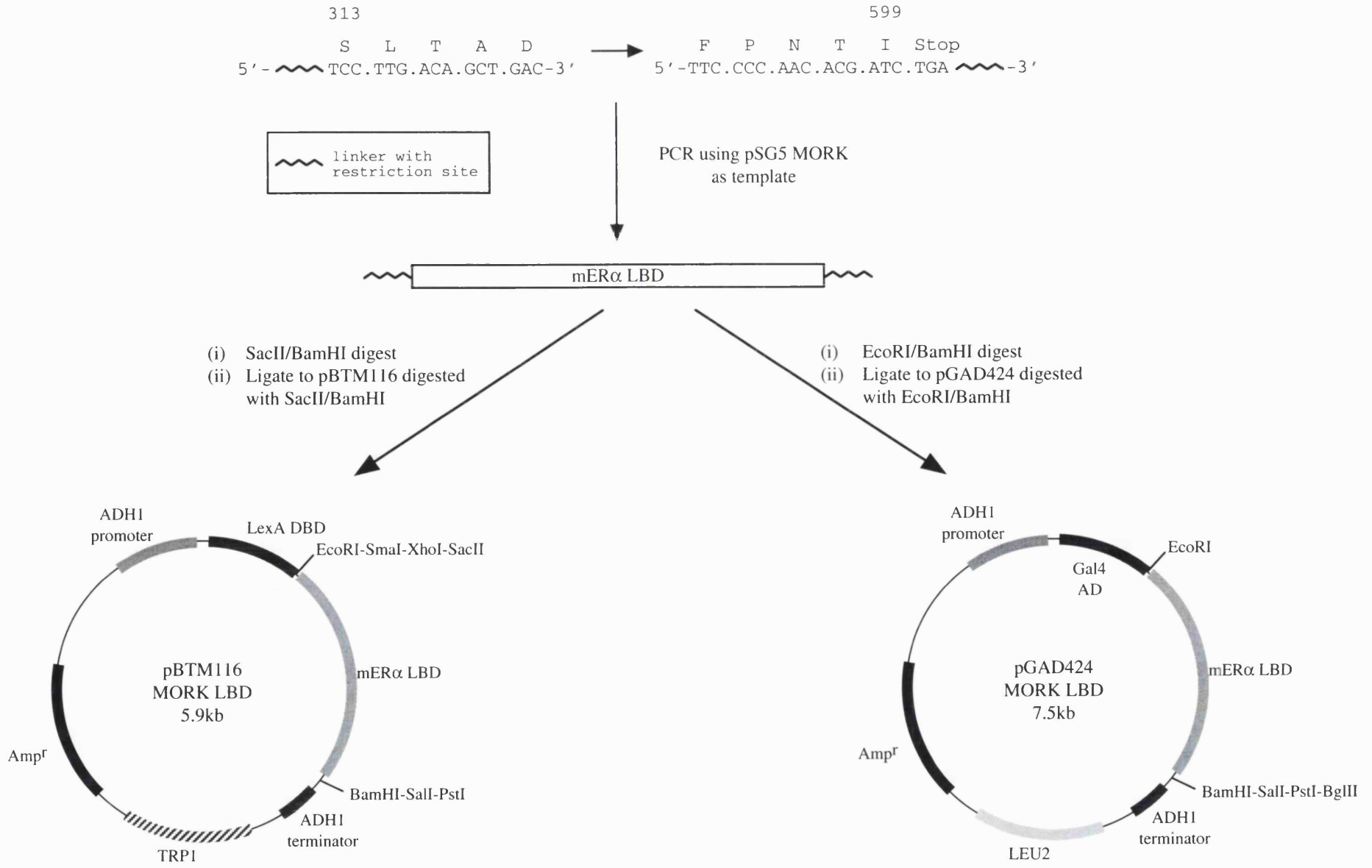

313
599
S L T A D
F P N T I Stop
5'-TCC.TTG.ACA.GCT.GAC-3'
5'-TTC.CCC.AAC.ACG.ATC.TGA-3'
linker with restriction site
PCR using pSG5 MORK as template
mERα LBD
(i) SacII/BamHI digest
(ii) Ligate to pBTM116 digested with SacII/BamHI
(i) EcoRI/BamHI digest
(ii) Ligate to pGAD424 digested with EcoRI/BamHI
ADH1 promoter
LexA DBD
EcoRI-SmaI-XhoI-SacII
mERα LBD
BamHI-SalI-PstI
ADH1 terminator
TRP1
Ampr
pBTM116
MORK LBD
5.9kb
ADH1 promoter
EcoRI
Gal4 AD
mERα LBD
BamHI-SalI-PstI-BglII
ADH1 terminator
LEU2
Ampr
pGAD424
MORK LBD
7.5kb

Figure AII.7 Construction of pSG FLAG SRC1e m13 and pSG FLAG SRCX1e m13

A SmaI/CelII restriction fragment from pSG FLAG SRC1e was subcloned into pSG5 SRC1e m13 digested with SmaI and CelII. As a result, a FLAG epitope tag is placed at the N-terminus of full length SRC1e m13. The pSG FLAG SRC1e m13 recombinants were verified by sequencing.

By recombinant PCR, silent mutations were introduced into pSG FLAG SRC1e m13 in order to generate a unique XhoI site 5' to the coding sequence of LXXLL motif 2. The PCR products were digested with EcoRI/BamHI and subcloned into pSG FLAG SRC1e m13, which has been prepared by BamHI/partial EcoRI digestion. The pSG FLAG SRCX1e m13 recombinants were verified by sequencing.

Outer primers	
SRC1 (5' primer)	5'-GAGACAGGTTACTTCTGGATTGGCAACAAG-3'
SRC2 (3' primer)	5'-TCTTCTGATTTACTCTGATTTATAGCTGTC-3'
Mutagenic primer	(only the sense strand is shown below, mutated bases are underlined)
SRCX1	5'-GAGGTTCTTGTCCCTCTTCTCACTCGAGCTT GACAGAACGGCATAAAATTC-3'

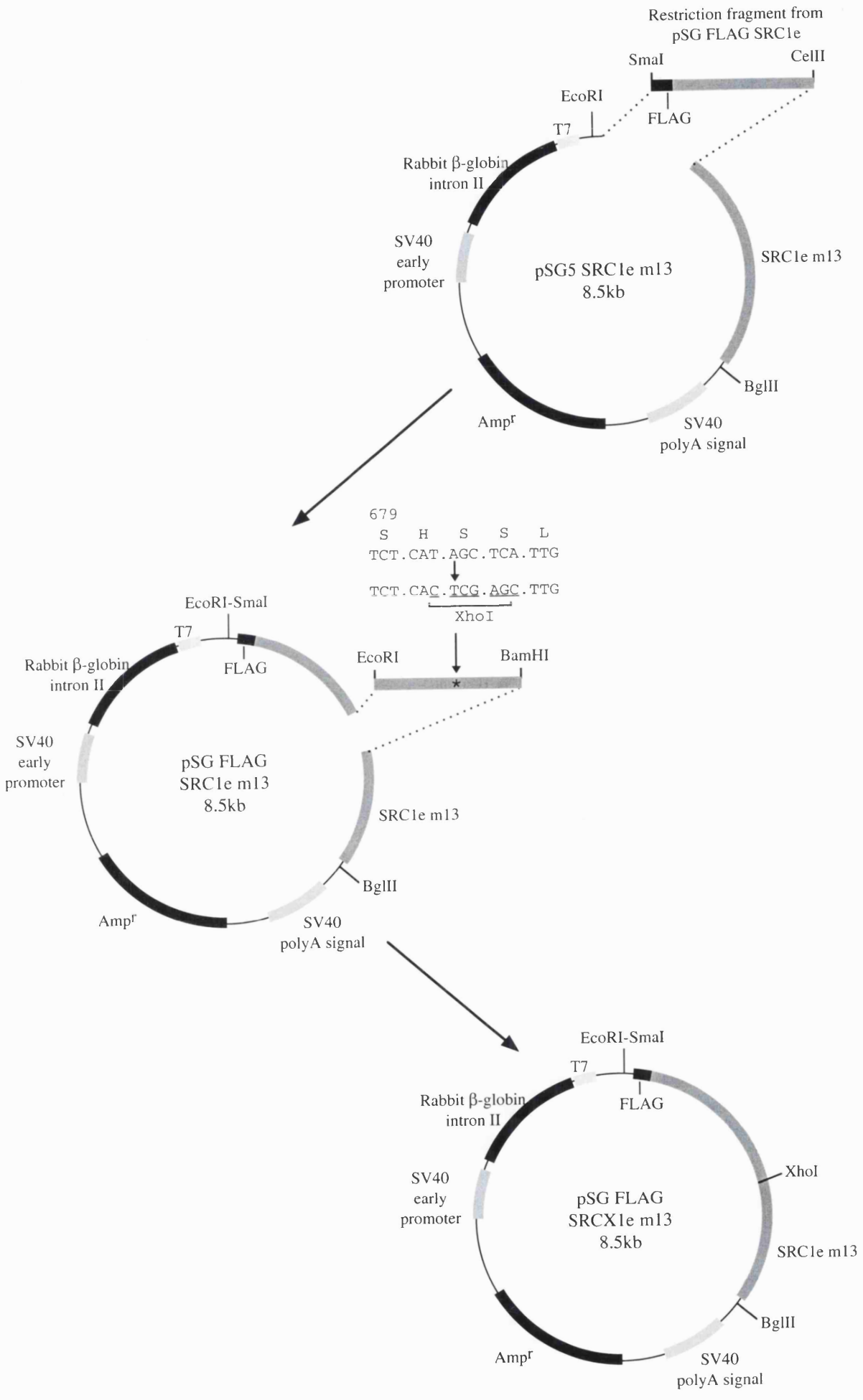

Restriction fragment from
pSG FLAG SRC1e
SmaI
CelII
EcoRI
FLAG
T7
Rabbit β-globin
intron II
SV40
early
promoter
pSG5 SRC1e m13
8.5kb
SRC1e m13
BglII
Ampr
SV40
polyA signal
679
S H S S L
TCT.CAT.AGC.TCA.TTG
TCT.CAC.TCG.AGC.TTG
XhoI
EcoRI-SmaI
T7
FLAG
EcoRI
BamHI
Rabbit β-globin
intron II
SV40
early
promoter
pSG FLAG
SRC1e m13
8.5kb
SRC1e m13
BglII
Ampr
SV40
polyA signal
EcoRI-SmaI
T7
FLAG
Rabbit β-globin
intron II
SV40
early
promoter
pSG FLAG
SRCX1e m13
8.5kb
XhoI
SRC1e m13
BglII
Ampr
SV40
polyA signal

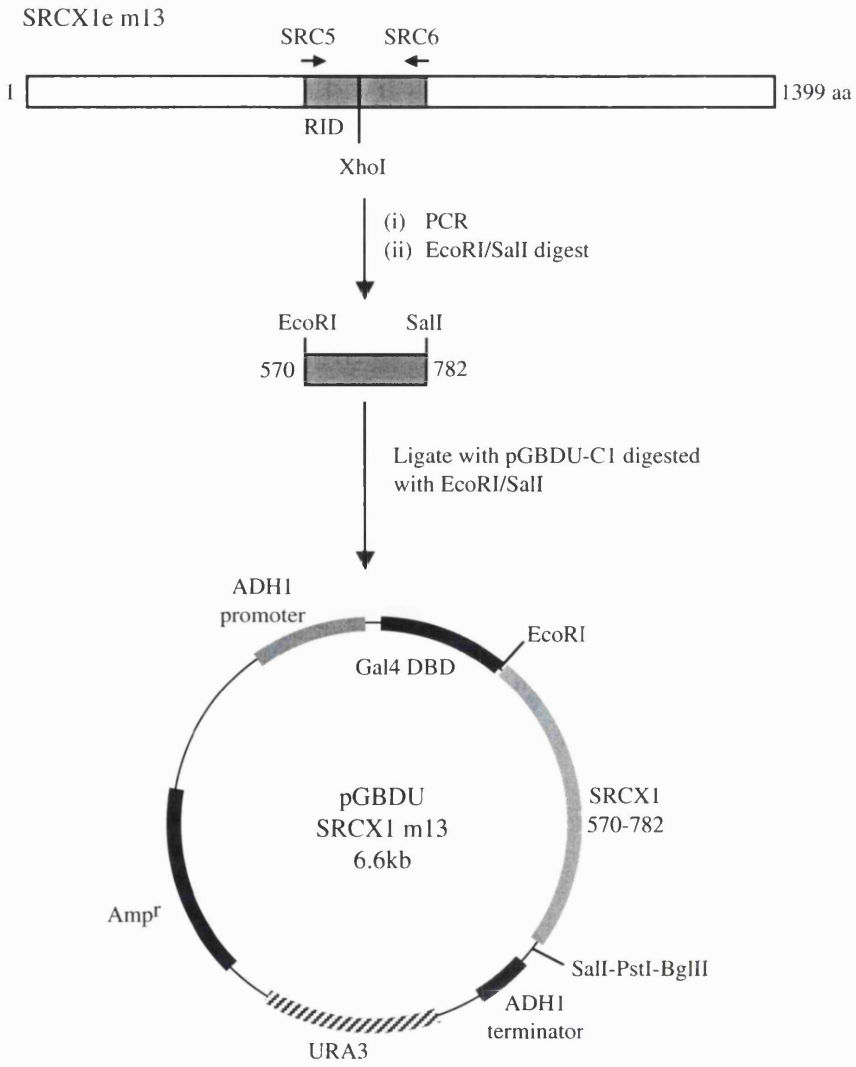
Figure AII.8 Construction of pGBDU SRCX1 m13 and pSG-Gal SRC1

In order to construct pGBDU SRCX1 m13, cDNA encoding the receptor interacting domain (RID) was amplified by PCR using primers SRC4 and SRC5 and pSG5 SRCX1e m13 as template. The PCR products were digested with EcoRI/SalI and subcloned into pGBDU-C1 (James *et al.*, 1996) digested with the same enzymes. The recombinants were verified by sequencing.

For pSG-Gal SRC1, cDNA encoding the receptor interacting domain (RID) was amplified by PCR using primers SRC5 and SRC39 (Eric Kalkhoven) and pSG5 SRC1e as template. The PCR products were digested with EcoRI/BamHI and subcloned into pSG-Gal digested with EcoRI/BglII. As a result, the BglII restriction site of the vector was destroyed upon ligation. The pSG-Gal SRC1 recombinants were verified by sequencing.

SRC5 (5' primer)	5'-CTAGAATTCCCTAGCAGATTAAATATAC-3'
SRC4 (3' primer)	5'-TAATGTCGACTAATCCATCTGTTCTTTC-3'
SRC39 (3' primer)	5'-AGCGTGGGCAGTAACTGATC-3'

Restriction sites in the primers are underlined.


SRCX1e m13
SRC5
SRC6
1
1399 aa
RID
XhoI
(i) PCR
(ii) EcoRI/SalI digest
EcoRI
SalI
570
782
Ligate with pGBDU-C1 digested
with EcoRI/SalI
ADH1
promoter
EcoRI
Gal4 DBD
pGBDU
SRCX1 m13
6.6kb
SRCX1
570-782
Amp^r
SalI-PstI-BglII
ADH1
terminator
URA3

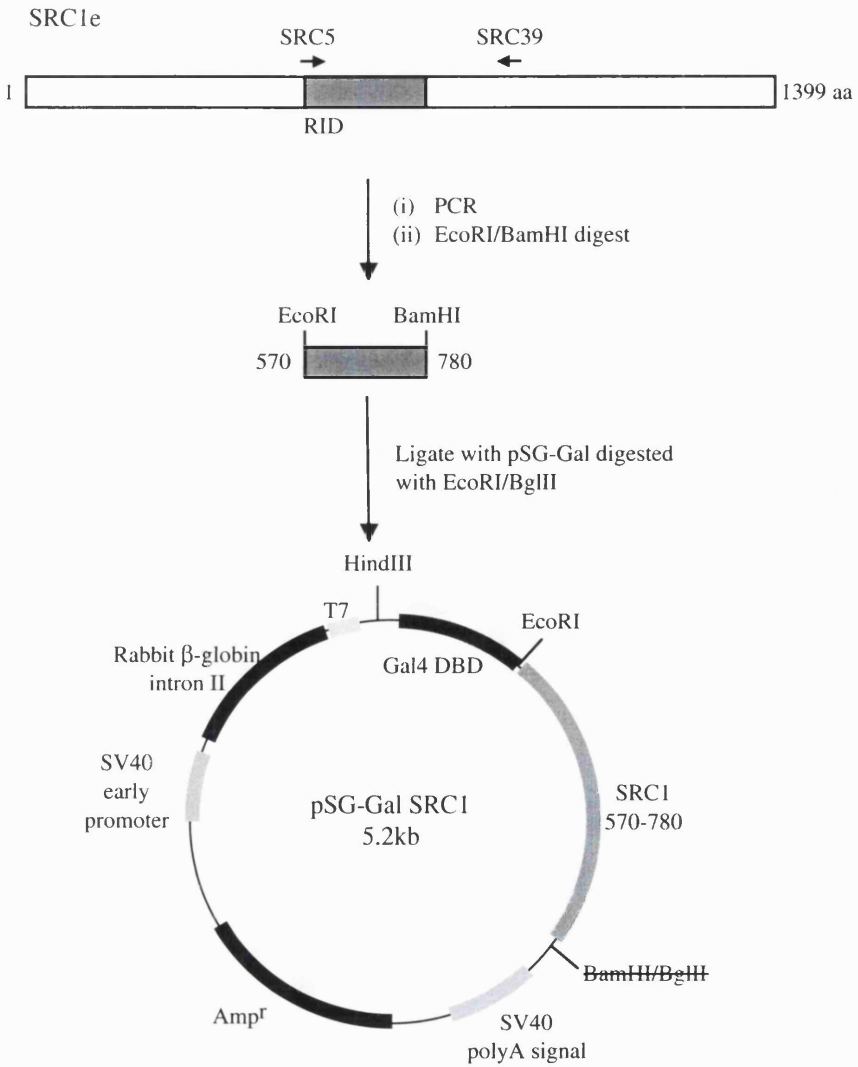

SRC1e
SRC5
SRC39
1
1399 aa
RID
(i) PCR
(ii) EcoRI/BamHI digest
EcoRI
BamHI
570
780
Ligate with pSG-Gal digested
with EcoRI/BglII
HindIII
T7
EcoRI
Rabbit β-globin
intron II
Gal4 DBD
SV40
early
promoter
pSG-Gal SRC1
5.2kb
SRC1
570-780
BamHI/BglII
Amp^r
SV40
polyA signal

Figure AII.9 Construction of pSG FLAG SRC1e VHC and pSG FLAG SRC1e VHC ΔAD1

Full length FLAG epitope tagged SRC1e VHC was generated by replacing an XhoI/EcoRV restriction fragment of pSG FLAG SRCX1e m13 with one from pGBDU SRCX1 VHC, which was recovered directly from the yeast two-hybrid screen. The pSG FLAG SRC1e VHC recombinants were verified by sequencing.

To generate the ΔAD1 construct, a BamHI/MscI restriction fragment of pSG FLAG SRC1e VHC was replaced with one from pSG5 SRC1e ΔAD1. The pSG FLAG SRC1e VHC ΔAD1 recombinants were subsequently verified by sequencing.

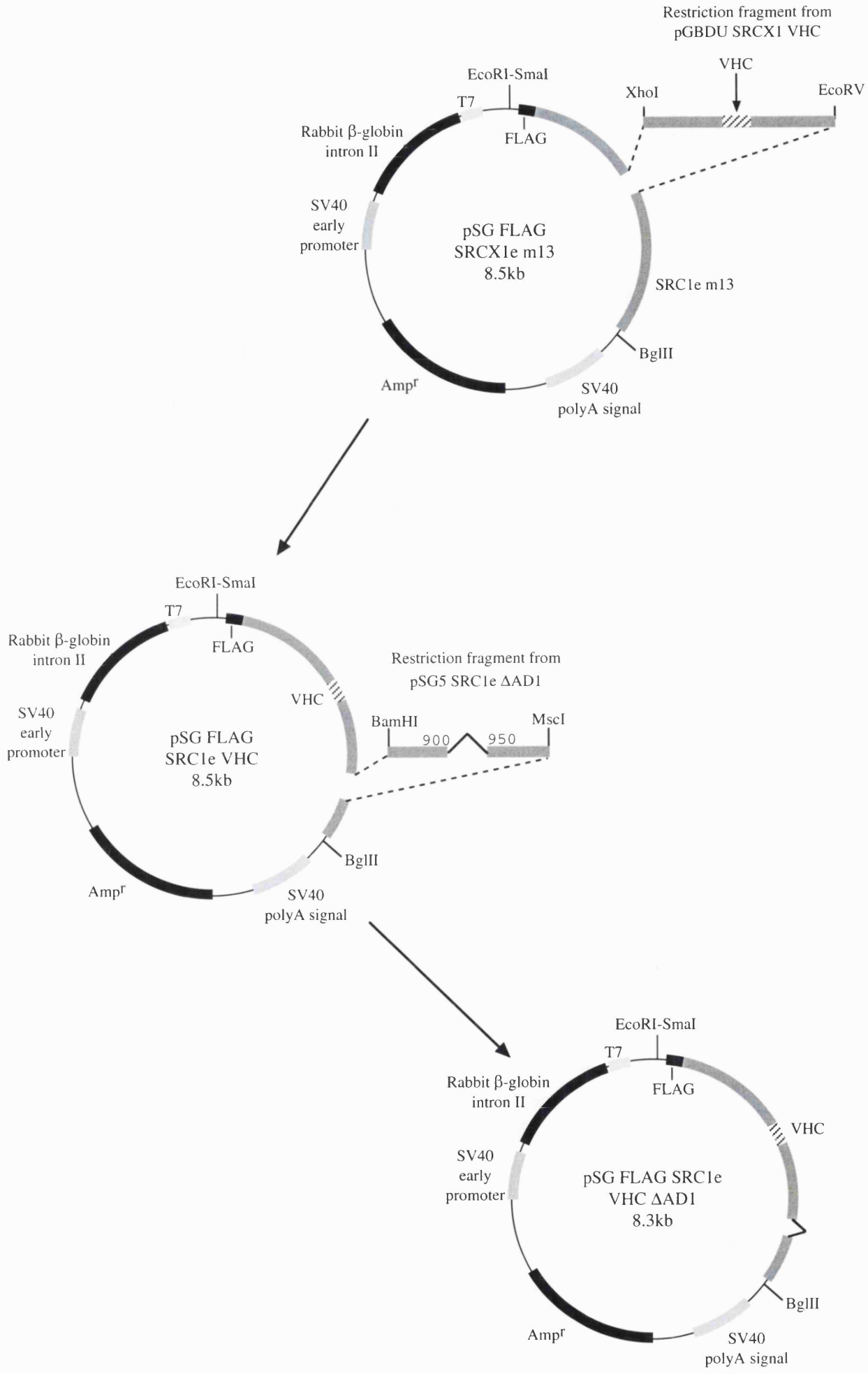

Restriction fragment from
pGBDU SRCX1 VHC
VHC
EcoRI-SmaI
XhoI
EcoRV
T7
FLAG
Rabbit β-globin
intron II
SV40
early
promoter
pSG FLAG
SRCX1e m13
8.5kb
SRC1e m13
BglII
Ampr
SV40
polyA signal
EcoRI-SmaI
T7
FLAG
Rabbit β-globin
intron II
SV40
early
promoter
pSG FLAG
SRC1e VHC
8.5kb
VHC
Restriction fragment from
pSG5 SRC1e ΔAD1
BamHI
MscI
900
950
BglII
Ampr
SV40
polyA signal
EcoRI-SmaI
T7
FLAG
Rabbit β-globin
intron II
VHC
SV40
early
promoter
pSG FLAG SRC1e
VHC ΔAD1
8.3kb
BglII
Ampr
SV40
polyA signal

Figure AII.10 Construction of pSG FLAG TIF2 m123

In order to put the N-terminal end of TIF2 in frame with the FLAG epitope tag present in the vector pSG-FLAG (Borja Belandia), cDNA encoding TIF2 (aa 1 to 385) was amplified by PCR using primers TIF2C and TIF2D and pSG5 TIF2 as template. The PCR products were digested with EcoRI/XhoI and subcloned into pSG5-FLAG digested with the same enzymes to yield pSG FLAG TIF2-N.

The pSG FLAG TIF2 m123 was then constructed by inserting an NdeI/BamHI fragment from pSG5 TIF2 m123 (Voegel *et al.*, 1998) into pSG FLAG TIF2-N, which has been prepared by BglII/partial NdeI digestion. The BglII restriction site in the vector was destroyed upon ligation, however a unique BglII site remained in the 3'UTR of the cDNA. The recombinants were verified by sequencing.

TIF2C (5' primer)	5'-TAGAATTCATGAGTGGGATGGGAGAAAATAC-3'
TIF2D (3' primer)	5'-TAACTCGAGTCCGGATTCATCACACACAC-3'

Restriction sites in the primers are underlined.

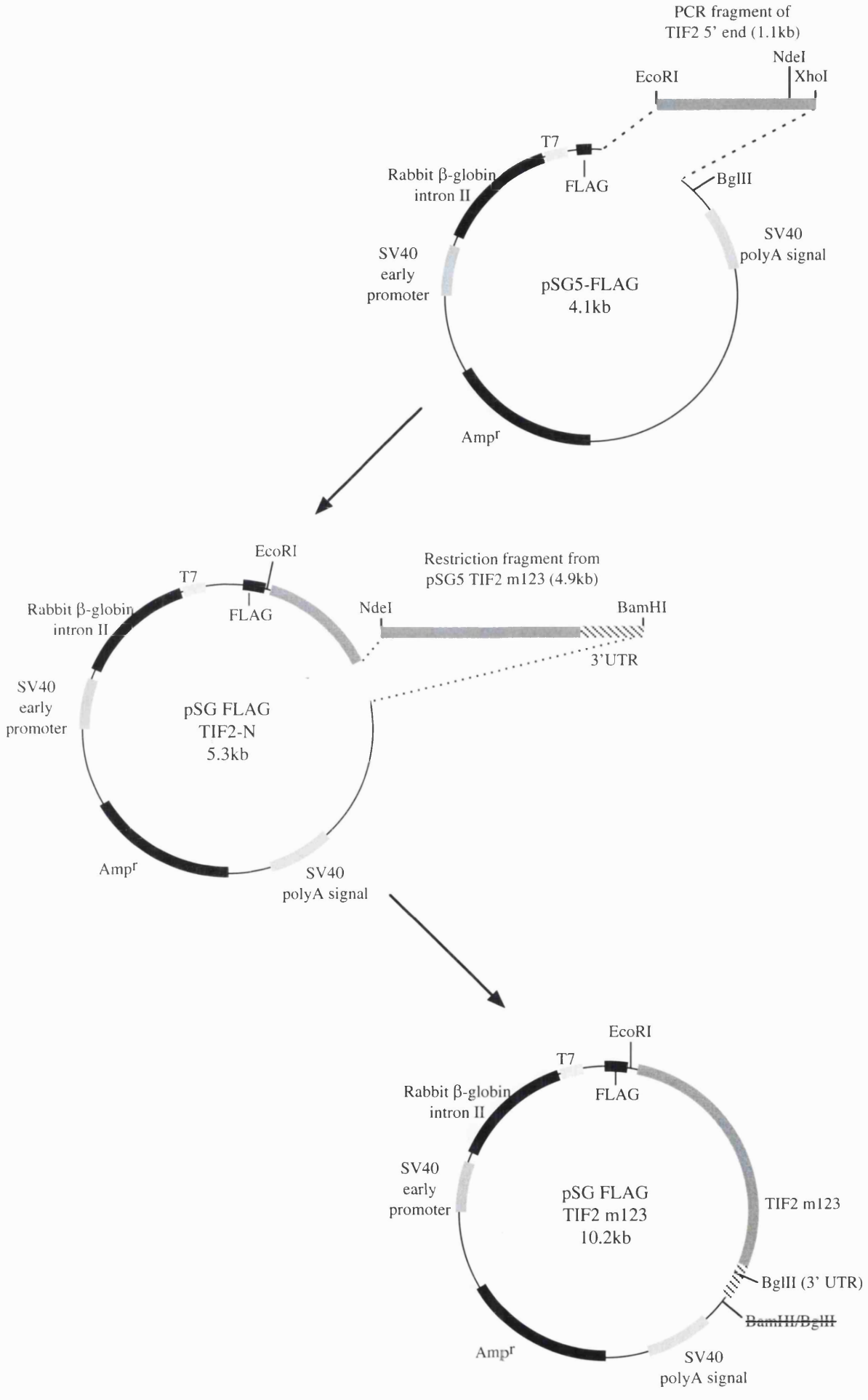

PCR fragment of
TIF2 5' end (1.1kb)
NdeI
EcoRI
XhoI
T7
Rabbit β-globin
intron II
FLAG
BglII
SV40
polyA signal
SV40
early
promoter
pSG5-FLAG
4.1kb
Amp^r
EcoRI
T7
Restriction fragment from
pSG5 TIF2 m123 (4.9kb)
Rabbit β-globin
intron II
FLAG
NdeI
BamHI
3'UTR
SV40
early
promoter
pSG FLAG
TIF2-N
5.3kb
Amp^r
SV40
polyA signal
EcoRI
T7
Rabbit β-globin
intron II
FLAG
SV40
early
promoter
pSG FLAG
TIF2 m123
10.2kb
TIF2 m123
BglII (3' UTR)
~~BamHI/BglII~~
Amp^r
SV40
polyA signal

Figure AII.11 Construction of pSG FLAG TIF2 m13 and pSG FLAG TIF2 VHC

Motif 2 of TIF2 m123 was reverted from LXXAA back to the wild-type sequence LXXLL by recombinant PCR using primers TIF2E, TIF2F, TIF2M2+ and TIF2M2- and pSG5 TIF2 m123 as template. The PCR products were digested with PstI (x = non-functional LXXAA motifs 1 and 3 and θ = functional LXXLL motif 2) and subcloned into pSG FLAG TIF2 m123 digested with PstI. An XhoI site 3' to the sequence encoding the LXXLL motif 2 was created for identification of recombinants which were verified by sequencing.

The VHC mutation was introduced to TIF2 m13 by recombinant PCR using primers TIF2E, TIF2F, TIF2VH+ and TIF2VH- and pSG FLAG TIF2 m13 as template. The PCR products were digested with PstI (* = VHC mutation) and subcloned into pSG FLAG TIF2 m13 digested with PstI. A BglII site between the sequence encoding the wild-type LXXLL motif and the 15 amino-acids insertion was created for identification of recombinants which were subsequently verified by sequencing.

Outer primers	
TIF2E (5' primer)	5'-TCACCAAGGCATCGCATGAGCCCTGGAGTGGC-3'
TIF2F (3' primer)	5'-ATCTCCTCCAGGCCATCAAAATTCCGCAAGGC-3'
Mutagenic primers	
TIF2M2+	5'-CAGACTCTTGCAGGACTCGAGTTCCCCTGTGGACTTGGCC-3'
TIF2M2-	5'-GGAACTCGAGTCCTGCAAGAGTCTGTGCAAAATTTTATGC-3'
TIF2VH+	5'-GAGGGTAGCCCCTCAGAACGGCATAAGATCTACCACCGG CTCAAGCAGGACTCGAGTTCCCCTGTGGACTTGGCCAAG-3'
TIF2VH-	5'-CTTGAGCCGGTGGTAGATCTTATGCCGTTCTGAGGGGCT ACCCTCCTGCAAGAGTCTGTGCAAAATTTTATGCTTCTC-3'

Restriction sites in the primers are underlined.

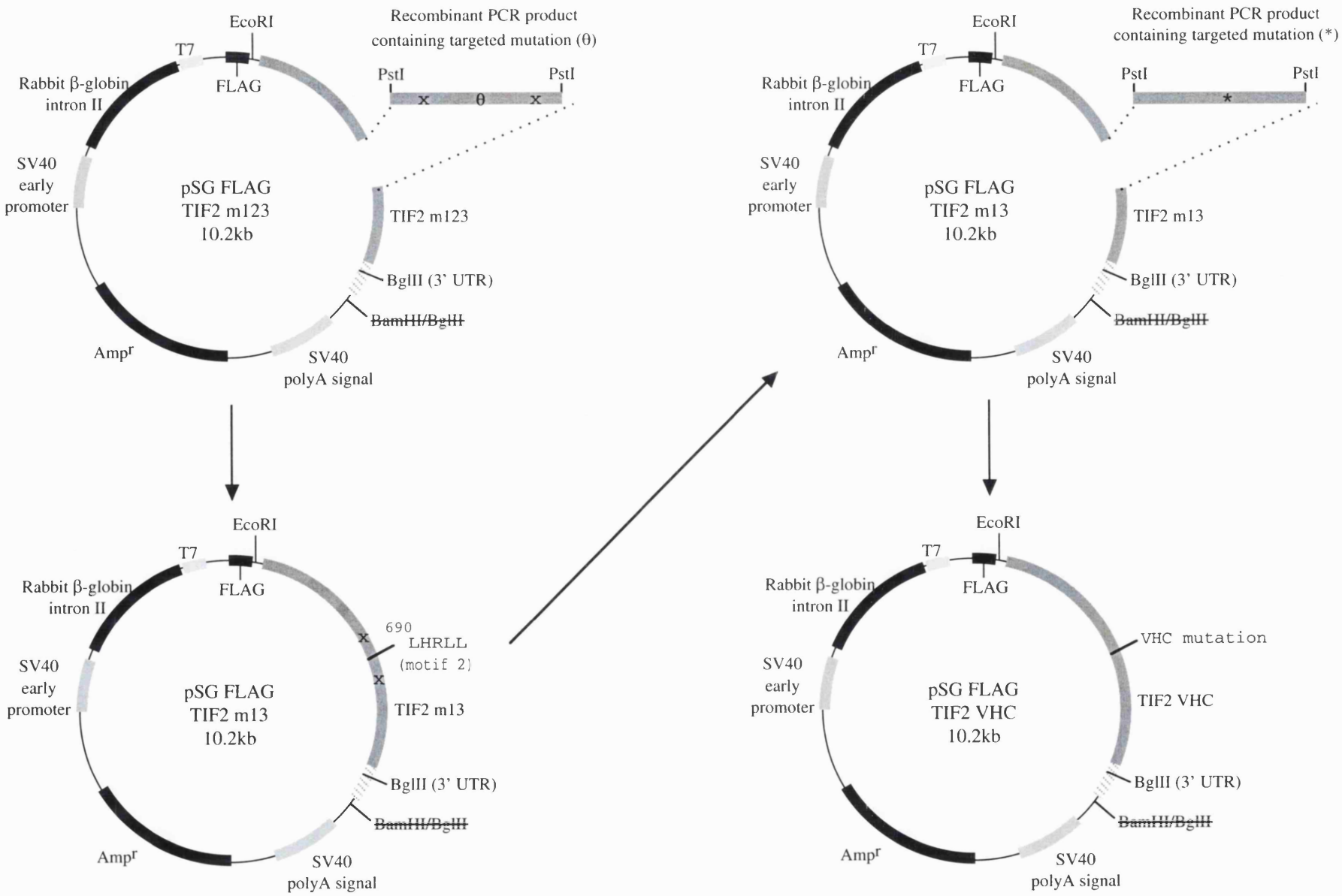

EcoRI
T7
FLAG
Rabbit β-globin intron II
SV40 early promoter
pSG FLAG TIF2 m123 10.2kb
Ampr
SV40 polyA signal
Recombinant PCR product containing targeted mutation (θ)
PstI
PstI
x
θ
x
TIF2 m123
BglII (3' UTR)
BamHI/BglII
EcoRI
T7
FLAG
Rabbit β-globin intron II
SV40 early promoter
pSG FLAG TIF2 m13 10.2kb
Ampr
SV40 polyA signal
Recombinant PCR product containing targeted mutation (*)
PstI
PstI
*
TIF2 m13
BglII (3' UTR)
BamHI/BglII
EcoRI
T7
FLAG
Rabbit β-globin intron II
SV40 early promoter
pSG FLAG TIF2 m13 10.2kb
Ampr
SV40 polyA signal
690
LHRLL
(motif 2)
x
x
TIF2 m13
BglII (3' UTR)
BamHI/BglII
EcoRI
T7
FLAG
Rabbit β-globin intron II
SV40 early promoter
pSG FLAG TIF2 VHC 10.2kb
Ampr
SV40 polyA signal
VHC mutation
TIF2 VHC
BglII (3' UTR)
BamHI/BglII

Figure AII.12 Construction of pSG FLAG RAC3

In order to put the N-terminal end of RAC3 in frame with the FLAG epitope tag present in the vector pSG-FLAG (Borja Belandia), cDNA encoding RAC3 (aa 1 to 327) was amplified by PCR using primers RacA and RacD and pCMX-RAC3 (Li *et al.*, 1997) as template. The PCR products were digested with SmaI/XhoI and subcloned into pSG5-FLAG digested with the same enzymes to yield pSG FLAG RAC3-N.

The pSG FLAG RAC3 was then constructed by inserting a HindIII/SalI fragment from pCMX-RAC3 into pSG FLAG RAC3-N, which has been prepared by HindIII/XhoI digestion. As a result. the XhoI restriction site in the vector was destroyed upon ligation. The recombinants were verified by sequencing.

RacA (5' primer)	5'-TATCCCGGGATGAGTGGATTAGGAGAAAAC-3'
RacD (3' primer)	5'-TTTCTCGAGATGGCCATTAAGATAAGCTTC-3'

Restriction sites in the primers are underlined.

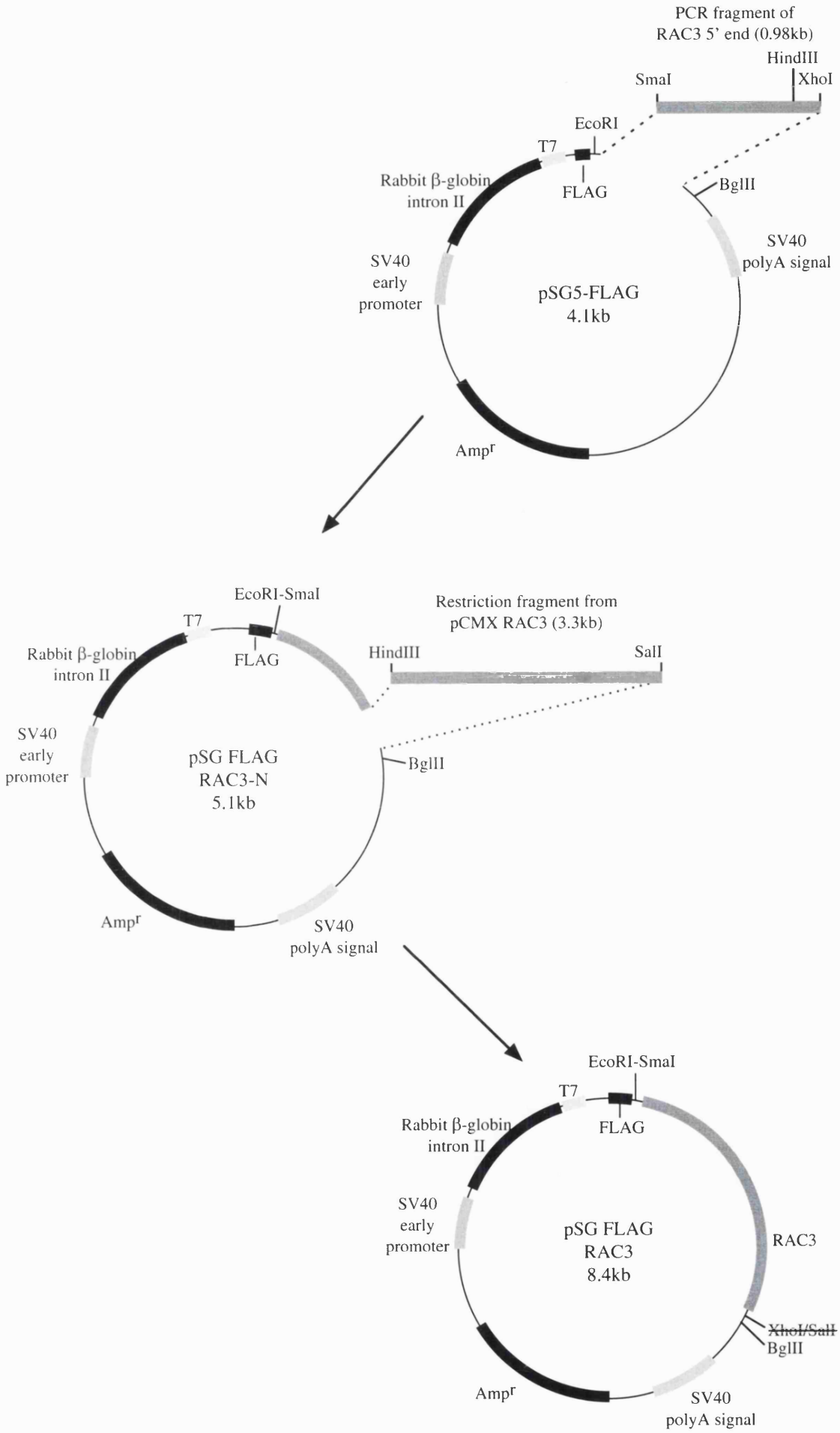

PCR fragment of
RAC3 5' end (0.98kb)
HindIII
SmaI
XhoI
EcoRI
T7
FLAG
Rabbit β-globin
intron II
BglII
SV40
polyA signal
SV40
early
promoter
pSG5-FLAG
4.1kb
Ampr
EcoRI-SmaI
T7
FLAG
Rabbit β-globin
intron II
Restriction fragment from
pCMX RAC3 (3.3kb)
HindIII
SalI
SV40
early
promoter
pSG FLAG
RAC3-N
5.1kb
BglII
Ampr
SV40
polyA signal
EcoRI-SmaI
T7
FLAG
Rabbit β-globin
intron II
SV40
early
promoter
pSG FLAG
RAC3
8.4kb
RAC3
XhoI/SalI
BglII
Ampr
SV40
polyA signal

Figure AII.13 Construction of pSG FLAG RAC3 m13 and pSG FLAG RAC3 VHC

RAC3 LXXLL motif 1 was mutated to LXXAA by recombinant PCR using primers RacD, RacE, RacM1+ and RacM1- and pSG FLAG RAC3 as template. The PCR products were digested with HindIII/SpeI and subcloned into pSG FLAG RAC3 digested with the same enzymes. An XhoI site 3' to the sequence encoding the mutated motif 1 was created for identification of recombinants. pSG FLAG RAC3 m13 was generated by recombinant PCR using primers RacH, RacE, RacM3+ and RacM3- and pSG5 FLAG RAC3 m1 as template. The PCR products were digested with XhoI/SpeI and subcloned into pSG FLAG RAC3 m1 digested with the same enzymes. (x = non-functional LXXAA motifs 1 and 3).

The VHC mutation was introduced to RAC3 m13 by recombinant PCR using primers RacH, RacE, Rac3VH+ and Rac3VH- and pSG FLAG RAC3 m13 as template. The PCR products were digested with XhoI/SpeI (* = VHC mutation) and subcloned into pSG FLAG RAC3 m13 digested with the same enzymes. Two PstI sites were destroyed for identification of recombinants which were subsequently verified by sequencing.

Outer primers	
RacD (5' primer)	5'-TATCAAGAAGCTTATCTTAATGGCC-3'
RacH (5' primer)	5'-CTCAGTGACAAAGAAAGTAAGGAG-3'
RacE (3' primer)	5'-CCAGTCTCCTGAGGAAGGAGTCTG-3'
Mutagenic primers	
RacM1+	5'-CAGGCAGCTACCTGCTCGAGTGATGACCGGGGTCATTCCTCCTTG-3'
RacM1-	5'-CACTCGAGCAGGTAGCTGCCTGCAGTAATTTTTTATGACCTTTGC-3'
RacM3+	5'-AGATACGCGGCGGACAGGGATGATCCTAGTGATGC-3'
RacM3-	5'-ATCCCTGTCCGCCGCGTATCTAAGAAGTGCATTATTC-3'
Rac3VH+	5'-CAAGAGAAGCACCGGATTTTGCACAAGTTG*CTGC*AAGAGGG TAGCCCCTCTGAACGGCATAAGATATACCACCGGCTC-3'
Rac3VH-	5'-GGCTT*CTGCGG*TAATCTTGGCTACCTCAGCTGGTGAATTCCC TTCCTGCTTGAGCCGGTGGTATATCTTATGCCGTTCAGA-3'

Restriction sites in the primers are underlined and destroyed sites are in italics.

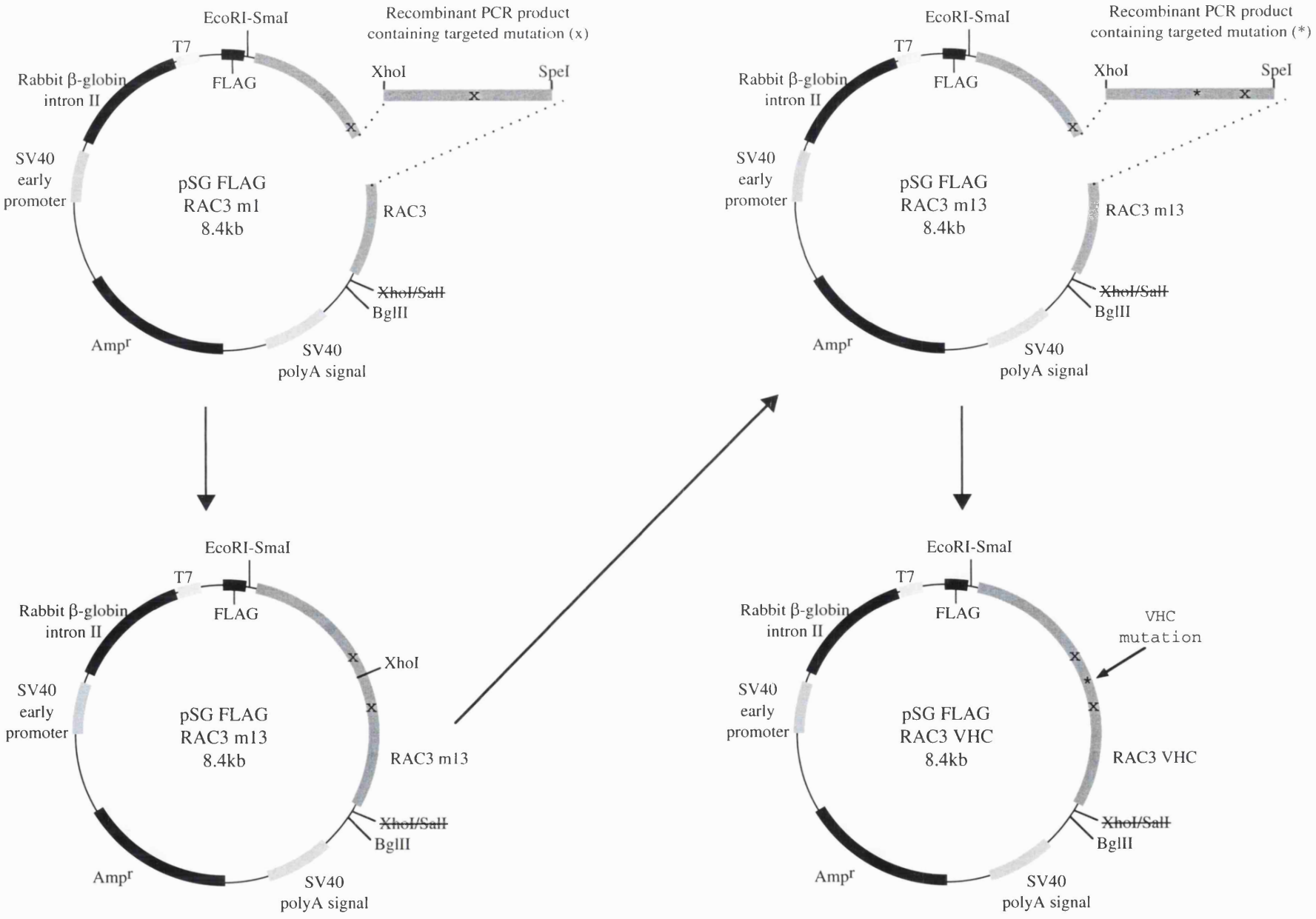

EcoRI-SmaI
T7
FLAG
Rabbit β-globin intron II
SV40 early promoter
pSG FLAG RAC3 m1 8.4kb
Ampr
SV40 polyA signal
Recombinant PCR product containing targeted mutation (x)
XhoI
SpeI
RAC3
XhoI/SalI
BglII
EcoRI-SmaI
T7
FLAG
Rabbit β-globin intron II
SV40 early promoter
pSG FLAG RAC3 m13 8.4kb
Ampr
SV40 polyA signal
XhoI
RAC3 m13
XhoI/SalI
BglII
Recombinant PCR product containing targeted mutation (*)
XhoI
SpeI
RAC3 m13
XhoI/SalI
BglII
EcoRI-SmaI
T7
FLAG
Rabbit β-globin intron II
SV40 early promoter
pSG FLAG RAC3 VHC 8.4kb
Ampr
SV40 polyA signal
VHC mutation
RAC3 VHC
XhoI/SalI
BglII

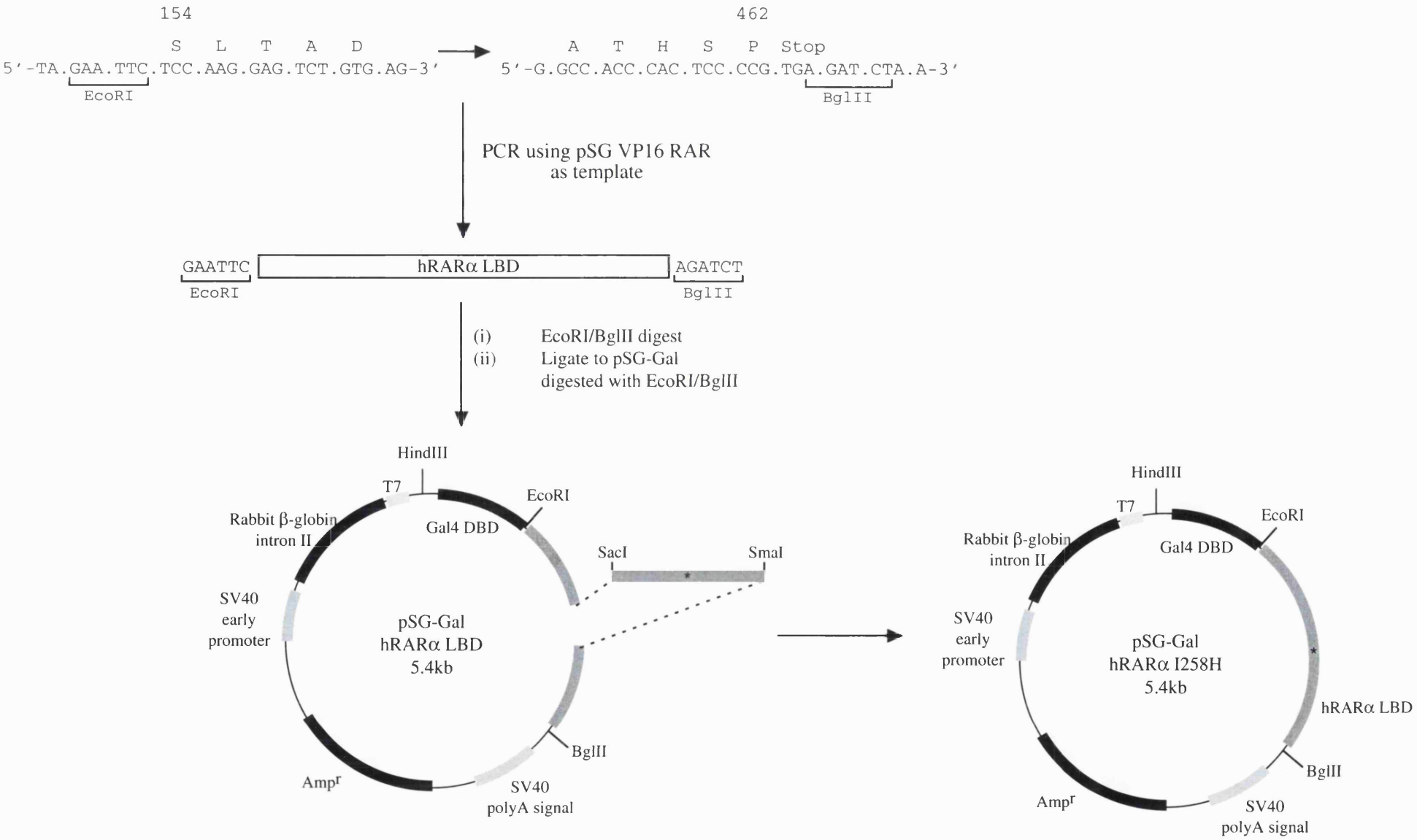
Figure AII.14 Construction of pSG-Gal hRARα I258H

The cDNA encoding hRARα LBD (aa 154 to 462) was amplified by PCR using primers RAA1 and RAA2 and pSG VP16 RAR (Butler and Parker, 1995) as template. The PCR products were digested with EcoRI/BglII and subcloned into pSG-Gal digested with the same enzymes. The pSG-Gal hRARα LBD recombinants were verified by DNA sequencing.

The I258H mutation (denoted by *) was introduced by recombinant PCR using primers RAA1, RAA2, RAA258+ and RAA258- and pSG-Gal hRARα LBD as template. The PCR products were digested with SacI/SmaI and subcloned into pSG-Gal hRARα LBD digested with the same enzymes. The pSG-Gal hRARα I258H recombinants were verified by DNA sequencing.

Outer primers	
RAA1 (5' primer)	5'-TAGAATTCTCCAAGGAGTCTGTGAG-3'
RAA2 (3' primer)	5'-TTAGATCTCACGGGGAGTGGGTGGCC-3'
Mutagenic primers	
RAA258+	5'-CACCATCGCCGACCAGCACACCCTCCTCAAGGC-3'
RAA258-	5'-GCCTTGAGGAGGGTGTGCTGGTCGGCGATGGTG-3'

Restriction sites in the primers are underlined.


154
462
S L T A D
A T H S P Stop
5'-TA.GAA.TTC.TCC.AAG.GAG.TCT.GTG.AG-3'
5'-G.GCC.ACC.CAC.TCC.CCG.TGA.GAT.CTA.A-3'
EcoRI
BglII
PCR using pSG VP16 RAR as template
GAATTC
hRARα LBD
AGATCT
EcoRI
BglII
(i) EcoRI/BglII digest
(ii) Ligate to pSG-Gal digested with EcoRI/BglII
HindIII
T7
EcoRI
Rabbit β-globin intron II
Gal4 DBD
SacI
SmaI
SV40 early promoter
pSG-Gal hRARα LBD 5.4kb
BglII
Ampr
SV40 polyA signal
HindIII
T7
EcoRI
Rabbit β-globin intron II
Gal4 DBD
SV40 early promoter
pSG-Gal hRARα I258H 5.4kb
hRARα LBD
BglII
Ampr
SV40 polyA signal

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