# Ligand regulated site specific recombination in mammalian cells

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This thesis is dedicated to

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# Abbreviations

FLP The conservative site specific DNA recombinase encoded by the 2 micron circle of *S.cerevisiae*

FRT The DNA sequence element that represents the binding sites and substrate for the FLP enzyme

EBD The estrogen receptors' ligand binding domain, comprising amino acids 251 to 595 unless otherwise specified

CRE The conservative site specific DNA recombinase encoded by the *E.coli* phage P1

# Abstract

Chapter 1 of this thesis summarizes the current knowledge about the nuclear receptor superfamily of transcription factors. Chapter 2 describes the materials and methods used to carry out the research presented in the next 4 chapters. In chapter 3, I demonstrate that the FLP site specific DNA recombinase can be regulated by steroid ligands. The kinetics of recombination mediated by FLP-steroid receptor ligand binding domain fusion proteins (FLP-LBD) are described in this chapter and the parameters affecting these kinetics are discussed. In chapter 4 the effect of synthetic steroid ligands on the kinetics of FLP-LBD mediated recombination are explored using a combination of site directed mutagenesis and dose response experiments. In chapter 5, the generation of EBDs with altered ligand specificities by amino acid changes at 2 positions in the EBD is described. Finally, in chapter 6, the capacity of a number of nuclear receptor superfamily members to conditionally repress FLP is analysed.

# Chapter 1 : INTRODUCTION

## On site specific recombinases and their uses

DNA is the molecular support of genetic information. Therefore altering the DNA of living cells manipulates the genetic information in a heritable manner. The more precise and predictable the DNA alteration is, the more straightforward the interpretation or prediction of the consequence(s) of the DNA modification will be.

Uptake and integration of exogenous DNA is an inefficient process. It is therefore common practice to use genetic markers, genes that confer either a distinctive property or a growth advantage to the cells that express them, ensuring the identification of cells that underwent the desired change in genotype.

In certain organisms, exogenous DNA introduced into cells will efficiently recombine with genomic DNA by means of homologous recombination, leading to the integration of the new DNA into the cells genome. This process relies on endogenous DNA metabolising enzymes that require a certain extent of complete sequence homology between the exogenous DNA.

Introduced DNA can also be integrated into chromosomal sequences at random locations. In this case, there is little control over the integration site of the new DNA. Thus, the newly integrated DNA may affect, and be affected by, the properties of the DNA sequences surrounding its integration site.

Site specific DNA recombinases (SSRs) are enzymes that catalyse recombination between two DNA segments that carry specific sequences that represent the target sites for the enzymes. The biological role of these enzymes include the de-catenation of circular replicons that underwent replication (Blakely *et al.*, 1993), the re-orientation of replication forks to promote replicon amplification (Russo *et al.*, 1992), the circularisation of long linear concatamerised phage replication intermediates (Hoess and Abremsky, 1990) and the integration of phages into given genomic loci to establish a pro-phage (Craig, 1988).

Although many of the known SSR systems have complex co-factor and DNA topology requirements, there is a class of SSRs that encode all the necessary functions to catalyse site specific recombination within one polypeptide. Some of the enzymes that belong to this class have been shown to function in all the organisms in which they have been tested. These enzymes include the phage P1 encoded CRE recombinase and the 2μ circle encoded FLP recombinase.

Both CRE and FLP catalyse recombination across target sites that consist of two head to head 13bp recombinase binding sites separated by an 8bp spacer (Hoess *et al.*, 1982), (Andrews *et al.*, 1985). The recombination reactions catalysed by FLP and CRE go through a Holliday junction intermediate and involve transient covalent 3' phosphotyrosyl bonds (Andrews *et al.*, 1985) between the substrate DNA and the enzymes. The Holliday junction established at one end of the spacer is thought to migrate across the spacer sequences before its resolution.

The polarity of the spacer dictates the directionality of the recombination event that occurs between two recombination target sequences (Cox, 1983), (Hoess *et al.*, 1986), (Senecoff and Cox, 1986). In practice this means that if two target sequences are located on the same DNA molecule, depending on the relative orientation of the spacers of the recombination targets, either an excision or an inversion of the intervening DNA will occur upon recombination. If the recombination targets are located on separate molecules and if at least one of these molecules is circular, site specific recombination will result in the integration of the circular molecule into the other DNA molecule in a spacer orientation dependent orientation. If both recombinase target site bearing molecules are linear, a translocation will take place, the products of which are again determined by the orientation of the spacer sequences.

CRE and FLP represent powerful tools to manipulate living genomes as they permit site specific deletions, integrations and translocations to be induced. The sole pre-requisite for such manipulations is that a recombinase target site be present at a suitable genomic location.

Once one target is present, it becomes possible to integrate DNA molecules bearing sequences of interest, as well as other recombination targets into it, opening the door for increasingly complex genome manipulations. One application being the removal of genetic markers from integrated transgenes, enabling the repeated use of one marker gene in consecutive rounds of transfection. An aspect of the site specific integration strategy outlined above is that position effects due to random integration of transgenes (Feinstein *et al.*, 1982) are reduced to those impinging on the locus that was initially targeted. If the locus into which the initial recombination target was integrated is carefully selected, a chromatin environment with the desired properties may be targeted.

To exploit the potential of site specific recombinases to precisely manipulate living genomes it is necessary to have ways and means to regulate their recombination reactions.

In order to control the activity of a site specific recombinase, I fused steroid receptor ligand binding domains to the FLP recombinase.

## Nuclear receptors molecular biology

The steroid receptors form a sub-family within the superfamily of nuclear receptors (Laudet *et al.*, 1992), (Evans, 1988). More than 200 different cDNAs of nuclear receptor superfamily members have been cloned from a variety of organisms (Kostrouch *et al.*, 1995). Below I will list a number of the members of this protein superfamily and summarise the current molecular knowledge pertaining to their mode of action. This thesis describes a new assay for one of the functions of the steroid receptors, namely the capacity of their ligand binding domains (LBDs) to function as heterologous, ligand dependency conferring, protein domains (Mattioni *et al.*, 1994). In chapter 6, I explore the possibility that this feature is not unique to the steroid receptors sub-family of the nuclear receptors.

## Nuclear receptors; a growing family of transcription factors

The nuclear receptor superfamily includes all the steroid hormone receptors; androgen (Lubahn *et al.*, 1988), estrogen (Walter *et al.*, 1985), glucocorticoid (Hollenberg *et al.*, 1985), mineralocorticoid (Arriza *et al.*, 1987) and progesterone receptors (Loosfelt *et al.*, 1986) as well as the thyroid hormone receptors (Sap *et al.*, 1986), the retinoic acid receptors (Dejean *et al.*, 1986), (Leid *et al.*, 1992), the vitamin D3 receptor (Baker *et al.*, 1988), the peroxisome proliferator activated receptors (Sher *et al.*, 1993), the receptors for the invertebrate hormone ecdysterone (Koelle *et al.*, 1991)and juvenile hormone (Harmon *et al.*, 1995), the receptor for farnesol metabolites (Forman *et al.*, 1995a), and an ever growing list of so called orphan receptors for which no ligand, if there is any, has been described as yet. These include the drosophila ultraspiracle protein (Oro *et al.*, 1990), MB67 (Baes *et al.*, 1994), COUP-TF factors and their drosophila homologue seven up (Sagami *et al.*, 1986), (Mlodzik *et al.*, 1990), the drosophila fushi tarazu regulatory factor and its mammalian homologue SF-1 (Lala *et al.*, 1992), the ecdysterone inducible genes E75A, E75B (Feigl *et al.*, 1989), (Segraves and Hogness, 1990) and E78 (Stone and Thummel, 1993), the estrogen related receptors (Giguere *et al.*, 1988), the human testicular receptor 2 (Chang *et al.*, 1989), NGFI-B also called Nak1 or nur77 (Milbrandt, 1988), the drosophila gap gene tailless and its vertebrate homologue Tlx (Yu *et al.*, 1994), the RLD-1 factor also called LXR and OR (Apfel *et al.*, 1994), (Teboul *et al.*, 1995), the DAX-1 gene product (Muscatelli *et al.*, 1994), the RZR orphan receptors also called RORs (Becker *et al.*, 1993), (Giguere *et al.*, 1994), Rev-ErbAα receptor related protein which is encoded on the opposite strand of the thyroid receptor α in both humans and rats (Miyajima *et al.*, 1989), (Lazar *et al.*, 1989), Rev-Erbβ (Dumas *et al.*, 1994) and the HNF-4 protein (Costa *et al.*, 1989) and its drosophila homologue (Zhong *et al.*, 1993).

All the members of this superfamily are transcription factors and share a common modular architecture (Laudet *et al.*, 1992), (Krust *et al.*, 1986). The function of these protein modules is discussed below.

The role of the members of the superfamily for whom ligands are known is quite clear; by virtue of their tissue specific distribution and activity, they enable the co-ordination of cellular responses in a whole organism by "endocrine tissues" that secrete their cognate ligands. Naturally, the ligand may emanate and be interpreted by the same cell (Forman *et al.*, 1995a), (Gottlicher *et al.*, 1992). Orphan receptors that have a ligand will function analogously. It is quite possible however, that some 'orphan' receptors do not have ligands and that they are simply tissue specific transcription factors that either modulate the action of ligand activated nuclear receptors or have their own target genes and mode of regulation.

The nuclear receptor superfamily can be subdivided into different classes according to a number of criteria. Three of these criteria are; (i) the mode of DNA binding; monomeric, homodimeric and heterodimeric, (ii) the core sequence of their DNA binding site; estrogen related or glucocorticoid related, and (iii) whether or not they are sequestered away from their genomic target sites by a repressor complex when they are not bound to their cognate ligands.

## Nuclear receptors can bind DNA either as monomers, homodimers or heterodimers

Theoretically, homo- and heterodimerisation of DNA binding proteins with different target specificities permits the recognition of more different sequences than monomer binding would allow without increasing the complexity of the individual DNA binding domains involved. In addition, dimerisation increases the specific DNA binding affinity and it doubles the number of base pairs required for specific DNA binding, two essential features for factors that regulate the expression of genomes that comprise several billions of base pairs.

To date, four orphan receptor subfamilies are known to encode nuclear receptors that bind to their targets as monomers, SF-1 (Wilson *et al.*, 1993), NGFI-B (Wilson *et al.*, 1993), RORα1 and the RZRs (Giguere *et al.*, 1995), (Carlberg *et al.*, 1994) and Rev-ErbAα (Harding and Lazar, 1993). The recognition sequence of these factors is at least 10bp long. NGFI-B can also bind to DNA as a heterodimer with RXR (Forman *et al.*, 1995b).

The steroid receptors bind their response elements as homodimers (Schwabe and Rhodes, 1991). Homodimerization allows the recognition site to be extended to two monomer recognition sites. In most cases the steroid response elements consist of an inverted repeat spaced by 3 nucleotides.

Most of the other nuclear receptors bind to DNA as heterodimers. The partner is always the retinoid X receptor (RXR) or its homologue in drosophila, ultraspiracle (Yao *et al.*, 1992), (Thomas *et al.*, 1993). The only exception to this is RXR itself, which can bind to specific DNA sequences as a homodimer (Zhang *et al.*, 1992). As mentioned above, dimer formation permits the specific regulation of many more different target DNA sequences by fewer factors than if each different target was bound by one DNA binding protein. If all the nuclear receptors heterodimerize with one same partner this argument loses its foundation. Heterodimerisation with one common partner may however provide a mode of regulation that relies on the competition of different proteins for this partner, thus a whole battery of responses may be modulated simply by modulating the availability of the common partner. The fact that RXR is the receptor for 9 cis retinoic acid (Heyman *et al.*, 1992), (Levin *et al.*, 1992) raises the possibility that all the heterodimerizing nuclear receptors will be affected by the presence of 9 cis retinoic acid. This is not always the case, because the intrinsic transcription activation capacity of RXR can be masked when it is bound to the retinoic acid- or thyroid receptors (Forman *et al.*, 1995b) and this in a response element specific fashion (Kurokawa *et al.*, 1994). In other cases however, for example when NGFI-B is complexed with RXR (Forman *et al.*, 1995b), when the peroxisome proliferator receptor is complexed with RXR (Keller *et al.*, 1993) or when the farnesol metabolite receptor is complexed with

RXR (Forman *et al.*, 1995a), 9 cis retinoic acid does influence the transcription activation capacity of the heterodimer positively. Thus, although nuclear receptors-RXR heterodimers do not require the presence of the RXR ligand 9 cis retinoic acid to function as transcription factors, their activity can be modulated by the presence of this vitamin A metabolite.

**DNA sequence specificity of the nuclear receptors**

The second criterion used to group nuclear receptors into different categories is their half site recognition sequence. Only the progesterone, androgen, mineralocorticoid and glucocorticoid receptors recognise elements with the AGAACA core sequence, all the other nuclear receptors, including the estrogen receptor, recognise half sites with the $AG^{G}/_{T}TCA$ core sequence. The above sequence specificity has been localised to a particular string of amino acids located inside the highly conserved DNA binding domain of the nuclear receptors that has been called the P box (Umesono and Evans, 1989), (Mader *et al.*, 1989).

What then stops the different members from activating the same response elements ? In the case of the monomer binders, the answer lies in their extended sequence requirements for tight binding to DNA; up to 11 base pairs for rev-ErbAα (Harding and Lazar, 1993). In the case of the glucocorticoid group the progesterone, androgen, mineralocorticoid and glucocorticoid receptors all bind the same inverted repeat. This poses a perplexing question, namely; how can one steroid receptor homodimer discriminate between its own response elements and those of the other three? The answer is largely provided by the different spatial and temporal expression patterns of these receptors in the body, since glucocorticoid inducible genes can be rendered progestin inducible by ectopic expression of the progesterone receptor (Strahle *et al.*, 1989). It is probable that tissue specific expression or activation of auxiliary transcription factors also required for the expression of genes whose transcription is controlled by steroid hormones limits the number of target genes that the 4 promiscuous steroid receptors can activate in any one cell type. In the cases where two or

more of the above 4 steroid receptors are expressed in the same cell, the capacity of the steroid receptor to co-operate or not with the transcription activation functions of the auxiliary factors may provide an additional layer of regulation that confers steroid specific inducibility onto target regulatory sequences. Furthermore, it has been shown that some tissues can selectively inactivate certain steroids (Funder *et al.*, 1988) and therefore do not rely on the lack of expression of the receptors for those steroids to avoid cross-activation of target genes by multiple steroid hormones. Cell type specific import or export of receptor ligands may also modulate the ligand responsiveness of a cell type (Kralli *et al.*, 1995). Tissue specific inactivation of the progesterone receptor through expression of a dominant negative, alternative splice form, of this receptor is another means by which ectopic activation of androgen, glucocorticoid or mineralocorticoid target genes by progesterone is avoided (Tora *et al.*, 1988). These strategies are probably also applied by nature to limit cross-activation by the heterodimerizing subclass of the nuclear receptors superfamily (Jow and Mukherjee, 1995). In the case of the heterodimerizing members, the orientation of the half sites; direct repeats, inverted repeats or everted repeats and the spacing of the repeats by -1 to 12 nucleotides appears to confer some factor specificity (Naar *et al.*, 1991), (Umesono *et al.*, 1991). The protein determinants that confer spacing specificity are located in the DNA binding domain as well, in a stretch of amino acids called the D box (Umesono and Evans, 1989). Overlap in the target specificity does occur and may have a regulatory role as has been reported for the COUP-TF sub-family members which seem to be able to repress transcription mediated by estrogens (Liu *et al.*, 1993), by retinoids (Tran *et al.*, 1992) and by the peroxisome proliferators (Miyata *et al.*, 1993). COUP-TF and HNF-4 bind the same response element in the phosphoenolpyruvate carboxykinase gene (Hall *et al.*, 1995). MB67 and RLD-1 have been reported to bind retinoic acid response elements as heterodimers with RXR and to activate transcription through those sites (Baes *et al.*, 1994), (Apfel *et al.*, 1994). Dax-1 can acts as a dominant negative regulator of transcription mediated by the retinoic acid receptor (Muscatelli *et al.*, 1994).

Together these observations suggest that the cellular promoter/enhancer regions that regulate the expression of nuclear receptor target genes may contain cis-acting sequences that discriminate between the available nuclear receptor dimers. The mechanisms underlying this discrimination are likely to be varied and include subtle sequence differences in the nuclear receptor binding sites themselves as well as the presence of additional sequence motifs that bind to other types of transcription factors that cooperate with or antagonise the function of the bound nuclear receptor.

**Sequestration of unliganded receptors into a repressor complex**

The early studies of steroid receptor structure demonstrated that the receptors could be recovered from hormone-free cells in two forms as defined by sucrose gradient centrifugation, sedimenting at either 4S or 9S. Hormone induced the formation of the 4S form at the expense of the 9S form. The extraction profile of the receptors from cells treated with hormone differed from that of untreated cells. The former requiring high salt and detergent to dissociate from the chromatin and the latter eluting at low salt concentrations from the cells. Since then, it has been shown that the 9S form consists of one steroid receptor molecule, two HSP90 molecules and one HSP56/p59 molecule (Joab *et al.*, 1984), (Tai *et al.*, 1986), (Rafestin *et al.*, 1989), (Perdew, 1988), (Rexin *et al.*, 1991), (Segnitz and Gehring, 1995). Recruitment of the newly translated glucocorticoid- (Dalman *et al.*, 1989) and progesterone receptors into this complex has been shown to be required to activate ligand binding activity (Bresnick *et al.*, 1989), (Scherrer *et al.*, 1992), (Schulman *et al.*, 1992). In yeast, reduced levels of HSP90 compromise steroid receptor mediated transcription activation (Picard *et al.*, 1990a), providing *in vivo* evidence that HSP90 is essential for signal transduction by steroid receptors. The HSP90 binding domain of the steroid receptors is their ligand binding domain (Pratt *et al.*, 1988) and several regions of the ligand binding domain seem to be required for this interaction (Chambraud *et al.*, 1990), (Schlatter *et al.*, 1992).

HSP90 is one of the most abundant cytoplasmic proteins inside cells comprising 1-2% of the total protein (Lindquist and Craig, 1988) and can be associated with tubulin and actin (Schlesinger, 1990). It can act as a chaperone, *in vitro*, preventing the aggregation and preserving the activity of proteins (Miyata and Yahara, 1992), (Wiech *et al.*, 1992), (Jakob *et al.*, 1995). HSP90 is also an ATPase (Hendrick and Hartl, 1993), ATP has been reported to induce an 'open to closed' conformational change in HSP90 (Csermely *et al.*, 1993).

The HSP56/p59 molecule associated with the steroid receptors is an immunophilin (Yem *et al.*, 1992), (Tai *et al.*, 1992), (Smith *et al.*, 1993), (Segnitz and Gehring, 1995). Immunophilins are peptidyl prolyl cis-trans isomerases. They fall into two classes which are named after the clinically important immunosuppressive agents that inhibit their isomerase activity; cyclophylins bind cyclosporin A and the FK506-binding proteins bind FK506 and rapamycin (Gething and Sambrook, 1992) All these compounds are fungal peptide antibiotics (Kunz and Hall, 1993). Peptidyl prolyl cis-trans isomerases have been found in most cellular compartments but no physiological role or substrate has been identified for these enzymes (Gething and Sambrook, 1992). The immunosuppressive properties of cyclosporin A, FK506 and rapamycin are due to their ability to inhibit T-cell activation or proliferation. Intriguingly, these effects are not due to inhibition of peptidyl prolyl cis-trans isomerase activities but rather to *de novo* association with, and inhibition of, the activity of proteins involved in signal transduction by the immunosuppressor-immunophilin complex. The target of both the cyclosporin A-cyclophilin complex and the FK506-FK506 binding protein is calcineurin, a serine/threonine phosphatase whose calcium dependent activity is necessary for activation of the autocrine interleukin 2 loop by inducing the nuclear translocation of the NF-AT transcription factor complex that acts on an AP-1 response element in the interleukin 2 regulatory sequence (Bierer *et al.*, 1993), (Etzkorn *et al.*, 1993). Rapamycin acts downstream of the cytokine/growth factor signal and impedes entry into S-phase by inhibiting phosphorylation events (Albers *et al.*, 1993). A direct cellular target of the rapamycin-FK506 binding protein complex has been described (Brown *et al.*, 1994). The

presence of peptidyl prolyl cis-trans isomerases in the 9S complex of unliganded steroid receptors raises the possibility that this catalytic activity is required to induce a conformational change in either the receptor, HSP90 or another component of the steroid receptor complex. However, FK506 does not inhibit the formation of the HSP90-GR complex *in vitro* (Hutchison *et al.*, 1993). The immunophilin in steroid receptor complexes is associated with HSP90 rather than the receptor itself, although the presence of two HSP90 molecules and of only one p59 molecule in association with one steroid receptor molecule either implies that the HSP90-p59 interaction requires a receptor moiety or that p59 interacts with a dimer of HSP90 (Renoir *et al.*, 1990). Last, a cyclophilin (CyP-40) has been reported to associate with the estrogen receptor complex (Ratajczak *et al.*, 1993) although another group failed to detect this protein in highly purified, crosslinked estrogen receptor complexes that contained p59 and HSP90 (Segnitz and Gehring, 1995).

HSP70 has also been recovered with the untransformed steroid receptors ((Kost *et al.*, 1989), (Sanchez *et al.*, 1990), (Ratajczak *et al.*, 1993). It seems to be required to establish the HSP90/glucocorticoid receptor complex but is not required after establishment (Pratt, 1993).

In yeast, a dnaJ chaperone homologue, YDJ1 has been shown to be necessary for maintaining the repressed state of the glucocorticoid and estrogen receptors as indicated by the steroid receptor's ligand independent transcriptional activity when only a point mutated version of this protein is present in the cell (Kimura *et al.*, 1995). Androgen receptor function has been shown to be greatly impaired in the absence of YDJ1 protein (Caplan *et al.*, 1995).

Apart from maintaining the steroid receptors in a ligand binding state, the HSP90 complex may also participate in the intracellular movements of the receptors (see below). Indeed, it has been postulated that HSP56 protein plays a role in microtubule directed intracellular trafficking (Walsh *et al.*, 1992), (Czar *et al.*, 1994).

FK506 and rapamycin have been reported to potentiate glucocorticoid receptor induced transcription at sub-saturating hormone concentrations, probably by increasing the nuclear concentration of receptor (Ning and Sanchez, 1993).

Nur77 is a member of the NGFI-B sub-family of the nuclear receptor superfamily. Cyclosporin A can inhibit Nur77 acticivity. The presence of Cyclosporin A apparently did not influence the phosphorylation status of the DNA binding domain of Nur77, a phosphorylation event previously shown to inhibit its DNA binding ability (Hirata *et al.*, 1993). The cyclosporin A effect required the presence of the A/B domain of Nur77 (Yazdanbakhsh *et al.*, 1995). This provides a new link between immunosuppressing drugs and nuclear receptor action.

There is ample evidence showing that the steroid receptor LBDs can confer ligand dependency onto intracellular proteins (Mattioni *et al.*, 1994). These proteins include the adenovirus E1A gene product (Picard *et al.*, 1988), a reverse transcriptase; the HIV Rev protein [Hope, 1990 #118], transcription factors; c-Myc (Eilers *et al.*, 1989), v-Myb (Burk and Klempnauer, 1991), various members of the Fos family ((Schuermann *et al.*, 1993), (Superti *et al.*, 1991), MyoD (Hollenberg *et al.*, 1993), p53 (Roemer and Friedmann, 1993), C/EBP (Umek *et al.*, 1991), LFB-1, HNF-4, HNF-3 (Drewes *et al.*, 1994), v-Rel (Boehmelt *et al.*, 1992), Gata-1, 2 and 3 (Briegel *et al.*, 1993), GCN4 (Fankhauser *et al.*, 1994) and Gal4 derivatives (Webster *et al.*, 1988), (Braselmann *et al.*, 1993) as well as protein kinases including c-Abl (Jackson *et al.*, 1993) and Raf1 (Samuels *et al.*, 1993). It is thought that the steroid receptor LBDs cause the inactivation of these factors' functions in the absence of hormone by binding to the HSP90 complex. Upon hormone binding the fusion proteins are released from the complex. The LBDs of steroid receptors also mediate homodimerization (see below). This may account for the regulation of the kinases for example (Jackson *et al.*, 1993). All the other factors that can be regulated by LBDs need to bind directly to nucleic acids, or chromatin associated factors, in order to carry out their function. Recruitment into the HSP90 complex appears to preclude this. The exact

mechanism underlying this repression is still unknown. It does not involve extensive 'melting' of the complexed protein, since enzymes such as β-galactosidase, galactokinase and diydrofolate reductase retain catalytic activities *in vivo* upon fusion with steroid receptor LBDs, whether or not the steroid cognate to the LBD are present (Mattioni *et al.*, 1994). The elution properties of steroid free receptors versus steroid bound receptors (75mM Tris-HCl pH 7.5 versus 0.4M KCl) indicate that HSP90 interaction results in the segregation of steroid receptors to a cellular compartment distinct from that in which they can be found upon steroid treatment, namely nuclear chromatin.

## Receptor structure-function relationships

The nuclear receptor superfamily members encode a distinctive and highly conserved DNA binding region, the so called Zn finger motif (Hard *et al.*, 1990), (Schwabe *et al.*, 1990). This domain is also called the C domain (Kumar *et al.*, 1986). On the amino terminal side of the DNA binding domain there is a non conserved domain, termed the A/B domain, that is responsible for part of the transcriptional activity of, at least, the steroid receptor members of the nuclear receptor superfamily (Tora *et al.*, 1989b), (Tasset *et al.*, 1990). At the carboxyl terminal side of the DNA binding domain of the steroid receptors there is a non conserved region, domain D, which often encodes nuclear localisation signals. These signals not only direct the receptors to the nucleus (Picard and Yamamoto, 1987), (Guiochon *et al.*, 1989), (Picard *et al.*, 1990b), (Ylikomi *et al.*, 1992), but, when it was analysed, also potentiate the capacity of the receptors to shuttle in and out of the nucleus and to exchange partners in the process of doing so, as was revealed in heterokaryon experiments (Guiochon *et al.*, 1991), (GuiochonMantel *et al.*, 1994), (Chandran and DeFranco, 1992). The next domain, domain E, is a complex domain. It is the ligand binding domain (LBD) and is followed by a variable region termed the F domain which has no known function, although, dominant negative estrogen receptors have been obtained by mutagenesis of the F domain (Ince *et al.*, 1993).

Besides mediating ligand binding (Green *et al.*, 1986), the LBD also encodes a dimerisation function (Kumar and Chambon, 1988) and a transcription activation function (Webster *et al.*, 1988), (Tora *et al.*, 1989b), (Tasset *et al.*, 1990), (Berry *et al.*, 1990). The steroid hormone receptor LBD is also responsible for the sequestration of the receptor into a repressor complex (Segnitz and Gehring, 1995) from which it dissociates upon hormone binding (Catelli *et al.*, 1985), (Tai *et al.*, 1986), (Nathan and Lindquist, 1995). Another form of repression is mediated by the LBDs of the thyroid receptors (Baniahmad *et al.*, 1992); it has been demonstrated that the ligand free thyroid receptor is able to repress the transcription of a gene linked to a thyroid hormone response element (Damm *et al.*, 1989). The silencing activity of the thyroid receptor α and β have been demonstrated *in vitro* . The thyroid receptors appear to inhibit transcription at an early step during transcription pre-initiation complex assembly, thyroid hormone relieves this inhibition (Fondell *et al.*, 1993), (Tong *et al.*, 1995). A similar silencing function has been documented for the retinoic acid receptors (Baniahmad *et al.*, 1992), (Casanova *et al.*, 1994).

The transcription activation functions of the A/B region and of the LBDs of the glucocorticoid and estrogen receptors and the transcription activation domain of VP16 were extensively analysed and compared by Pierre Chambon and his co-workers. This was done using synergism assays, whereby two activation domains were brought together onto one promoter (Tora *et al.*, 1989b), (Tasset *et al.*, 1990) and squelching assays whereby the capacity of one activation domain to squelch the transcriptional activity of another was measured in mammalian cells in culture (Meyer *et al.*, 1989), (Tasset *et al.*, 1990) and biochemically in transcription reactions *in vitro* (Pfitzner *et al.*, 1993). These studies showed that all 5 transcription activation domains (VP16, ER and GR A/B and E-F domains) activated transcription differently, even though certain combinations of activation domains did not result in synergism and other combinations did result in squelching. These findings strongly supported the idea that some transcription activation domains act on the same co-factors of the RNA polymerase II holo-enzyme, but via more co-factors (Brou *et al.*, 1993),

(Shemshedini *et al.*, 1992). The A/B domain of the estrogen receptor has been subdivided into 2 domains that can both independently synergise with the LBD transcription activation domain of the estrogen receptor in Hela cells (Metzger *et al.*, 1995). In yeast, the A/B region of the estrogen receptor can be subdivided into 3 regions which are almost as active on their own as the whole A/B region (Metzger *et al.*, 1995).

There is evidence that transcriptional stimulation by the estrogen, glucocorticoid and progesterone receptors occurs not simply by direct stimulation of the RNA polymerase or its associated co-factors but rather by a remodelling of the chromatin structure of target genes (Zaret and Yamamoto, 1984), (Pham *et al.*, 1991), (Richard and Hager, 1987), (Mymryk and Archer, 1995). These chromatin effects are presumably mediated by mammalian homologues of the yeast SW1, SWI2 and SWI3 proteins (Yoshinaga *et al.*, 1992), (Muchardt and Yaniv, 1993) and associated factors (Laurent *et al.*, 1992) and maybe the retinoblastoma protein (Singh *et al.*, 1995). Chromatin remodelling events such as nucleosome displacement may allow sequence specific and/or general transcription factors access to their DNA response elements (Cordingley *et al.*, 1987). It may also allow the juxtaposition of enhancers and promoters leading to the formation of tertiary structures essential for the efficient transcription of target genes (Grunstein, 1990). The steroid receptor mediated chromatin remodelling process does not require DNA replication, and in the case of the glucocorticoid receptor, it can readily be reversed by the administration of an antiglucocorticoid, namely RU38486 (Reik *et al.*, 1991).

## Nuclear receptor co-factors

One known protein ligand of the estrogen receptor A/B region is SSN6 (McDonnell *et al.*, 1992), a protein known to be the mediator of the α2-Mcm1 heterodimer mediated repression of the a cell type specific genes in α type yeast cells (Keleher *et al.*, 1992). It has been reported that the VP16 transcription activation domain and other 'acidic activation domains',

tethered to the Gal4 DNA binding domain, bind to the general transcription factor TFIIB (Lin *et al.*, 1991), (Lin and Green, 1991) and can recruit it to a DNA template containing Gal4 binding sites, where it then facilitates the formation of an RNA polymerase II holo-enzyme transcription pre-initiation complex. A ubiquitously expressed human protein, Trip 1, with homologies to an ATPase involved in yeast transcription activation, Sug 1, has been cloned in a yeast two hybrid screen using the hormone bound thyroid receptor as a bait. This protein also interacts with 9-cis bound RXR, the Gal4 acidic transcription activation domain and VP16, but not with the glucocorticoid receptor (Swaffield *et al.*, 1995) and has been shown to also interact directly with the TATA box binding protein TBP. It has been reported that some members of the steroid hormone superfamily interact with TFIIB (Ing *et al.*, 1992), (MacDonald *et al.*, 1995), (Cavailles *et al.*, 1995). Transcription activation by the retinoic acid receptor in teratocarcinoma cells has been shown to be potentiated by an E1A-like activity, allowing it to co-operate with TFIID in transactivation at a retinoic acid response element (Berkenstam *et al.*, 1992). The estrogen receptor LBD has been shown to rely on a hTAF$_{II}$30 factor containing sub-population of holo-TFIID complexes for transcription activation *in vitro* (Jacq *et al.*, 1994). In the same 2 hybrid screen used to isolate Trip1, D.D.Moore and colleagues isolated 15 different cDNAs, 11 of which encoded protein factors that only interacted with the ligand bound thyroid hormone receptor and 4 of which only interacted with the hormone free receptor. Furthermore, 12 of these proteins were also shown to interact with the RXR (Lee *et al.*, 1995). One study has revealed the existence of an auxiliary factor, RAF, which, *in vitro*, enhances sequence specific DNA binding by the glucocorticoid and androgen receptor homodimers (Kupfer *et al.*, 1993). Calreticulin, a major calcium binding protein of the endoplasmatic reticulum has been shown to bind the DNA binding domain of the glucocorticoid receptor and thus negatively modulate its DNA binding capacity (Burns *et al.*, 1994). An HIV-1 gene product, vpr, whose function in the viral life cycle is not yet clear, has been shown to interact with a 41 kDa protein, Rip-1 and to induce its translocation to the nucleus. Nuclear localisation of Rip-1 could also be induced by

glucocorticoid agonists and was inhibited by the glucocorticoid antagonists, RU38486. Moreover, vpr, and Rip-1 could be co-immunoprecipitated by antibodies against the receptor, but only when the receptor was loaded with agonists. Interestingly, vpr complementation of a *vpr* mutant virus was mimicked by glucocorticoid agonists and HIV-1 replication in U937 cells could be inhibited by RU38486 (Refaeli *et al.*, 1995). This discovery may have relevance for the clinical management of HIV infection, and uncovers yet another steroid receptor interacting protein. One laboratory reported the existence of three proteins that interact with the estrogen binding domain in a ligand dependent fashion (Cavailles *et al.*, 1994), (Halachmi *et al.*, 1994). Another laboratory reported the existence of a different estrogen receptor co-factor, TIF1. TIF1 interacts with the LBDs of RXR α, of the retinoic acid receptor α and of estrogen receptor. It was cloned by virtue of its ability to enhance transcription by the RXR LBD fused to the estrogen receptor DNA binding domain in yeast. TIF1 bears homologies to conserved domains found in transcriptional regulatory proteins, such as the bromodomain also found in the SWI2/SNF2 protein, a protein which has been implicated in chromatin remodelling (Cote *et al.*, 1994), (Kwon *et al.*, 1994). One study (Moghal and Neel, 1995), conducted on several human lung cancer cells with a panel of retinoic acid response elements uncovered two different genetic defects that lead to an impairment of the retinoic acid receptor LBD transactivation function in a response element dependent manner (Nagpal *et al.*, 1993), (Nagpal *et al.*, 1992) demonstrating the existence of two retinoic acid receptor LBD co-factors in this cell type.

The hunt for proteins that bind and mediate the action of nuclear receptors is still ongoing. It should result in the cloning of genes encoding ubiquitous and tissue specific proteins, some of which will interact with a large proportion of the nuclear receptor superfamily, and some of which will be receptor or even response element specific. The isolation of steroid receptor co-factors is an important avenue to further our understanding of the molecular mode of action of the nuclear receptors.

## Steroid receptor phosphorylation

The members of the nuclear receptor superfamily are phosphoproteins (Orti *et al.*, 1992). Serine phosphorylation in the DNA binding domain has been shown to modulate DNA binding by the vitamin D receptor (Hsieh *et al.*, 1993) and by the orphan receptor NGFI-B (Hirata *et al.*, 1993). It has been shown that the thyroid receptor β isoform induces gene expression more efficiently upon phosphorylation (Lin *et al.*, 1992). This stimulatory effect was further analysed and attributed to a more efficient homodimerisation of the receptor (Sugawara *et al.*, 1994). Different serine, threonine and tyrosine phosphorylation patterns have been observed for the different splice forms of the retinoic acid receptor β (Rochette *et al.*, 1992). Four serine residues of the progesterone receptor can be phosphorylated. Two of these sites show substantial phosphorylation in the absence of hormone, the other two are only phosphorylated upon hormone binding (Poletti and Weigel, 1993). Furthermore, one of these phosphorylations did not occur if the progesterone receptor was bound by an anti-progestin, ZK98299, a ligand that inhibits DNA binding (Takimoto *et al.*, 1992). A DNA dependent kinase has been implicated in this process (Weigel *et al.*, 1992), and phosphorylation has been shown to be important for transcription activation by the progesterone receptor (Denner *et al.*, 1990). The glucocorticoid receptor A/B domain bears not less than 7 serine residues that can be phosphorylated. In a transient transfection context, mutation of all seven residues to alanines did not affect its transcription activation function (Mason and Housley, 1993). In another experimental context, a correlation was made between the inability of glucocorticoids to induce TAT gene expression in the G2 phase of the cell cycle with changes in phosphorylation status and changes in the nuclear retention of the glucocorticoid receptor (Hsu *et al.*, 1992). This indicates that phosphorylation of the glucocorticoid receptor may affect its intra-cellular localisation. Interestingly, the authors report that RU38486 bound glucocorticoid receptor was efficiently retained in the nucleus during the G2 phase of the cell cycle. RU38486 bound receptor is not phosphorylated (Orti *et*

*al.*, 1989). Okadaic acid, a cell permeable inhibitor of serine/threonine phosphatases inhibits glucocorticoid receptor recycling, i.e. okadaic acid must be present while the receptor is exported from the nucleus to block its subsequent nuclear re-import (DeFranco *et al.*, 1991). Together, these data indicate that phosphorylation is not required for nuclear import but that dephosphorylation is important for receptor shuttling between the cytoplasm and the nucleus. Moreover, they provide indirect evidence that the LBD conformation of this receptor affects the phosphorylation status of the A/B domain. Okadaic acid has been reported to increase retinoic acid induced transcription, while over expression of the phosphatase catalytic subunit inhibited retinoic acid induction of various response elements, albeit not to the same extents, leading the authors to conclude that subsets of retinoic acid controlled genes will be differentially affected by the phosphorylation status of the receptors (Lefebvre *et al.*, 1995). The human estrogen receptor serine phosphorylation status is increased by (i) estrogen, hydroxytamoxifen and ICI164.384 and (ii) by activation of either protein kinase A or C signal transduction pathways. The ligand and PKA induced phosphorylation events occurred mainly in the A/B region while the PKC induced phosphorylation site was located elsewhere. The targets in the A/B domain were all putative proline directed protein kinase targets. Mutation of the A/B serine residues resulted in a partial impairment in transcription activation in transient transfection experiments (Le Goff *et al.*, 1994). Phosphorylation of the mouse estrogen receptor is induced by ligand binding and DNA binding (Lahooti *et al.*, 1994). Casein kinase II phosphorylates serine 167 of the human estrogen receptor in vitro, and this post translational modification is stimulated by estrogen binding (Arnold *et al.*, 1994). Site directed mutational analysis defined serine 118 of the human receptor as important in modulating the transcription activity of the A/B region. Phosphorylation of this serine was induced by estradiol and hydroxytamoxifen and to a lesser extend by ICI 164.384. Moreover, it was shown that the phosphorylation event did not affect the intranuclear localisation of the receptor (Ali *et al.*, 1993). A uterine, estradiol dependent, tyrosine kinase has been described. It phosphorylates tyrosine 537 of the human estrogen receptor in an

estrogen bound-estrogen receptor and calcium bound calmodulin dependent fashion. This kinase has specificity for tyrosine residues with acidic amino acids close to the carboxyl side (Castoria *et al.*, 1993). Very rapid (10 seconds) and transient stimulation of protein tyrosine phosphorylation by estradiol has been described in MCF-7 cells. The estrogen stimulation is inhibited by co-administration of ICI 164.384, and therefore seems to be mediated by the activated estrogen receptor (Migliaccio *et al.*, 1993).

In summary, it appears that phosphorylation of steroid receptors can affect DNA binding, dimerisation, nucleo-cytoplasmic recycling, and transcription activation. On the other hand, ligand binding, DNA binding and intracellular localisation have also been reported to induce phosphorylation or dephosphorylation events. Steroid receptor phosphorylation sites and the consequences of the phosphorylation events would appear to be receptor specific. Last, a tissue specific kinase that is estradiol inducible has been reported and induction of phosphorylation of cellular proteins by a steroid receptor dependent kinase has been proposed (Castoria *et al.*, 1993), (Migliaccio *et al.*, 1993).

**Interaction between steroid signalling and other signal transduction pathways**

Intercellular communication is not only mediated by small lipid soluble molecules such as the ligands for the nuclear receptor superfamily but also by protein factors. These protein factors interact with receptors located on the cell surface. There are two main ways in which cell surface signals are relayed into the cell; intracellular second messengers production and/or activation of phosphorylation cascades. These transduction pathways result not only in the second messengers induced activation of- or post translational modification of proteins pre-existing in the cell but also in the transcriptional induction of new sets of genes.

Many peptide hormones activate the cyclic AMP signalling pathway which results in activation of the catalytic subunit of protein kinase A, which, in turn phosphorylates a number of transcription factors (de Groot and Sassono-Corsi, 1993). These transcription

factors can affect steroid regulated genes, usually by binding to so called cyclic AMP response elements (Comb *et al.*, 1986), (Montminy *et al.*, 1986), (Bokar *et al.*, 1988), (Quinn *et al.*, 1988) (Weih *et al.*, 1990). Thus, multiple hormone signalling pathways may converge onto the regulatory sequences of one same target gene. Another type of interaction involves the dependency of cyclic AMP induced increases in human aromatase P450 in human ovary cells on the presence of the orphan receptor SF-1 whose binding site acts as a cyclic AMP response element in the promoter of the gene encoding this enzyme (Michael *et al.*, 1995).

One of the major nuclear targets of the growth factor induced protein kinase cascades is the AP1 complex. AP1 consists of two subunits, both are encoded by nuclear proto-oncogenes, namely, c-jun and c-fos (Bohmann *et al.*, 1987), (Rauscher *et al.*, 1988). Three papers, published in the same issue of Cell, showed that the glucocorticoid receptor has the ability to down-modulate AP1 activity on the collagenase promoter AP1 response element. These three reports showed that antagonism between AP1 and the glucocorticoid receptor was mediated by protein-protein interactions between AP1 and GR (Jonat *et al.*, 1990), (Yang-Yen *et al.*, 1990), (Schule *et al.*, 1990). Furthermore, it was demonstrated that the retinoic acid receptor DNA binding domain could be substituted for that of the glucocorticoid receptor without greatly affecting the down modulation potential (Schule *et al.*, 1990). Interestingly, RU38486 bound glucocorticoid receptor lost its capacity to interfere with AP1 stimulated transcription on the collagenase promoter response element. Interference was also shown to be reciprocal when tested on the well described glucocorticoid response elements of the MMTV promoter and TAT enhancer (Jonat *et al.*, 1990) (Reik *et al.*, 1994). In a study where lymphocytes were stably transfected with various glucocorticoid receptor mutants, glucocorticoid hormone mediated repression of AP1 activity, inhibition of the interleukin 2 mediated autocrine feedback loop and inhibition of c-Myc expression, were demonstrated to be mediated by inhibition of AP1 activity rather than by activation of *bona fide* glucocorticoid response elements. This led the authors to conclude that glucocorticoid induction of apoptosis

in lymphocytes is at least in part mediated by interference with AP1 induced transcription events (Helmberg *et al.*, 1995). In Jurkat cells, a cytotoxic T cell model system, negative regulation of the interferon γ promoter by glucocorticoids was shown to occur through an AP1 response element. Furthermore, this effect could be mimicked by dominant negative forms of c-Jun (Cippitelli *et al.*, 1995). Spi-1, also known as PU.1, is an ets related proto-oncogene expressed in various hematopoietic cell lines. It was shown to interfere with glucocorticoid as well as thyroid hormone and retinoic acid receptor transcription activation even when its DNA binding domain was deleted. Moreover, the glucocorticoid receptor was shown to antagonise the transcription activation capacity of Spi-1 and the glucocorticoid receptor DNA binding domain was necessary for this inhibition (Gauthier *et al.*, 1993).

Together, the above data, show that different transduction pathways can antagonise each other at the gene expression level through the same response elements. They also suggest a molecular mechanism for the anti-tumour and anti-inflammatory activity of glucocorticoids that does not involve transcriptional induction by the glucocorticoid receptor and thus reveal a repressive function for this receptor which may be as important as its activation function in mediating the anti-tumour and anti-inflammatory activity of glucocorticoids.

AP1 antagonism mediated by retinoic acid was demonstrated (Schule *et al.*, 1991). This led to the isolation of synthetic ligands for the retinoic acid receptors that have AP1 antagonistic activity but do not stimulate the transcription activation function by the retinoic acid receptor α. This represents a step forward in the development of new therapeutic drugs which specifically activate a single function of the receptor, hence potentially circumventing the pleiotropic effects usually entailed by natural ligand administration (Nagpal *et al.*, 1995).

AP1 has also been shown to be a target of the estrogen receptor, although in this case, the interaction seems to be of a positive nature. An estrogen response element in the chicken ovalbumin gene was shown to bind AP1, and the estrogen response at this site did not require the estrogen receptor DNA binding domain, suggesting that a tertiary complex involving AP1 and the estrogen receptor was formed on this element (Gaub *et al.*, 1990). A

similar mode of estrogen regulation at the insulin-like growth factor I promoter has been reported (Umayahara *et al.*, 1994). In a recent study (Webb *et al.*, 1995), AP1 dependent estrogen stimulation of transcription at the collagenase promoter was determined to require the A/B domain but not the DNA binding domain of the estrogen receptor and co-immunoprecipitation of c-Jun and the estrogen receptor only required the presence of the A/B domain of the receptor. Moreover, the anti-hormone hydroxytamoxifen was shown to induce higher levels of transcription in this system than estradiol. However, this effect required the DNA binding domain of the receptor, as did a weaker stimulation observed with another anti-estrogen, ICI 164.384. The authors concluded that there are at least two distinct ways in which AP1 and the estrogen receptor can interact. These two pathways appeared to be tissue specific because the estrogen receptor DNA binding domain dependent (hydroxytamoxifen induced) pathway was more active in cervix derived Hela and endometrium derived Ishikawa cells while the DNA independent pathway predominated in the breast cancer cell line MCF7. In a Chinese hamster ovary derived cell line, both pathways were operative. The tissue specificity described above correlates well with the known tissue specificities of hydroxytamoxifen antagonism and agonism in vivo. This validates the proposition that tissue specific agonist effects of anti-estrogens may be due to tissue specific estrogen receptor-AP1 interactions. The positive interaction between the antihormone bound estrogen receptor and AP1 depends on the AP1 response element since Phillips *et al* found that only estrogens can stimulate AP1 driven transcription levels and that tamoxifen and ICI 164 384 antagonised this estrogen effect when they used a different AP1 response element, derived from the polyoma virus enhancer in MCF7 cells (Philips *et al.*, 1993). Overexpression of c-jun or c-fos proteins in MCF7 cells inhibits estrogen stimulation of transcription through an estrogen response element (Doucas *et al.*, 1991). The c-fos but not the c-jun mediated inhibition could be relieved by overexpression of the estrogen receptor, arguing that the AP1 subunits interfered with the estrogen receptor in at least two different fashions.

Mice devoid of c-Fos protein suffer of osteopetrosis, a disease caused by the failure of bone macrophages, the osteoclast precursors, to differentiate (Grigoriadis *et al.*, 1994). Overexpression of c-fos in mice results in osteosarcomas and chondrosarcomas ((Grigoriadis *et al.*, 1994). Since a lack of estrogens results in osteoporosis (Korach, 1994), a disease characterised by an altered balance in osteoclast and osteoblast mediated bone remodelling, and since two anti-estrogens, raloxifene (Evans *et al.*, 1994) and hydroxytamoxifen (Isserow *et al.*, 1995) have estrogen like bone sparing properties, it is tempting to speculate that the bone specific estrogen agonism of these two compounds is due to an AP1 mediated estrogen response.

The above discussion favours the concept that, as has been demonstrated for the glucocorticoid receptor and AP1, there is a direct interaction between AP1 and the estrogen receptor. It is however possible that the AP1-hormone effects that have been described were caused by an indirect activation of the transcriptional activity of AP1, for example by estrogen induction of a phosphorylation cascade (Aronica *et al.*, 1994), (Migliaccio *et al.*, 1993).

Both inhibitory and stimulatory effects on androgen and progesterone transcription mediated by AP1 were observed in a cell type specific fashion (Shemshedini *et al.*, 1991). On the other hand, only negative effects of AP1 on glucocorticoid receptor action were observed in the same study. This tends to argue against a direct interaction between the progesterone and androgen receptors and AP1. Mechanisms invoked to rationalise trans-repression and stimulation between AP1 and nuclear receptor superfamily members other than a direct interaction, include squelching (Ptashne, 1988) and receptor or AP1 induced changes in the transcription pre-initiation complexes or co-factors available in the nucleus (Moyer *et al.*, 1993).

## High resolution map of the interdigitated functions of the LBD at the primary sequence level

Although the multiple functions that the steroid receptor LBDs encode are interdigitated, mutational analyses of the LBD of the mouse and human estrogen receptors have highlighted amino acid stretches that determine ligand binding (Danielian *et al.*, 1993), (Fawell *et al.*, 1990a), (Pakdel and Katzenellenbogen, 1992), others that are important for receptor homodimerisation (Fawell *et al.*, 1990a),(White *et al.*, 1991), (Lees *et al.*, 1990) and yet others that are essential for the LBD's transactivation potential (Danielian *et al.*, 1992). Interestingly, these mutations localise to the carboxyl terminus part of the E region of the estrogen receptor and delineate three adjacent regions, amino acids 500-508, 514-531 and 538-545 (human amino acid sequence) as being responsible for dimerisation, ligand binding specificity and transcription activation respectively.

The ligand binding function of amino acids 514-531 was inferred earlier from chemical cross linking studies (Harlow *et al.*, 1989). Chemical cross-linking studies with the rat or mouse glucocorticoid receptors highlighted amino acids corresponding to residues 386, 420 and 530 of the human estrogen receptor as being close to the bound steroid (Carlstedt *et al.*, 1988), (Simons, 1987), (Smith *et al.*, 1988). One study wherein the chicken progesterone receptor, which is unable to bind RU38486, a steroid molecule bearing a large side group at position 11β of the steroid ring, was mutated to allow RU38486 binding identified amino acids corresponding to amino acid 350 of the human estrogen receptor as being close to this part of the bound steroid ligand (Benhamou *et al.*, 1992). Together, these data imply that the residues that make up the ligand binding pocket of steroid receptors are distributed all along the E domain of the receptors.

The crystal structure of a homodimer of the RXR LBD was published very recently (Bourguet *et al.*, 1995), it shows that the LBD is constituted of 11 α-helices and of 1 two-stranded β-sheet located between helices 5 and 6. The dimerisation region discussed above

(500-508) is located at the end of helix 9 and the beginning of helix 10. The ligand specificity sites and the cross linking sites are located on helices 5, 7 and 11. The ligand specificity region (514-531) is located on the end of helix 10. The transcription activation mutants are situated on helix 11.

The availability of crystal structures for steroid receptors will greatly help the rational design of synthetic drugs that affect specific physiological functions of the nuclear receptors that have ligands. Nevertheless, there is a need to functionally test the molecular consequences of ligand binding. The agonistic or antagonistic properties of these synthetic compounds can be tested in transcription assays. However, transcription activation is the last step of the molecular events that ensue ligand binding.

This thesis presents a new assay for the early steps of steroid receptor (in)activation by natural and artificial ligands. It relies on the repressor function of the steroid receptor LBDs which is generally thought to be due to complex formation between the steroid receptor LBDs and 2 HSP90 molecules and an FK506 binding protein (see above). I replaced the sequences coding for the A/B and DNA binding domains of nuclear receptors by sequence encoding the FLP site specific recombinase. These fusion proteins transduce the transient event of ligand binding into a DNA recombination event. The major differences between the nature of the signal obtained using these chimaeric receptors and the natural steroid receptors upon ligand exposure are (i) that the induction of the production of messenger RNAs by the full length steroid receptors requires cooperation between the receptor and a host of nuclear factors associated with the DNA template and the RNA polymerase in a fashion that is not fully understood to this date, while ligand induced site specific recombination is catalysed solely by the FLP-LBD fusion proteins and (ii) that for site specific recombination to occur, the only functions attributed to steroid receptor LBDs that need to be intact are repression and ligand induced relief of this repression while transcription assays employing the full length steroid receptors and their derivatives require that all the LBD encoded functions that result in transcriptional stimulation need be intact. This system therefore has the potential to more

accurately report the transient event of ligand binding to the steroid LBDs by virtue of its simplicity. In addition, this system may be quantitatively affected by events such as changes in nucleo-cytoplasmic shuttling, dimerisation and other protein-protein contacts that would occur during the transformation of steroid receptors from an unliganded, repressed state, into that of active transcription factors and therefore has potential as a means to study the receptors' functions that come into play after ligand induced release of the receptors from the HSP90 repressor complex.

The above LBD-recombinase fusion strategy can be used to investigate the possibility that the repressor function of the steroid receptor LBDs is not a characteristic unique to this sub-family of the nuclear receptor superfamily but that it is shared by more of its members. If this were the case for a so-called orphan receptor, a recombinase based selection scheme could be applied to discover the biomolecules that reverse this repression, namely new ligands or activating signals that regulate these nuclear receptors.

The work presented in this thesis demonstrates that the activity of the FLP site specific recombinase can be regulated by steroid receptor ligand binding domains. The effect of synthetic steroid ligands on the kinetics of FLP-LBD mediated recombination are documented and were analysed further using mutant LBDs in which the homodimerisation, transcription and nuclear localisation functions had been disrupted by site directed mutagenesis.

Finaly, the capacity of the LBDs of a number of nuclear receptor superfamily members to conditionally regulate FLP recombinase activity is analysed.

# Chapter 2 : MATERIALS AND METHODS

## MATERIALS

### Chemicals

Bacterial media, were purchased from Gibco-BRL, USA. Mammalian cell culture media were supplied by Sigma, Germany or Gibco-BRL, UK as was G418 sulphate (referred to as neomycin in this thesis). Hygromycin B was from Calbiochem, USA. All other organic and inorganic reagents not listed here were obtained from Biorad, Fluka, Merck, Serva or Sigma, Germany. The Enhanced Chemi-Luminescence Western blotting detection kit was purchased from Amersham, Germany.

### Steroids and other hormonal compounds

Dexamethasone, diethylstilbesterol, estradiol, hexesterol, tamoxifen, 5α-dihydrotestosterone and testosterone, were ordered from Sigma, Germany. Mibolerone was purchased from Dupont, USA. 4-OH tamoxifen was obtained from Günther Schutz, DKFZ Heidelberg, Germany. ICI compounds were the generous gifts of Zeneca pharmaceuticals, UK or ICI, UK. RU compounds were donated by Roussel Uclaf, France. CGP52608, melatonin, raloxifen and 1,25-$(OH)_2D_3$ were gifts from Ciba-Geigy, Basel Switzerland. FK506 was the generous gift of Fujisawa, Germany. Cyclosporin A was the gift of Sandoz, Switzerland. All these chemicals were dissolved in 95% ethanol apart from CGP52608 and melatonin which were dissolved in DMSO.

### Radioactive isotopes

[α-$^{35}$S]dATP (5000Ci/mmol) [α-$^{32}$P]UTP (800Ci/mmol) were obtained fresh each week from Amersham, UK.

### Enzymes

Restriction enzymes, calf intestinal phosphatase, *E. coli* DNA polymerase I Klenow fragment and T4 DNA ligase were purchased from New England Biolabs, USA, Fermentas, Lituania or Boehringer Mannheim , Germany. β-galactosidase was from Sigma, Germany. T7 and T3 RNA polymerases were obtained from Stratagene, Germany and RQ1 DNase and recombinant human RNasin were from Promega USA.

### Synthetic oligonucleotides

All the oligonucleotides used in this work were synthesised by the oligo service at EMBL.

## METHODS

### *Bacterial techniques*

### Bacterial strains used

The *E. coli* strains HB101 (genotype: *supE44 ara14 galK2 lacY1* Δ(*gpt-proA)62 rpsL20 (Str$^r$) xyl-5 mtl-1 recA13 Δ(mcrC-mrr)* HsdS$^-$ (r$^-$m$^-$)) and XL1-blue (genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI$^q$ZΔM15* Tn*10* (Tet$^r$)]$^c$were used for propagation and amplification of double stranded DNA plasmids.

**Maintenance and media**

*E. coli* cells were at 37°C in L-broth or on L-broth agar plates as described (Sambrook, 1989) except that ampicilin was present at 100μg/ml.

**Preparation of competent cells**

HB101 or XL1-blue were prepared for transformation by the following procedure. Bacteria were plated on L-agar without antibiotics and grown overnight. One colony was innoculated into 50ml of L-broth and grown without antibiotics overnight. Then 1ml of the 50ml overnight culture was used to innoculate 100ml of L-broth. The culture was grown at 37°C in a 500ml flask shaking at 300rpm until an $OD_{600}$ of 0.6 was attained. After chilling the culture in an ice-water bath for 10 minutes, the bacteria were harvested at 4°C by centrifugation at 5,000rpm for 5 minutes in a Sorval centrifuge. All subsequent steps were performed at 4°C in the cold room. The cells were gently resuspended by pipetting up and down in 25 ml of 0.1M $MgCL_2$. The resuspension of cells was harvested exactly as before and resuspended in 0.1M $CaCl_2$ and left on ice for 20 minutes after which the cells were spun as before and resuspended in 4.3 ml ice cold 0.1M $CaCl_2$ containing 14% (v/v) glycerol. Aliquots of 100 μl were then flash-frozen in liquid nitrogen and stored at -70°C.

**Transformations**

The 100μl frozen aliquots of competent cells were thawed on ice and split into two Eppendorf tubes. About one half of a pre-chilled ligation reaction or approximately 50ng of supercoiled plasmid was mixed with the bacteria and left on ice for 10 minutes. The cells were then subjected to a 2 minutes heat shock at 37°C or 42°C. 600μl of L-broth without antibiotics were added to the transformation mix which was then incubated at 37°C for 45

minutes. The cells were harvested by a flash-spin in an Eppendorf microfuge, resuspended in approximately 50 µl and plated out onto room temperature L-agar plates containing antibiotics as appropriate. After inversion the plates were incubated overnight at 37°C.

## *Recombinant DNA techniques*

### Restriction enzyme digests

Digests of double stranded DNA were performed under the conditions recommended by the supplier, using the buffers supplied. Standard recombinant DNA techniques were employed (Sambrook, 1989).

### Ligations

Ligations were carried out in a volume of 20µl in the standard ligation buffers (67mM Tris-HCl, pH 7.6, 1mM $MgCl_2$ 1mM DTT and 0.1mM ATP) with 1 unit of T4 DNA ligase for 2 to 24 hours at room temperature.

### Plasmid DNA mini-preparations

To obtain DNA of sufficient quality for the screening of recombinant plasmids by restriction analysis the following protocol was used. Single colonies from one L-agar plate were used to innoculate 700 µl of L-broth supplemented with 100µg/ml ampicillin in 2ml Eppendorf tubes and grown overnight at 37°C shaking at 400rpm. The cultures were lysed by addition of 700µl of 50% equilibrated phenol - 50% chloroform and shaking for 8 minutes in a bench top shaker. Then the samples were spun for 8 minutes at 14,000rpm in an Eppendorf microfuge and 500µl of the aqueous phase were isolated and mixed with an equal volume of

isopropanol. After a 10 minutes centrifugation at room temperature at 14,000rpm, the DNA containing pellet was washed with 70% ethanol, dried in a speed vac and resuspended in 50 µl of TE (10mM Tris-HCl pH 8.0, 1mM EDTA). 5µl of this solution were then used for restriction analysis. 1µl was then used for transformation of *E coli.* cells.

**Plasmid DNA maxi-preparations**

To obtain large quantities of DNA of appropriate quality for transfection into mammalian cells the following protocol was used. 500ml overnight cultures of a transformed *E. coli* clone were harvested by centrifugation, resuspended in 10ml buffer P1 buffer (RNAase A 100µg/ml, 50mM Tris pH 8, 10mM EDTA) and mixed gently with 10ml buffer P2 (200mM NaOH, 1% SDS) followed by incubation at room temperature for 5 minutes. Then, 10ml of buffer P3 (2.55M KAc pH 4.8) were mixed in gently and the lysate was incubated for 20 minutes on ice. After which the sample was centrifuged twice in a Sorval SS-34 at 16,000rpm for 15 minutes. The supernatant was then applied onto 500µg Qiagen columns, equilibrated with 10ml 750mM NaCl, 50mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100, and allowed to flow through by gravity flow. The Qiagen collumn 500 was washed 3 times with 30ml 1.0M NaCl, 50mM MOPS, pH 7.0 followed by elution with 15ml of 1.25M NaCl, 10mM Tris-Cl, 1mM EDTA, pH 8.0 solution into 30ml glass corex tubes. The eluant was then mixed with 11.5ml isopropanol and centrifuged at 8,000rpm, the supernatant was discarded and the pellet washed with 3ml 70% ethanol, dried and resuspended in 400µl TE. The $OD_{260}$ of the DNA solution was determined and the plasmid sample was further diluted to obtain either a 1 or 0.5mg/ml solution.

## DNA sequencing reactions

Dideoxy sequencing was employed to sequence all DNA constructs. 5µg of double-stranded templates were annealed to 1pM of primer in 142mM NaOH in a volume of 7µl by heating to 70°C. 2µl of TES buffer (560mM N-Tris [hydroxymethyl] methyl-2 amino ethane sulphonic acid (Sigma #T6152), 240mM HCl, 100mM $MgCl_2$) were then added to each reaction which was then left to anneal at room temperature ( TES acts to neutralise the NaOH and brings the reaction to pH 7). Each annealing reaction was mixed with 5.5µl labelling mix, containing 1µl 100mM DTT, 2µl 7.5µM dATP/dCTP/dGTP/dTTP 0.5µl [$\alpha$-$^{35}$S]dATP (5000Ci/mmol) and 1.5 units of T7 DNA polymerase, and incubated for 5 minutes at room temperature to allow extension of labelled products from the primer. 3.5µl aliquots of the labelling reaction were then added to 2.5µl of each of 4 termination mixes (80µM dATP/dCTP/dGTP/dTTP, 8µM ddXTP (where X is the terminating dideoxynucleotide) and incubated at 37°C for 5 minutes, after which 4µl stop solution (95% formamide, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue, 10mM EDTA) were added to stop the reactions. Samples were heated to 95°C for 2 minutes before loading on a 6% denaturing polyacrylamide gel.

## Polymerase chain reactions

The conditions used for polymerase chain reactions (PCR) were as follows; 50mM KCl, 10mMTris-HCl pH 6.8, 0.5mM $MgCl_2$, 0.2mM dNTPs, 10ng plasmid template, 2.5U amplitaq DNA polymerase (Cetus) and 50pM of each primer. The reactions were performed in a final volume of 100µl overlaid with 60µl mineral oil in 0.5ml PCR tubes. The amplifications were carried out on a thermal cycler with 30 cycles of; 1 minute at 90°C (denaturation), 2 minutes at 42°C (annealing), 1.5 minute at 72°C (extension). The last cycle was followed by a 5 minute incubation at 72°C.

### Site directed mutagenesis

All point mutations were generated using the method of Baratino *et al.* in 1994. This system relies on two rounds of PCR and two template plasmids into one of which the final PCR product is cloned. The first round of PCR was carried out with a "mutagenic" primer which encoded the desired mutation and a primer whose annealing site was (i) not present on the second template plasmid and (ii) lied beyond of restriction site suitable for sub-cloning. The product of this round of PCR was then gel purified. The second round of PCR was carried out using the second template plasmid, the product of the first PCR round as a "mega-primer", the above 3' primer now unique to the "mega-primer", and a primer whose annealing site on the template lied beyond a second restriction site suitable for sub-cloning. Thus a PCR product encoding a directed mutation and two suitable restriction sites, one 5' and one 3' of the mutation, was produced. This DNA was sub-cloned in the desired vector and the integrity of the construct was confirmed by restriction analysis and sequencing.

## ***Electrophoresis***

### Agarose gel electrophoresis

Agarose gel electrophoresis was performed essentially as described by (Sambrook, 1989). For the restriction analysis of plasmids and the isolation of DNA restriction fragments, 0.7-2% agarose gels were prepared in 1xTBE (90mM Tris-HCl pH 8.3, 90mM boric acid, 1mM EDTA) by boiling the agarose for several minutes followed by readjustment of the volume. Ethidium bromide was added to a concentration of 0.05µg/ml before pouring. Before loading samples, 1/10th volume of agarose sample buffer (40% (w/v) Ficoll, 1mM EDTA pH 8.0, 0.1% (w/v) bromophenol blue) was added. The gels were run submerged in 1xTBE at 15-20 volts/cm. Nucleic acids were visualised under UV irradiation and the relevant restriction

fragments were excised using a scalpel blade. For Southern analysis of genomic DNA, 0.8x20x20cm 0.86% agarose gels were prepared in 1xTAE (40mM Tris-acetate, 1mM EDTA, pH8.0) by boiling 3g of agarose in 350ml of gel solution for several minutes followed by readjustment of the volume. The DNA samples were resuspended in 70µl 1x agarose sample buffer and loaded onto the gel which was run overnight at 2.5 volts/cm.

**DNA sequencing gels**

Sequencing reactions were analysed on 0.2mm, 6% acrylamide (29:1 acrylamide:bis-acrylamide), 7M urea, 1xTBE pH 8.3 gels. The notched plate was treated with dimethyldichlorosilane solution and the back plate with technical grade ethanol, before polishing. To polymerise 30ml of gel 180 µl 10% ammonium persulphate and 40µl TEMED were added. Gels were prerun at 60 volts/cm for 30 minutes. Stopped sequencing reactions were heated to 95°C for 2 minutes and loaded. The gels were run at 60 volts/cm for as long as appropriate and then soaked in 10% acetic acid 10% methanol for 30 minutes prior to transfer to 3MM paper. The gel was dried and then exposed to Kodak XAR5 film at room temperature.

**SDS polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed essentially as described by Laemmli (Laemmli, 1970) using a Mini-Protean II gel kit from Biorad. 7.5-12% polyacrylamide (29:1 acrylamide:bis-acrylamide) 375mM Tris-HCl pH 8.8, 0.1% SDS (Biorad) running gels were used with a 3.75% polyacrylamide (29:1 acrylamide:bis-acrylamide) 1.25mM Tris-HCl pH 6.8, 0.1% SDS (Biorad) stacking gel. 10ml of gel solution were polymerised by addition of 60µl 10% ammonium persulphate and 10µl TEMED. Before loading, samples were mixed with 1/5 volume 6x sample buffer (0.25M Tris-HCl pH 6.8, 36% (w/v) glycerol, 10% (w/v) SDS

0.12% (w/v) bromophenol blue and 600mM DTT) gels were run at 10 volts/cm in running buffer (25mM Tris-HCl, 200mM glycine, 0.1% SDS).

## *Mammalian cell culture*

### Cell lines

E25B2 cells were derived from CV1 cells (ATCC CCL70, simian kidney cells) by O'Gorman *et al.* (O'Gorman *et al.*, 1991). Both these and 293 cells (ATCC, CRL 1573, human embryonic kidney cells) grew as adherent monolayers in cell culture dishes (Nunc or Falcon).

### Fœtal calf serum stripping

To extract steroid/lipophilic compounds from fœtal calf serum, a 5% (w/v) charcoal (Serva) - fœtal calf serum solution was stirred for 20 minutes at room temperature in 400ml Sorval centrifuge tubes. The charcoal was then removed by centrifugation at 10,000rpm for 30 minutes at 4°C. The supernatant was filtered once through a paper filter and then through a 0.22μm Millipore filter to sterilise the stripped serum.

### Maintenance of 293 and CV1 cells

Cells were grown at 37°C, 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) without phenol red (Sigma), supplemented with 10% stripped fœtal calf serum, 100 units/ml penicillin (Gibco-BRL), 100μg/ml streptomycin (Gibco-BRL) and 2mM glutamine. Cells were fed every three days and passaged about every seven days. Cells were passaged by removal of old media by aspiration, rinsing the confluent monolayer of cells shortly with 5

ml PBS (0.8% NaCl, 0.02% KCl, 0.02% $KH_2PO_4$, 0.115% $NaHPO_4$ pH 7.4) and adding 5 ml trypsin EDTA solution (0.025% trypsin, 1mM EDTA in PBS, (Gibco-BRL)) pre-warmed to 37°C. The plates were then incubated at 37°C for 2-5 minutes to allow the onset of cell detachment. The cells were taken up with an additional 5 ml of growth medium into a 15ml Falcon tube and spun down at 800rpm in a Hereaus bench top centrifuge for 3 minutes. The pellet of cells was resuspended and $1/5^{th}$ to $1/10^{th}$ of this cell suspension was used to innoculate fresh dishes.

**Freezing and thawing cells**

For freezing, a confluent plate of cells was treated with trypsin and spun down as described above. The cells were resuspended in 4ml of ice cold growth medium containing 10% DMSO and stored as 1ml aliquots in cryo-vials overnight at -70°C in a cardboard box to ensure slow freezing. The next day the vials of cells were transferred to a -180°C liquid nitrogen storage tank. For thawing, a vial of cells from the liquid nitrogen container was incubated in a 37°C water bath until the cell suspension was liquid. The cells were diluted in 10ml culture medium, pelleted as above, resuspended and seeded into one 10cm dish. The next day, the growth medium was replaced to eliminate all traces of DMSO.

**Dilution and administration of chemicals to cell cultures**

Chemicals were serially diluted tenfold by diluting 1 volume of ligand solution into 9 volumes of vehicle solution (95% ethanol or 100% DMSO) and vortexing followed by a quick centrifugation before the next dilution step was undertaken. These dilution series were then used as 333x and 1000x working solutions to generate an array of measurements evenly spaced out on a logarithmic scale. All the time courses of ligand induced recombination included a negative control that consisted of vehicle only, added to the growth medium.

These samples were collected at the end of the time course and were used as the time=0 point. Chemicals were always first diluted into growth medium which was then added to the cell culture dish(es).

***Mammalian cell transfection protocols***

**Transient DNA transfection by lipofection**

Lipofections were performed essentially as described by Boehringer Mannheim in the protocol accompanying the lipofection reagent DOTAP (N-[1-(2,3-Dioleoyloxy) propyl] -N, N, N-trimethyl-ammoniummethylsulphate). 30µl DOTAP reagent were added to 70µl 1xHBS (20mM HEPES, 150mM NaCl; pH 7.4). This solution was mixed with 100µl HBS containing 5µg of supercoiled plasmid DNA and incubated at room temperature. After 10 minutes the transfection-reagent-mixture was added to 6 ml pre-warmed culture medium. A 3.5cm dish containing a 70% confluent mono-layer of CV1 derived cells growing on a 2x2cm glass coverslip was exposed to 3ml of the above transfection mix. When more than two dishes were to be transfected with one same plasmid, the above procedure was repeated in parallel as many times as necessary and all the transfection-reagent-mixtures were mixed into the appropriate volume of growth medium which was then added to the individual culture dishes. After 8 to 24 hours the cells were rinsed twice with 1xPBS and medium containing either vehicle or hormones dissolved in vehicle was added for approximately 50 hours. Only CV1 cells and their derivatives were transfected by lipofection as 293 cells proved to be refractive to this transfection protocol.

**DNA transfection by the calcium phosphate co-precipitation method**

Both 293 and CV1 derived cells were transfected by the calcium phosphate co-precipitation method. Four hours before transfection, the medium of 70% confluent monolayers of cells was replaced with fresh medium. The co-precipitate was formed in a 15ml Falcon tube by dropwise addition of 250mM $CaCl_2$ to an equal volume of 2xHBS (50mM HEPES, 1.5mM $Na_2HPO_4$, 280mM NaCl, pH 7.13) containing 1 to 15µg of DNA whilst gently vortexing the Falcon tube. The precipitate was left to stand for 30 minutes at room temperature, mixed thoroughly and applied dropwise to the cells. After overnight incubation the old medium was removed, the cells were washed three times with PBS and fresh medium was added. For transient (co-)expression studies the DNA used was in the form of supercoiled circular plasmids and the cells were exposed to medium containing either vehicle or the compound under study dissolved in vehicle for a total of 50 hours after transfection. When stable chromosomal integration of the DNA constructs was desired, the DNA encoding both the transgene and a drug resistance marker was in a linearised form. The cells were treated with trypsin as described above following the overnight incubation with the co-precipitate and re-plated onto a new 10cm cell culture dish. The following day selection was imposed by inclusion of selection drug(s) in the growth medium. The concentration of drug used was determined beforehand to be lethal in 6 days by carrying out a titration of drug concentration in the growth medium of untransfected cells and monitoring the cultures daily. These concentrations were 0.4 and 1.6mg/ml G418 and 0.6 and 0.4mg/ml hygromycin B for 293 and CV1 derived cell lines respectively. Drug resistant clones were picked and plated out into single duplicate or triplicate wells of 24 well plates to test them for the desired properties and for further expansion. Typically 6 to 12 clones were picked per transfected construct.

**DNA transfection by electroporation**

Electroporations were carried out essentially as described in Current Protocols for Molecular Biology. 5 or 10 million cells were treated with trypsin and resuspended in 450µl 1xPBS (room temperature). This was mixed in a 1ml mammalian cell electroporation cuvette (Biorad) with 5µg linearised (for stable transfection) or 5µg supercoiled (for transient expression) plasmid DNA diluted in 50µl 1xPBS. Electroporation was performed at 350V and 960µF with a Biorad gene pulser with a Biorad capacitance extender. The electrocuted cells were then dispersed by vigorous pipetting in 10ml growth medium and plated out onto a 10cm dish. All subsequent steps were identical to those described above for the calcium phosphate mediated transfection .

***Genomic DNA isolation and analysis***

**Genomic DNA purification**

Genomic DNA purification for Southern analysis was performed by removing the medium from the cell culture dishes and squirting 2ml lysis buffer (20mM Tris-Cl pH 8.0, 50mM EDTA, 1% SDS) onto the cell monolayer. The lysate was collected in a 14 ml Falcon 2059 tube and incubated overnight at 37°C in the presence 200µg/ml proteinase K (Boehringer Mannheim). The next day 4ml of phenol equilibrated against 100nmM Tris, pH 8.0, was added to each tube and gently shaken for 5 minutes. Phase separation was carried out by centrifugation in a Beckman bench top centrifuge at ambient temperature for 10 minutes at 3000rpm. The supernatant was collected in a new Falcon 2059 tube and mixed thoroughly with 1ml of 7.5M ammonium acetate. 6ml of 100% ethanol were then mixed in and the samples were stored for at least 20 minutes at -20°C. Then the samples were subjected to a centrifugation as above and the pellets were rinsed with 3ml 70% ethanol. The inside of the

tubes was dried with a tissue to remove as much ethanol as possible and the pellet was resuspended in 2ml TE. The DNA was left to dissolve for at least 1 day on a rotary shaker at room temperature and a second ammonium acetate precipitation was performed as described above followed by re-suspension in a 250-1000µl of TE which would yield a DNA solution of 0.1-0.5mg/ml (apparent concentration as determined by $OD_{260}$ of 0.4-2.0mg/ml).

**Genomic DNA restriction digest**

Restriction digests of 20µg genomic DNA were carried out in 1.5ml eppendorf tubes in a final volume of 250µl. 40 units of the appropriate restriction enzyme, the restriction enzyme buffer recommended by the enzyme manufacturer and 0.5µg of acetylated bovine serum albumin (BSA, New England Biolabs) were used. The reactions were incubated at the appropriate temperature overnight after which 10µl of 0.5M EDTA pH8.0, 25µl 3M NaAc and 625µl 100% ethanol were added to each reaction. After vortexing, the samples were left at -20°C for 20 minutes and spun in an Eppendorf microfuge at 14,000rpm for 10 minutes at 4°C. The precipitates were rinsed once with 70% ethanol. All the ethanol was carefully removed and the DNA was dissolved in 70µl 1x agarose sample buffer by shaking at 37-50°C for 20 minutes.

**Southern blotting**

After electrophoresis, genomic DNA gels were immersed in 0.25M HCl for 9 minutes precisely (this results in a partial de-purination of the DNA fragments) followed by three 15 minute washes in 0.4M NaOH (resulting in the denaturation of the DNA duplex and in the conversion of the de-purinated bases into strand breaks). The gel was then equilibrated in 20xSSC (3M NaCl, 0.3M NaCitrate). DNA transfer onto Biodyne B membranes (negatively charged nylon) was mediated by capillary action in 20xSSC and was left to happen

overnight. The next day, the membrane was floated DNA side down onto 25mM $NaPO_4$ pH 7.2, 1mM EDTA solution for 2 minutes, dried between 3MM paper sheets and baked at 80°C in a vacuum oven for at least 4 hours. The filters were not exposed to UV.

**Riboprobe synthesis**

1 µg of plasmid templates from which T3 or T7 RNA polymerase transcripts could be generated (pBluescribe or pBluescript plasmid derivatives (Stratagene)) were linearised with the appropriate restriction enzyme to allow for the generation of 300-800 base transcripts corresponding to the genomic region under scrutiny (only restriction enzymes which generate blunt ends or 5'overhangs were used). The linear template DNA was precipitated with 1/5 volume of 3M NaAc and 0.6 volumes isopropanol by centrifugation in an Eppendorf microfuge, washed with 70% ethanol and dried in a speed vac. The DNA pellet was resuspended in 2µl $ddH_2O$, 3µl 5x transcription buffer (Stratagene) 1µl RNasin, 6µl [$\alpha$-$^{32}$P]UTP (800Ci/mmol), 2µl 3.3mM ATP, GTP, TTP and 20 units T3 or T7 RNA polymerase as appropriate and incubated at 37°C for 15 minutes. 0.3µl of the reaction was spotted onto 0.1mm cellulose polyethyleneimine thin layer chromatography paper before and after polymerisation. The reaction products were resolved by chromatography with 0.75M $KPO_4$, pH 3.5, buffer to check for label incorporation. After polymerisation, 2µl RQ1 DNase were added to the reaction and the reaction was incubated for 15 minutes at 37°C (this serves to eliminate the template DNA which would act as a competitor for the probe during the hybridisation step). To terminate the reaction, 40µl of Church and Gilbert buffer (7% SDS (Biorad), 0.25M $NaPO_4$ pH 7.2, 1% BSA, 1mM EDTA) were added to the reaction. The probe was added directly to the pre-heated hybridisation solution.

### Hybridisation of riboprobes to Southern blots

Hybridisations were performed in glass tubes (30cm long, 2.5cm radius) in an oven fitted with a rotating wheel and an adjustable thermostat (Bachofer, Laboratoriumsgeräte, Reutlingen, Germany). Up to four membranes pre-equilibrated in 25mM $NaPO_4$ pH 7.2 were pre-hybridised together in a glass tube in 8-10 ml Church and Gilbert buffer at the final hybridisation temperature (72°C for the LacZ probe and 70°C for all other probes used in this work) for at least 30 minutes, after which the riboprobe was simply added to the pre-hybridisation reaction. After at least 16 hours of hybridisation, the hybridisation solution was poured off and the membranes were rinsed briefly with 200ml washing buffer (10mM $NaPO_4$ pH 7.2 1% (w/v) SDS, 1mM EDTA) at room temperature, and 4 times in 1L washing buffer at 70°C (72°C for the LacZ probe). The membranes were then exposed to Phosphorimager screens (Molecular Dynamics) or Kodak XAR5 film at -70°C for 1 to 14 days. Phosphorimager quantification of radioactive signals was carried out with the ImageQuant software using the rectangle object to define background and volume integration functions.

## *Western blotting*

### Sample preparation

Between 5 and 10 million cells were scraped off a 10cm cell culture dish and rinsed once in 10ml PBS. The cell pellet was then resuspended in 100µl lysis buffer (0.1% Triton X-100, 400mM NaCl, 25mM Tris-Cl, pH 8.0, 1mM EDTA, 0.1mM PMSF, pepstatin, leupeptin) and left on ice for 20 minutes, followed by a 15 minutes centrifugation at 14,000rpm in an Eppendorf microfuge at 4°C. The supernatant was collected and the protein concentration was determined using the Bradford assay. Samples typically contained 0.5-2.0mg/ml protein.

10µg of protein sample were mixed with sample buffer and boiled for 5 minutes before resolution by electrophoresis as described above.

**Blotting and immunoprobing**

After electrophoresis, proteins were transferred from the SDS-polyacrylamide gel to a nitrocellulose membrane by electroblotting in transfer buffer (25mMTris-HCl, 192mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) at a current of 500mA for 4 hours at 4°C. The filter was pre-blocked in the blotting solution (4% (w/v) dry fat milk, 1xPBS 0.02% sodium azide) by shaking gently at room temperature for 2 hours. The filter was then rinsed briefly in 1xPBS. Mouse monoclonal antibody raised against the F domain of the human oestrogen receptor (Mab-EF-3 provided by Dr. H. Gronemeyer, Strasbourg, France) was diluted 1/1000 in blotting solution and incubated with the filter as described above. Dried mouse monoclonal antibody solution raised against the carboxyl half of the human oestrogen receptor (Mab-17, kindly provided by Dr. R. Miksicek, Stony Brook, USA) was reconstituted with 1ml $H_2O$ and diluted further with 1ml 1xPBS and hybridised to the pre-blocked filter in a sealed hybridisation bag for two hours by shaking gently at room temperature for 2 hours. After giving the filter three 5 minute washes in blotting solution, the horse radish peroxidase conjugated second anti-mouse antibody was diluted 1/1000 in blotting solution (without Na azide) and incubated with the filter as above for 2 hours. Then the filter was washed once for 10 minutes in 1xPBS and 3 times 5 minutes in blotting solution (without Na azide). The blot was exposed for 1 minute to a 50/50 mix of the ECL detection solutions 1 and 2, wrapped in Saran and exposed for 10 seconds to 10 minutes to Fuji RC film.

***Reporter gene enzymatic activity detection***

**In situ β-galactosidase assay**

Histochemical detection of β-galactosidase activity was performed essentially as described by Sanes *et al.* Confluent monolayers of cells were rinsed with 1xPBS, exposed for 2 minutes to fixing solution (2% formaldehyde, 0.1% glutaraldehyde, in PBS), washed three times with PBS in the course of the next 5 minutes and then overlaid with staining solution (1mg/ml X-gal, 2mM $MgCl_2$, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, in PBS) for 20 hours after which the cells were rinsed with PBS and examined. The number of blue cells on a 2x2cm glass slide mounted on a microscope slide was obtained using a microscope fitted with a slide controlling device either by sweeping across the whole of the area, thus yielding the absolute number of blue cells in $4cm^2$, or by counting the number of blue cells in five blindly chosen ocular field and extrapolating from those numbers by multiplying the average number of blue cells by the quotient of $4cm^2$ and the surface area of one ocular field. The error bars shown correspond to the standard deviations of those five measurements.

**Liquid β-galactosidase activity assay**

Quantitative β-galactosidase activity was determined essentially as described by Herbomel *et al.* (1984). Cells were washed with PBS, scraped with a rubber policeman in 1 ml of 40mM Tris-HCl pH 7.4, 1mM EDTA, 150mM NaCl into Eppendorf tubes, pelleted, and resuspended in 150µl of lysis buffer (250mM Tris-HCl pH 7.4 1mM EDTA, 0.2% saponin, 1mM DTT) and freeze-thawed 3 times by incubation for 5 minutes at -70°C followed by an incubation in water at room temperature for 5 minutes. The samples were then centrifuged for 10 minutes at 14,000rpm in an Eppendorf microfuge and the supernatant was collected. The

protein concentration of each sample was determined for each sample by the method of Bradford and used later to normalise the β-galactosidase activity measurements. β-galactosidase activity was measured by mixing 100µl of extract with 400µl LacZ buffer (100mM $NaPO_4$ pH 7.0, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol) and with 100µl ONPG (4mg/ml in 100mM $NaPO_4$, pH 7.0) to start the reaction. Reactions were carried out at 30°C as 293 cells contain a pseudo β-galactosidase activity which is less active at 30°C then at 37°C. Before the most active reactions turned deep yellow (between 10-30 minutes) all the reactions were stopped by the addition of 500µl stop solution (1M $Na_2CO_3$). The negative control consisted of 400µl LacZ buffer mixed with 100µl mock extract and 100µl ONPG. The optical density reference was generated by mixing 500µl LacZ buffer with 100µl 100mM $NaPO_4$, pH 7.0 and 500µl stop solution. Under these conditions β-galactosidase activity was directly proportional to the optical density of the samples measured at 420nm and it was linear with time.

**Luciferase assay**

Cells were washed with PBS, scraped with a rubber policeman in 1ml lysis buffer (100mM $KPO_4$, pH 7.8, 1mMDTT) into Eppendorf tubes, pelleted, and resuspended in 150µl lysis buffer and freeze-thawed three times. The lysate was spun 10 minutes at 14,000rpm in an Eppendorf microfuge in the cold room and the supernatant was collected in a new Eppendorf tube and kept on ice. A reaction mix which contained 9ml of 25mM glycylglycine pH 7.8, 1ml of 20mM ATP and 100µl 1M $MgSO_4$ was prepared and kept at room temperature. The luminometer (Bethold, Biolumat LB 9500 C) was set to measure the light emitted by the luciferase reaction for 30 seconds. An injection mix was prepared that consisted of 4ml of 25mM glycylglycine and 1ml of 1mM D-Luciferin (Sigma, dissolved in distilled water). ATP and D-Luciferin are the substrates for the enzyme. 10µl of extract was added to 350µl of the reaction mix in a luminometer tube and this was inserted into the luminometer, which then

automatically added 100µl of injection mix. The luciferase activity expressed in light units was digitally displayed and recorded manually.

***Recombinant DNA constructions***

All FLP recombinase fusion protein encoding constructs used in this study are derived from a modified pOG44 plasmid (O'Gorman *et al.*, 1991) wherein the stop codon of FLP was replaced by a BsiWI site followed by a BamHI, an EcoRI and a KpnI site. The recombinase fusion proteins therefore consisted of a variety of nuclear receptor superfamily members' ligand binding domains (LBDs) fused onto the carboxyl terminus of FLP recombinase. The DNA sequences written below are all in the 5' to 3' orientation.

**FLP-ER and FLP-GR constructs**

pOG44 was cut (i) with HindIII, the overhang was filled in (FI) using the *E. coli* DNA polymerase I Klenow fragment and (ii) with SphI. A 767bp fragment consisting of the 3' end of the FLP gene and of part of the polyadenylation signal was sub-cloned into pBS (Stratagene) which had been cut with (i) EcoRI (FI) and (ii) SphI. The resulting plasmid was was cut NsiI and Asp718 and a double stranded adaptor was cloned in to generate the following NsiI-SphI sequence interval; ATGCATATGCGTACGCGGATCCGAAT TCGGTACC containing an NdeI, a BsiWI, a BamHI and an EcoRI site. This plasmid was cut NsiI and BsiWI to introduce a PCR fragment generated using the following two primers (GGTACTATGCATATGATCC and GAAATGCGTACGGATATGCGTCT) that encoded the 3' end of the FLP gene where the stop codon of FLP was replaced by a BsiWI site. This plasmid was called pBSFLPHBD3, it was cut with BamHI and EcoRI to permit the sub-cloning of the BamHI-EcoRI fragments of pHE63 and pHG7 (Kumar *et al.*, 1986) encoding the human estrogen LBD (amino acids 252 to 595) and glucocorticoid LBD (amino acids 485

to 777). These plasmids were then cut with NdeI and NarI to release a 1.2 or a 1.4kbp fragments which could be sub-cloned into pOG44 cut with the same restriction enzymes to yield plasmids p44HE1 and p44HG1. The estrogen binding domain above contains a point mutation (G400V). To back-mutate this mutation, the NcoI-BglII fragment spanning this mutation was replaced with the homologous NcoI-BglII fragment from the mouse estrogen receptor encoded on plasmid J.MOR121-599 (Danielian *et al.*, 1992), kindly provided by Dr. D. Baratino. This did not result in any further alterations of the open reading frame. The plasmid thus obtained was called $p44HE^{WT}$. pHFE1, pHFE2 and pHFG (Hygromycin Flp receptor) plasmids were built by digesting pCNH2 (Schmidt *et al.*, 1991) with restriction enzymes SnaBI and HpaI and inserting the SnaBI-NarI (fill in), FLP fusion encoding fragments of $p44HE^{WT}$, p44HE1 and p44HG1 respectively.

**FLP-AR constructs**

Plasmid p44PCR was generated by cutting plasmid pOG44 and pBSFLPHBD3 NsiI-SphI and introducing the modified FLP 3' end back into pOG44. To introduce the human androgen receptor LBD (amino acids 624 to 919) into p44PCR, the PCR products generated with primer (i) GGCACCGTACGGATGCATATGACTCTGGGAGCCCGAAG and primer (ii) GGGCTCCGTACGTCACTGGTGTGGAAATAGATG and using plasmid pMS56XahAR [De, 1994 #288] as the template. pHFA was made by introducing the NarI (Fill in)-SnaBI, FLP-hAR encoding, fragment into the HpaI-SnaBI linearised pCNH2 plasmid.

**FLP-hRXRα and FLP-USP constructs**

p44hRXRα was constructed by cutting pBSFLPHBD3 with BamHI (Fill in) and EcoRI and introduction of the NcoI (Fill in)-EcoRI fragment, encoding the human RXRα LBD from pSG5hRXRα (Bugge *et al.*, 1992) and then insertion of the BsiWI-SphI, FLP-hRXRα encoding, fragment into likewise cut p44HE1. pHF-hRXRα was generated by cutting p44hRXRα BsiWI and Eco47III and introduction of this FLP-hRXRα encoding fragment into likewise cut pHFA. pSG5USP* was generated by introducing an adaptor into the BspMI site of pSG5USP (Thomas *et al.*, 1993) to create a BglII site right in front of methionine 220. (adaptor sequence; CATGAAGAGATCTATGAAGCGC). pSG5USP* was cut BglII and EcoRI (Fill in), the USP LBD containing fragment was then sub-cloned into p44HE1 which had been linearised with BamHI and StuI to generate p44USP

**Deletion of the nuclear localisation of the estrogen receptor ligand binding domain**

p44HE2 was cut with BamHI and EagI and a linker was inserted (GGATCCAACAGCCTGGCCTTGTCCCTGACGGCCG) thus deleting amino acids 251-303 of the estrogen ligand binding domain which encode the major nuclear localisation determinants of the human estrogen ligand binding domain (Ylikomi *et al.*, 1992)

**Construction of mutations affecting transactivation, dimerisation and ligand specificity properties of the estrogen receptor ligand binding domain**

Point mutations were generated by site directed mutagenesis as described above. The sequence of the 5' primer was; CCACCGAGTCCTGGACAAG and that of the 3' primer was; CCAGTAGTAGGTTGAGGCCGTTG. The template for the first round of

amplification cycles using one of the mutagenic primers and the 3' primer was p44HE1. The sequences of the "mutagenic" primers encoding the R503A-L507R, L507R, G521R, G521V, V534Stop and LL540-541AA mutations were; g.cac.cag.GCg.ctg.gcc.cag.AGG.ctc.ctc.atc, g.cgg.ctg.gcc. cag.GAc.ctc.ctc.atc, gg.cac.atg.agC.aac.aaa.AgA.atg.gag, gg.cac.atg.agt.aac.aaa.gtc.atg.gag, g.tgc.aag.aac.gtg.TAG.ccc.ctc.tat.g and c.gtg.gtg.ccc.ctc.tat.gac.GCg.GCg.ctg.aga.tg respectively. The second template, lacking sequence homology for the 3' primer, was pHFE1. The products of the second PCR reactions were digested with StuI and Eco47III to generate a 499bp fragment which were inserted into StuI-Eco47III linearised pHFE1. In all cases but one, the amino acid mutation (upper case) was tagged by creating a silent point mutation that would knock out a restriction site in its vicinity (underlined sequences). These were MspAI and AluI, AluI only, MaeIII, not tagged, BanI and BspMI respectively.

**Other FLP-LBD fusion proteins**

The ligand binding domains of the following members of the nuclear receptor superfamily were amplified by PCR in order to fuse them to the amino terminus of FLP. The origin of the cDNAs used can be found either through the EMBL DNA sequence database accession number provided or in the publication cited.

Drosophila ecdysone receptor (EcR, accession number; m:74078) full length: 5' primer; gcgatgtatgtacaagtgggtatgcggccggaatgcgtcgtcccggagaac and 3' primer; taccgcggatccctatgcagtcgtcgagtgctccgacttaac or truncated at amino acid 679: 3' primer; taccgcggatccctacgaagtggaggcagagtcgcaatc. Mouse estrogen related orphan receptor 2 (Err2, Berkenstam, unpublished): 5' primer; gcgatgtacgtacgagtggggatgctgaaccaacctgtgcaccttg and 3' primer; taccgcggatcctcacaccttggcctccagcatc . Human retinoid receptor RARα (accession number; x06538) 5' primer; gcgatgtacgtacgagtgggcatgtccaaggagtctgtg and 3' primer; taccgcggatcctcacggggagtgggtggccgggctgcttc. Human RZRα and rat RZRβ (accession

numbers; 114611 and 114610). Mouse steroidogenic factor, SF-1 (sequence available from ref Mol Endo 7 pp852-60): 5' primer; gcgatgtacgtacgagtgggcatgcgcctggaag, 3' primer; taccgcggatcctcaagtctgcttggcctgcagcatc. The mouse homologue of the drosophila tailless orphan receptor (Monaghan *et al.*, 1995): 5' primer; gcgatgtacgtacgcatgaacaaagatgccgtgcagcac, 3' primer; taccgcggatccttagatgtcactggatttgtac . Human thyroid receptor B (accession number; x04707): 5' primer; taccgcggatccctaatcctcgaacacttc and 3' primer; gcgatgtacgtacgagttggcatggcaacagatttggtgctggatg. Human vitamin D3 receptor (accession number; j03258): 5' primer; gcgatgtacgtacggatgatgaaggagtcattctg and 3' primer; taccgcggatcctcaggagatctcattgccaaacacttc.

# Chapter 3 : RESULTS AND DISCUSSION

## Steroid regulated conservative site specific DNA recombination

SUMMARY

To attempt to create conditionally active site specific recombinases, three steroid receptor ligand binding domain - FLP recombinase fusion protein encoding genes were constructed; FLP-human androgen receptor (FLP-hAR), FLP-human glucocorticoid receptor (FLP-hGR) and FLP-human estrogen receptor (FLP-hER). The ability of these new proteins to catalyse recombination was analysed, first by transient expression in cell lines engineered to report a chromosomal site specific recombination event and second by stable expression in recombination reporter cell lines. With each of the three proteins, ligand regulated site specific recombination was observed. The rate of site specific recombinase mediated excision of a 1300bp genomic DNA segment was measured in homogenous transgenic cell populations. Regardless of the genomic integration locus of the recombination target, initially, 8% of the cell population underwent excision recombination per hour, amounting to two events per cell per generation. Parameters that could affect the rate of production of recombination products within a population of cells stimulated with steroids are discussed. Plasmids bearing one or two FRTs were integrated into pre-existing chromosomal FRTs at high frequencies by ligand modulated site specific recombination.

INTRODUCTION

Steroid dependency has been conferred onto the function of a diverse collection of transcription factors through fusion to a steroid receptor ligand binding domain (Picard *et al.*, 1988). This strategy was also used successfully to render the transforming activity of the nuclear adenoviral oncogene, E1A, steroid dependent (Mattioni *et al.*, 1994). I was interested to find out whether the catalytic activity of a site specific recombinase could also be controlled through fusion to a steroid ligand binding domain. In 1991, O'Gorman *et al.* (O'Gorman *et al.*, 1991) showed that the site specific recombinase encoded by the nuclear 2μm plasmid of *S.cerevicsae*, FLP, is able to catalyse the excision of a FRT flanked DNA segment from a chromosome and to catalyse the integration of a plasmid bearing one FRT into a chromosome bearing a FRT itself in mammalian cells. The chromosomal FRT in question had been introduced in the open reading frame of the *E.coli* LacZ gene (figure 3.1) so as to permit the translation of functional β-galactosidase enzyme. This provided a system whereby site specific recombination could be scored histochemically. The induction or disappearance of β-galactosidase activity in cells can be monitored by in situ X-gal staining which stains β-galactosidase positive cells blue. Briefly; FLP mediated excision of a neomycin resistance conferring transcription cassette which disrupts the open reading frame of the LacZ gene results in the transcription of functional LacZ mRNA ($LacZ^-$,$Neo^r$ {white}-> $LacZ^+$,$Neo^s$ {blue}) while integration of a neomycin resistance encoding, FRT bearing, plasmid into the FRT located in the open reading frame of the LacZ gene will disrupt the open reading frame and result in the generation of β-galactosidase negative, neomycin resistant cells ($LacZ^+$,$Neo^s$ {blue} -> $LacZ^-$,$Neo^r$ {white}). This assay was used to study the recombinase activity of FLP-LBD fusion proteins. Two types of expression strategies were used; (i) transient transfection of fusion protein encoding plasmids (p44LBD) and (ii) stable transfection of plasmids bearing both a drug resistance gene and the recombinase fusion gene (pHFL) in clonal cell lines maintaining a chromosomal FLP recombination reporter transgene

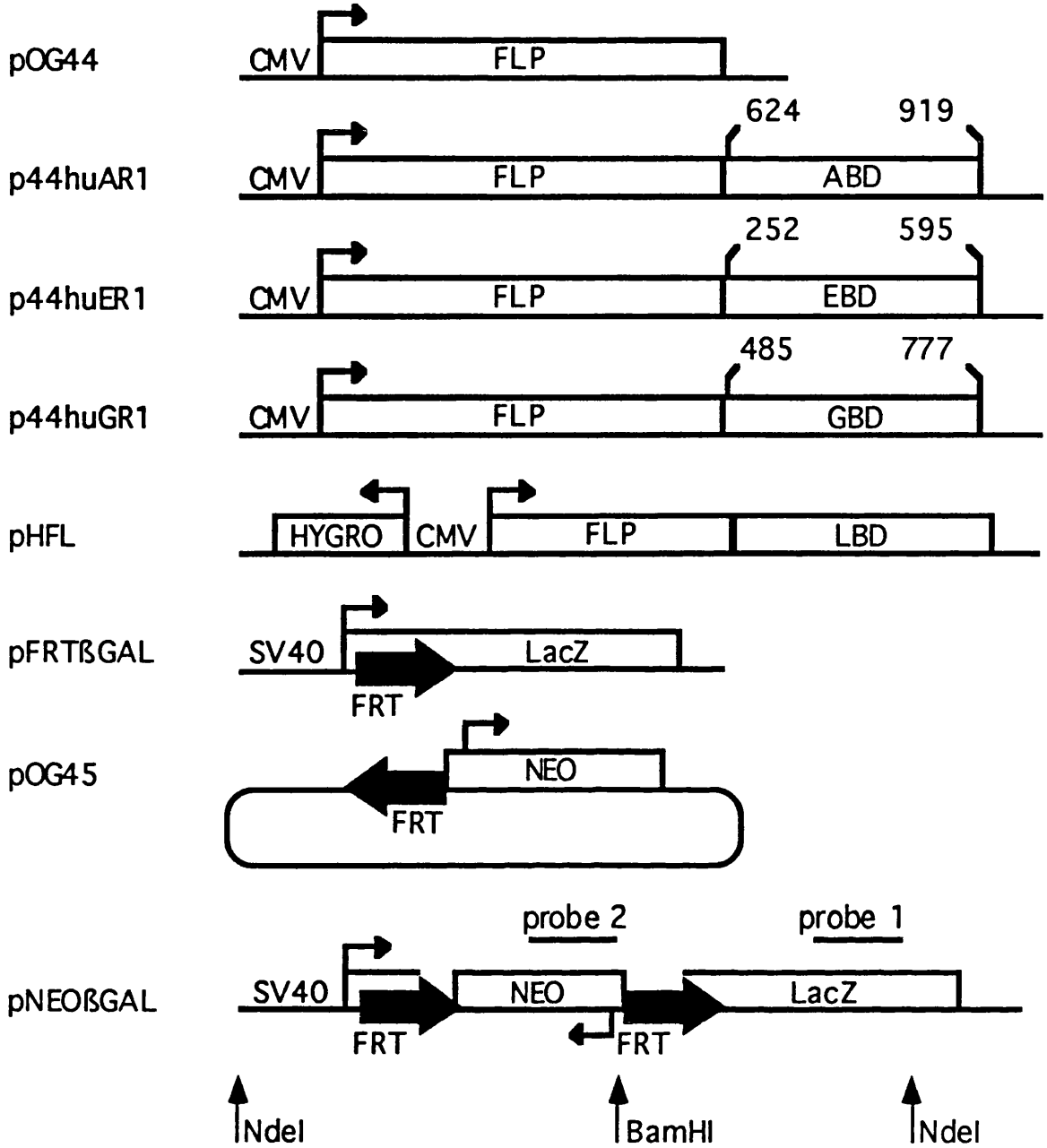



Figure 3.1 DNA constructs used in this study. p44LBD vectors are FLP fusion protein expression vectors, derived from pOG44 by replacing the FLP stop codon with a BsiWI a BamHI, an EcoRI and a KpnI site and fusing LBDs from the human androgen, estrogen or glucocorticoid receptors. The bi-directional CMV.tk cassette and hygromycin resistance gene of pHFL (L stands for androgen, estrogen or glucocorticoid) were derived from pCNH2 (Schmidt). pFRTβGAL, pNEOβGAL and pOG45 are FRT bearing plasmids, constructed by O'Gorman *et al.* The FLP recombination targets (FRTs) are depicted as large arrows and transcription start sites as small arrows. Restriction sites and probes relevant for the Southern analyses are shown on pNEOβGAL.

(figure 3.1). Recombinase activity in mammalian cells was measured by two means; indirectly by in situ staining for β-galactosidase activity and directly by Southern analysis of the recombination substrates and products.

RESULTS

## Generation of reporter cell lines for FLP mediated excision recombination

### Construction of CV1 derived reporter cell lines for FLP mediated excision recombination

In order to monitor the recombinase activity of FLP-LBD fusions, a FLP mediated excision reporter cell line was constructed. A CV1 derived cell line (E25B2) that contained one copy of pFRTβGAL (figure 3.1) inserted in its genome was subjected to co-transfection of a FLP expression plasmid (pOG44, figure 3.1) and pOG45 (figure 3.1), a neomycin resistance encoding, FRT bearing plasmid, in order to integrate the pOG45 plasmid into the chromosome via FLP mediated recombination. O'Gorman *et al.* (O'Gorman *et al.*, 1991), reported that under these conditions, FLP mediated integration of pOG45 into the chromosomal FRT is almost as frequent as is the random integration of the pOG45 into the genome. This was also true in my hands (see table 3.1).

Site specific integration was confirmed by two criteria; (i) concomitant gain of a $Neo^r$ phenotype with a loss of the $LacZ^+$ phenotype and (ii), restoration of the $LacZ^+$ phenotype upon exposure to FLP enzyme activity. In the course of 2 independent experiments, 12 neomycin resistant clones were picked, 5 were β-galactosidase negative, of which 4 clones could be induced to re-express functional β-galactosidase upon transient expression of FLP (Table 3.1). One of the 4 cell lines that displayed LacZ induction upon transfection of FLP expression plasmid (E25B2/B2), was used later as an excision reporter cell line.

| Clone | number of copies | Recombi-nation | Clone | number of copies | Recombi-nation |
|---|---|---|---|---|---|
| P1 | 1 | +++ | R5 | 3 | +++ |
| P2 | 1 | NT | R6 | 1 | ++ |
| P3 | 1 | NT | R7 | 2 | NT or + |
| P7 | 1 | NT | R7* | 2 | NT or + |
| P9 | 1 | NT | R8 | 1 | + |
| P10 | 2 | NT | R9 | 3 | +++ |
| Q1 | 3 | NT | R10 | 1 | +++ |
| Q2 | 1 | NT | R11 | 3 | + |
| Q3 | 4 | + | R12 | 2 | NT |
| Q4 | 2 | +++ | S1 | 3 | NT |
| Q5 | 1 | +++ | S2 | 3 | ++ |
| Q6 | 1 | NT | S3 | 1 | NT |
| Q7 | see R7 | see R7 | S4 | 1 | ++ |
| Q8 | 1 | ++ | S5 | 3 | ++ |
| Q9 | 1 | ++ | S6 | 2 | + |
| Q10 | 2 | + | S7 | 2 | + |
| Q11 | 2 | NT | S8 | 3 | +++ |
| Q12 | 2 | ++ | S9 | none** | - |
| Q13 | 2 | +++ | S10 | 1 | + |
| R1 | 2 | NT | S11 | 2 | ++ |
| R3 | 2 | ++ | S12 | 1 | + |
| R4 | none** | NT | | | |

Table 3.2 Screen of 42 pNEOβGAL cell lines by Southern analysis andby FLP expression. Neomycin resistant clones were screened for the number of pNEOβGAL inserts and for FLP mediated induction of β-galactosidase. * indicates that there was a confusion about which cells were Q7 or R7. ** indicates that no band could be detected with a probe directed against the LacZ gene1 (probe 1, figure 3.1) but that a specific band could be seen when a probe directed against the Bluescript plasmid backbone was used. NT means that the clone was not tested by transfection of FLP expression plasmid.

| Experiment | $Neo^r$ clones analysed | $LacZ^-$ clones | LacZ inducible |
|---|---|---|---|
| 1 | 7 | 2 | 2 |
| 2 | 5 | 3 | 2 |

Table 3.1 Generation of FLP mediated genomic excision reporter cell lines by co-transfection of 20μg pOG44 and 1μg pOG45 into the E25B2 cell line. Twelve clones were analysed. Of these; seven clones had a $LacZ^+$,$Neo^r$ phenotype, as would be expected if pOG45 had been integrated randomly into the genome. Five clones had switched from a $LacZ^+$,$Neo^s$ to a $LacZ^-$,$Neo^r$ phenotype and could be re-induced to assume a $LacZ^+$ phenotype upon exposure to FLP enzyme, as would be expected if pOG45 had been integrated into the genomic pFRTβGAL locus via FLP mediated site specific integration. One clone was $LacZ^-$,$Neo^r$ but did not display β-galactosidase activity upon exposure to FLP recombinase activity.

## Generation of 293 cell lines containing chromosomal FLP excision recombination substrates

To establish that FLP recombination was not only limited to the E25B2/B2 cell line or to the particular chromosomal locus where the recombination substrate was integrated in that cell line, 293 cells (human embryonic kidney cell line) were electroporated with 5μg linear pNEOβGAL plasmid and a total of 40 neomycin resistant clones was isolated. Electroporations were carried out at 4 voltages, 200V, 250V, 300V and 350V and the colonies picked from each experiment were named $P_X$, $Q_X$, $R_X$ and $S_X$ respectively.

Genomic DNA was isolated from each of these clones and analysed by Southern analysis with a restriction enzyme that cut only once within the transgene. The autoradiograms of the resulting Southern blots are not shown here but the information they yielded is summarised on table 3.2.

No sister clones were picked as the restriction pattern obtained was unique to each clone. The copy number of the transgene varied from one partial copy to 4 copies per cell line. In certain cases the copy number indicated may be an overestimate because the cell line analysed may have been of a polyclonal origin, as could be deduced from the differences in intensity of distinct restriction fragments originating from one cell line.

None of the cell lines contained concatamers of the pNEOβGAL plasmid (neither head to head, nor tail to tail, nor head to tail), as no band of a size indicative of these was observed.

Of the 42 clones, 27 were subjected to transfection with the FLP expression plasmid, pOG44 (figure 3.1), to check whether or not FLP could excise the neomycin transcription unit flanked by directly repeated FRTs from the integrated pNEOβGAL transgene, this data is also summarised on table 3.2. Of the 27 clones, 8 showed high numbers of blue cells, 9 showed intermediate numbers of blue cells, 9 showed lower numbers of blue cells upon transfection of pOG44 and 1 clone, S9, did not display any β-galactosidase positive cells after exposure to FLP activity. By Southern analysis the S9 clone was shown to lack the LacZ part of the pNEOβGAL transgene. One cell line, R5, already contained blue β-galactosidase positive cells, this probably indicated that it was derived from more than one neomycin resistant clone, one of which contained a re-arranged copy of pNEOβGAL which drove the expression of functional β-galactosidase enzyme.

The above data indicated that the pNEOβGAL construct could be utilised as an excision recombination substrate at most chromosomal loci from which selectable levels of neomycin phosphotransferase could be expressed. The variability in numbers of blue cells obtained after transfection of FLP expression plasmid is not necessarily indicative of differences in efficiencies of the recombination reaction at different chromosomal loci because there are other parameters which could affect the number of β-galactosidase positive cells obtained in this experiment. One of these parameters is that different 293 derived clones did not absorb calcium phosphate precipitated DNA with the same efficiency. This was demonstrated by exposing two of the above clones, P1 and S12, carefully grown to the same level of

confluence, to one same calcium phosphate precipitate of pFRTβGAL or of pOG44 plasmid and counting the number of blue staining cells obtained after 48 hours. The results of this experiment are shown on table 3.3.

| Clone | blue cells from pFRTβGAL (a) | blue cells from pOG44 (FLP) (b) | % ratio (b)/(a) |
|---|---|---|---|
| P1 | 483 | 294 | 61% |
| S12 | 98 | 63 | 64% |

Table 3.3 5μg of pFRTβGAL or of pOG44 were transfected as described in the text. The percentage of recombined cells is calculated as the ratio of blue cells obtained by transfection of pOG44 over that obtained by transfection of pFRTβGAL.

It is apparent from the experiment described in table 3.3 that the difference in blue cell number is almost entirely due to a difference in the transfection efficiency between these two clones. For both clones, the frequency of FLP mediated induction of the LacZ gene (calculated as the ratio of blue cells obtained by transfection of the FLP expression plasmid over the number of blue cells obtained by transfection of the LacZ expression plasmid) is around 60%.

Parameters that may explain the differences in the β-galactosidase response of table 3.2 include; (i) that clones containing multiple inserts of the pNEOβGAL transgene might undergo recombination more often, thus yielding a more recombinogenic cell line; (ii) clones should yield different numbers of blue cells merely because the expression levels of β-galactosidase differ; (iii) the genomic position of the pNEOβGAL integration locus might have influenced the observed recombination frequency.

To circumvent the above experimental caveats and to find out whether there was a correlation between the expression levels from a pNEOβGAL insert and its capacity to recombine, I decided to study the properties of the recombination system in a number of the

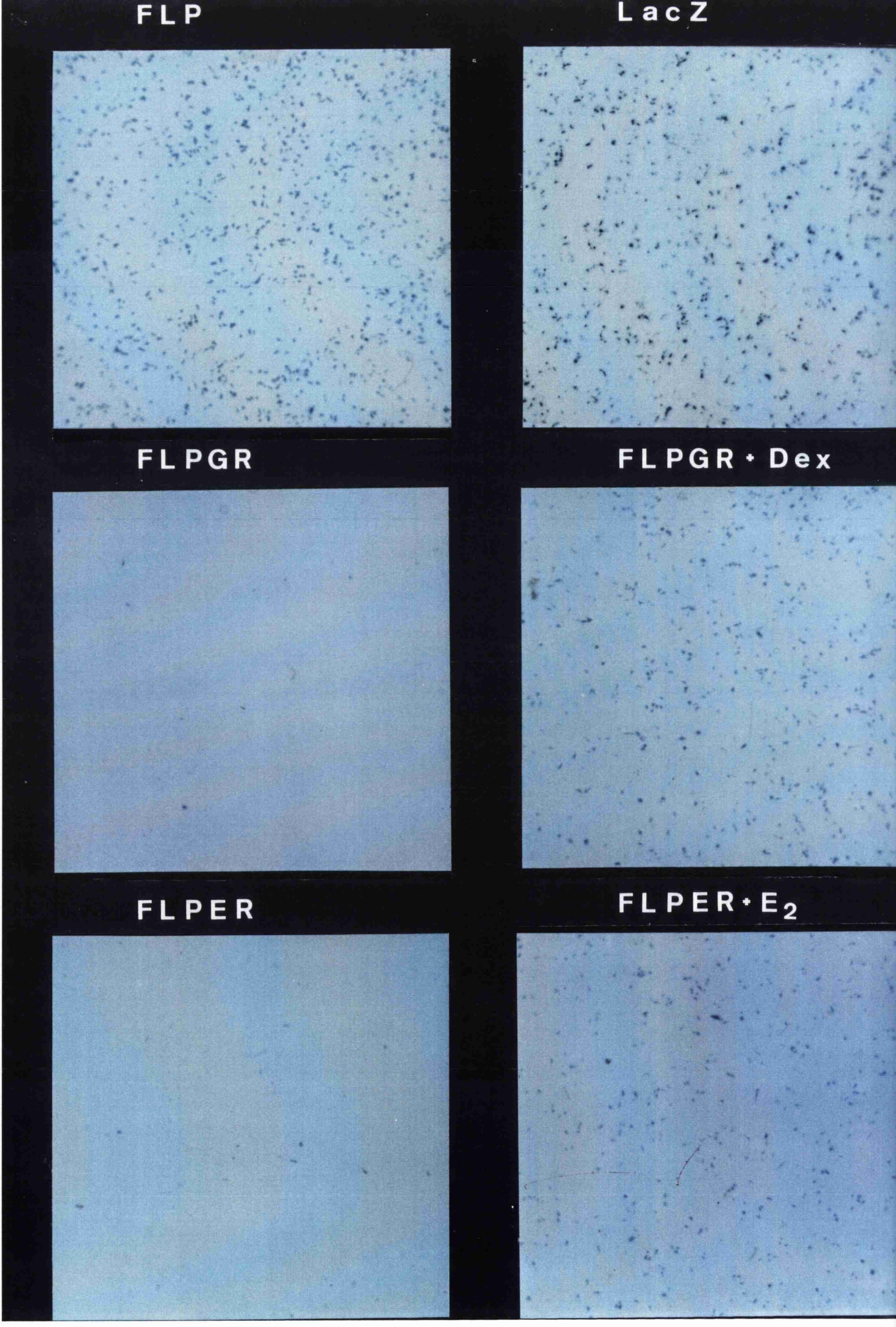

FLP
LacZ
FLPGR
FLPGR + Dex
FLPER
FLPER + E2

Figure 3.2 70% confluent E25B2/B2 cells were lipofected with pOG44, a FLP expression plasmid (top left), pFRTβGAL, a LacZ expression plasmid (top right), p44HG1, a FLP-GBD expression plasmid (middle pannels) or p44HE1, a FLP-EBD$^{G400V}$ expression plasmid (bottom panels). Ethanol vehicle or 1μM of hormone was added to the cells culture medium for 50 hours after which the confluent cell layers were stained for β-galactosidase activity using the X-gal substrate. Cells expressing β-galactosidase stain blue under these conditions.

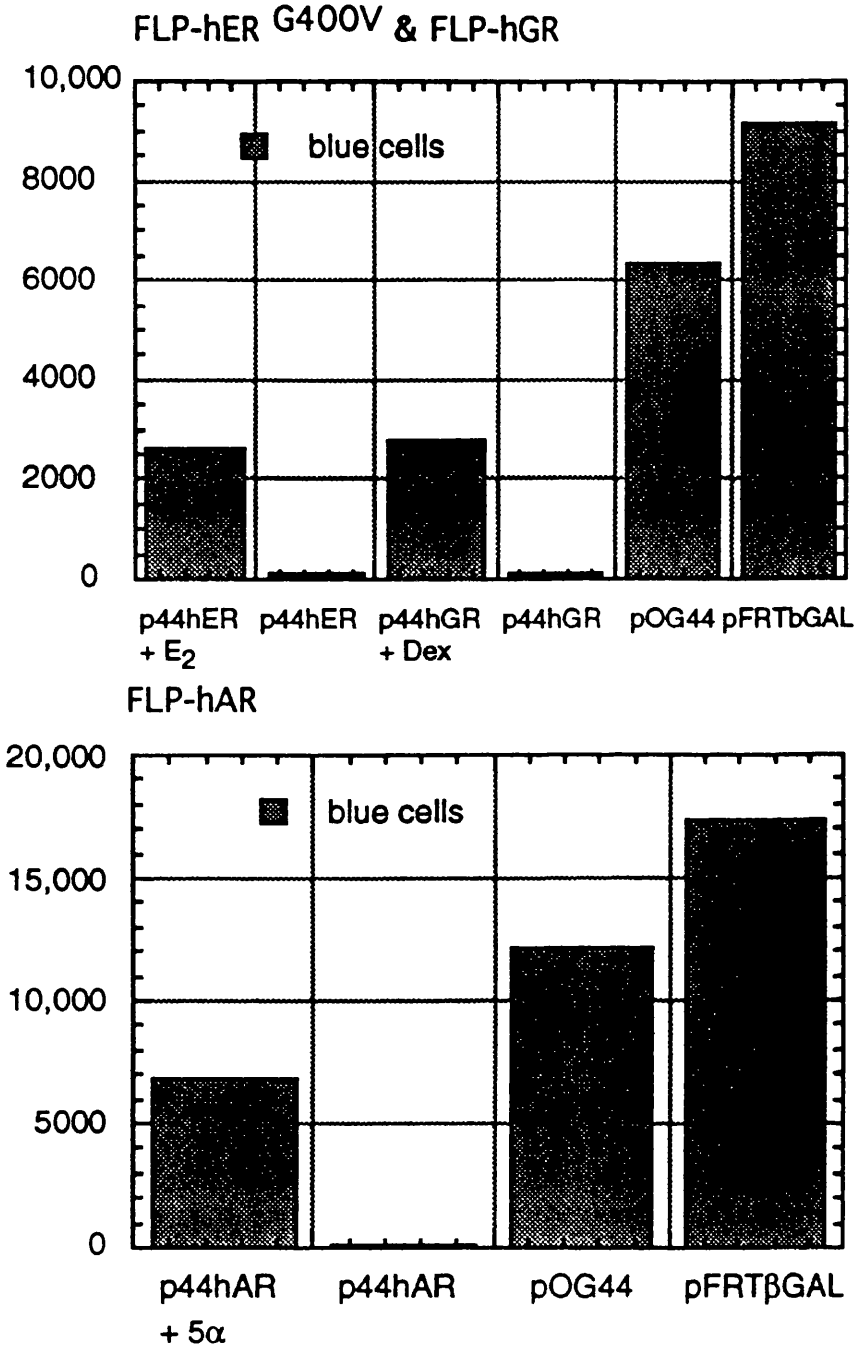
*Neo*$^r$ 293 cell lines by integration of a pHFL plasmid (figure 3.1) into their genome to yield homogenous populations of cells expressing FLP-LBD fusion protein.

## Ligand dependent chromosomal deletions

### Transient expression studies with FLP-hAR, -hEr and -hGR proteins in the E25B2/B2 cell line

Firstly, FLP-LBD fusion proteins were tested for ligand responsiveness in the E25B2/B2 cell line (see above) in a transient expression experiment. After DNA transfection, the cells were exposed for 50 hours, either to a ligand cognate to the ligand binding domain or to ethanol vehicle. As well as the fusion protein expression constructs, E25B2/B2 cells were also transfected with the unmodified FLP recombinase expression plasmid, pOG44, to enable us to compare the recombinase activity of the FLP fusion proteins with that of unmodified FLP protein. pFRTβGAL, a plasmid which expresses β-galactosidase (and is indeed identical to the chromosomal product of the FLP catalysed excision recombination reaction in the E25B2/B2 cell line) was also transfected into E25B2/B2 cells, to give an indication of the efficiency of transfection and to serve as a positive control for the histochemical detection of β-galactosidase activity.

Figures 3.2, 3.3a, b and c show that steroid receptor ligand binding domains render the recombinase activity of FLP ligand dependent. Figure 3.3d shows that in this experimental context, the ligands used, did not affect recombination mediated by unmodified FLP enzyme. About half of the transfected cells (as given by transfection of pFRTβGAL) undergo recombination when pOG44 is transfected and about one quarter of the transfected cells undergo recombination when FLP is fused to a ligand binding domain and when inducing ligand has been added to the cells' culture medium. FLP-LBD mediated recombination in the absence of inducing ligand occurs in about 3% of transfected cells in the case of the




Figures 3.3a and 3.3b Plasmids encoding FLP-LBD fusion proteins or unmodified FLP or simply driving b-galactosidase expression (pFRTβGAL) were transiently transfected into the E25B2/B2 cell line. The cells were then exposed to 1μM of ligand or ethanol vehicle for 50 hours. The cells were then stained with X-Gal and the number of blue staining cells was counted manually on a surface of $4cm^2$. Ligands: E2; estradiol, Dex; Dexamethasone, 5α; dihydrotestosterone.

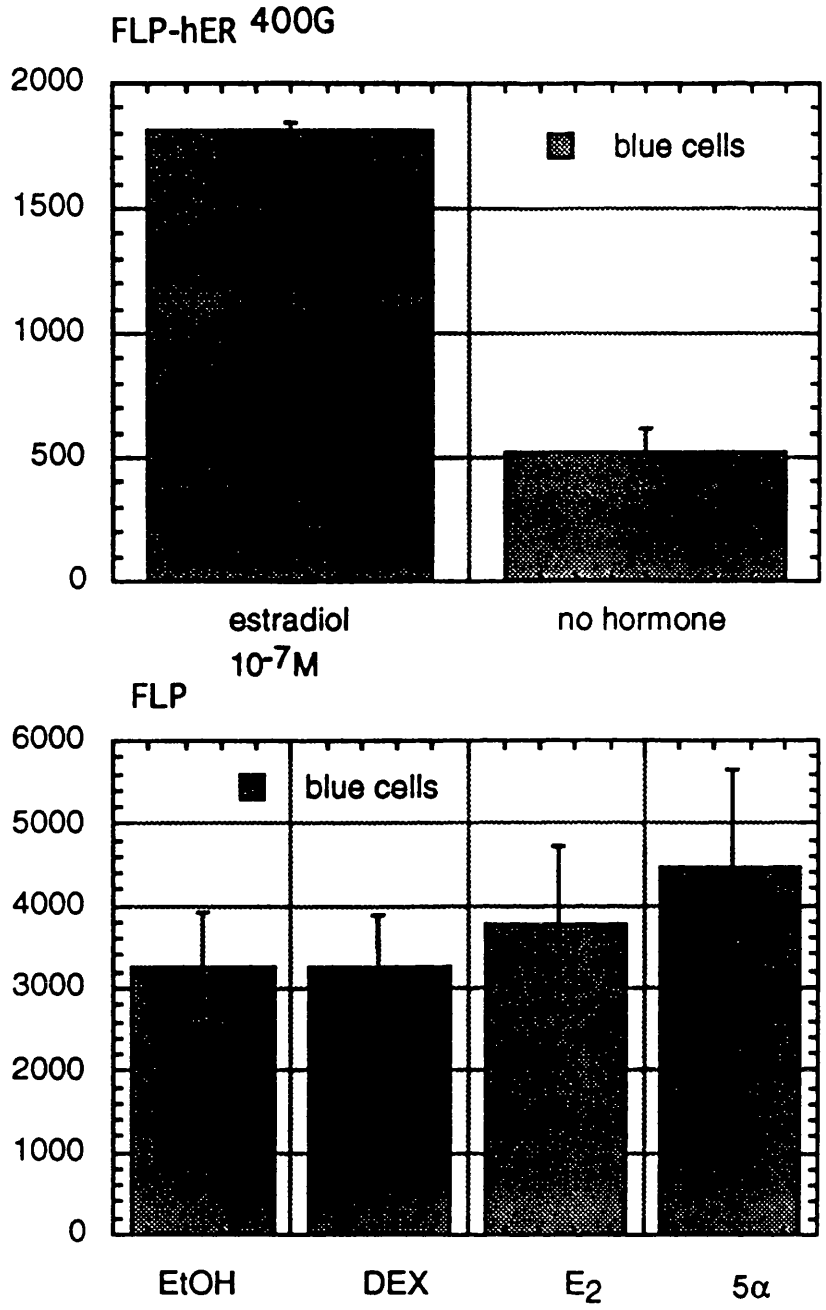
androgen, estrogen$^{G400V}$ and glucocorticoid receptor fusion proteins (figures 3.3 a and b) but reaches 22% in the case of the wild type estrogen ligand binding domain (figure 3.3c). The high frequency of recombination observed in the absence of added ligand with the wild type human estrogen receptor ligand binding domain can be explained either by the high affinity of this receptor for estrogens 0.1nM (Westley *et al.*, 1984) combined with the presence of residual amounts of estrogens in stripped fœtal serum or in the cell culture medium, or by postulating a ligand independent nuclear activity for the native receptor (Reese and Katzenellenbogen, 1992), (Zhuang *et al.*, 1995), (Tzukerman *et al.*, 1990). I would favour the former explanation since the G400V mutation (Tora *et al.*, 1989a) and a number of other mutations in the estrogen binding domain that lower the apparent affinity for estrogens of the estrogen ligand binding domain, also lower the levels of recombination observed in the absence of ligand (see chapter 4).

Therefore, in a transient transfection assay, the LBDs of the human androgen, estrogen and glucocorticoid receptors are able to confer ligand dependency on the activity of a fused FLP moiety.

**Establishment of cell lines which constitutively express FLP-EBD proteins and maintain an unrecombined chromosomal excision recombination substrate**

To evaluate the regulatory potential of FLP-LBD fusion proteins, we introduced expression plasmids for stable expression of these fusion proteins into the genome of 293 derived FLP recombination reporter cell lines (table 3.2) that contained the pNEOβGAL recombination reporter stably integrated into their genome.

Since the neomycin resistance gene of pNEOβGAL can be excised by FLP mediated recombination, continued selection for neomycin resistance could select against recombination. To evaluate this potential, pools of P1 or R10 cells (table 3.2) transfected




Figures 3.3c and 3.3d Transient transfection of plasmids driving the expression of FLP-EBDwt (3.3c) or unmodified FLP (3.3d) into E25B2/B2 FLP recombination reporter cells followed by exposure to 1μM of the indicated ligand (3.3d) for 50 hours. After staining with X-gal, the number of blue cells was determined in 5 randomly chosen occular fields. The number of blue cells counted was then extrapolated to obtain the total number of blue cells on a $4cm^2$ surface area. Error bars represent the standard deviation of 5 measurements. Ligands: EtOH; ethanol, E2; estradiol, Dex; Dexamethasone, 5α; dihydrotestosterone.

with pHFE1 or pHFE2 (G400V and wild type estrogen LBD encoding pHFL plasmids respectively) were divided into two and grown under selection for hygromycin resistance in the presence or in the absence of neomycin. The result of one such experiment is shown in table 3.4. Approximately the same number of colonies were obtained with both selection protocols, indicating that the effect of neomycin selection was negligible under these conditions.

| Construct | Selection | n° of colonies | colonies with blue sectors |
|---|---|---|---|
| pHFE1 (G400V EBD) | hygromycin | 440 ± 80 | 2 |
| pHFE1 (G400V EBD) | hygromycin + neomycin | 370 ± 70 | 2 |
| pHFE2 (wild type EBD) | hygromycin | 460 ± 70 | 30 |
| pHFE2 (wild type EBD) | hygromycin + neomycin | 480 ± 60 | 33 |

Table 3.4 Assesment of the impact of continuous neomycin selection on the number and phenotype of pHFE transgenic clones. The colony number was determined by counting colonies in 4 randomly chosen $2cm^2$ areas and extrapolation to the total surface area of the culture dish. The errors shown represent the standard deviation of the 4 measurements. Colonies were deemed to contain a blue sector if a patch of blue cells could be seen with the naked eye.

X-gal staining of the plates bearing these colonies, showed that the number of colonies containing blue sectors, indicative of the excision of the FRT flanked neomycin resistance gene, was higher in the case of pHFE2 than in the case of pHFE1 transfected P1 cells under both selection protocols. This means that under the experimental conditions used, counter selection of cells which had undergone recombination was not absolute in nascent clones which constitutively expressed a FLP recombinase-EBD fusion protein.

Individual *Neo$^r$,Hygro$^r$* clones were picked and analysed for β-galactosidase expression before and after ligand administration (for example see figure 3.4 A). I concentrated firstly on comparing the FLPER$^{wt}$ (pHFE2) and FLP-ER$^{G400V}$ (pHFE1) constructs. As shown on figure 3.4, pHFE2 transformed clones are generally inducible but show a substantial amount of β-galactosidase expression in the absence of ligand. In contrast, pHFE1 transformed cells showed much lower levels of ligand independent site specific recombination (figure 3.4B). The capacity of pHFE1 and pHFE2 derived 293R10 clones to undergo recombination upon exposure to estradiol was analysed again (table 3.5).

| plasmid | estradiol | White cells only | Few blue cells | Many blue cells | Mainly blue cells |
|---|---|---|---|---|---|
| pHFE2 (wild type) | none added | 5, 12 | 4, 6, 8 | 1 ,2, 3, 7, 9, 10, 11 | none |
| pHFE2 (wild type) | + 1μM | 5,12 | 6, 8 | 1, 4 | 2, 3, 7, 9, 10, 11 |
| pHFE1 (G400V EBD) | none added | 4, 11, 8 | 1, 2, 3, 5, 6, 7, 9, 10 12 | none | none |
| pHFE1 (G400V EBD) | + 1μM | none | none | 2, 5, 10, 12 | 1, 3, 4, 6, 7, 8, 9, 11 |

Table 3.5 Analysis of the recombination competence of R10 cells (table 3.2) transformed either with pHFE1$^{(G400V)}$ or pHFE2$^{(400G)}$ and grown under selection for both neomycin and hygromycin. Numbers 1 to 12 represent individual, randomly picked clones. The recombination status of each clone was monitored by staining for X-gal. Four categories of blue cell contents were arbitrarily chosen and are indicated. Each clone was analysed after growth for 4 days in growth medium supplemented with 1μM estradiol or ethanol vehicle only.

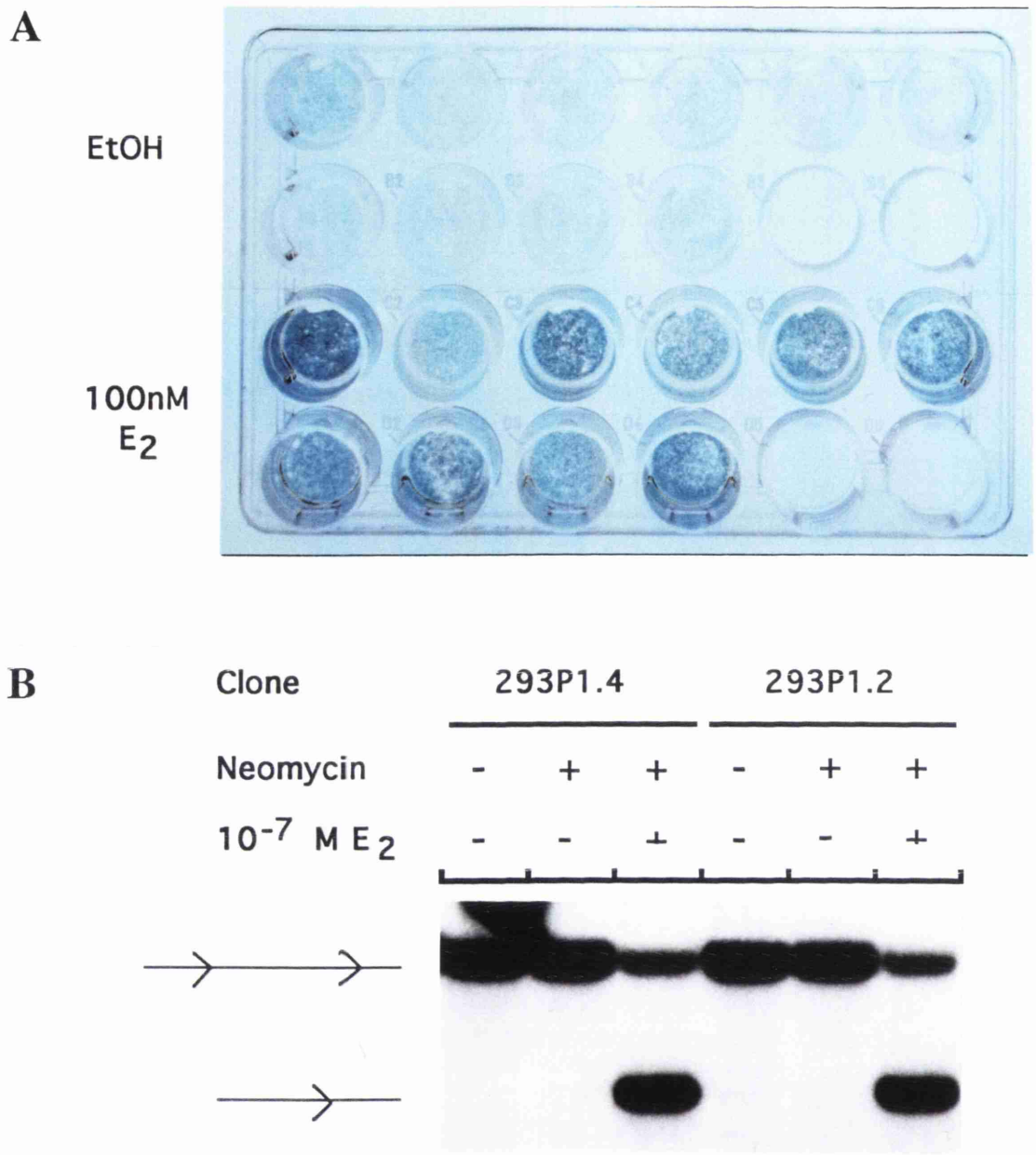



Figure 3.4 A. 293P1 cells were transformed with linearised pHFE2 vector (wild type EBD) and cultured under neomycin selection. After 10 days, 10 colonies were picked, split into 2 equal parts and transferred onto a 24 well plate. The duplicates were cultured for 5 days with neomycin and then exposed to ethanol vehicle (top 10 wells) or 100nM estradiol (bottom 10 wells). Note (i) that even in the absence of added ligand, the populations contained many blue cells and (ii) that upon addition of ligand all the clones responded by expressing the LacZ gene, indicating the all 10 clones underwent recombination efficiently. B. 293P1 derived clones transformed with pHFE1 (G400V EBD) cultured for 14 days in the presence or absence of neomycin and then exposed to estradiol for 48h. Note the absence of recombination in the absence of ligand.

Of 12 *Hyg$^r$,Neo$^r$* pHFE1 clones, every one contained no or only a few blue cells in the absence of estrogen. When grown in the presence of estradiol, all 12 showed a significant increase in blue cells. Of 12 *Hyg$^r$,Neo$^r$* pHFE2 clones, 7 already displayed a large proportion of blue cells in the absence of inducing ligand, indicating that this construct encodes a poorly regulated recombinase. Of the 5 *Hyg$^r$,Neo$^r$* pHFE2 clones which did not display large numbers of blue cells, 4 did not respond to estradiol treatment by an increase in blue cell number, indicating that the recombination system was somehow compromised in these clones.

Generally, about one in four cells of pHFE2 clones grown in the presence of hygromycin only were blue. 10 of 10 such pHFE2 clones (which had never been exposed to neomycin) underwent recombination in the presence of estradiol (figure 3.4, panel A). Since the only difference between this experiment and the one shown on table 3.5 was the addition of neomycin to the selection medium of the transfected cells, this ruled out the possibility that pHFE2 clones were less recombinogenic (table 3.5) because the pHFE2 plasmid encoded a less active recombinase fusion protein.

The last three experiments indicated that, when neomycin is included in the growth medium, there is a stronger selection pressure on pHFE2 derived 293-pNEOβGAL sub-clones than on pHFE1 derived ones. This happens presumably, through genetic or epigenetic alteration of either the recombinase transgene or in the recombinase substrate transgene.

Overall, the above observations indicated that the likelihood of obtaining a cell line containing an aberrant site specific recombination system should be low when pHFE1 is used to establish 293-pNEOβGAL cell lines which maintain both an unrecombined excision recombination substrate and constitutively express FLP-hER protein. As a further test for the effect of neomycin selection on recombination, two pHFE1 derived hygromycin resistant subclones of clone 293P1 (figure 3.1 and table 3.2) were cultured for 14 days with or without neomycin followed by 48 hours of culture in the presence of 0.1μM estradiol or

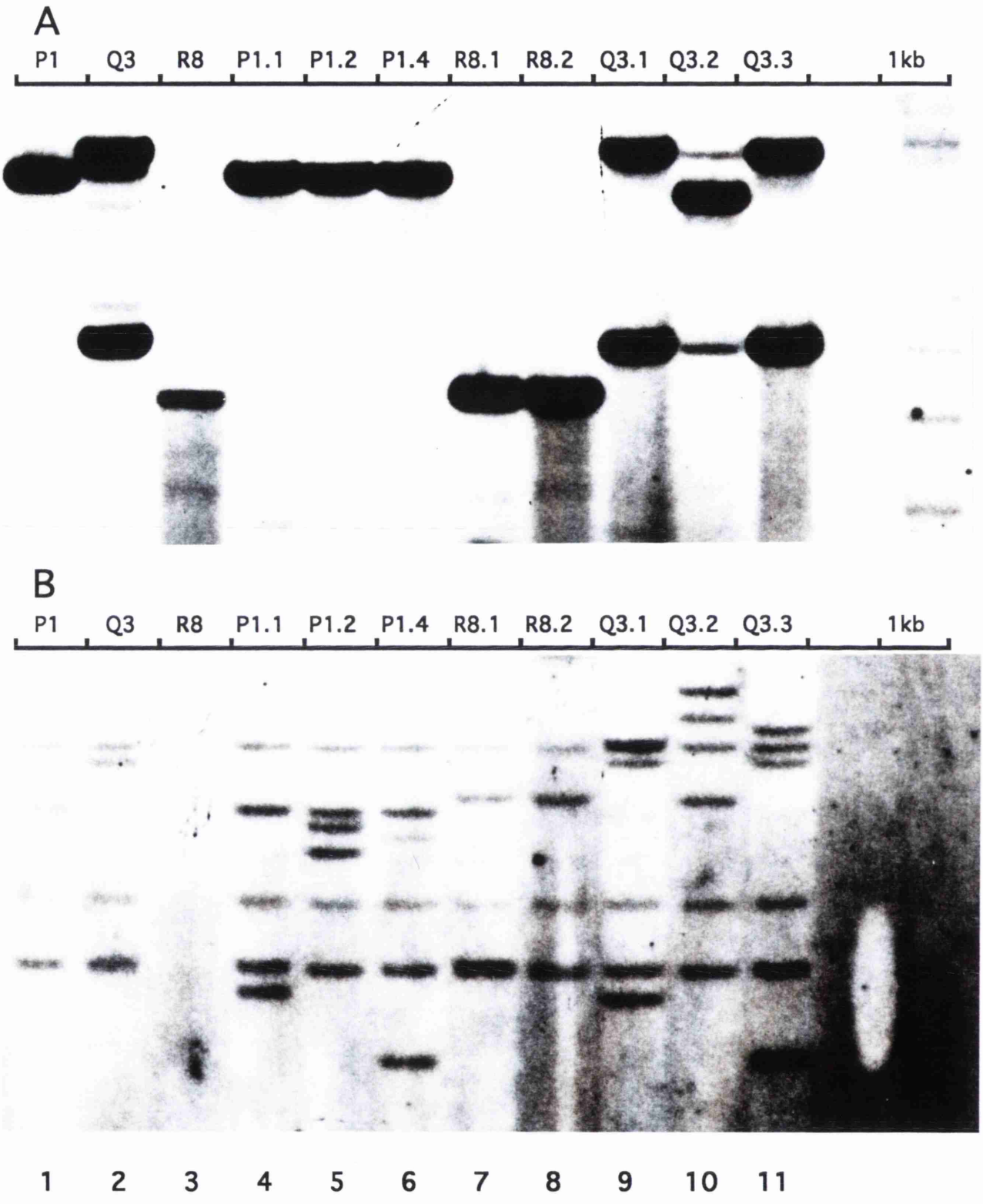



Figure 3.5 A. Southern blot of HindIII cut genomic DNA from the indicated clones probed with probe 1 shown on figure 3.1. (The weak bands in lane 10 are due to sample overflow from the adjacent wells) B. Same blot as above but hybridised with a probe directed against the estrogen ligand binding domain.

ethanol vehicle. Southern analysis showed no recombination in the absence of neomycin and/or hormone and recombination in the presence of hormone (figure 3.4 panel B).

To study the properties of FLP mediated recombination in mammalian cell culture, 3 *Neo*$^r$ 293 cell lines were chosen for stable transfection with the pHFE1 plasmid (table 3.2). These cell lines were chosen (i) to represent the range of LacZ expression levels/recombination frequencies observed in the previous section and (ii) to include a cell line which contained more than one copy of the chromosomal pNEOβGAL recombination substrate. The second criterion was included as it was of interest to find out to what extent FLP is capable of catalysing recombination between pNEOβGAL transgenes located in different parts of the genome. The ranking order in terms of decreasing levels of β-galactosidase gene induction upon transient expression of FLP enzyme was P1 > Q3 > R8. The P1 and R8 cell lines contained one copy of the pNEOβGAL transgene and the Q3 cell line contained 4 copies of it (with the possible caveat that the Q3 cell line was of a polyclonal origin). The 3 chosen cell lines were transfected with 5μg of linearised pHFE1 plasmid. Twelve *Neo*$^r$,*Hyg*$^r$ clones were picked, they were named P1.1, P1.2, P1.3, P1.4, Q3.1, Q3.2, Q3.3, Q3.4, R8.1, R8.2,R8.3 and R8.4. The 8 fastest growing ones were expanded, these were P1.1, P1.2, P1.4, Q3.1, Q3.2, Q3.3, R8.1, and R8.2. All contained less than 0.1% blue cells as determined by X-gal stains. The copy number of the pNEOβGAL recombination reporter was again determined by Southern analysis using probe 1 shown on figure 3.1. All the P1 and R8 derived clones had 1 copy, yielding a HindIII restriction fragment identical to that of their parental cell line (figure 3.5 panel A, lanes 1, 4, 5 and 6, and lanes 3, 7 and 8). The Q3.1 and Q3.3 clones contained two copies of the pNEOβGAL transgene, both of the same size as two of the bands seen for the parental Q3 cell line (figure 3.5 panel A, lanes 2, 9 and 11). The Q3.2 cell line yielded one HindIII restriction fragment, of the same size as one of the minor HindIII fragments seen in the parental Q3 cell line (figure 3.5 panel A, lanes 2 and 10). The independence of the clones was established by stripping probe 1 from the filter and re-probing with a probe directed against the ligand binding domain of the estrogen receptor.

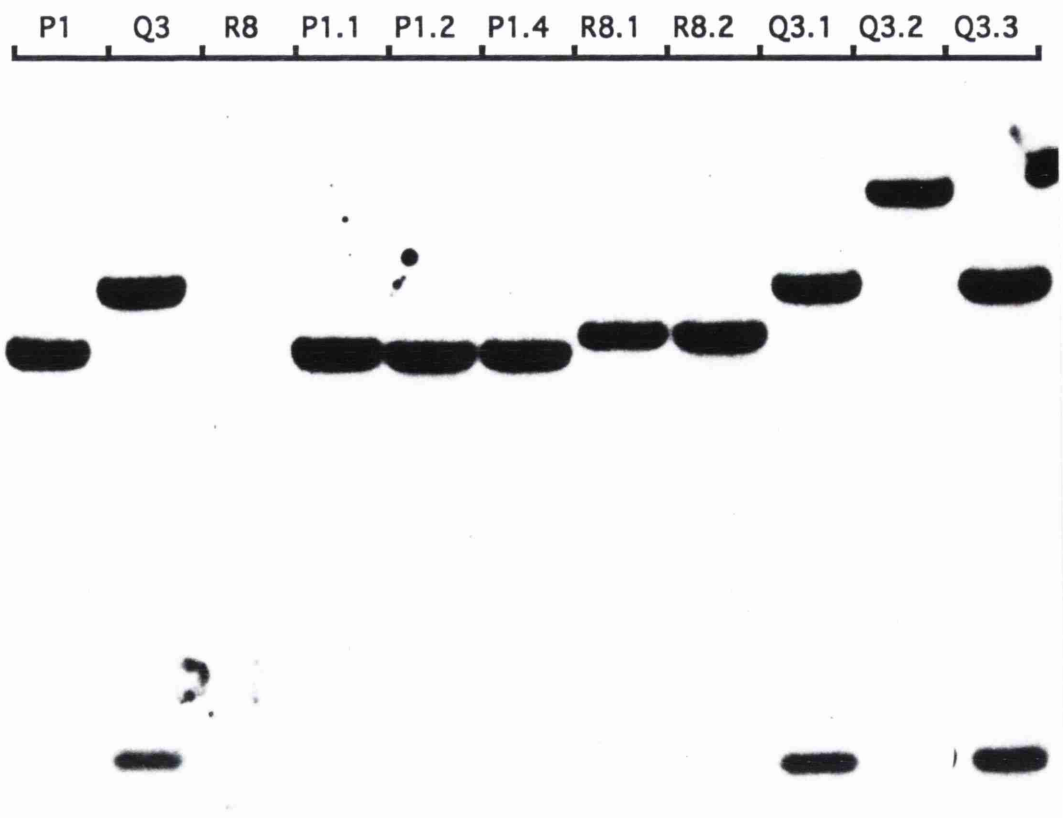



Figure 3.6 Same Southern blot as on figure 3.5 but probed with probe 2 shown on figure 3.1 which is directed against the neomycin gene. Note the short size of the lowest band in the Q3.1 and Q3.3 lanes (1.8 kb)

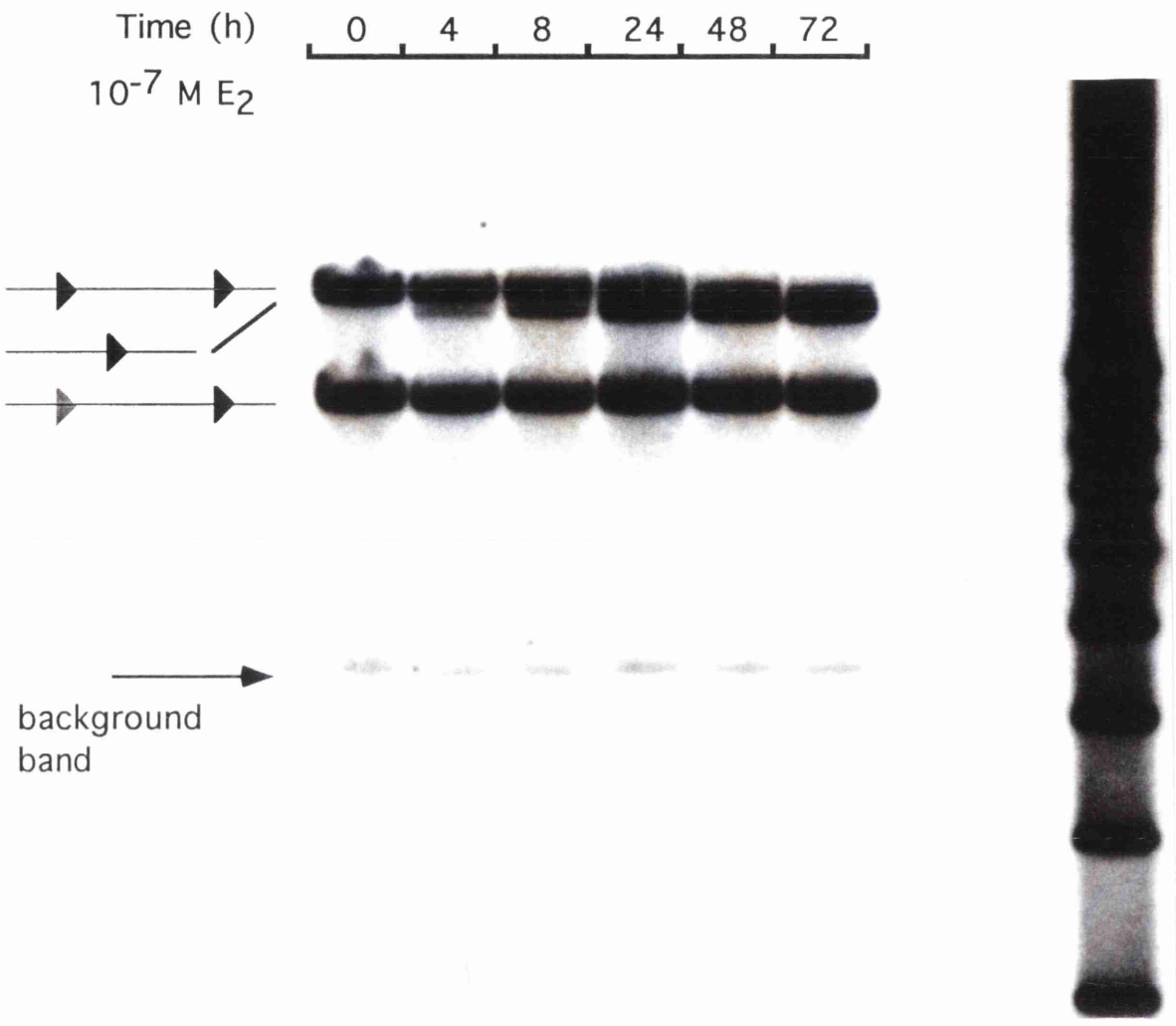



Figure 3.7 Southern blot analisis of a time course of estradiol induced site specific recombination in cell line 293Q3.1. The 2 pNEOβGAL loci are indicated, as is the recombination product for the recombining pNEOβGAL locus. The identity of the background band is unknown, it probably stems from a plasmid contamination. Note the absence of pairs of restriction fragments which would have been indicative of a reciprocal recombination event between the 2 pNEOβGAL loci. Probe 1 shown on figure 3.1 was used.

As can be seen from figure 3.5 panel B, each clone yielded a different banding pattern, providing positive evidence that all 8 clones were independent in terms of the chromosomal location of the pHFE1 construct. The bands common to all the clones are due to cross hybridisation to genomic HindIII restriction fragments bearing exons 5 to 9 of the human estrogen receptor. It is of note that this endogenous restriction pattern is different between the parental *Neo*$^r$ clones P1 and Q3, indicating that the genome of 293 cells is subject to frequent alterations in the course of cloning experiments such as those described above. The fact that two different pNEOβGAL restriction patterns were derived by re-cloning of the Q3 *Neo*$^r$ cell line indicated again that it was of a polyclonal origin. Hence 8 independent cell lines which represented 5 different pNEOβGAL integration sites were generated.

Cell lines derived from the P1 clone were also transformed with pHFA and pHFG plasmids (these encode a hygromycin resistance gene and a FLP-hAR or FLP-hGR gene respectively). It was more difficult to isolate hormone responsive clones with those constructs. Nevertheless, such clones were isolated. Androgen and glucocorticoid induced FLP mediated site specific recombination are discussed in chapter 4.

**Failure to detect site specific recombinase mediated inter-loci recombination**

The cell lines Q3.1 and Q3.3 contained two copies of the pNEOβGAL recombination substrate. Theoretically it was therefore possible that recombination would not only occur at each recombination reporter substrate between the FRTs flanking the neomycin transcription unit but also between FRTs located on the two copies or even that a circle excised from one pNEOβGAL locus would be integrated into the other pNEOβGAL locus.

The Q3.1 cell line was further characterised by stripping the membrane used to produce figures 3.4 and 3.5, and then probing it with a probe which covers the neomycin gene, probe 2 on figure 3.1. The resulting hybridisation pattern is shown in figure 3.6. HindIII cuts only once within the pNEOβGAL sequence, 3' of the second FRT, in the 5' end of the LacZ

gene. Probe 2 lit two fragments up in the Q3.1 and Q3.3 lanes. One of these is only 1800bp long, indicating that the pNEOβGAL insert is truncated somewhere between the two FRTs. A time course of estradiol induced site specific recombination revealed that only one of the two genomic copies of the pNEOβGAL plasmid underwent recombination (figure 3.7).

To detect FLP catalysed site specific translocations or transpositions we used an enzyme which does not cut the pNEOβGAL transgene, therefore, if recombination had occurred between the two pNEOβGAL inserts, new restriction fragments due to the juxtaposition of the 3' and 5' genomic BglII sites of each insert should have appeared. Such restriction fragments are not apparent on figure 3.7, nor is there an increase in size of either of the parental BglII restriction fragments which would have been indicative of the integration of the circle excised from the recombination competent pNEOβGAL locus, into itself (this could occur during the G2 phase of the cell cycle) or into the excision incompetent pNEOβGAL locus. On none of all the Southern blots performed during this thesis did we detect restriction fragments which would have been indicative of the transposition of a circle from one chromatid into its sister chromatid.

Since the detection level of these Southern analyses does not exceed 0.25% of the total signal, we can say that circle transposition and inter-locus recombination do not occur at frequencies above $2.5x10^{-3}$ events per cell per generation. It should be noted however that it was not demonstrated that the recombination incompetent pNEOβGAL locus of this cell line still contained a functional FRT sequence. This could have been done either by direct sequencing of the cloned genomic DNA segment or in a more functional assay by attempting to site specifically integrate a FRT bearing plasmid into it.

The frequency of translocations mediated by a site specific recombinase in mammalian cells has been reported to be of the order of $5.10^{-8}$ events per cell. This was achieved by transient transfection of a CRE recombinase expression plasmid into an appropriately engineered cell line with a dominant selection strategy that enabled the isolation cell lines bearing the product

of such a reaction (Smith *et al.*, 1995). Such low levels of inter-locus recombination would not have been detected in the present study.

## Quantitative analysis of FLP mediated recombination in homogeneous mammalian cell populations

The estradiol induced recombination rate was measured in 8 independent cell lines. Autoradiograms of the Southern analyses are shown in figures 3.8, 3.9, 3.10 and 3.11. Cell lines P1.1 and R8.1 did not undergo recombination at all. A western blot using samples from the 8 cell lines and an antibody directed against the F domain of the human estrogen receptor is shown on figure 3.12 A. The upper band runs at the predicted size for the FLP-EBD fusion protein (85kD). It can be seen that the cell lines P1.1 and R8.1 are devoid of FLP-EBD protein. This explains why they did not undergo site specific recombination of the pNEOβGAL locus upon exposure to estradiol.

A phosphoimager generated quantification of the Southern blot analyses shown on figures 3.8 to 3.11 yielded the curves shown on figure 3.12 B, upper panel. Remarkably, all but one of the recombination competent cell lines underwent excision of the FRT flanked neomycin resistance cassette with very similar kinetics. Because cell line Q3.1 underwent recombination at a lower frequency than the 5 other cell lines I re-cloned it, as well as its sibling cell line, Q3.3, by plating out 100 cells under neomycin and hygromycin selection and then picking nascent clones. A time course of recombination on two randomly chosen sub-clones of Q3.1 and Q3.3, $Q3.1_8$ and $Q3.3_6$, showed that both cell lines behaved similarly (figure 3.12 B, lower panel). Therefore, in all recombination competent cell lines examined, recombination occurred with very similar kinetics, irrespective of the genomic integration sites of either the pNEOβGAL or the pHFE1 constructs.

The generation time of cell lines P1, P1.2 and P1.4 was determined by monitoring their proliferation rate at three different cell densities and was determined to be approximately 27

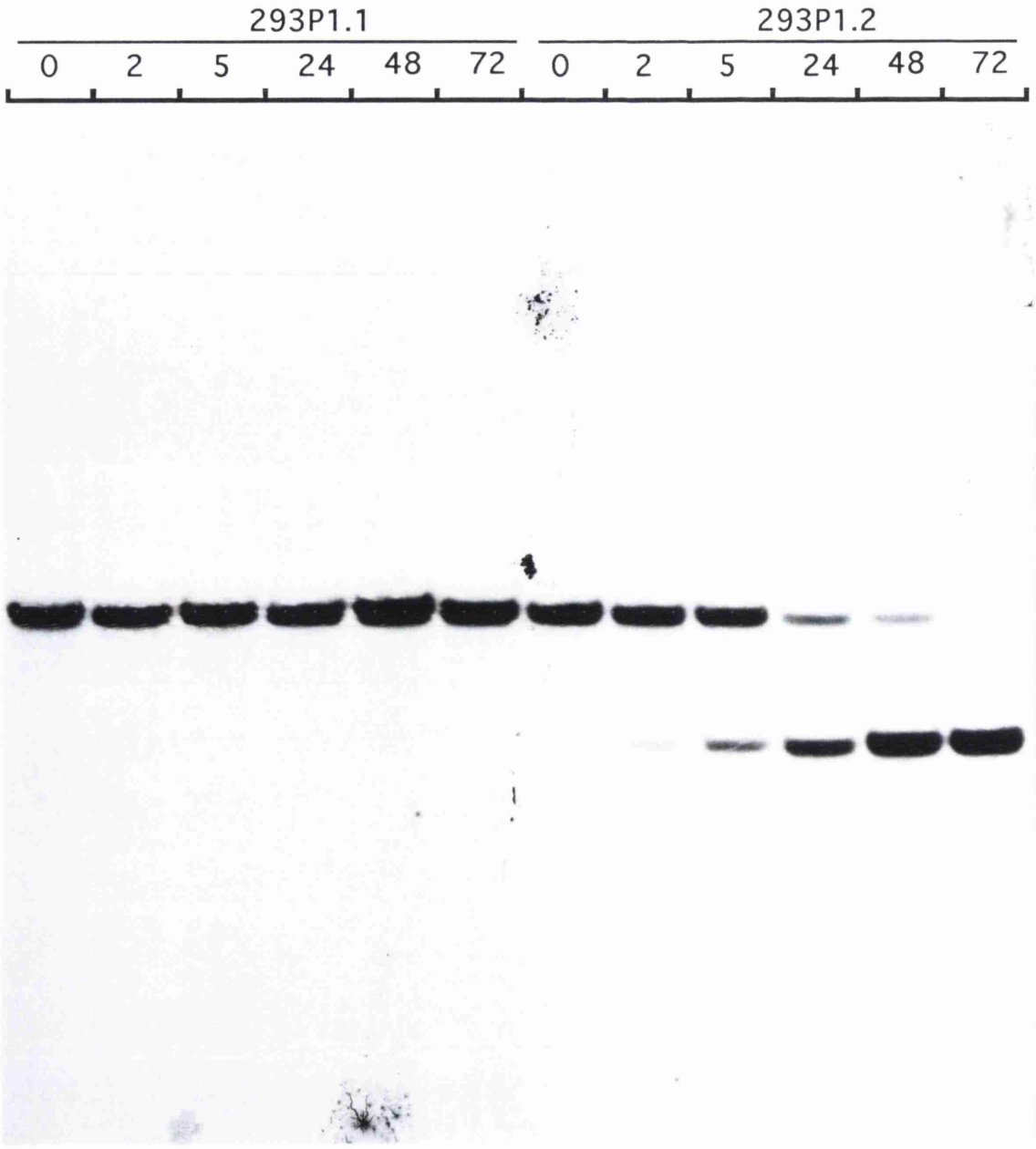



Figure 3.8 Time course (hours) of induced recombination in cell lines 293P1.1 and 293P1.2. 100nM estradiol was used. The DNA samples were digested with Nde1, whose restriction sites lie outside of the excised FRT flanked DNA segment. The Southern blot was hybridised to probe 1 shown on figure 3.1. The upper and lower bands represent the unrecombined and recombined chromosomal pNEOβGAL locus respectively.

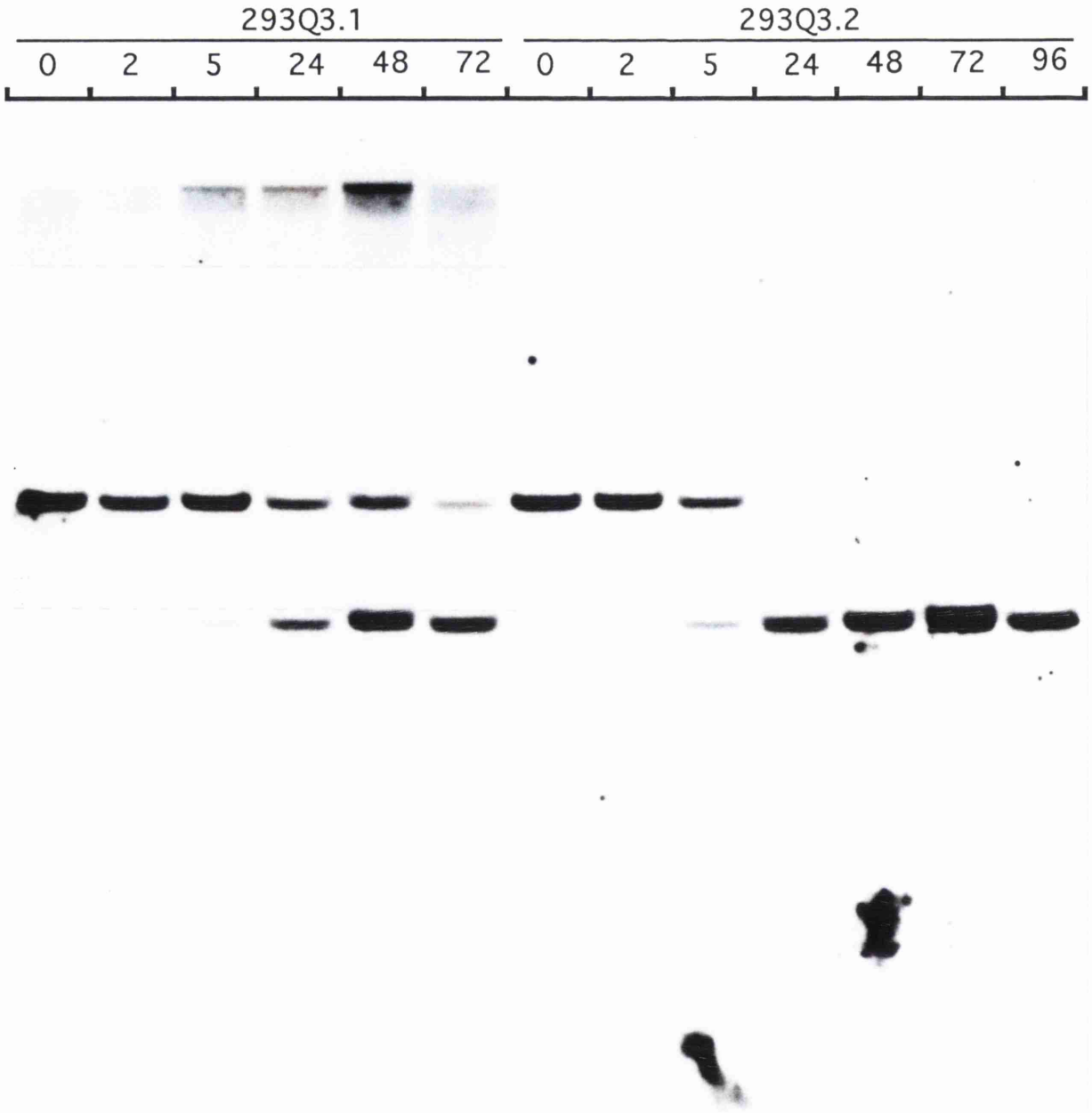



Figure 3.9 Time course (hours) of induced recombination in cell lines 293Q3.1 and 293Q3.2. 100nM estradiol was used. The DNA samples were digested with Nde1, whose restriction sites lie outside of the excised FRT flanked DNA segment. The Southern blot was hybridised to probe 1 shown on figure 3.1. The upper and lower bands represent the unrecombined and recombined chromosomal pNEObGAL locus respectively. The high molecular weight band in the Q3.1 samples represents the second pNEOβGAL locus, in which one of the Nde1 sites has been lost.

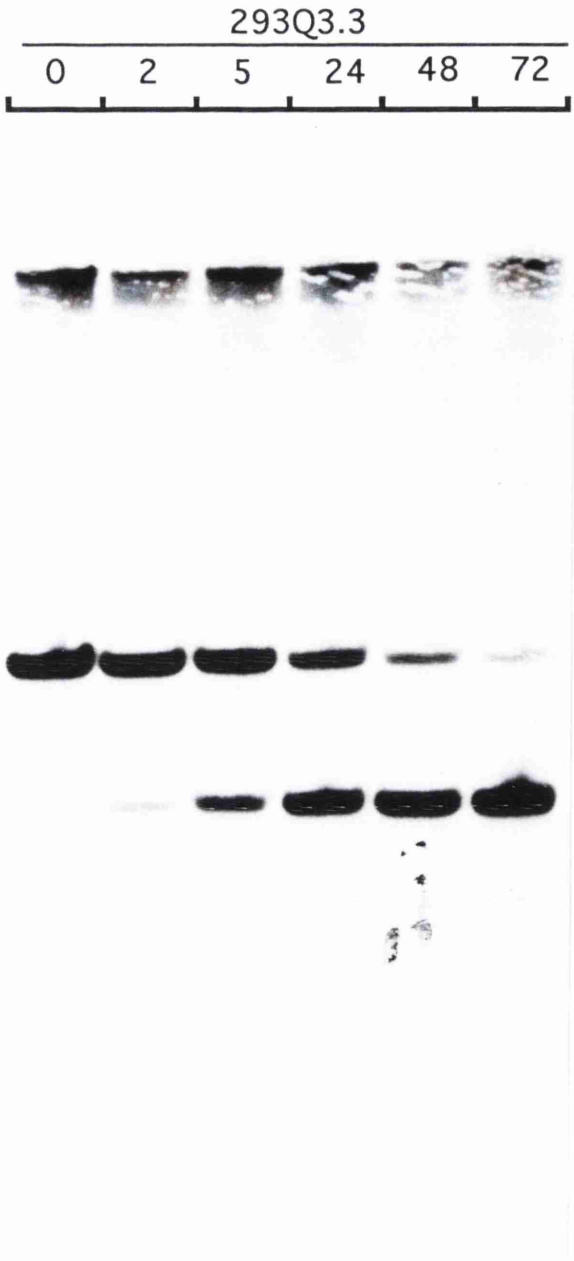



Figure 3.10 Time course (hours) of induced recombination in cell line 293Q3.3. 100nM estradiol was used. The DNA samples were digested with Nde1, whose restriction sites lie outside of the excised FRT flanked DNA segment. The Southern blot was hybridised to probe 1 shown on figure 3.1. The upper and lower bands represent the unrecombined and recombined chromosomal pNEObGAL locus respectively. As on figure 3.9, the upper band is due to a second truncated pNEObGAL locus

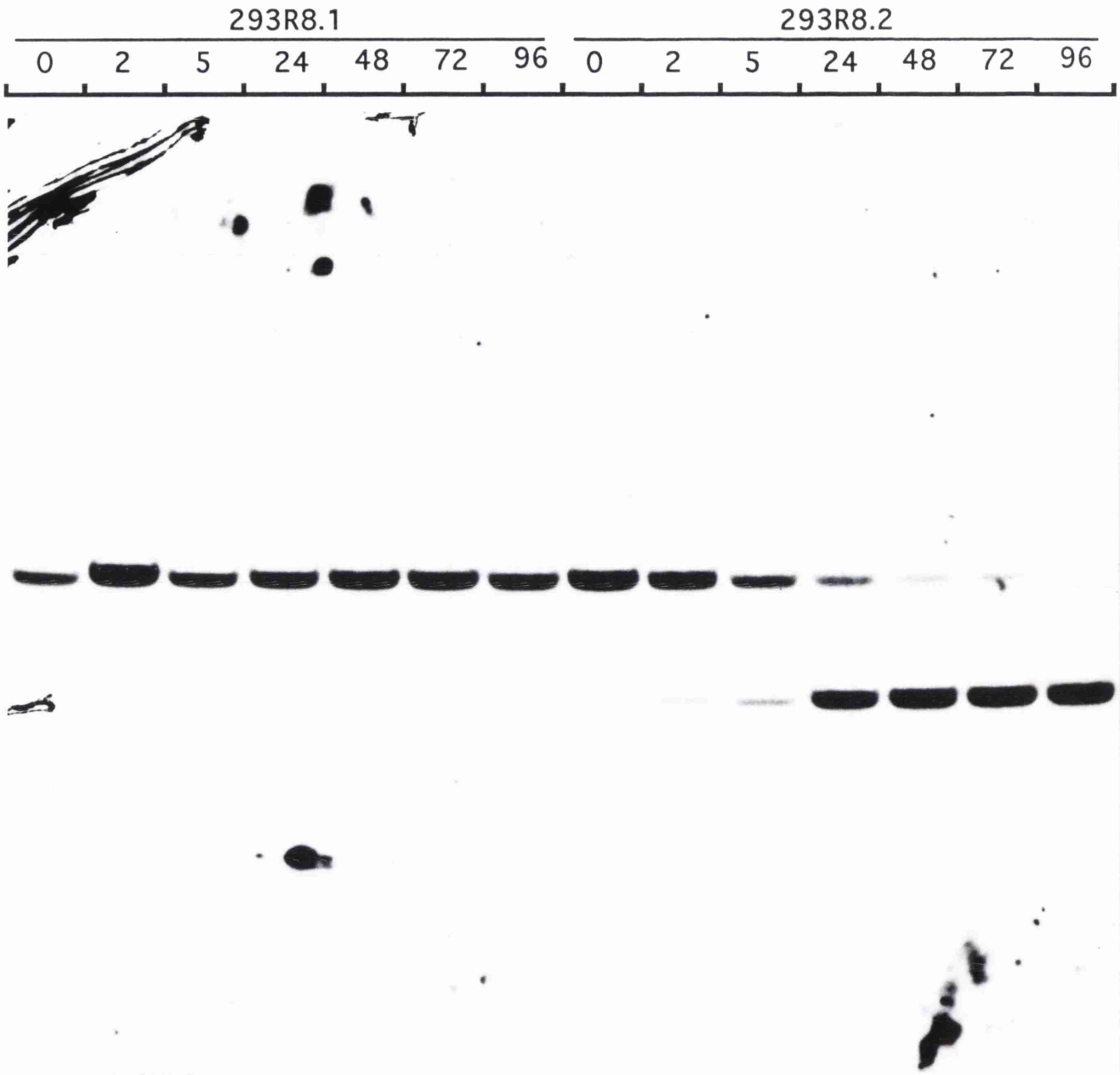



Figure 3.11 Time course (hours) of induced recombination in cell lines 293R8.1 and 293R8.2. 100nM estradiol was used. The DNA samples were digested with Nde1, whose restriction sites lie outside of the excised FRT flanked DNA segment. The Southern blot was hybridised to probe 1 shown on figure 3.1. The upper and lower bands represent the unrecombined and recombined chromosomal pNEObGAL locus respectively.

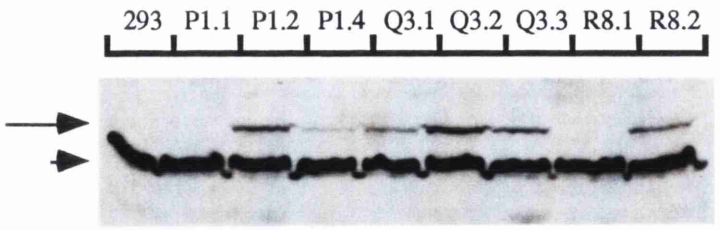
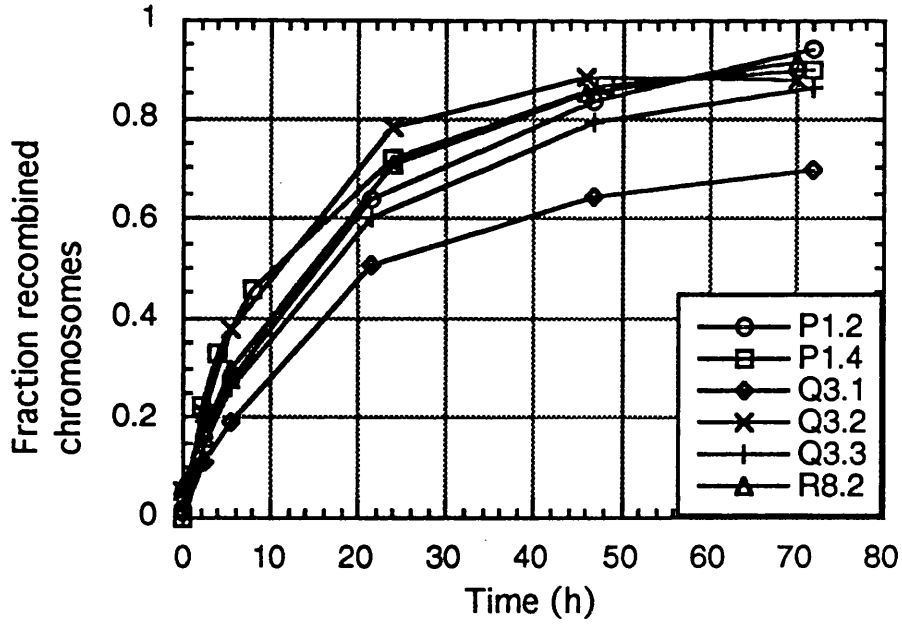



Figure 3.12 A Western blot analysis on the 8 cell lines described by Southern analysis on figures 3.5-11. Monoclonal Antibody Mab-EF-3 directed against the F domain of the human estrogen receptor was used. The molecular weight of the FLP-EBD fusion protein is indicated by the arrow (85kDa). The arrowhead designates an epitope present in 293 cells that migrates at 65kDa.

hours (figure 3.13). The average initial excision recombination rate for the six cell lines was 8% of the population per hour. Therefore the initial frequency of excision upon exposure to estradiol was 2.1 events per cell per generation.

After about 10 hours, 50% of the cells bore a recombined pNEOβGAL locus, after 20 hours 70%, after 50 hours 85% and after 70 hours 90%. It is apparent that the recombination frequency dropped dramatically during the course of the experiment. One explanation for this is that the kinetics were exponential. i.e.; since there were fewer and fewer cells that had not excised the neomycin gene as the time course went on we observed less and less excision events per unit time as the time course went on. This type of curve is mathematically expressed as $N_t=N_0.e^{-kt}$ where N is the number of cells at time t, e is the natural exponential number and k is the rate (expressed in $s^{-1}$ or $h^{-1}$). Figure 3.14 shows a graph where a curve obtained using this formula and the experimentally obtained rate of 8% per hour is compared to the experimentally determined curve. Comparison of the two curves suggests that the experimental curve does lag behind the predicted curve, although both curves seem to be of a similar nature.

The reactions catalysed by site specific recombinases are reversible and ideally will reach equilibrium at 50% if the substrate and products are equivalent (Senecoff and Cox, 1986), (Beatty *et al.*, 1986), (Cox, 1983). In excision recombination, the substrates and products differ, in that the forward reaction (excision) is intramolecular and the reverse (integration) is intermolecular. Moreover, in a living system, the excised product, unlike the chromosomal product and the substrate, may not replicate and can be degraded. I reason that the progress of excision recombination beyond the equilibrium between forward and reverse reactions will reflect the parameters that decrease re-integration, such as physical separation of the products or loss of the excised DNA. Hence, the lag observed in figure 3.14 could be explained by postulating that in a proportion of the cells the excised circle was re-integrated into the chromosomal FRT. This issue was approached by carrying out a Southern analysis on a more detailed time course of estradiol induced recombination. This was done in the P1.4 cell



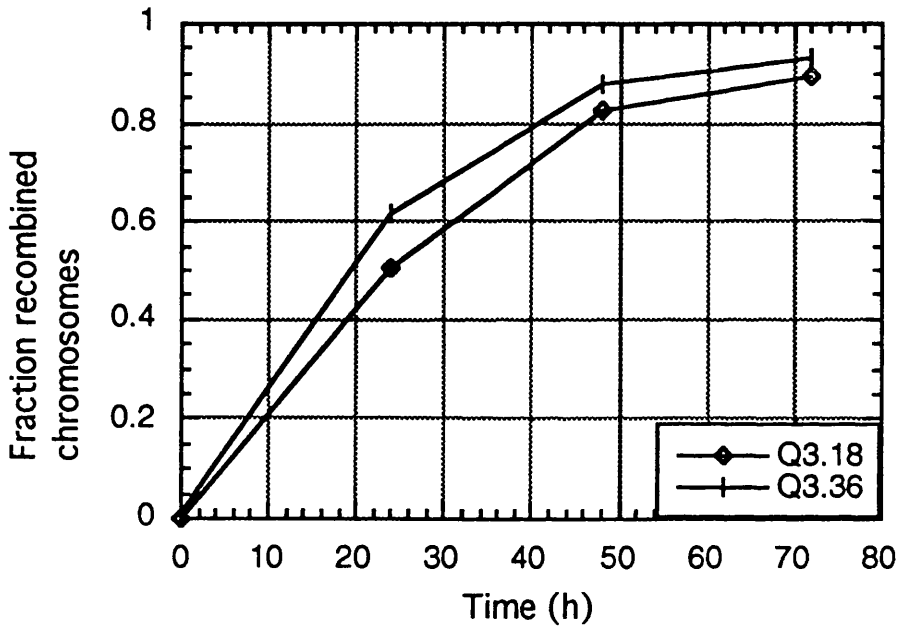




Figure 3.12 B Time courses of estrogen induced recombination in the 6 cell lines stably transfected with both pNEOβGAL and pHFE1 plasmids. The Q3.1 and Q3.3 cell lines were recloned and subjected to a new time course to confirm that both underwent recombination with the same frequency. The fraction recombined chromosomes was calculated as recombined signal over total signal.

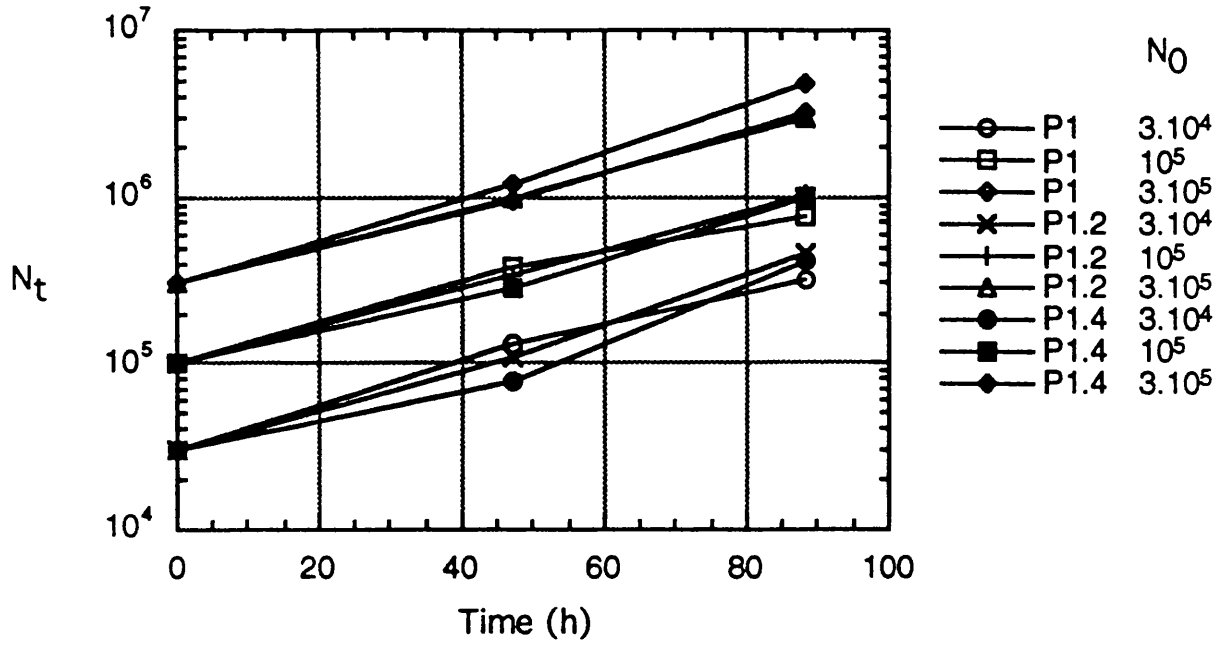



Figure 3.13 The generation time of cell line P1 and two of its descendants was derived by counting cell numbers at two time points from plates seeded at three different cell densities. The generation time is given by; $t.\log 2/(\log N_t - \log N_0)$ where t is time, $N_t$ the number of cells at time t and $N_0$ the number of cells at $t_0$. The average generation time calculated from the 18 data points presented is 26.6h ± 3.3

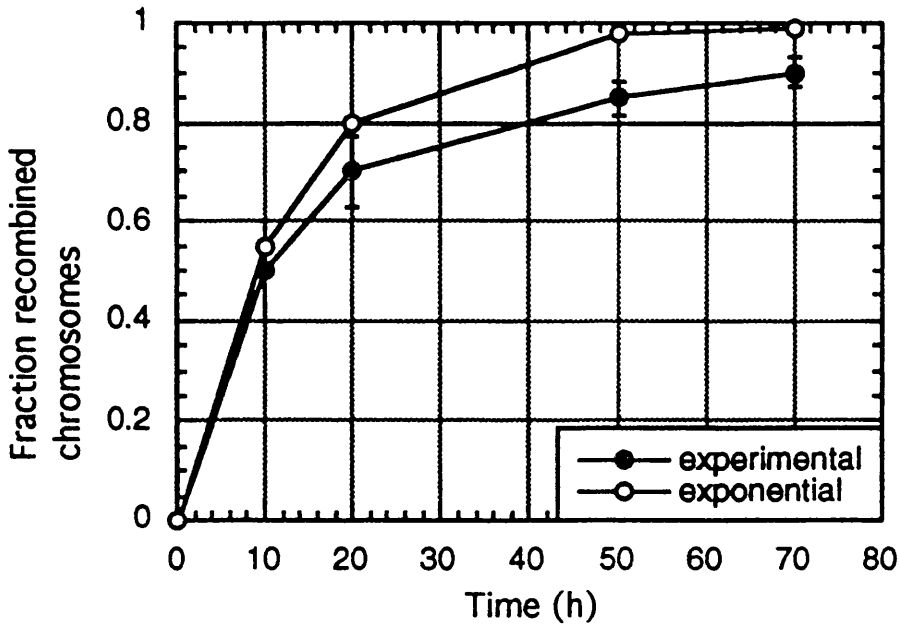



Figure 3.14 Comparison of an exponential curve calculated with the observed average initial frequency of recombination in 5 cell lines representing 4 pNEOβGAL loci and 5 different pHFE1 integrants and the observed average frequency of recombination in those same 5 cell lines. Error bars are the standard deviation from the mean of the five samples. The theoretical curve was calculated with the following formula;
Fraction recombined chromosomes=$1-e^{-(0.08.t)}$ where e is the natural exponential number and t is time. This formula differs from the one used in the text because that formula expresses decay (in our case this would be the disappearance of unrecombined chromosomes [100% or 1 at time zero]) while I desired to plot the accumulation of recombined chromosomes.

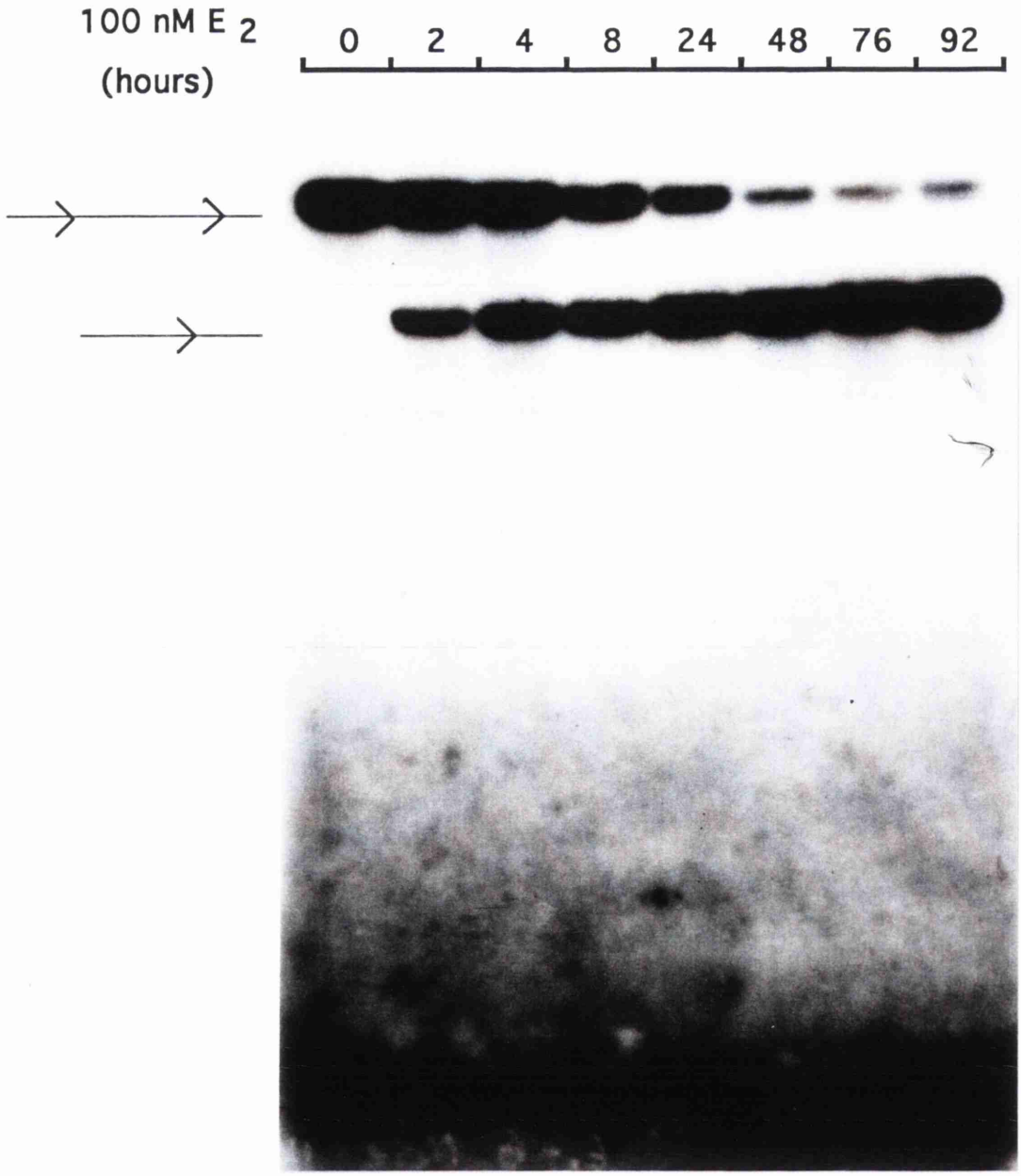



Figure 3.15 Southern blot of a time course of estradiol induced site specific recombination in clone 293P1.4. The restriction and probing strategies are identical to those used to generate figure 3.8. The lower band represents the recombined chromosomal pNEOβGAL locus. The "0" hour time point consisted of cells that were not exposed to estradiol and was collected at the 96 hours time point.

line. Figures 3.15 and 3.16 show Southern blots of a time course of estrogen induced recombination, hybridised with probe 1 (directed against the LacZ gene, figure 3.1) or with probe 2 (directed against the neomycin gene, figure 3.1) to visualise either both chromosomal recombination products or the excised circle and the "unrecombined" chromosome respectively. The data set used to generate figure 3.17 included the data of figures 3.15 and 3.16 and was composed of triplicate samples collected every $1^{1/2}$ hour for 28 hours and one final point at 51 hours.

Beyond 10 hours of recombination the kinetics were slightly slower than in the experiments shown in figure 3.14, even though the initial rate of recombination (54% ± 8% in 10 hours) was similar to that observed previously. The signal quantification for the excised circle is two to three times more noisy than the chromosomal signals. This is most likely due to the usage of data derived from two Southern blots for each sample to obtain the relative amount of circle to total chromosomes (see the legend of figure 3.17) but might also have been due a DNA amplification phenomenon discussed later.

The relative amount of circle peaked between 7 and 12 hours, coincident with the time when about half of the cells had excised the FRT flanked DNA insert. After this, the relative level of circle diminished at approximately the same speed as the rate of accumulation of recombined chromosomes. It was therefore concluded that the kinetics of recombination in this system are bi-phasic. During the first phase, the kinetics are mainly governed by the frequency of excision of the FRT flanked insert while in the second phase, the rate of accumulation of cells harbouring a recombined chromosome is affected by a combination of four parameters; (i) as in the first phase, by the frequency of excision of the insert, (ii) by the frequency of re-integration of the excised circle into the chromosomal FRT, (iii) by the rate at which the cells divide, and (iv) by the rate of degradation of the excised circle. This kinetic scheme is shown on figure 3.18.

Assuming that, beyond the initial phase of excision, the accumulation of cells harbouring a recombined chromosome is affected positively by the rate of proliferation of the cells and by

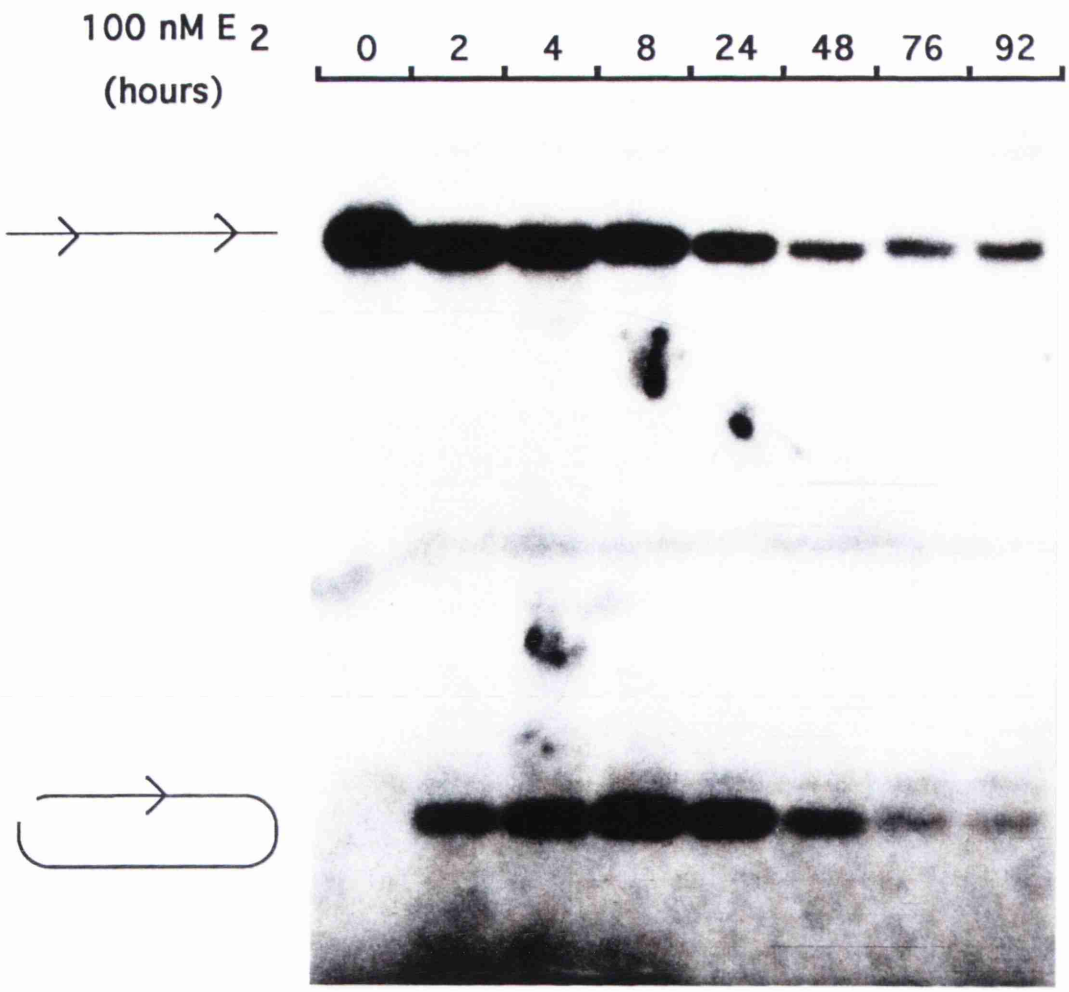



Figure 3.16 Southern blot of a time course of estradiol induced site specific recombination in clone 293P1.4. The genomic DNA samples were digested with BamHI, which linearised the excised circle (1.3 kb), and generates a 5.2 kb restriction fragment representing the unrecombined pNEOβGAL locus. The filter was probed with probe 2 shown on figure 3.1 that hybridises to the neomycin phosphotransferase coding sequence. The samples used were the same as those used to generate the Southern blot shown on figure 3.15.

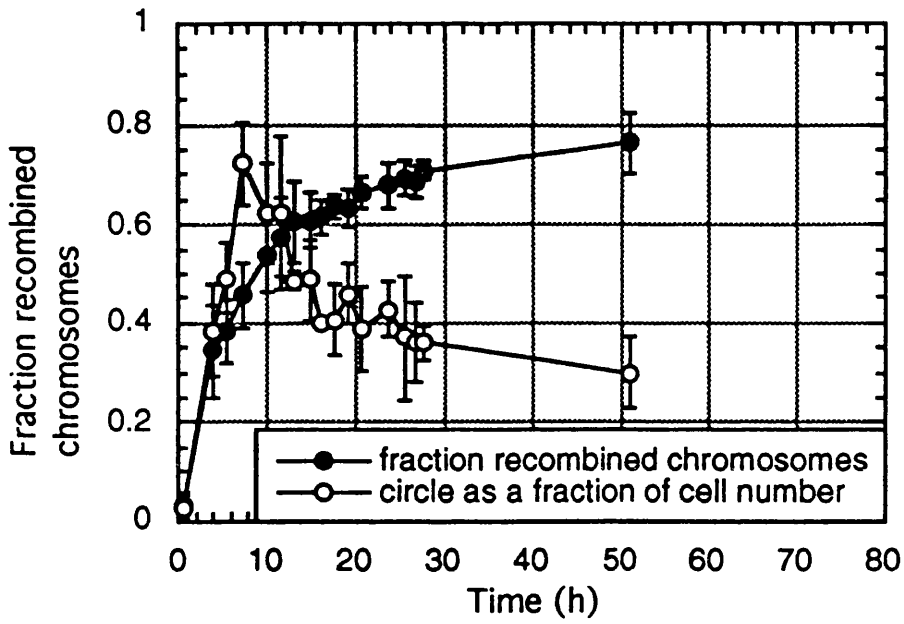
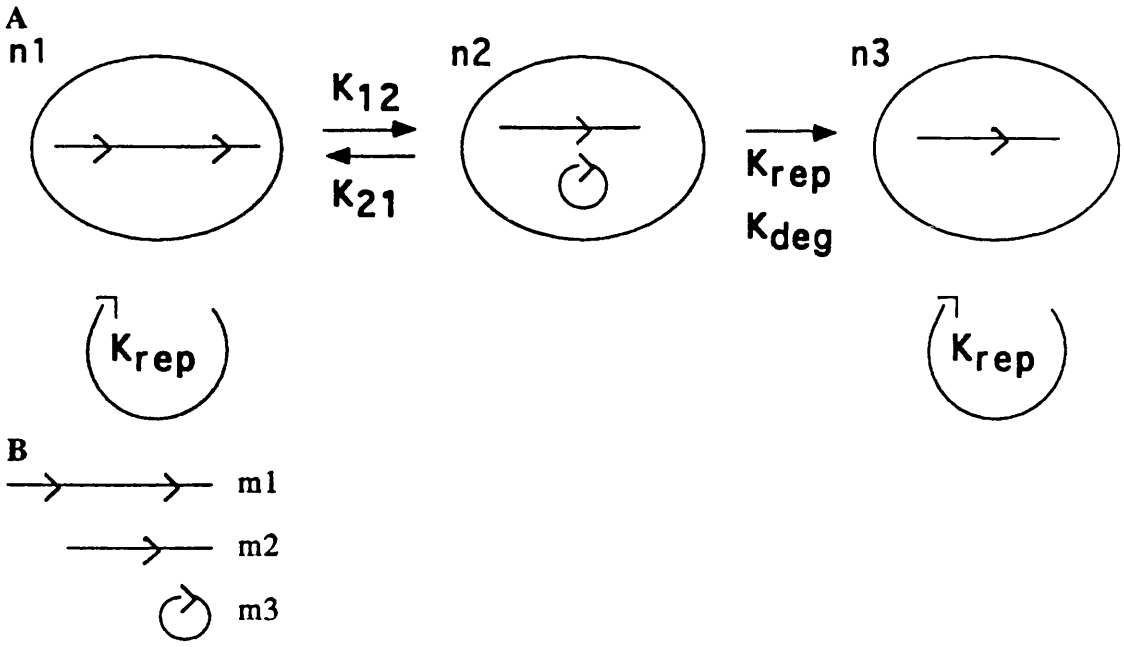



Figure 3.17 Triplicate samples taken in parallel every $1^{1/2}$ hour during a time course of ligand induced site specific recombination in cell lineP1.4. The fraction recombined chromosomes was obtained by dividing the recombined signal by the sum of recombined and unrecombined signal (see figure 3.15). The amount of circle as a fraction of total cell number was calculated by dividing the circle signal (see figure 3.16) by the sum of unrecombined signal (see figure 3.16) and recombined signal (from figure 3.15) that had been multiplied by the ratio of unrecombined signal seen with probe 2 (see figure 3.16) to unrecombined signal seen with probe 1 (see figure 3.15). Error bars are the standard deviation calculated from three samples.




**C**

$n1 = m1 \qquad n2 = m3 \qquad n3 = m2 - m3$

$n1 + n2 + n3 = m1 + m2 = 1$

**D**

| | | | |
|---|---|---|---|
| $\delta n1 =$ | $-K12.n1 + Krep.n1$ | $+K21.n2$ | |
| $\delta n2 =$ | $+K12.n1$ | $-K21.n2 - Kdeg.n2$ | |
| $\delta n3 =$ | | $+Kdeg.n2 + Krep.n2$ | $+Krep.n3$ |

**E**

| | | | |
|---|---|---|---|
| $\delta m1 =$ | $-K12.n1 + Krep.n1$ | $+K21.n2$ | |
| $\delta m2 =$ | $+K12.n1$ | $-K21.n2 + Krep.n2$ | $+Krep.n3$ |
| $\delta m3 =$ | $+K12.n1$ | $-K21.n2 - Kdeg.n2$ | |

Figure 3.18 K12, K21, Krep and Kdeg stand for the excision and integration frequencies, the replication rate and the degradation rate of the circle respectively (all expressed as time unit $^{-1}$). **A.** Kinetic scheme showing the main parameters thought to govern the accumulation of cells harbouring an intact pNEOβGAL insert (n1), a recombined pNEOβGAL insert and a circle bearing the neomycin transcription unit (n2) or only a recombined pNEOβGAL insert (n3). **B.** m1, m2 and m3 represent the 3 DNA forms; intact pNEOβGAL insert, recombined pNEOβGAL insert and circle bearing the neomycin transcription unit respectively. **C.** The relations between the 3 cell types and the relation between the proportions of the 3 cell types and the 3 DNA forms are listed. **D.** The factors that can be used to simulate the time course of recombination are shown in terms of which cell type they affect. **E.** The factors that can be used to simulate the time course of recombination are shown in terms of the strand form they affect.

the rate of degradation of the circle and negatively by the frequency of re-integration of the excised circle into the chromosome. Figure 3.18 shows a set of mathematical relations that can be used to simulate the process of accumulation of cells whose genotype has been modified by site specific deletion at one chromosomal locus. This exercise in developing computational simulations remains untested since I was not able to measure directly three required parameters. Namely the absolute excision, and re-integration frequencies as well as the degradation rate of the circle.

To characterise the hormone inducible site specific recombination system further, a hormone withdrawal experiment was performed. 293P1.4 cells, grown at three different densities, were exposed to 100nM estradiol for twelve hours, and then either cultured in the continued presence or in the absence of hormone. Analogously to the experiment displayed on figure 3.13, the data obtained with the 3 cellular densities were equivalent. The quantification of this experiment is shown on figure 3.19. Thirty hours after hormone withdrawal, an increase in the relative amount of recombined chromosomes of 14% had occurred. When estradiol had not been removed, an increase of the relative amount of recombined chromosomes of 35% was measured. After this time point no more recombination was observed in the absence of hormone. The reduction in the relative amount of circle was of 31% and 39% for the samples grown in the continued presence and absence of hormone respectively. The fact that the amount of circle present in these cultures decreased concomitantly with an increase in the number of recombined chromosomes and therefore of circles, argues that the excised circle does not replicate and is in fact diluted within the population of cells that proliferate. The experiment displayed on figure 3.19 could theoretically have served to determine the degradation rate of the circle since the proliferation rate of the cells is known. However, it is clear from comparing the graphs on figures 3.12, 3.17 and 3.19 that there are variations in the measured amount of circle from experiment to experiment. Furthermore, there is an additional phenomenon which potentially affects the amount of circle measured, namely the capacity of FLP mediated excision recombination to initiate a gene amplification event. We therefore did not attempt to carry out experiments to evaluate the half life of the excised circular DNA.

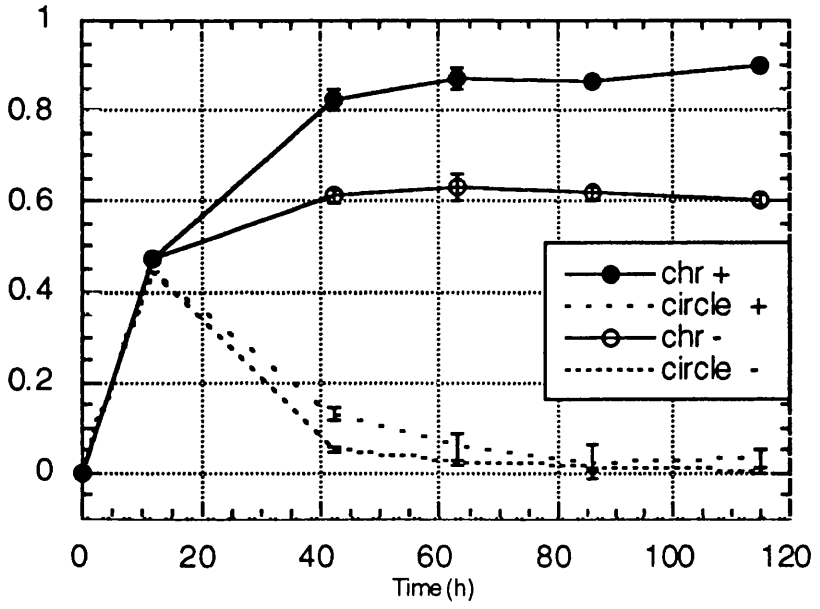



Figure 3.19 Time course of recombination wherein estrogen was or was not withdrawn after 12 hours. chr + and chr - stand for fraction of recombined chromosomes in the continued presence or after removal of estrogen respectively. circle + and circle - similarly stand for the proportion of circle to total number of chromosomes.

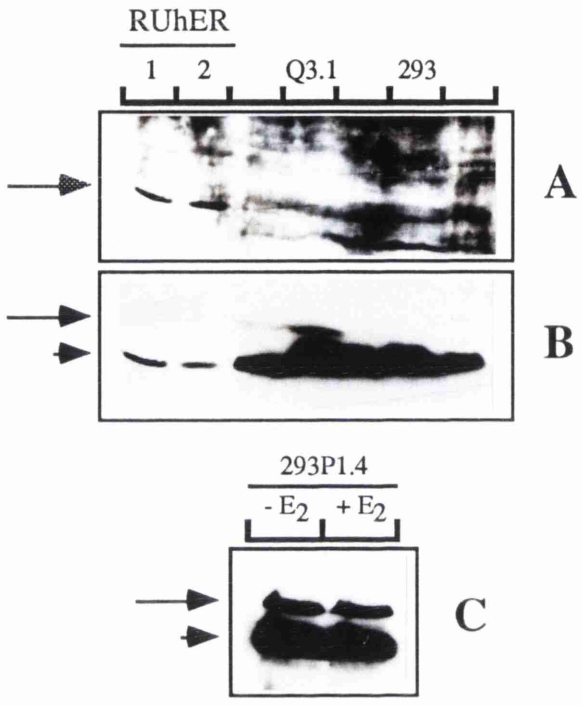



Figure 3.20 A. Western blot of recombinant full length human estrogen receptor and of lysates from 293Q3.1 cells or 293 cells probed with Mab-17, directed against the amino terminus of the human estrogen receptor. Either 1 or 2 femptomoles (3.1 or $6.2x10^8$ molecules) of recombinant human estrogen receptor were used and protein from $2x10^6$ cells was loaded. Note the absence of a band migrating at the level of the arrowhead in 293Q3.1 and 293 cell extracts. B. Same Western blot as for panel A but probed with Mab-EF-3 which is directed against the F domain of the human estrogen receptor. Note the prominent band running at the level of the arrowhead in 293Q3.1 and 293 cell extracts. C. Western blot of lysates from 293P1.4 cells cultured for 48 hours either in the presence of ethanol vehicle or 1μM estradiol.

therefore did not attempt to carry out experiments to evaluate the half life of the excised circular DNA.

What was concluded from the experiment shown on figure 3.19 though, is that the present steroid inducible recombination system could be modulated by withdrawing hormone.

In a further effort to characterise the present estradiol regulated recombination system, a western analysis was performed. As a control, recombinant human estrogen receptor was used. It had been produced in a baculoviral expression system and was kindly provided by Roussel Uclaf, who also provided the concentration of estrogen receptor present in their sample. Panel A of figure 3.20 shows a western blot, probed with Mab-17, which is directed against an amino-terminally located epitope in the human estrogen receptor. This epitope is not present in the FLP-EBD molecules which was therefore not recognised. The Roussel Uclaf protein however, was detected and ran at 65kDa as predicted. Panel B of figure 3.20 shows the same blot as panel A, probed with Mab-EF-3, a monoclonal antibody raised against the F domain of the estrogen receptor, whose epitope is present in the FLP-EBD fusion protein. The Roussel Uclaf estrogen receptor is detected by Mab-EF-3. FLP-EBD fusion protein (86kDa) is only detected in the Q3.1 cell line extract and not in 293 cell extract. The identity of the 65kDa Mab-EF-3 epitope is presently unknown. The blot on panel A shows that the major Mab-EF-3 antigen running at 65kDa (see panel B) is not bona fide human estrogen receptor since the 65kDa signal observed with Mab-17 is lower than the signal observed with Mab-EF-3.

About $3.18 \times 10^8$ molecules of Roussel Uclaf estrogen receptor were used. Lysate of $2 \times 10^6$ 293Q3.1 cells was loaded two wells further and lysate from $2 \times 10^6$ 293 cells was loaded another two wells further. The equivalent intensities of the Roussel Uclaf estrogen receptor and fusion protein bands suggests that there were between 100 and 1000 FLP-EBD molecules per cell in the 293Q3.1 clone. Furthermore, this analysis indicates that the 293 cells did not contain more than 200 estrogen receptor molecules per cell (Figures 3.20a and b).

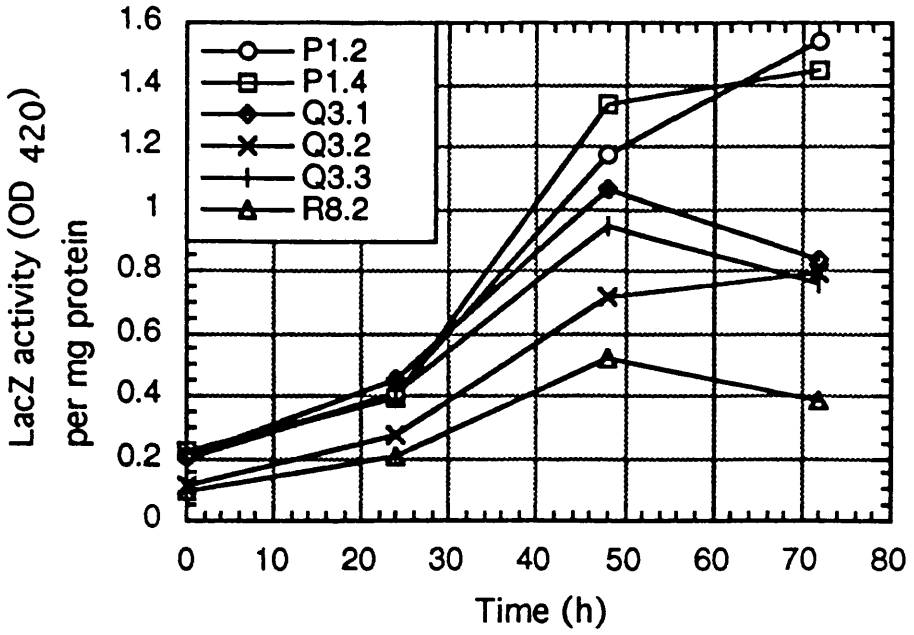



Figure 3.21 β-Galactosidase activity was measured using a liquid assay, on cell extracts derived from cells exposed to 100nM estradiol for the indicated period of time. The background of pseudo β-Galactosidase activity in 293 cells resulted in an $OD_{420}$ of 0.09 per mg protein. The assays on all the cell lines but Q3.1 and Q3.3, were performed in parrallel. The decrease in enzymatic activity observed after 50 hours for Q3.1 and Q3.3 was probably due to unfavourable culture conditions.

The effect of estradiol on FLP-EBD levels was examined by western blotting total lysates from 293P1.4 cells, exposed to 1μM estradiol or ethanol vehicle for 48 hours, were loaded. Under both growth conditions a band with the same intensity can be seen. Estradiol therefore did not significantly alter FLP-EBD protein levels in 293P1.4 cells (figure 3.20, panel C)

## Kinetics of estradiol induced, site specific recombinase mediated, gene expression

Temporal control of a chromosomal site specific recombination reaction permits temporal control of the phenotype of a cell population. To analyse the kinetics of such an event, we quantified the induction of β-galactosidase activity in the 6 *Neo$^r$,LacZ$^-$* pHFE1 transformed 293 cell lines discussed above. The result of this experiment are shown on figure 3.21. Two sets of sister clones, P1.2/P1.4 and Q3.1/Q3.3, showed similar levels of β-galactosidase activity, indicating that in the cloning process, the transgene expression levels had remained faithful to the potential expression levels in their respective parental cell lines. By 70 hours, the R8.1 and Q3.2 cell lines displayed rather low levels of β-galactosidase activity, the Q3.1 and Q3.3 cell lines displayed intermediate levels of β-galactosidase activity and the P1 derived clones displayed the highest levels of β-galactosidase activity. These differences in expression levels are due to so called genomic position effects on transgene expression levels since the number of chromosomes bearing a reconstituted LacZ gene was virtually identical in all the cell lines (figure 3.12). Therefore, it was concluded that FLP catalysed site specific recombination is insensitive to the genomic integration site effects that affect transgene expression. It should be noted however, that the recombination substrates were selected to be within genomic regions that were permissive for gene expression and that the FRTs were situated within a transcribed region of the transgene.

In contrast to the kinetics of hormone induced chromosomal DNA deletion (fig 3.12), the main increase in site specific deletion induced gene expression occurred between 24 and 48

| Expt | Estradiol, hours pre or post transfection | | Recipient cell line | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | P1.4B | | P1.2B | | Q3.2B | |
| | pre | post | w/b | % | w/b | % | w/b | % |
| 1 | - | - | 0/160 | 0 | 0/248 | 0 | 0/230 | 0 |
| 2 | - | 22 | 17/104 | 14.0 | 16/98 | 14.0 | 10/101 | 9.0 |
| 3 | - | 28 | 16/155 | 9.4 | 11/226 | 4.6 | 7/220 | 3.1 |
| 4 | 2 | 22 | 54/169 | 24.2 | 35/190 | 15.6 | 25/204 | 10.9 |
| 5 | 2 | 28 | 44/220 | 16.7 | 29/311 | 8.5 | 11/170 | 6.1 |
| 6 | - | - | 0/116 | 0 | | | | |
| 7 | - | 22 | 4/44 | 8.3 | | | | |
| 8 | - | 28 | 3/120 | 2.4 | | | | |
| 9 | 2 | 22 | 8/67 | 10.7 | | | | |

Table 3.6 Frequencies of site specific and random integration. 5μg of supercoiled FRT bearing plasmids pOG45 (expts 1-5) or pFTkNeoF (expts 6-9) were electroporated into $5x10^6$ *Neo$^S$,LacZ$^+$* cells (P1.2B, P1.4B or Q3.2B). The cells had been treated with estradiol (expts 4, 5 and 9) or with ethanol vehicle (expts 1, 2, 3, 6, 7 and 8) for 2 hours prior to transfection. After transfection, the cells were plated out in medium containing estradiol (expts 2-5 and 7-9) or ethanol vehicle (expts 1 and 6). After 22 hours (expts 2, 4, 7 and 9) or after 28 hours ( expts 3, 5 and 8) the estradiol was removed by washing the cells 3 times. This experiment was carried out in duplicate. Media containing neomycin was applied to all the plates for 10 days, after which one half of the culture plates were stained for β-galactosidase expression and scored as white, indicative of site specific integration into the open reading frame of the genomically located LacZ gene, or blue, indicative of random integration. The total number of white (w) and blue (b) colonies on each plate is given, as is the number of white colonies expressed as a percentage of the total number of colonies.

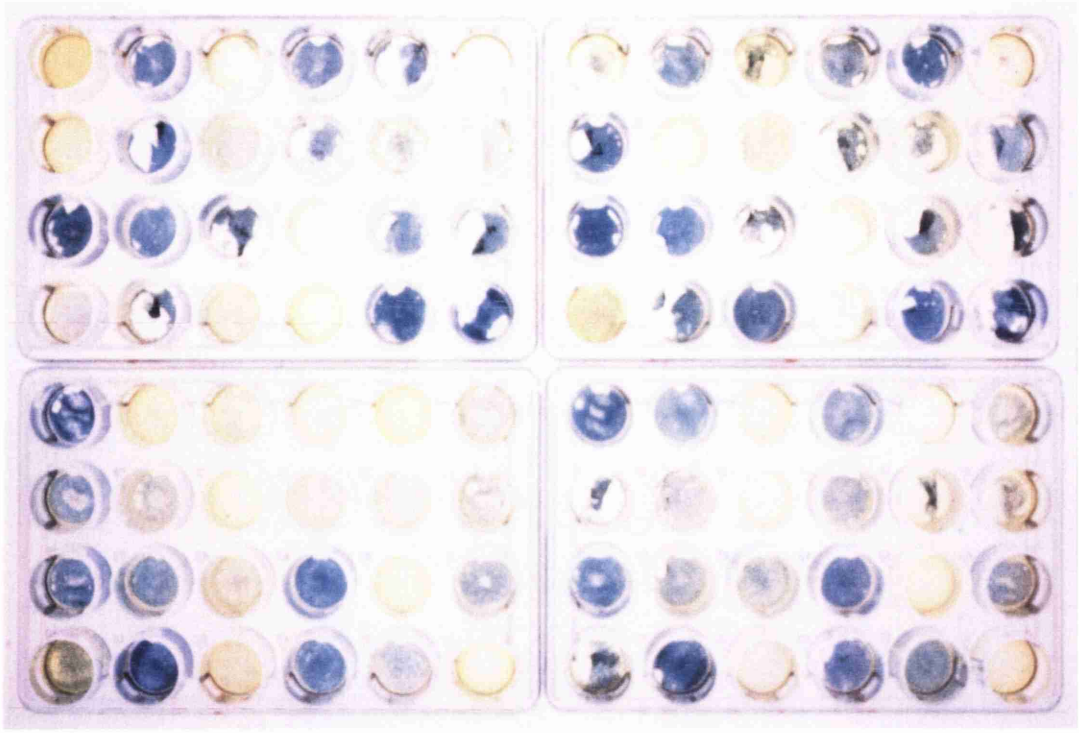
hours after estrogen treatment. This is probably due to the need to accumulate the gene product in order to obtain an increase in enzyme activity. This delay may, however, also reflect the initial instability of the new genotype due to the reversibility of FLP mediated site specific recombination reactions (excision versus re-integration).

**Ligand modulated chromosomal integration of plasmids bearing FRTs**

Experiments were undertaken to assess the frequency of FLP-EBD-mediated site specific integration of introduced plasmids carrying one or two FRTs. Site specific recombinases can integrate transfected DNA in mammalian cells (O'Gorman *et al.*, 1991), (Sauer and Henderson, 1990), (Fukushige and Sauer, 1992), (Baubonis and Sauer, 1993). The ability of endogenous FLP-EBD to site specifically integrate transfected DNA was measured by transfection of pOG45 (figure 3.1) or of pFTk-NeoF into $Neo^{S},LacZ^{+}$ derivatives of cell lines P1.2, P1.4 and Q3.2 (see below). pOG45 is described on figure 3.1 and bears only one FRT. pFTk-NeoF is a plasmid that bears both a neomycin and a Herpes Simplex thymidine kinase transcription cassette flanked by directly repeated FRTs ie; it bears two FRTs.

Neomycin sensitive, β-galactosidase positive cell lines were derived from $Neo^{r},LacZ^{-}$ parental lines P1.4, P1.2 and Q3.2, by plating 100 cells on a 10cm cell culture dish in the presence of 100nM estradiol and picking clones 10 days later. All those sub-clones were β-galactosidase positive and neomycin sensitive, indicating that the FRT flanked neomycin resistance gene flanked by FRTs had been excised, leaving a single FRT in the reconstituted open reading frame of the genomic LacZ gene. These new cell lines were named P1.2B, P1.4B or Q3.2B.

Since both plasmids, pOG45 and pFTkNeoF, contain a complete neomycin resistance gene including its own promoter and polyadenylation signal, transfections followed by selection for neomycin resistance compared the frequencies of random integration to those of site




| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S | M | M | B | B | M | | | | | | |
| M | B | M | M | B | S | ! | ! | | | | |
| B | B | B | S | B | B | | | | | | |
| M | B | S | S | B | B | | | | | | |
| B | S | W | S | X | M | | | | | | |
| M | M | M | S | M | M | | | | | | |
| B | M | B | B | X | M | | | | | | |
| M | B | M | B | M | X | | | | | | |

Clones 1->24

Clones 25->48

Figure 3.22 X-gal analysis of *Neo*$^r$ clones derived from cell line P1.4B by transfection of pOG45 as described on table 3.6 experiment 4. The lower panel schematises the microscopic observations on the plates shown in the upper pane. Symbols; S: switched from white to blue upon hormone treatment, B: always 100% blue cells, M: mix of white and blue cells, no hormone effect, W: 100% white cells and no effect of hormone, X: clone did not survive picking, !; switched wells.

specific integration. Random integration would yield neomycin resistant clones that are still β-galactosidase positive, while site specific integration would disrupt the LacZ open reading frame and yield neomycin resistant clones that are β-galactosidase negative.

Figure 3.13 shows that recombinase activity could be regulated by the addition and subsequent removal of estradiol. For site specific integration, recombinase activity must be regulated to disfavour the re-excision of integrants. Recombinase activity was therefore modulated by the addition of estradiol, either before, to potentially pre-load the genomic FRT with FLP-EBD protein, or after transfection. The estradiol was then removed by washing the cells either at 22 or 28 hours, whereupon neomycin selection was imposed. The results of these experiments are shown on Table 3.6. All colonies obtained from transfections that were not treated with estradiol stained blue (experiment 1). With pOG45, for each estradiol protocol, the frequencies of white colonies observed varied consistently according to the host cell line (P1.4 > P1.2 > Q3.2). Since P1.2 and P1.4 shared the same genomic FRT integration site, it was not possible to attribute this difference to genomic position effects. Within each host cell line, the frequencies of white colonies observed, consistently varied according to the estradiol treatment protocol (22 hours post transfection > 28 hours, 2 hours pre-treatment > no pre-treatment). In the case of the plasmid bearing two FRTs, pFTk-NeoF, the proportion of white colonies was 10.7% of the total colony number when the cells were exposed to estradiol 2 hours prior to transfection and it was 8.3% when the cells had not been pre-treated with estradiol.

To confirm that the white colonies did represent site specific integration events, 48 randomly chosen neomycin resistant colonies from the P1.4B experiment 4 and 36 neomycin resistant colonies from P1.4B experiment 9 (table 3.6) were isolated and cultured on 24 well plates in the presence or absence of estradiol for 4 days (figure 3.22)

In agreement with the frequencies observed by staining primary colonies (24.2%), 8 isolated clones showed β-galactosidase re-expression only in the presence of estradiol (17.8% of the total number of viable colonies picked). Eighteen of the clones that were

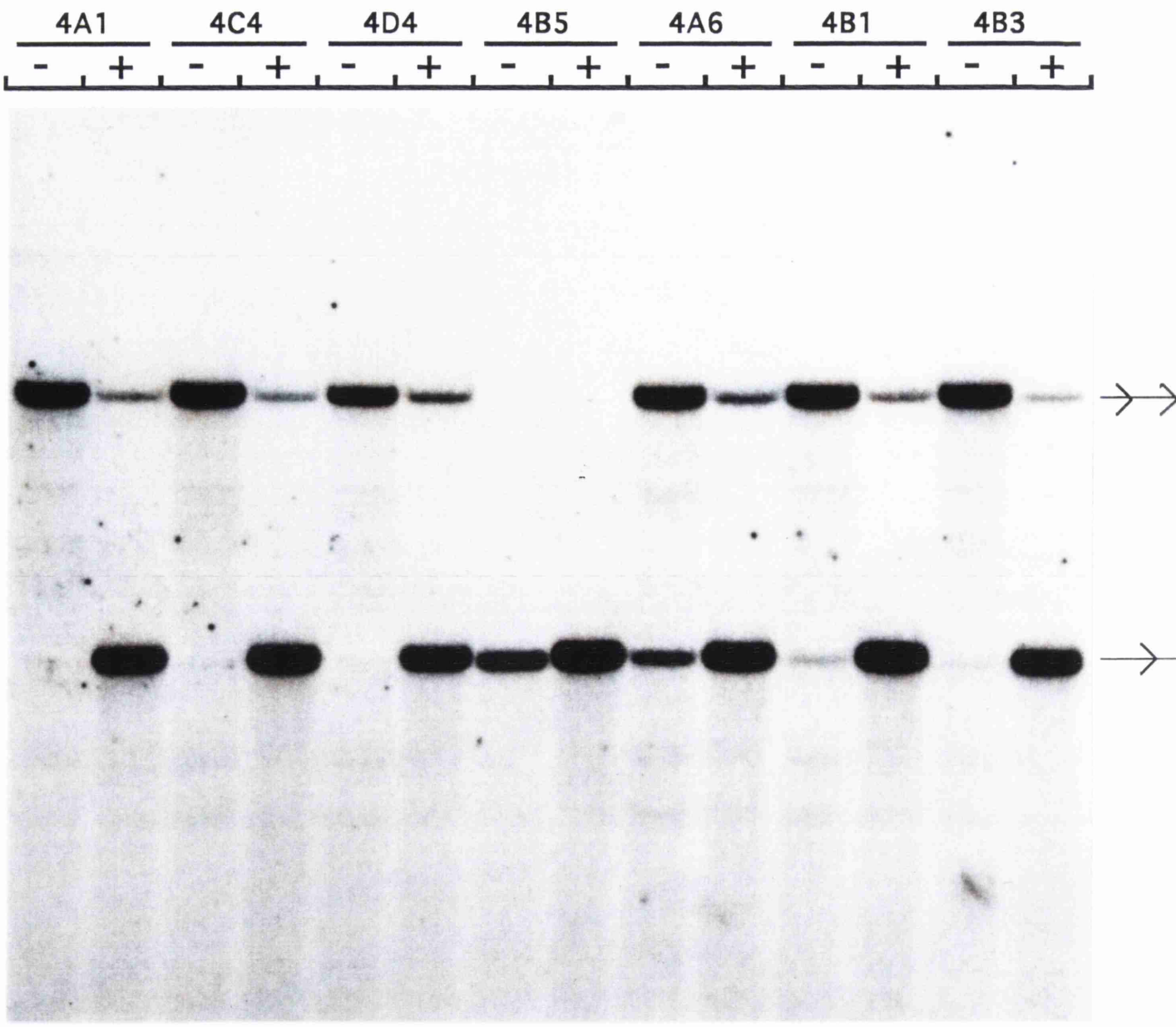



Figure 3.23 Southern blot analysis using probe 1 shown on figure 3.1, directed against the LacZ gene. Seven neomycin resistant clones obtained by transfection of circular pOG45 plasmid into the P1.4B cell lines as described on table 3.6, expt 4, were amplified and then grown for 60 hours in the presence or absence of 1μM estradiol. The genomic DNAs were digested with NdeI, an enzyme that does not cut the pOG45 plasmid. Note that all clones, except clone 4B5, yield a high molecular weight restriction fragment and that a fragment of the same size as those seen with clone 4B5 DNA is produced at the expense of the high molecular weight fragment upon hormone treatment, indicative of FLP mediated excision of the pOG45 plasmid from the pNEObGAL locus in those cells.

isolated contained a mixture of blue and white cells in both culture conditions (40%). One clone was completely devoid of blue cells whether cultured in the presence or absence of estradiol and 18 clones contained only blue cells in the absence or presence of estradiol, indicative of random integration of the pOG45 plasmid, leaving the LacZ gene undisrupted (40%). Three clones did not grow (figure 3.22).

Of the 36 FtkNeoF neomycin resistant transformants, 4 (11.1% of the total) showed β-galactosidase re-expression, again in agreement with the frequencies of site specific integration observed by staining primary colonies (10.6%).

Seven of the 48 P1.4B derived neomycin resistant clones were chosen for Southern analysis, 3 of these were able to switch from a $LacZ^-$ to a $LacZ^+$ phenotype in the experiment shown on figure 3.22, these were 4A1, 4C4 and 4D4. Three clones belonged to the "mixed" class, which contained both blue and white cells before and after estradiol treatment, these were 4A6, 4B1 and 4B3. Clone 4B5 was chosen as control as it constitutively expressed β-galactosidase, indicative of random integration of the pOG45 plasmid. A Southern blot of DNA extracted from these clones after exposure to estradiol or ethanol vehicle for 60 hours is shown on figure 3.23. The blot was probed with a probe directed against the LacZ gene (probe 1, figure 3.1), to observe the chromosomal products of the recombination reaction. All the clones apart from clone 4B5 display the restriction fragments indicative of site specific integration of one copy of the pOG45 plasmid and of its excision upon hormone treatment. As expected, in clone 4B5, the restriction pattern indicated that the LacZ locus had not been altered. This blot was stripped and re-probed with probe 2 (directed against the FRT flanked neomycin resistance gene, figure 3.1) to look for randomly integrated copies of pOG45 (figure 3.24). The DNAs were digested with a restriction enzyme that does not cut the pOG45 plasmid. One of the excision recombination competent clones, 4A6, shows a minor band, possibly indicating a random integrant of the pOG45 plasmid. The 5 other excision competent clones did not display additional neomycin positive restriction fragments and therefore did not contain other copies of the pOG45 elsewhere in their genome. Clone 4B5

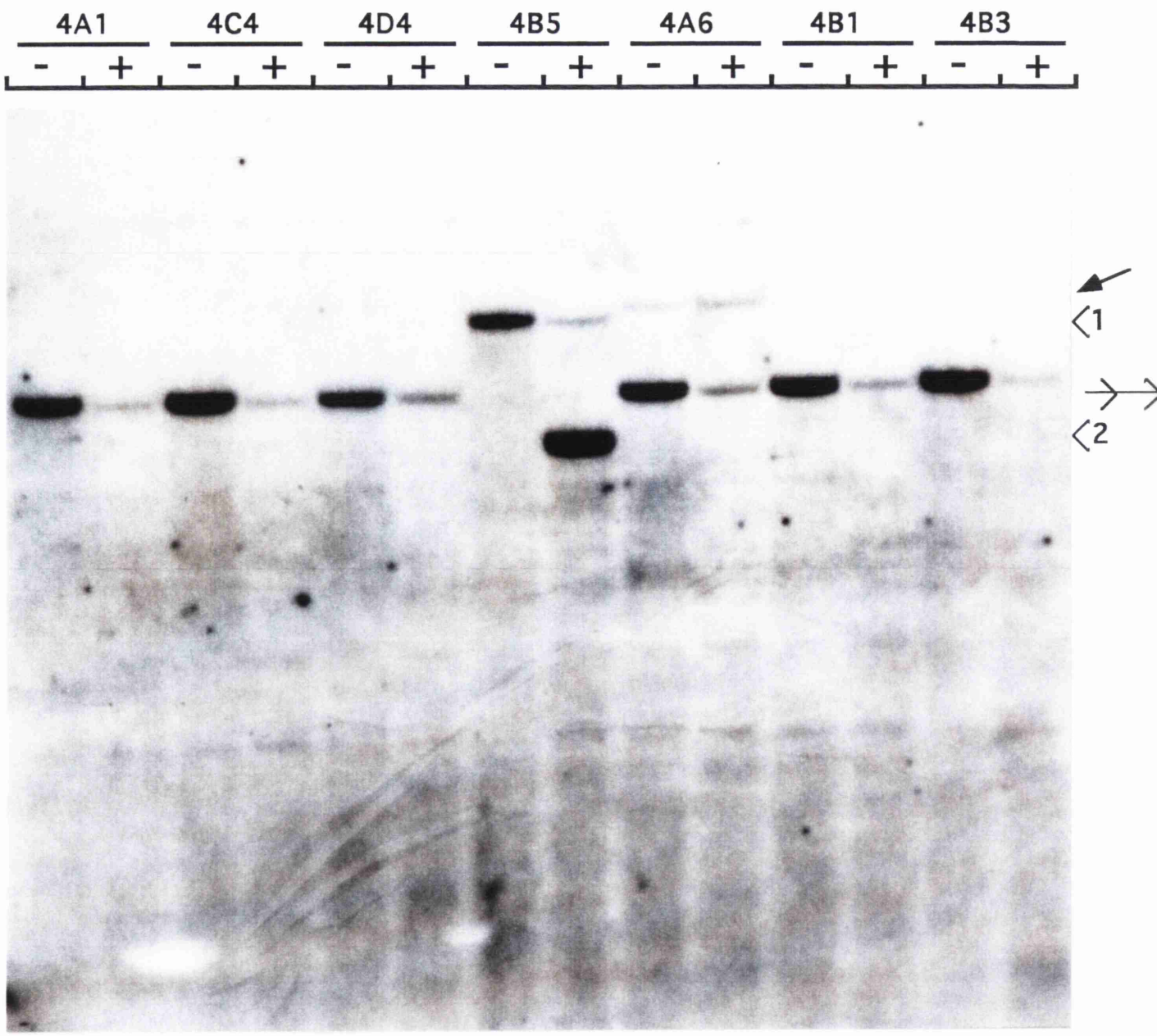



Figure 3.24 Southern blot analysis. The blot shown on figure 3.23 was stripped and probed with probe 2 shown on figure 3.1. This probe is directed against the neomycin phosphotransferase gene. NdeI does not cut within the excised circle and therefore, remaining excised circles in the estradiol treated samples do not resolve as a single band on this blot. The random integrant in 4B5 was subject to recombination in the presence of estradiol. Note the absence of additional bands in clones 4A1, 4C4, 4D4, 4B1 and 4B3, indicating that no copies of the pOG45 plasmid were present in the genome of these cells apart from the one integrated in the pNEObGAL locus. A faint bands in clone 4A6, indicates that it was of a polyclonal origin where one of the parental cells contained a randomly integrated pOG45 plasmid. The directly repeated arrow denotes the site specifically integrated pOG45 plasmid, the arrowheads 1 and 2 denote the random integrant in clone 4B5 containing 2 or 1 copies of pOG45 respectively and the arrow shows the position of the faint band in clone 4A6.

did yield a major restriction fragment indicative of random integration of the pOG45 plasmid. Surprisingly, estradiol caused a reduction in the size of this restriction fragment equal to the size of the pOG45 plasmid, suggesting that a dimer of pOG45 was present in the genome of this cell line and that one copy could be excised by the FLP-EBD recombinase. This dimer of pOG45 could have arisen either by FLP-EBD mediated co-integration of two pOG45 plasmids prior to random integration or by site specific integration of one pOG45 plasmid into a randomly integrated copy of pOG45.

Surprisingly, the three "mixed" clones for β-galactosidase expression had integrated the pOG45 plasmid into the chromosomal pNEOβGAL FRT and could excise it upon exposure to estradiol as demonstrated by the above Southern analysis (figures 3.23, clones 4A6, 4B1 and 4B3). Since 2 of 3 such clones had not integrated additional copies of pOG45 (figure 3.24), it can be concluded that neomycin resitance was conferred to a majority of this category of clones by site specific integration of the neomycin resistance conferring plasmid. Site specific integration of pOG45 therefore occured in 25 of the 45 neomycin resistant colonies (55%) that were picked (figure 3.22).

Three of the 4 pFtkNeoF plasmid derived P1.4B clones able to undergo β-galactosidase induction upon estradiol treatment were picked, expanded and subjected to a time course of hormone induced recombination for Southern blot analysis (FC6, FD1 and FD6). This analysis was carried out so as to distinguish between the several site specific circle integration events that could have resulted in the generation of neomycin resistant $LacZ^-$ clones. Since the pFtkNeoF plasmid bears two FRTs, there are potentially 8 different DNA species to observe, 3 of which are circular (figure 3.25). Figure 3.26 shows the Southern blot, probed with probe 1 (figure 3.1) which should visualise the 5 possible chromosomal recombination products. The recombination rate observed in this particular experiment is very low, probably

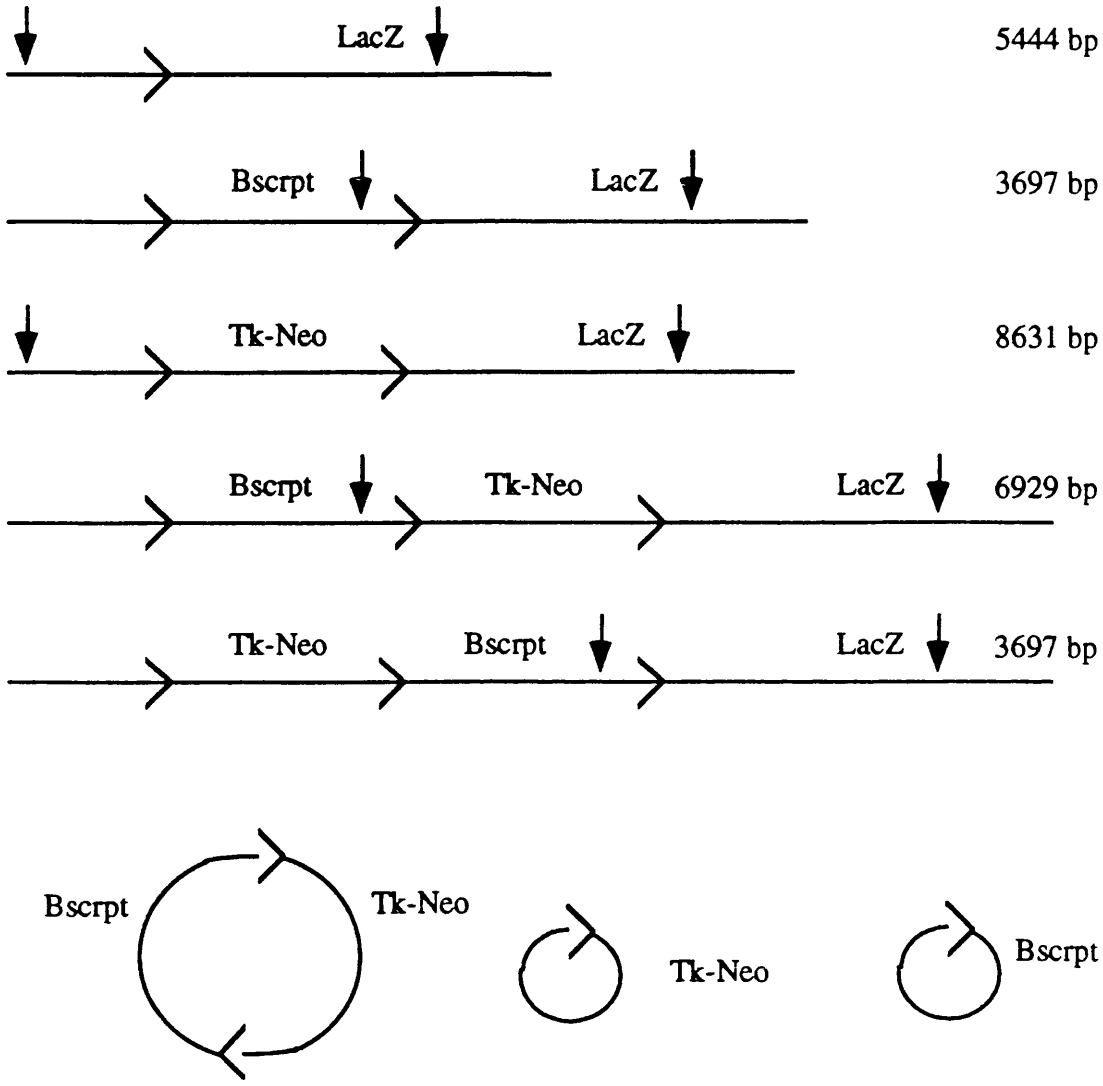



Figure 3.25 The eight DNA species that can occur upon introduction of FTk-NeoF plasmid into P1.4B cells and exposure of those cells to estradiol, are shown. The FRTs are drawn as arrowheads, which also denote the orientation of the spacers. The vertical arrows represent the VspI restriction sites used to generate the restriction fragments that can be seen on figure 3.24. The size of the expected restriction fragments is indicated. LacZ indicates the location of the LacZ gene, which is also the target of the probe used to generate the autoradiogram shown on figure 3.24, Bscrpt stands for pBluescript vector sequences, Tk-Neo stands for the drug resistance expression cassettes. Tk-Neo and Bscrpt are separated by an FRT.

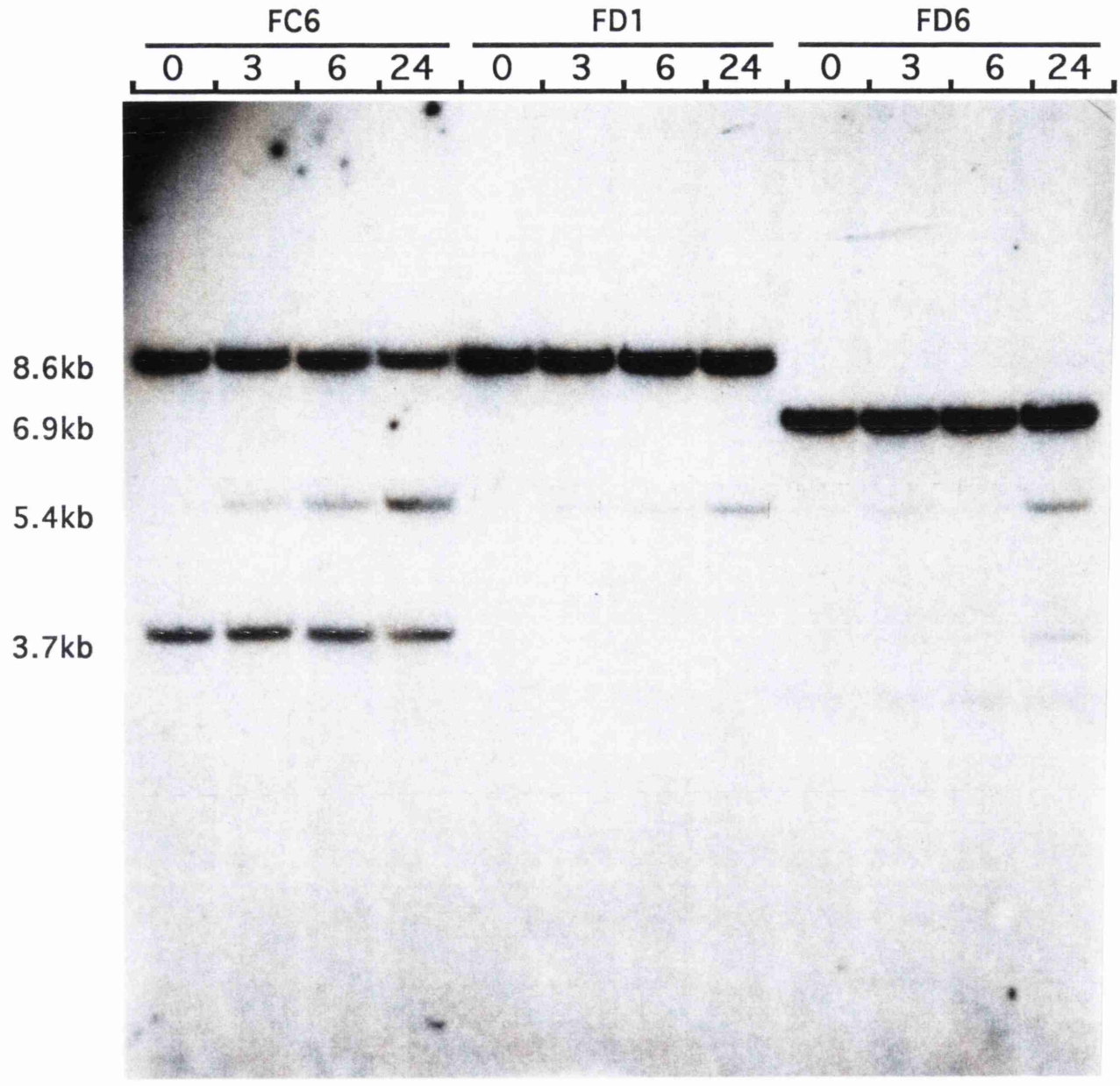



Figure 3.26 Southern blot analysis of the pFTkNeoF plasmid DNA integrated in the 293P1.4B pNEOβGAL locus using probe 1 shown on figure 3.1. See figure 3.23 for a detailed interpretation of the observed restriction patterns. The size of the restriction fragments is indicated on the right. Note that all possible, predicted, chromosomal DNA restriction fragments (figure 3.23) are present. The slow recombination kinetics are probably due to adverse culturing conditions.

because of adverse culture conditions rather than because of the presence of 3 FRTs since, in a blue/white test analogous to that shown on figure 3.22 (data not shown) these clones were able to excise the integrated DNA efficiently. It is apparent from figure 3.25 that the possible integration events resulting in the production of *Neo$^r$,LacZ$^-$* cells are represented in the 3 clones. Clone FC6, contains a majority of cells which bear the Tk-Neo cassette only, yielding a restriction fragment of 8631bp, and a minority of cells which bear either the whole or the "vector half" (these two recombination products are indistinguishable on this blot) of the pFtkNeoF plasmid integrated in the chromosomal LacZ FRT, yielding a 3697bp restriction fragment. Hormone treatment induces the appearance of a third restriction fragment of 5444 bp, which represents the original P1.4B LacZ locus. Clone FD1 cells, bear only the "Tk-Neo half" of the pFtkNeoF plasmid integrated in the chromosomal LacZ FRT, yielding the 8631bp fragment. Upon hormone treatment this cassette is excised, resulting in the appearance of the 5444 bp restriction fragment indicative of the regeneration of the original P1.4B, LacZ locus. Clone FD6, is constituted of cells which bear the whole pFtkNeoF plasmid integrated in the chromosomal LacZ FRT through the other FRT than the one used in clone FC6 as the order of the two FRT flanked plasmid halves is opposite to that in a minority of the cells present in clone FC6. Recombination in this cell line, again resulted in the appearance of the original P1.4B LacZ locus, but also in the appearance of a band at 3697bp, indicative of cells which either bear the whole pFtkNeoF plasmid or its "vector half" integrated in the LacZ FRT. No "Neo only" restriction band (8631bp) is visible.

DISCUSSION

In the above chapter, evidence is presented showing that the activity of the yeast 2μm episome encoded site specific recombinase, FLP, can be regulated by steroids through fusion of steroid receptor LBDs to its carboxyl terminus. This extends the list of proteins onto which steroid dependency can be conferred by this strategy (Mattioni *et al.*, 1994), to a recombinase.

FLP-fusion mediated site specific recombination was monitored in two established mammalian cell cultures models, human embryonic kidney cells (293 cells) and African green monkey cells (CV1 cells). The capacity of FLP fusions to carry out the excision of 1.3kb and 4.3kb DNA segments from chromosomes was tested. We also assayed the capacity of the FLP-estrogen LBD fusion protein to catalyse the integration of plasmids bearing either one or two FRTs into genomic FRTs. Below I will summarise the conclusions that emerged from those experiments.

## Steroid receptor LBD mediated repression of site specific recombinase activity

The repressive power of the estrogen, glucocorticoid and androgen receptor ligand binding domains fused onto FLP enzyme activity, was measured by transient transfection of plasmids encoding the fusion proteins into CV1 cells, containing a chromosomal 4.3kb DNA segment flanked by directly repeated FRTs. In all three case repression of FLP activity was observed. The wild type estrogen receptor LBD did not repress the fused recombinase tightly. This was probably due to the very high affinity of this receptor for estradiol combined to the inability of the serum stripping protocol used to totally remove the estrogens present in fœtal calf serum. The repression observed with the androgen, estrogen$^{G400V}$ and glucocorticoid receptor LBDs could be relieved by the addition of ligands cognate to the steroid receptor

LBD to the growth medium of the cells and resulted in recombinase activity levels equivalent to half the activity levels of native FLP enzyme (figure 3.3). This result was confirmed and extended by the generation of cell lines that maintained both a constitutively expressed FLP-estrogen LBD protein and an intact 1.3kb FRT flanked chromosomal excision substrate. Such cell lines maintained an intact excision substrate until excision was induced by the addition of estradiol (figure 3.4, panel B) to their growth medium (figure 3.5).

**The efficiency of FLP at excising chromosomal DNA**

In transient recombinase expression experiments, FLP mediated chromosomal excision was detected in 24 of 25 randomly chosen, 293 cell derived, clones which contained 1 to 4 randomly integrated genomic FLP recombination substrates. The single clone which was refractory to FLP mediated recombination harboured one truncated transgene which presumably no longer contained two directly repeated FRTs and could therefore not serve as a substrate for excision recombination by FLP. This experiment leads me to conclude that all FRT bearing transgenes able to drive the expression of selectable levels of a drug resistance conferring cassette can serve as substrates for FLP. Recombination frequencies however, varied greatly amongst the 24 clones (table 3.2). In two of the clones, this was shown to be due largely to a difference in the efficiency with which calcium phosphate precipitated DNA was absorbed (table 3.3) but apparent variations in recombination frequencies amongst the 24 clones could also have been due to differences in the number of inserts or to differences in reporter gene expression levels or to actual genomic position effects on the chromosomal FLP recombination substrates.

In an attempt to unmask a position effect, four of the above recombination reporter cell lines, two of which only expressed low levels of the reporter gene hooked onto the FLP recombination targets, were stably transfected with a FLP-estrogen LBD fusion protein encoding plasmid. Recombination frequencies and reporter gene expression levels were

monitored in parallel, in a time course using 8 independent cell lines representing 4 different reporter loci. I found that recombination occurred with remarkably similar kinetics in all the cell lines (figure 3.12). In contrast, reporter gene expression levels, faithfully reflected the expression levels from each locus (figure 3.21). I conclude that the ability of FLP fusion proteins to catalyse excisions at a chromosomal locus does not reflect genomic position effects that affect transgene expression. This data does not exclude however, that chromatin structure could have an effect on the efficiency of FLP mediated chromosomal recombination, but strongly suggests that any transgene available for transcription is also available for excision recombination.

**The efficiency of FLP at integrating FRT bearing plasmids into pre-existing chromosomal FRTs**

I carried out experiments to evaluate the capacity of FLP to site specifically integrate FRT bearing plasmids into pre-existing chromosomal FRTs in cell lines constitutively expressing FLP-EBD protein. I showed that by pre-treating cells for 2 hours with estradiol before and for 22 hours after transfection it was possible to integrate plasmids at frequencies equivalent to those of random integration (table 3.6). This exceeds the frequency of homologous recombination by several orders of magnitude (Zheng and Wilson, 1990), (Hasty *et al.*, 1991). It may be possible to improve on this experiment and to increase the frequency of site specific recombination events by changing experimental parameters such as the durations of pre- and post transfection hormone treatment and the amounts of plasmid transfected so as to decrease the frequency of random integration, as well as by using ligands which increase the activity of the recombinase fusion (see chapter 4) or by using a more active recombinase. I also showed that it is possible to site specifically integrate plasmids that bear two FRTs (table 3.6), allowing strategies to be devised whereby further FLP mediated recombination events on an integrated plasmid will yield an engineered chromosomal locus free of bacterial plasmid

maintenance sequences and of selectable markers. In other words, this system makes it feasible to modify chromosomal loci at will and without the permanent introduction of more heterologous sequences than those that serve as targets for the site specific recombinase.

**Kinetics of site specific excision in mammalian cell lines harbouring both a chromosomal FLP deletion substrate and conditional FLP activity**

Time courses of recombination are shown on figures 3.12 and 3.17. Initially, recombination occurred in 8% of the population per hour, equivalent to 2.1 events per cell per cell cycle. The rates observed in these experiments lag behind the predicted rate of accumulation of chromosomes bearing the FLP-EBD catalysed deletion, (figure 3.14). There are a number of parameters that could cause the difference between the observed and the predicted rates of accumulation of chromosomes bearing the FLP-EBD catalysed deletion, I will discuss these in the following paragraphs.

First of all there is the fact that the theoretical basis for the prediction, exponential decay (figure 3.14), is only valid for an infinitely large population, since the DNA samples analysed were obtained by lysis of 2 to 20 million cells, we might expect a deviation from the ideal values. However, we would expect this deviation to manifest itself in the other direction. i.e.; since initially deletions occurred at an apparent frequency equivalent to two events per cell cycle, we would expect that, after 2 cell cycles, all of the cells should have undergone the FLP mediated chromosomal deletion. The curves on figures 3.12, 3.17 and 3.19 show that this is not the case; 10% of the cells still bear an un-rearranged chromosome even after 100 hours of hormone induction. There may be a trivial explanation for the observed discrepancy; for example, a proportion of the cells in the cultures may have been moribund or they may simply not have expressed sufficiently high levels of recombinase or they may have lost the recombinase encoding transgene.

A parameter that may have affected the kinetics of accumulation of recombined chromosomes in the population would have been that the recombination process could not take place throughout the cells' growth cycle. From the biology of the 2μm episome, it is known that FLP is active during the S phase of the cell cycle (Russo *et al.*, 1992). Furthermore, it has been shown in *Drosophila* that FLP can catalyse recombination between homologues so as to generate genetic mosaics (Vincent *et al.*, 1994), (Xu and Rubin, 1993). Such events must occur either during the S or G2 phases of the cell cycle. There is no good reason to exclude that FLP functions during the G1 phase of the cell cycle. This only leaves the M phase of the cell cycle during which the chromosomes are highly condensed and therefore may not be accessible to the recombinase. The M phase of the cell cycle only represents 10% of the cycling time of mammalian cells however. This cannot account for the lag observed on figure 3.14.

The levels of FLP-EBD fusion proteins were measured before and after 50 hours of estradiol treatment because a decrease in recombinase levels could have explained the observed lag shown on figure 3.14. However, estradiol did not affect the levels of recombinase significantly, as can be seen on figure 3.20, panel C, also eliminating this explanation for the presence of unrecombined cells so late in the time courses.

The reactions catalysed by conservative site specific recombinases are reversible. It is therefore likely that one major cause for the lag shown on figure 3.14 is due to re-integration of previously excised DNA circles into the chromosomal FRT. If the substrates and products of the recombination reaction were equivalent, ideally, an equilibrium should be reached at 50% recombination, however, the excision reaction and the integration reactions differ. Firstly, in the case of the excision reaction, the diffusion of the two FRTs involved, is limited to one dimension because they are located on the same DNA molecule while the integration reaction involves two FRTs, located on separate DNA molecules, diffusing freely in three dimensions. Secondly, since we are dealing with a proliferating population of cells and since the excised DNA circle may not replicate, we would expect the proliferation rate of the cells

to affect the rate at which the excised circle is diluted within the population, this in turn would affect the frequency at which re-integration events can happen. If the half life of the circle were much smaller than the doubling time of the cells, this would not be true, but as DNA plasmids are known to remain present in cultured mammalian cells for prolonged periods (Krysan *et al.*, 1989), (Heinzel *et al.*, 1991), it is believed that it is the proliferation rate which dictates the rate of loss of the circle in this system. Replication of the circle by the cellular replication machinery is not significant since we did observe a loss of insert relative to total chromosome signal in Southern analyses (figures 3.17 and 3.19). The average initial frequency of excision calculated from the data displayed on figure 3.12 probably largely reflects the real excision frequency since it was measured in a population that could only undergo excision (at time zero, all the chromosomes were un-rearranged and no circle was present). The rate of accumulation of chromosomes later in the time courses is probably a reflection of the excision frequency together with an interplay between the frequency of re-integration and the proliferation rate of the cells. This is schematised on figure 3.18. One phenomenon which may affect the frequency of re-integration is the theoretical capacity of FLP to catenate the excised DNA circle to the chromosome. This could have led me to introduce three more factors into the equations shown on figure 3.18, namely the frequency with which excision reactions catenate the DNA circle to the chromosome, the integration rate of catenated circles and thirdly, the rate at which topoisomerase type two enzymes would free catenated circles allowing them to diffuse away.

Strategies could be devised to impede the re-integration of excised circles if this were the major cause of our inability to switch 100% of a cell population's genotype with the FLP-LBD/FRT system. But first it should be ascertained that re-integration of excised circles is the cause of the lag shown on figure 3.14. The frequency of re-integration may be found by studying the re-integration of circles bearing more than one FRT into a chromosomal FRT (figure 3.24). Integration of a plasmid bearing 2 FRTs can occur through either one of the two FRTs on the plasmid, resulting in one of two structurally distinct integration patterns

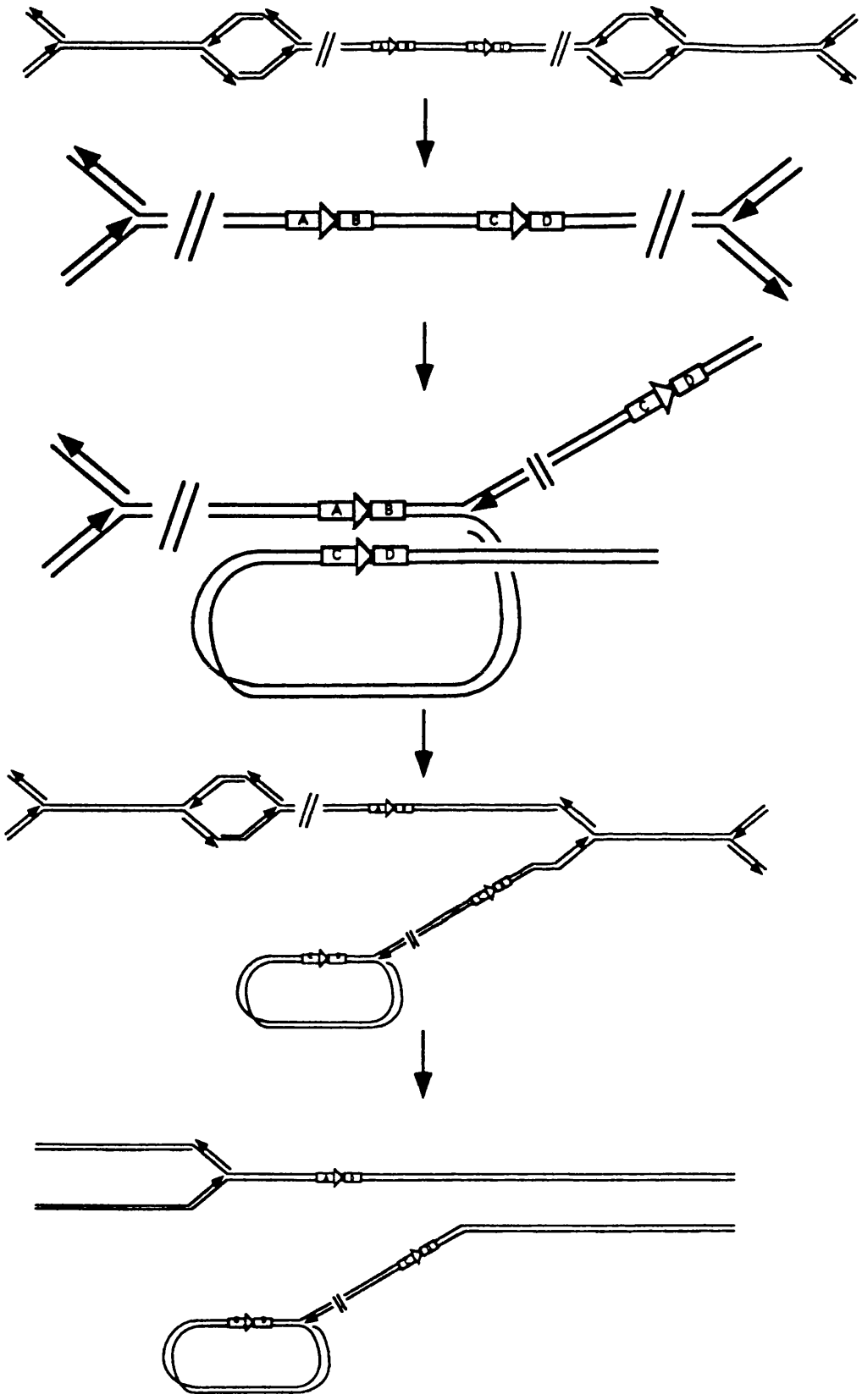



Figure 3.27 Recombination accross a replication fork during S-phase, isolates the replication fork. This leads to the formation of one replicated but truncated chromosome at whose end the FRT flanked DNA is re-replicated multiple times and of one chromosome which is potentially re-replicated by the fork towards which the excised fork was converging.

(figure 3.23, forms 3 and 4). By taking a population of cells carrying one such "3 FRT" structure, and inducing the cells to excise it, it should be possible to quantify the frequency with which the order of the 2 integrated, FRT-flanked DNA segments, is reversed. Although this order reversal can occur in three different ways (either by transposition of circles 7 or 8 (figure 3.23) into the other remaining chromosomal FRT or by re-integration of circle 6 through the other FRT), the simple observation of this phenomenon would provide a measure of the frequency of re-integration events. Measurements of circle re-integration into FRTs located elsewhere in the genome would also be informative, but would not answer the question at hand, which deals with the frequency of re-integration of circles into the chromosome locus from which they have been excised. To date this experiment has not been performed adequately, although the presence on figure 3.24 of a band at 3697bp in the extreme right lane, taken together with the absence of a band at 8631bp in the same lane, could suggest that re-integration of pre-excised plasmids bearing 2 FRTs can occur.

One other phenomenon which may be at the basis of the presence of unrecombined chromosomes in a population induced to undergo a FLP mediated chromosomal deletion is the potential of FLP to excise a replication fork during S phase. This would lead to the re-replication of the FRT flanked DNA sequences by the excised fork through a rolling circle mechanism. Such structures have been postulated, but never observed, for FLP mediated recombination of replicating DNA (Russo *et al.*, 1992). With the FRT configuration in our site specific recombination system, the rolling circle would be located at the end of one chromatid. The proximal end of the other chromatid would eventually be re-replicated by the fork towards which the excised one was converging. This would lead to the production of three chromatids; one truncated chromatid with one normal telomere and with one end that would consist of direct repeats of the FRT flanked insert, and two chromatids, where the FRT flanked insert is deleted, one of which whose length would depend on the ability of a single fork to synthesise it (figure 3.25). The visualisation of such structures is possible (Brewer and Fangman, 1987) but would have required much more material then what we had

in hand. On figure 3.17, the amount of circle generated until 12 hours into the time course consistently exceeds the amount of recombined chromosomes. This is paradoxical since the amount of circle should never exceed the amount of recombined chromosome (see figure 3.18). Although the excess of circle observed on figure 3.17 is likely to be an artefact due to the usage of data derived from two Southern blots for each sample to obtain the relative amount of circle to total chromosomes and/or of the isolation procedure and subsequent manipulation of the genomic DNA used to generate the Southern blots, it could nevertheless be considered as weak evidence for the DNA amplification mechanism outlined above. The consequence of such an amplification event would be that a single cell could end up containing many circles, since the rolling circle generated concatemers of the FRT flanked DNA insert could be recombined into individual circle monomers. The descendants of this cell would probably all inherit circles, which could potentially be re-integrated into the chromosomal FRT, thereby accounting for the presence of 5 to 10 % of cells harbouring intact pNEObGAL loci, even after 5 days of exposure to estradiol.

# Chapter 4 : RESULTS AND DISCUSSION

## Artificial steroid receptor ligands and FLP-LBD mediated recombination

SUMMARY

In the previous chapter I showed how steroid ligands could be used to regulate a site specific recombinase by virtue of the conditional repressor function of their cognate receptors' LBDs. In this chapter I describe the effect of various receptor ligands on site specific recombination mediated by the human estrogen, glucocorticoid and androgen receptor - FLP fusions.

All the estrogen ligands tested induced recombination albeit to different extents. One putative androgen receptor ligand did not induce recombination. One known anti-glucocorticoid did not induce any measurable levels of recombination in a FLP-GR recombination reporter cell line, all the other receptor ligands did induce recombination. It therefore appears that most synthetic steroid receptor ligands, including known anti-steroids dissociate the receptor from the HSP90 containing complex described in chapter 1. Currently available anti-steroids therefore seem to exert their inhibitory effects beyond this stage of receptor activation.

1. **Steroid import into the cell**
2. **Steroid binding to the receptor**
3. **Dissociation of the ligand bound receptor from the repressor complex**
4. **Intracellullar shuttling of the ligand bound receptor complexes**
5. **Interaction of the receptor with cellular proteins**
    a. **Dimerisation**
    b. **Post-translational modifications**
    c. **Heteromeric complex formation**
6. **DNA binding by the receptor complex**
7. **Transcription stimulation or repression via the recruitment of effector proteins to the receptor bound chromosomal locus**

Figure 4.1 Steroid signaling occurs by interaction of steroids with their nuclear receptors and the subsequent receptor mediated alterations in cellular gene expression patterns. The order and exact nature of steps 2 to 7 may well differ in different cellular contexts and for different steroid receptors, furthermore, some of the events listed may require a previous step to be achieved before they can occur. Compounds that inhibit the steroid response will interfere with any one of the steps shown on figure 4.1. The last 4 steps of the cascade are disposable in the FLP-LBD fusions since FLP enzyme is able to catalyse genomic recombination by itself. Drugs that inhibit steps 1 to 3 or that induce LBD fusion protein degradation will act as antagonists in the steroid reporter FLP recombination system. Drugs that inhibit any of the latter steps may affect the activity of the FLP-LBD fusions. The same holds true for mutations that impair any of the activation steps. Finally, it is always possible that drugs or mutations in the LBD cause a gain of function, that is not shown in the above cascade.

## The estrogen receptor

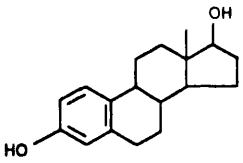
Estrogen is a steroid hormone that plays crucial roles in female embryonic, fœtal and pubertal development as well as being an essential component of the endocrine system that governs the adult female reproductive cycle and various stages of pregnancy and lactation. Not surprisingly, the major known target sites of estrogens in the female body are the reproductive tract, breast and neuroendocrine tissues in which it elicits a variety of specific responses. The lack of estrogens in post menopausal women has been associated with osteoporosis and cardiovascular disease. Estrogens are also implicated in the progression of a variety of cancers that affect the ovaries, breasts and endometrium (Henderson *et al.*, 1993). Estrogen signalling has therefore been a major focus of both clinical and biochemical research. These endeavours have resulted in the early cloning of the gene encoding the estrogen receptor (Walter *et al.*, 1985), in the production of many artificial ligands for the estrogen receptor by pharmaceutical companies, and last but not least, in the generation of mice devoid of the estrogen receptor (Lubahn *et al.*, 1993). Mice of both genders, devoid of estrogen receptor protein are viable but sterile. Female mice, homozygous null for the estrogen receptor have haemoraghic, cystic ovaries, indicating over stimulation by gonadotropins due to the absence of the estrogen mediated negative feedback from the ovaries to the hypothalamo-pituitary axis. As expected, no corpora lutea are present either. They also have undeveloped mammary glands. Unexpectedly, the testes of homozygous null males are reduced in size, and contain few seminiferous tubules and very few germ cells, providing evidence for a previously unsuspected requirement of estrogen signalling in male germ cell generation. The bone density of both males and females is 20-25% lower than in wild type mice. Together these findings confirm what was known about the target tissues of

estrogens. This mouse strain will provide a useful model system for reverse genetic studies of the receptor (Korach, 1994).

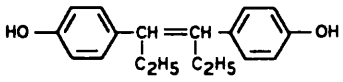
We were interested to observe the effects of synthetic steroid receptor ligands on recombination mediated by FLP-steroid receptor fusion proteins. Firstly because these compounds may serve to regulate hormone dependent recombination more subtly and secondly because most of the intracellular functions of the full length receptors that steroid hormone antagonist are likely to inhibit, should not compromise, but might measurably affect, the recombinase activity of FLP-steroid receptor LBD fusion proteins. I surmised that the estrogen responsive recombination reporter cell lines would, in combination with site directed mutagenesis of the steroid LBDs, provide a sensitive tool to study the mode of action of steroid receptor antagonists. The rationale of these experiments was to introduce mutations known to affect a steroid LBD functions in cis, and to compare the activity of the mutant LBD-recombinase fusion bound by its natural ligand to that of the wild type LBD-recombinase bound by the steroid antagonists under scrutiny. Thus, correlations could be drawn between particular receptor LBD functions and the mode of action of synthetic steroid (ant)agonists.

The chemical structures of the synthetic estrogens used in this study are displayed on figure 4.2. There are a number of operational definitions used to classify an estrogen receptor ligand as an agonist or an antagonist. Among these there is; (1) the capacity of a compound to stimulate or antagonise estrogen receptor mediated transcription from an estrogen response element in cell culture, (2) the capacity of a compound to stimulate or antagonise estradiol stimulated proliferation of breast cancer cells that require estrogens for growth, (3) the effect of a compound on immature, ovaryectomised rats in which it does or not stimulate uterine growth and fails, or not, to inhibit uterine growth stimulated by estradiol and (4) the bone protective effects of a compound in ovaryectomised rats, indicative of bone specific estrogenic activity (Oursler *et al.*, 1993), (Horowitz, 1993).

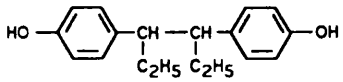
# Estrogen receptor ligands



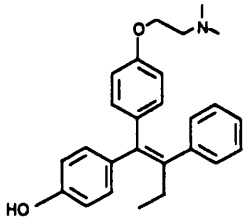

estradiol



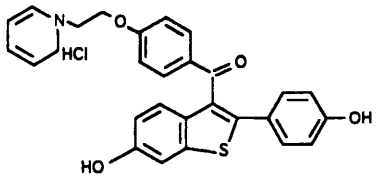

diethylstilbestrol



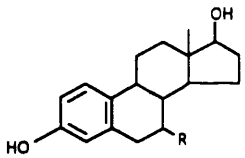

hexestrol




tamoxifen




raloxifene




ICI 182.780 R=$(CH_2)_9S(CH_2)_3C_2F_5$ (O)

ICI 164.384 R=$(CH_2)_{10}CON(CH_2)_3CH_3$ ($CH_3$)

Figure 4.2 The chemical structures of the 7 estrogen recptor ligands used for the present study are shown.

Diethylstilbestrol and hexesterol are bona fide estrogen agonists by all of the above criteria. Hydroxytamoxifen has been classified as an antagonist with weak agonistic activity by criterion 1 (Dana *et al.*, 1994) although on certain promoters (Tzukerman *et al.*, 1994) and in certain cell types (Berry *et al.*, 1990) it is an agonist. Hydroxytamoxifen is an estrogen antagonist in breast cancer cells (Gottardis and Jordan, 1987), (Gottardis *et al.*, 1988), (Westley *et al.*, 1984), (Philips *et al.*, 1993). Hydroxytamoxifen has been shown to be an estrogen agonist in the uterus (Gottardis *et al.*, 1988), (Huynh and Pollak, 1994), (Anzai *et al.*, 1989), (Gottardis *et al.*, 1990), (Kedar *et al.*, 1994), (Webb *et al.*, 1995), (Sundstrom *et al.*, 1990). Hydroxytamoxifen is an estrogen agonist in terms of estrogen induced bone protection (Isserow *et al.*, 1995). Both ICI 164.384 and ICI 182.780 are antagonists by all four criteria (Bondy and Zacharewski, 1993), (Wakeling *et al.*, 1991), (Musgrove *et al.*, 1993), (Gottardis *et al.*, 1990),(Huynh and Pollak, 1994), (Gibson *et al.*, 1991). Raloxifene has also been called keoxyfene or LY 156 758 and is not an agonist of estrogen receptor mediated transcription activation via estrogen response elements (Dana *et al.*, 1994). Raloxifene is a weaker anti-estrogen than hydroxytamoxifen in a chemically induced rat mammary carcinoma model (Gottardis and Jordan, 1987). Raloxifene is as effective as hydroxytamoxifen at inducing the uterine complement C3 genes in luminal and glandular epithelial cells (Sundstrom *et al.*, 1990), it partially antagonised hydroxytamoxifen induced growth of a human endometrial adenocarcinoma (Gottardis *et al.*, 1990). Raloxifene does not prevent uterine atrophy upon ovariectomy of rats indicating that it is not an estrogen agonist in this tissue (Evans *et al.*, 1994). In the bone however, raloxifene has estrogenic properties (Evans *et al.*, 1994).

All these compounds stimulated recombination in the estrogen inducible site specific recombination system described in the previous chapter, albeit to different extends, indicating that they did not inhibit steps 1 2 and 3 of the transduction cascade presented on figure 4.1.

The stage was therefore set to attempt to correlate the different agonistic and antagonistic effects of these compounds with three of the defined molecular events that constitute the

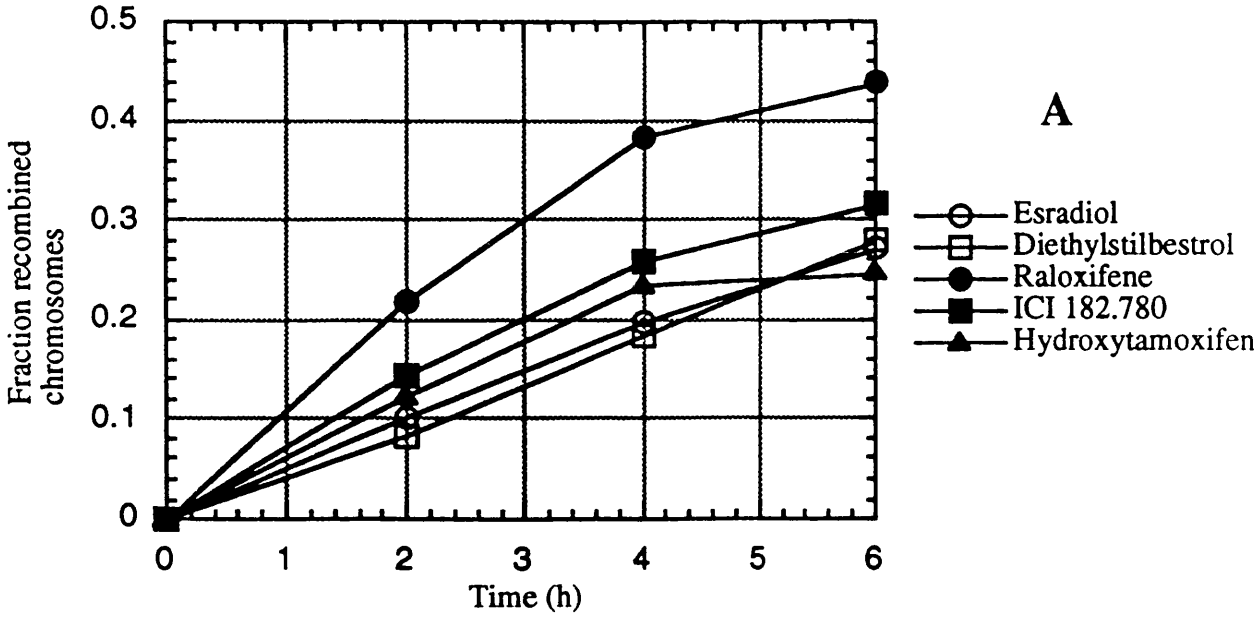
intracellular estrogen transduction pathway, namely estrogen receptor nuclear localisation, homodimerisation and transcription activation. At the time of redaction, the nuclear localisation signal disruption experiments were not completed.

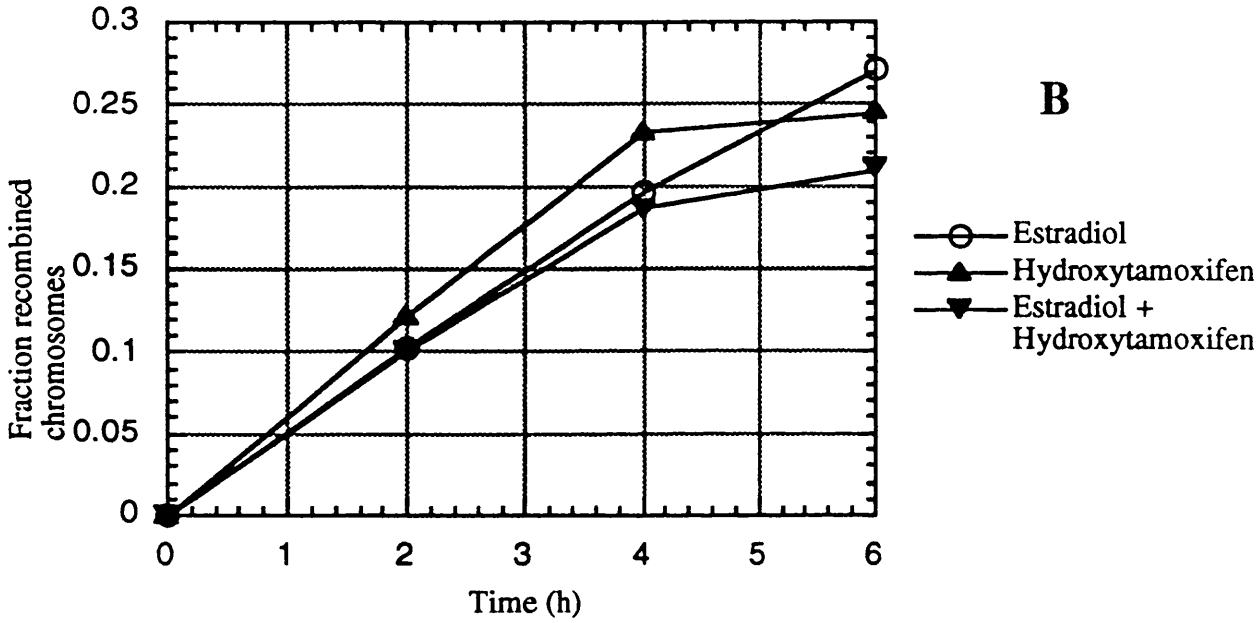
RESULTS

**Artificial estrogen receptor ligands induce the FLP-EBD recombinase to different extents**

293 cells that bore a chromosomal copy of pNEOβGAL and constitutively expressed the FLP-EBD$^{G400V}$ fusion protein were exposed to 1μM estradiol, diethylstilbestrol, raloxifene, ICI 182.780 or hydroxytamoxifen for 2, 4 and 6 hours. A Southern analysis was performed to quantify the amount of FLP-EBD mediated recombination that had occurred. As can be seen on figure 4.3, upper panel, all 5 ligands induced site specific recombination. The magnitude of the response was identical for estradiol and diethylstilbestrol treated cells. Cells treated for 2 hours with raloxifene, ICI 182.780 or hydroxytamoxifen displayed levels of site specific recombination 184%, 143% and 116% those attained by estradiol treated cells. After 6 hours the differences were reduced to 152%, 113% and 92%. Interestingly, the reduction in hydroxytamoxifen induced recombination after 6 hours, could also be seen when cells were treated with both estradiol and hydroxytamoxifen (figure 4.3, middle panel). The cause of this phenomenon remains an open question.

To better visualise the differences in the extent of recombination induced by estradiol versus ICI 164.384 and ICI 182.780, a ligand titration was carried out and samples were collected after 4 hours of ligand exposure. The result of this experiment is shown on figure 4.3, lower panel. This experiment demonstrates, firstly, that 1μM is a saturating concentration of ICI 182.780, since a plateau is reached at 300nM, secondly, that the apparent affinity of the two ICI compounds (figure 4.2) for the EBD$^{G400V}$differs approximately 5 fold, resulting in the


A
Fraction recombined chromosomes
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
Time (h)
Esradiol
Diethylstilbestrol
Raloxifene
ICI 182.780
Hydroxytamoxifen


B
Fraction recombined chromosomes
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
6
Time (h)
Estradiol
Hydroxytamoxifen
Estradiol + Hydroxytamoxifen

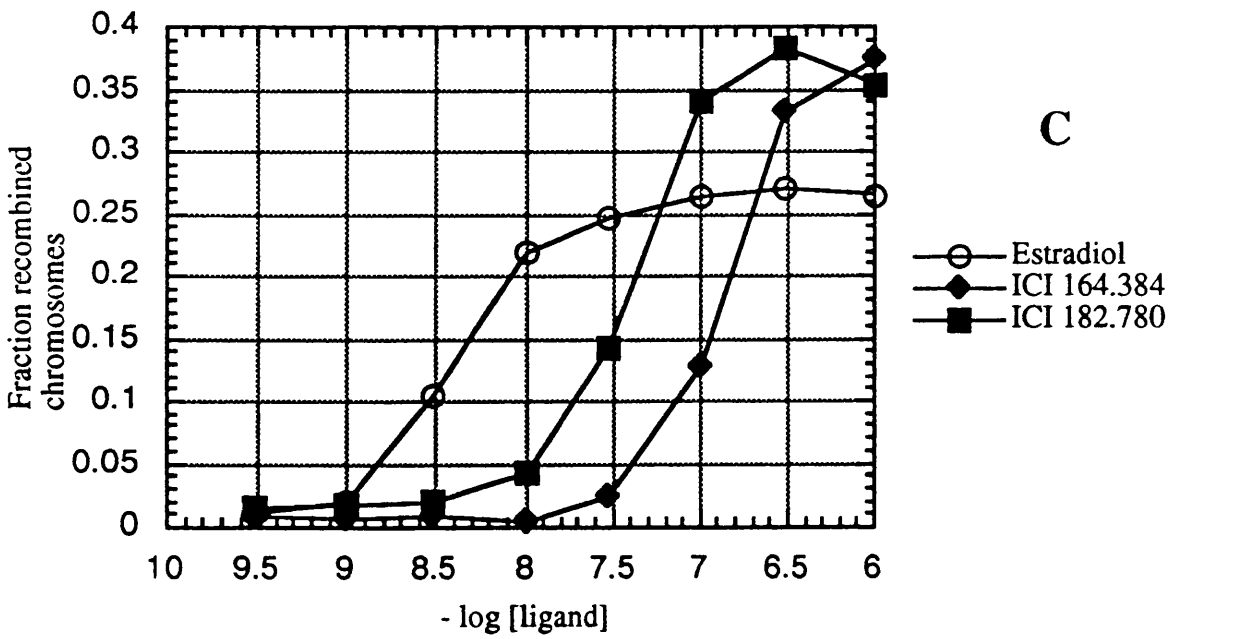

C
Fraction recombined chromosomes
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
10
9.5
9
8.5
8
7.5
7
6.5
6
- log [ligand]
Estradiol
ICI 164.384
ICI 182.780

Figure 4.3 A. Time course of recombination induced with the indicated ligands at a 1μM concentration, in the 293P1.4 recombination reporter cell line (cf. chapter 3). B. Same time course as in panel A but displaying only the estradiol as and tamoxifen data as well as the data obtained from cells exposed to both estradiol and hydroxytamoxifen. C. Ligand titration with estradiol and the two ICI compounds indicated. The cells were exposed to ligands for 4 hours.

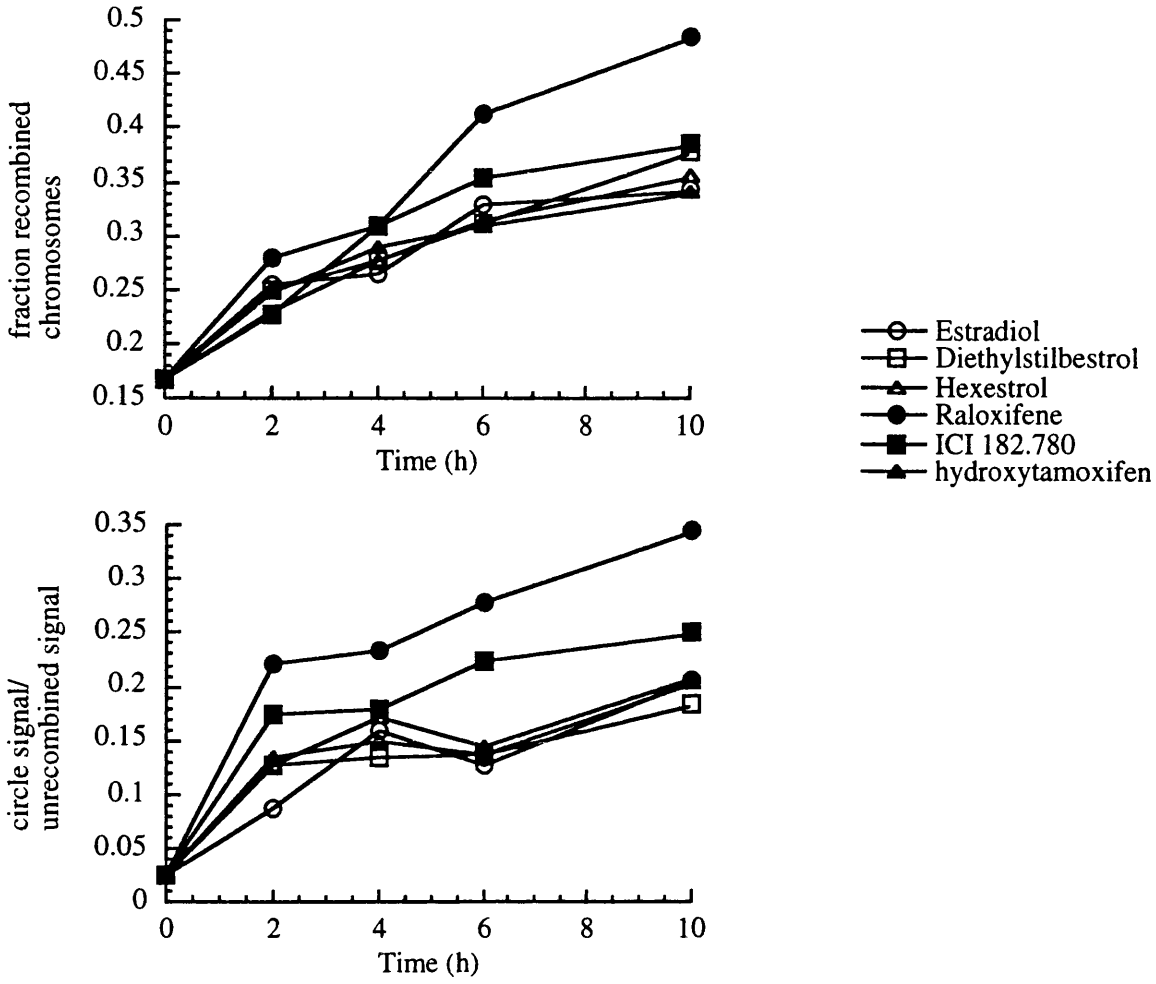



Figure 4.4 Time course of recombination in 293R10 cells expressing the FLP-EBD$^{wt}$ fusion protein grown for 5 passages in 2% fœtal calf serum and neomycin. The upper panel shows a phosphoimager quantification obtained by probing a Southern blot with probe 1 shown on figure 3.1 that labels the chromosomal recombination substrate and product. On the lower panel, the same blot was probed with probe 2 shown on figure 3.1 that labels the excised circle and the unrecombinaed chromosome. Note that in the upper panel, 16% of the chromosomes are in the recombined state while in the lower panel, only ~3% of the cells contain excised circles in the absence of added ligand.

same ranking order in affinities as determined by others (Wakeling *et al.*, 1991). Thirdly, it shows that ICI 164.384 and ICI 182.780 both caused an identical increase in recombinase activity relative to that induced by estradiol. This is not an unexpected result, since both these estrogen derivatives bear 7α side chains that render them anti-estrogenic (Wakeling *et al.*, 1991), (Wakeling, 1992).

The EBD used in the experiments described above (figure 4.3A, B and C) bears a point mutation, G400V, which modifies its ligand binding properties (Tora *et al.*, 1989a) and the response to the anti-estrogen hydroxytamoxifen (Jiang *et al.*, 1992), (Ramkumar and Adler, 1995). Hence, I tested whether the ligand specific effects observed with the G400V EBD could be reproduced with the wild type estrogen receptor LBD. This analysis was complicated by the fact that the wild type EBD-FLP recombinase was not tightly regulated in our cell culture system (cf. chapter 3). Nevertheless, induced recombination levels could be measured above background recombination levels. The analysis was carried out using 100nM estradiol, hexestrol, raloxifene, ICI182.780 and hydroxytamoxifen in the context of a time course. Figure 4.4 shows the quantification by Southern blot and phosphoimage analysis of the appearance of chromosomes from which the circle was excised (upper panel) and of the appearance of excised circle versus total chromosome signal (lower panel) during a time course of ligand induced recombination. It is evident from both panels that raloxifene and ICI 182.780 treated cells underwent more recombination than cells treated with estradiol, hexestrol or hydroxytamoxifen. It was concluded that the ligand specific levels of recombination observed when the FLP-EBD$^{G400V}$ recombinase fusion was exposed to various ligands could also be seen with the FLP-EBD$^{wt}$ fusion with one exception. The exception consisted of the slight increase in recombination with hydroxytamoxifen after 2 and 4 hours, which was EBD$^{G400V}$ specific.

Figure 4.5 shows a western blot analysis that was carried out to determine the effect of the ligands used above, on the steady state levels of FLP-EBD$^{wt}$ protein in the same cell line as that used to generate the data shown on figure 4.4. There is no more than a two fold variation

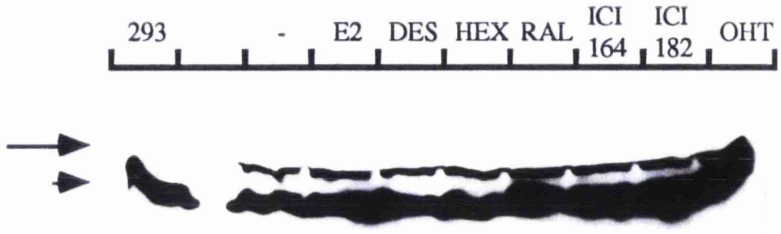



Figure 4.5 Western blot analysis on lysates from 293R10 cells expressing FLP-EBD$^{wt}$ protein. The cells were exposed to 100nM of the indicated ligands for 50 hours. Note the that none of the ligands affected the levels of fusion protein present in the extracts. E2; estradiol, DES; diethystilbestrol, HEX; hexestrol, RAL; raloxifene, ICI 164; ICI164384, ICI182; ICI 182780, OHT; hydrotamoxyfen.

in the levels of FLP-EBD$^{wt}$ in cells treated for 50 hours with 100nM of all the ligands used above.

**Mutations that impede EBD homodimerisation abolish the ICI 182.780 but not the raloxifene specific increase in recombination events**

It has been reported that ICI 164.384 and ICI 182.780 are anti-estrogens because they inhibit estrogen receptor homodimerisation through the ligand binding domain. This was suggested by experiments whereby homodimerisation of ICI 164.384 loaded estrogen receptors or estrogen receptors bearing mutations in the LBD which inhibit dimerisation could only be imposed by co-incubation with a particular antibody preparation (Fawell *et al.*, 1990b). I introduced point mutations (R503A+L507R or L507R) into the human estrogen ligand binding domain. These mutations have been reported to greatly compromise mouse estrogen receptor homodimerisation (White *et al.*, 1991), (Fawell *et al.*, 1990a), (Lees *et al.*, 1990). A site specific recombination reporter cell line (293R10, see table 3.2) was transformed with a vector driving the expression of either FLP-EBD$^{L507R}$, FLP-EBD$^{R503A+L507R}$ or FLP-EBD$^{G400V}$ protein. Chosen clones expressing the recombinase fusions were subjected to dose response experiments with the 6 estrogen receptor ligands (figure 4.2). The results shown on figure 4.6 indicate that the two EBDs impaired for homodimerisation did not sustain the increase in recombination events caused by ICI 182.780 on the wild type- and G400V FLP-EBD fusions (figures 4.3 and 4.4). As is shown on figure 4.7, this effect could have been due either to an increase in the activity of the estrogen loaded recombinase fusion impaired for LBD mediated homodimerisation or to a decrease in the activity of the mutant EBD-FLP fusion when it is bound by ICI 182.780. Formally it is not possible to discriminate between these two possibilities since two independent clones are compared. It is notable however, that both mutants still display a difference between the anti-estrogen raloxifene and the three agonists; estradiol, hexestrol

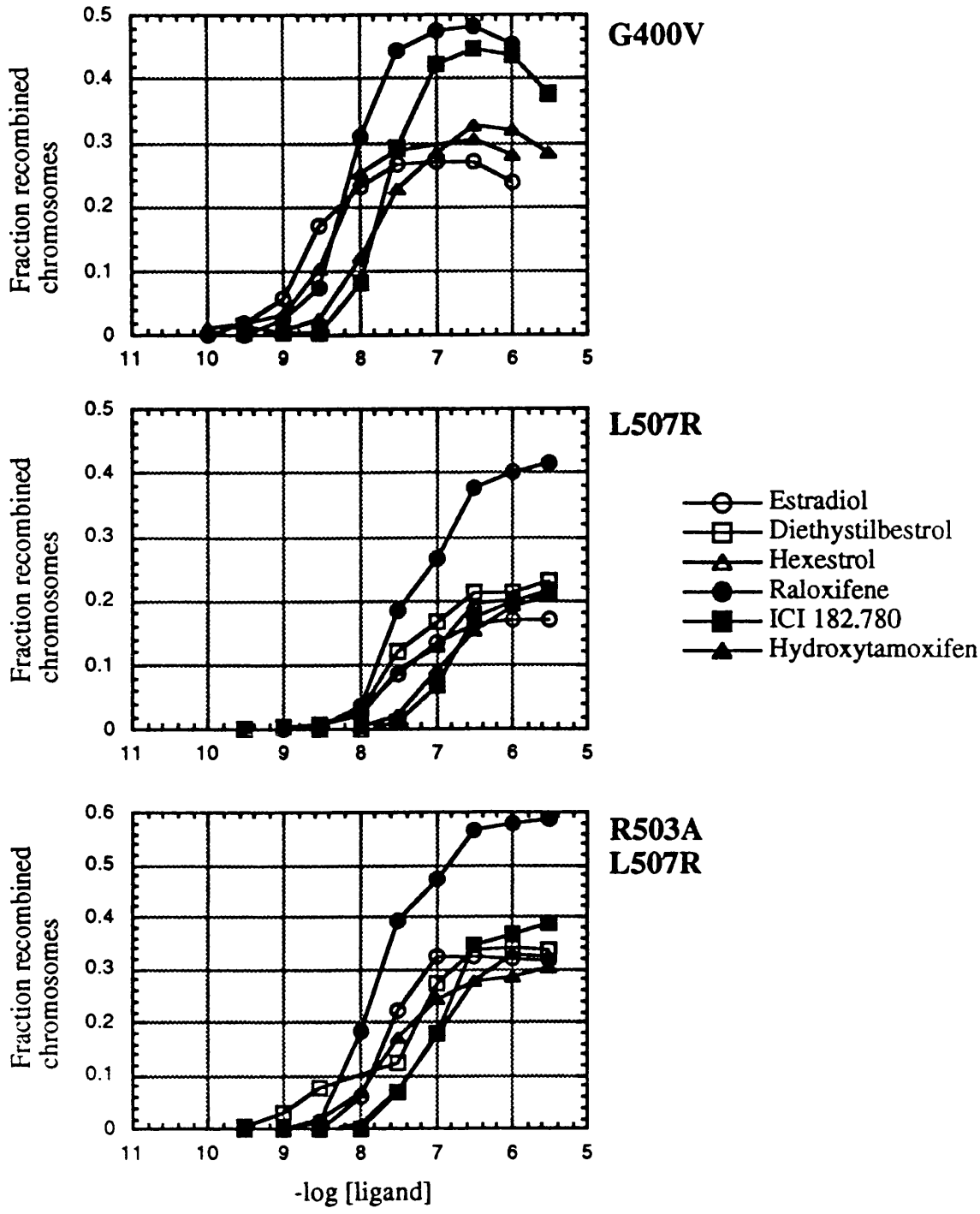



Figure 4.6 Phosphoimager quantification of the recombination signal observed after ligand titrations were carried out on recombination reporter cell lines expressing FLP-EBD fusion proteins G400V (upper panel), L507R (middle panel) or R503A/L507R (lower panel). Ligand exposure lasted 4 hours, after which the cells were lysed and genomic DNA was isolated and subjected to a Southern analysis. Note the reduction in recombination induced by ICI 182.780 compared with estradiol, diethylstilbestrol and hexestrol in the bottom 2 panels.

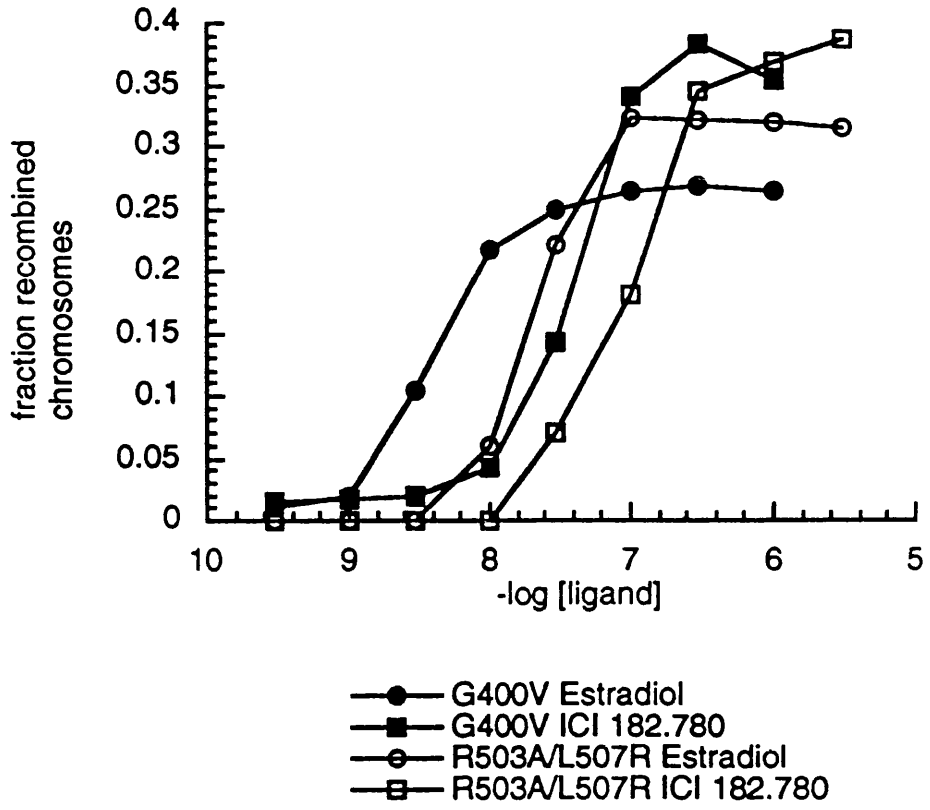



Figure 4.7 Comparison of estrogen and ICI 182.780 induced recombination in 293R10 derived cell lines expressing the FLP-EBD$^{G400V}$ or FLP-EBD$^{R503A/L507R}$ fusion proteins, in a ligand titration experiment. Note the similar shift in affinities for both ligands in the FLP-EBD$^{R503A/L507R}$ cell line. Note also the higher level of recombination achieved by the FLP-EBD$^{R503A/L507R}$ expressing cells compared to the FLP-EBD$^{G400V}$ cells upon exposure to estradiol and the similarity in levels of recombination achieved by both cell lines upon treatment with ICI 182.780.

and diethylstilbestrol, indicating that the increase in recombinase activity seen with raloxifene is not due to a loss of the dimerisation properties of the EBD of the type imposed by the mutation used. Alternatively the mutations introduced here may only partially abolish dimerisation and ICI compounds also only partially impede dimerisation. Then the effects of the ICI anti-estrogens could be obscured by the mutations and the raloxifene effect would still be observable.

**Mutations reported to eliminate the ligand binding domain transcription activation function (AF-2) impede regulation of, and ligand mediated activation by, the FLP-EBD recombinase fusions**

The ligand binding domain of the estrogen receptor encodes a ligand dependent transcription activation function. One protein motif essential for this function is highly conserved among the nuclear receptor superfamily (Danielian *et al.*, 1992), (Barettino *et al.*, 1994) and spans amino acids 535-549 of the human estrogen receptor. This region adopts an amphipathic α-helix conformation (Bourguet *et al.*, 1995). It is probably involved in protein-protein interactions that mediate the transcription activation function of the estrogen receptor LBD (Halachmi *et al.*, 1994), (Cavailles *et al.*, 1994).

In a further effort to correlate the differential effect of anti-estrogens on FLP-EBD mediated site specific recombination with the loss of protein-protein interactions involving the human estrogen receptor LBD, I introduced 2 mutations in the EBD that have been reported to abolish the transcription activation function of the mouse EBD without affecting either the homodimerisation potential or the hormone binding capacity of the EBD (Danielian *et al.*, 1992). In a first mutant, two adjacent leucines, at positions 539 and 540 of the human estrogen receptor, were substituted for alanines. A stop codon was introduced at amino acid 534 to generate a second transcription activation mutant. These two FLP-EBD$^{TXN-}$ expression vectors were integrated into the genome of the 293R10 site specific recombination

reporter cell line and in the course of two experiments 17 clones were isolated for each construct. The result of theses experiments are summarised on table 4.1.

| expt | mutation | inducible clones | uninducible clones |
|---|---|---|---|
| 1 | 534stop | 9 | 2 |
| 1 | LL539-40AA | 9 | 2 |
| 2 | 534stop | 5 | 1 |
| 2 | LL539-40AA | 6 | 0 |

Table 4.1 Hygromycin resistant 293R10 clones transformed with pHFE$^{534stop}$ or pHFE$^{LL539\text{-}40AA}$ were picked and grown for 5 days in 24 well cell culture dishes, in the presence of estradiol or of ethanol vehicle. Induction was measured by counting blue cells after X-gal staining of the cultures. Induction never exceeded a 10 fold increase in blue cell numbers. Clones grown in stripped serum containing medium often contained 2 to 10% blue cells.

The major conclusion of the experiments described in table 4.1 was that it seemed impossible to generate clones that efficiently underwent the FLP-EBD mediated chromosomal deletion. At most, a 10 fold induction was seen in any one pHFE$^{LL539\text{-}40AA}$ clone after five days of hormone induction. To confirm and extend this puzzling result, 5 pHFE$^{534stop}$ and 7 pHFE$^{LL539\text{-}40AA}$ clones were grown for 5 days in the presence of 1μM estradiol, hexesterol, raloxifene, ICI 182.780 or tamoxifen before being stained with X-gal. As above, the maximal induction observed did not exceed a ten fold increase in the number of blue cells, and as before, most of the cells remained white, indicative of the inability of these clones to efficiently undergo ligand induced site specific recombination. One pHFE$^{534stop}$ clone and one pHFE$^{LL539\text{-}40AA}$ clone were chosen, both of which appeared to be responsive, albeit weakly, to hormones, and were grown for 30 days (8 passages) in the presence of hygromycin and in the presence or absence of neomycin. Since neomycin selects against cells

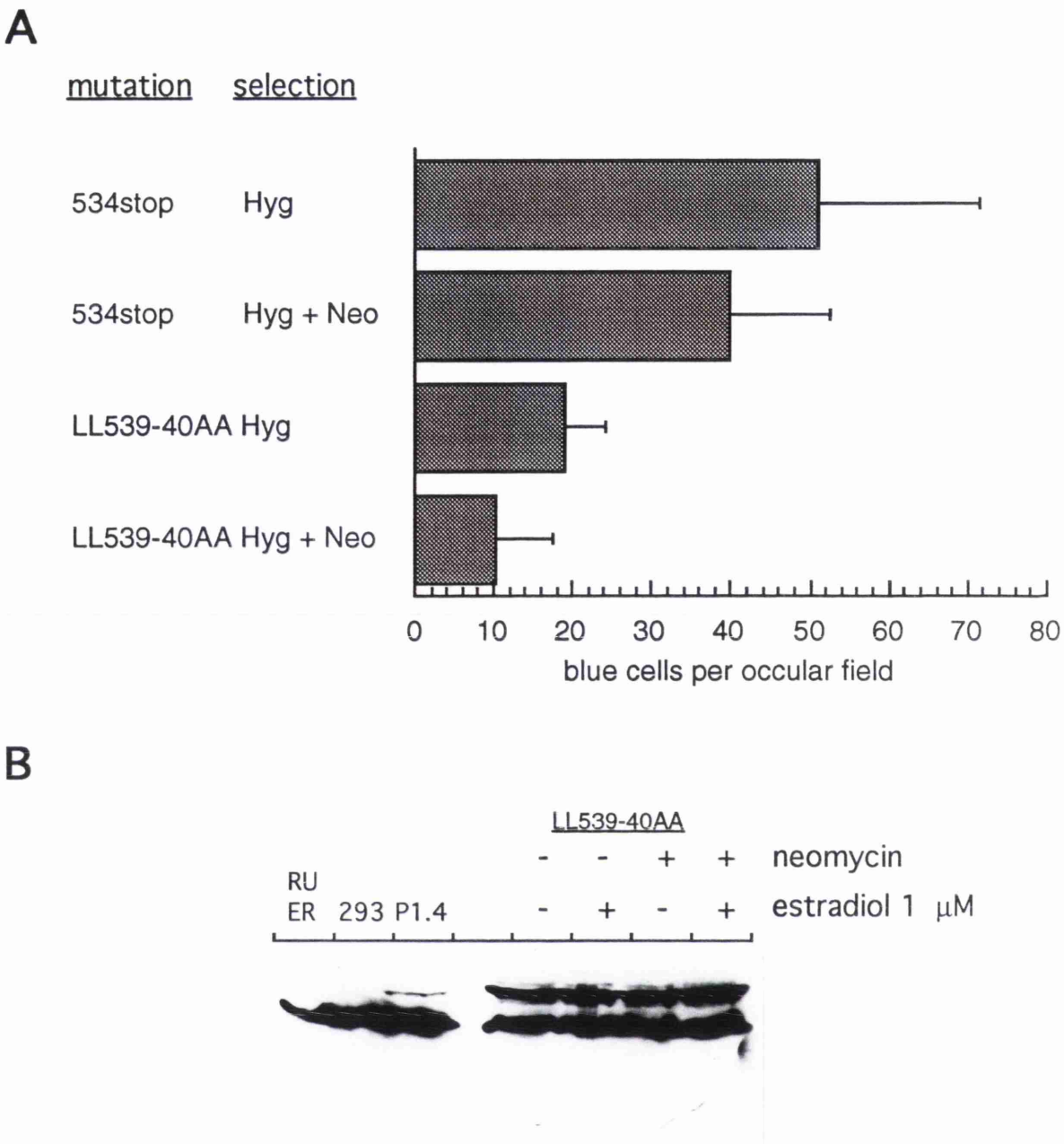



Figure 4.8 **A.** The number of blue cells in 4 randomly chosen occular fields of wells containing 293R10 expressing FLP-EBD$^{TXN-}$ cells. The cells had been grown for 8 passages in the presence of hygromycin and in the presence or absence of neomycin **B.** Western analysis on lysates of the FLP-EBD$^{LL539\text{-}40AA}$ clone after 8 passages of growth in the presence of hygromycin and in the presence or absence of neomycin and 50 hours of estradiol or ethanol vehicle exposure using Mab-EF3 directed against the F domain of the estrogen receptor. RUER; Roussel Uclaff bacculaviral estrogen receptor.

that have undergone recombination, this experiment evaluated the amount of uninduced recombination that took place in these clones. Cells from the two clones were stained at the end of this period. The effect of neomycin selection was noticeable, since for both clones, the number of blue cells is lower after 8 passages in the presence of neomycin than after 8 passages in the absence of neomycin selection (figure 4.8, panel A). Both clones were then grown for 50 hours in the presence or absence of 1μM estradiol and cells were collected for Southern and western analysis. The result of the western analysis on the LL539AA clone is shown on panel B of figure 4.8. It demonstrates that there are more than 2000 recombinase fusion proteins per cell (cf. figure 3.20) and that the level of the FLP-EBD$^{LL539AA}$ recombinase fusion protein was not affected by the neomycin selection procedure nor by the presence of 1μM estradiol. The amount of recombinase fusion present in the 534$^{stop}$ clone could not be determined as the epitope for Mab-EF3 is deleted in this protein.

The Southern analysis is shown on figure 4.9 panels A and B. The effect of neomycin selection is clearly visible here as well (compare lanes 1, 2, 5 and 6 to lanes 3, 4, 7 and 8) indicating that a substantial amount of uninduced recombination took place in 30 days of culture. Site specific recombination is only marginally induced in the 534$^{stop}$ cell line; 51 to 57% and 16 to 23% in 50 hours of estrogen treatment. In the LL539-40AA cell line, recombination is induced; 2 to 34% and 1 to 33%. This is significantly lower than in the cases of the wild type, G400V, L507R or R503A/L507R FLP-EBD cell lines, which typically would yield 70-80% recombination in 50 hours of hormone treatment. I concluded from these experiments that the transactivation mutations compromise the capacity of estrogen receptor ligands to activate the FLP-EBD fusion protein. It is unlikely that the transcription mutations caused a reduction in the affinity of the receptor for estrogen, since two groups generated or isolated the same (Danielian *et al.*, 1992) or similar mutations (Wrenn and Katzenellenbogen, 1993), (Tzukerman *et al.*, 1994) in this region of the EBD, and determined that although the transcriptional activity of these mutants is compromised, it is not due to a reduction in their affinity for estradiol.

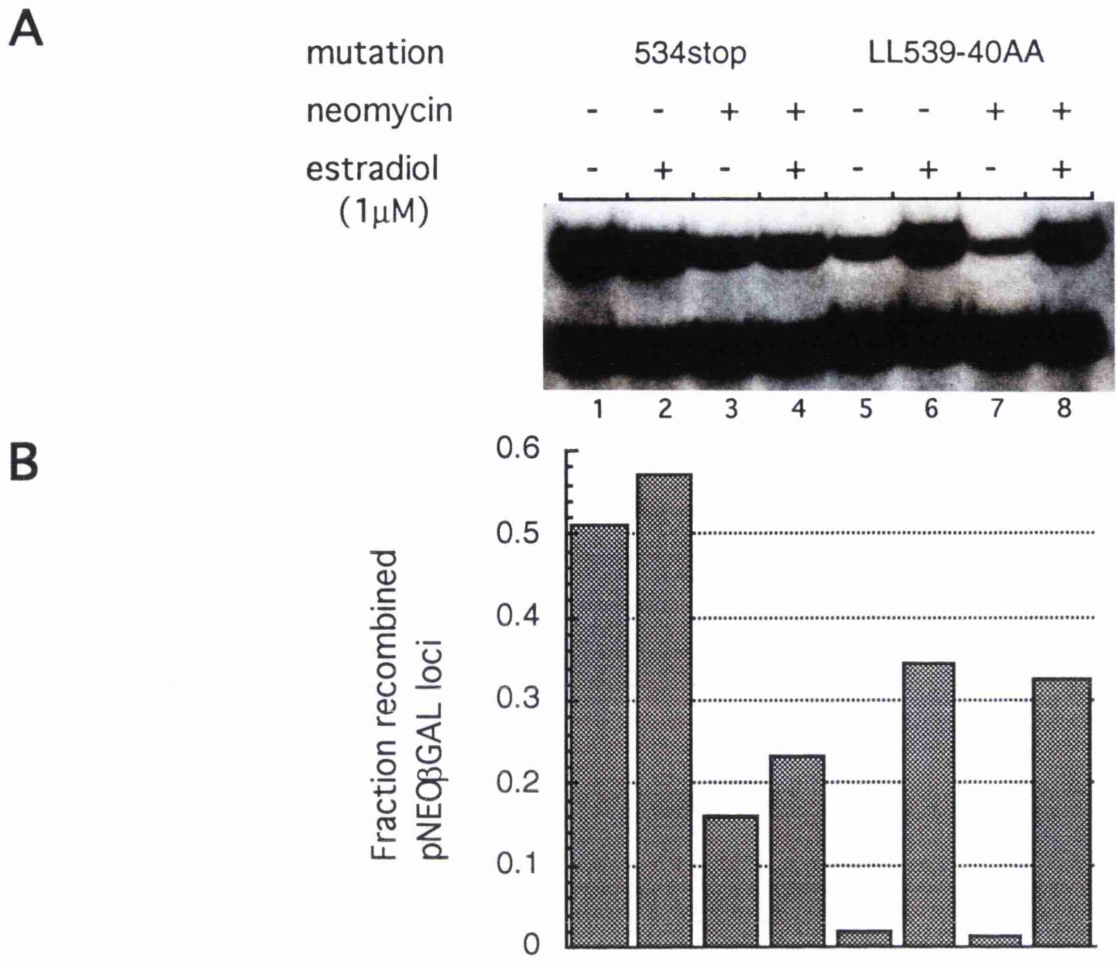



Figure 4.9

**A**. Southern analysis of 293R10 cells expressing the FLP-EBD$^{534stop}$ (samples 1-4) or FLP-EBD$^{LL\text{-}539\text{-}40}$ (samples 5-8) fusion proteins. The cells were grown for 8 passages in the presence of hygromycin and the presence (samples 3, 4, 7 and 8) or absence (samples 1, 2, 5 and 6) of neomycin and then treated for 50 hours with 1μM estradiol (samples 2, 4, 6 and 8) or ethanol vehicle (samples 1, 3, 5 and 7). Because of the restriction strategy used the product of recombination migrates above the intact pNEOβGAL restriction fragment. **B**. Phosphoimager quantification of the Southern blot shown on panel A.

The results shown on figure 4.8 and 4.9 indicate that none of the estrogens or anti-estrogens used here are able to efficiently activate the FLP-EBD$^{TXN-}$ recombinases when they are expressed from a chromosomal transgene.

In contrast, when plasmids encoding these FLP-EBD fusions were transiently transfected into a recombination reporter cell line I did observe a ligand response (figure 4.10). This is in agreement with data (Mahfoudi *et al.*, 1995) which indicated that full length estrogen receptor encoding the LL->AA mutation could be activated by estradiol, in transient transfection experiments.

Tzukerman *et al.* (1994) reported that estrogen receptors bearing mutations in the same region as those described above, exhibited significant activity in the absence of added estrogens. This result parallels the results shown on figure 4.9, panel A. This experiment highlights the poor repressor property of the EBD when this region is mutagenised. Because the Tzukerman study was based on transient transfections and analysed the transcriptional activity of the mutants, they did not however, uncover the poor ligand mediated activation capacity of these mutants seen in the present analysis, where the behaviour of these mutants was analysed under more physiological conditions in homogenous populations of cells expressing the proteins from a small number of chromosomal transgenes.

**Nuclear localisation signals appear to be responsible for the poor regulation conferred by the wild type estrogen receptor LBD onto FLP**

Ylikomi *et al* . (1992) reported that the estrogen receptor LBD encodes two constitutive and one estrogen inducible nuclear localisation proto-signals. These protein motifs were included into the estrogen receptor moiety that was initially fused onto FLP and span amino acids 250 to 303. Because it is possible that the nuclear localisation signals contribute to the anti-estrogen effects reported above, I deleted this region from the FLP-EBD fusion protein. To date the dose response experiments that would answer this question have

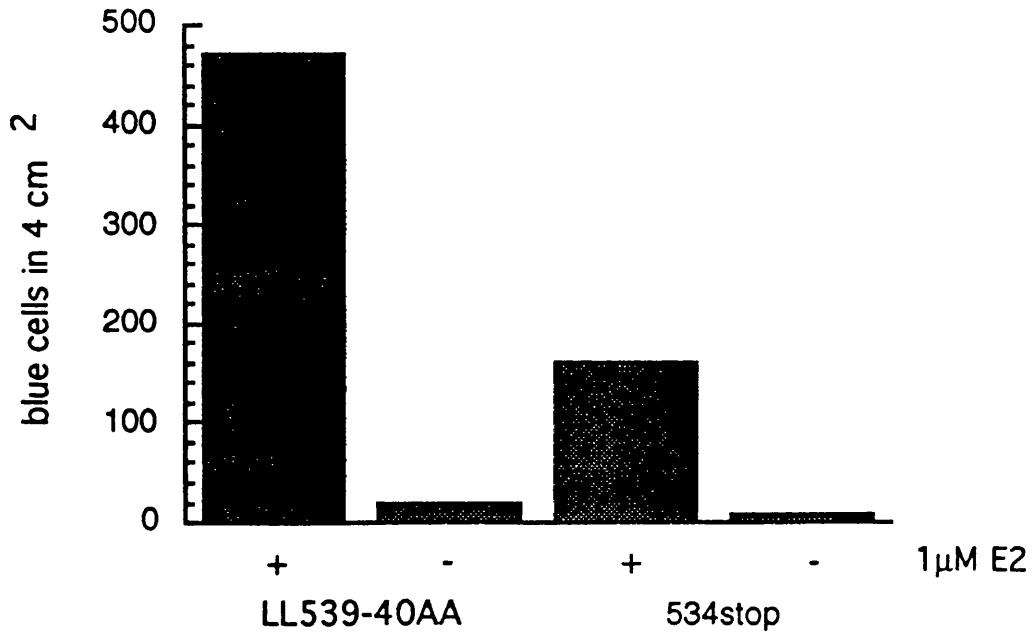



Figure 4.10 Transient transfection of the pHFE$^{LL539\text{-}40AA}$ and pHFE$^{534stop}$ plasmids into the recombination reporter cell line E25B2/B2. The cells were exposed for 50 hours to 1μM estradiol after which they were stained with X-Gal. All the blue cells in a 4cm$^2$ surface area were counted manually.

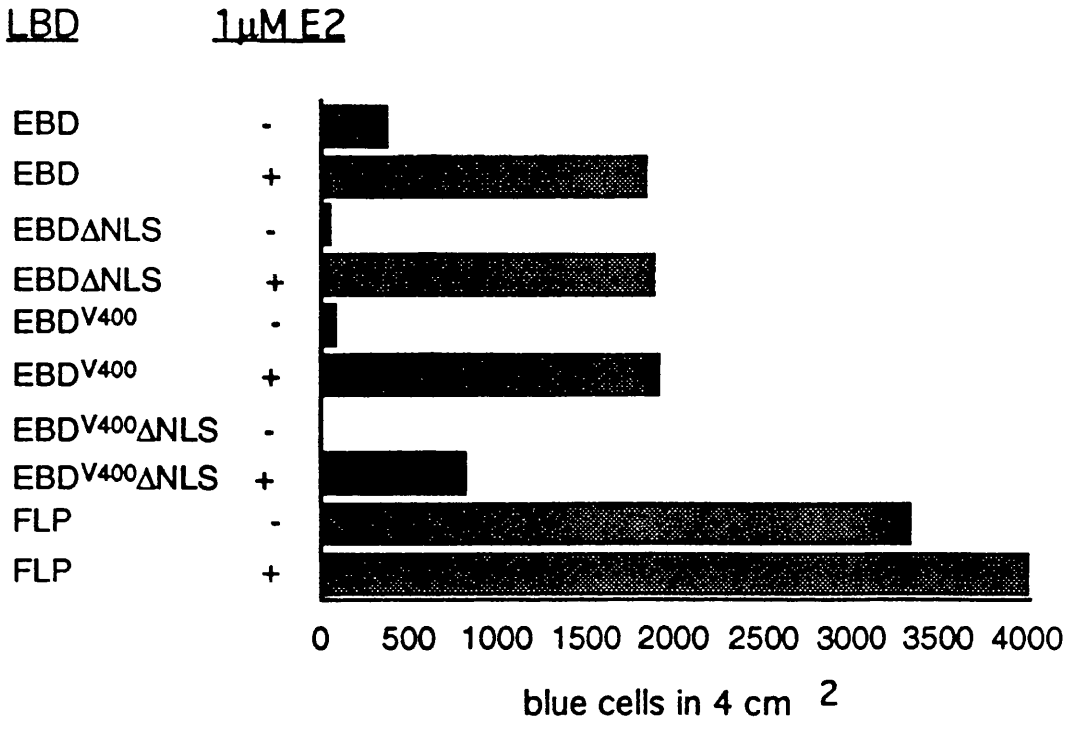



Figure 4.11 Constructs encoding the indicated FLP fusion proteins were transiently transfected into the E25B2/B2 FLP recombination reporter cell line. The cells were stained with X-Gal after 50 hours of hormone treament and the total number of blue cells on a 4 $cm^2$ surface area was counted manually.

not been carried out. The result of a transient transfections of these plasmids into the E25B2/B2 site specific recombination cell line described in chapter 3 is shown on figure 4.11. As has been mentioned earlier, the wild type EBD including amino acids 252-302 is a poorly repressed recombinase. When amino acids 252-302 are deleted from this construct (ΔNLS), the number of transfected cells that switch on the LacZ gene in the absence of added estrogens is strongly reduced. Addition of hormone, results in the excision of the pOG45 plasmid from the LacZ open reading frame at the same frequency for the $EBD^{wt}$, $EBD^{wt}\Delta NLS$ and the $EBD^{G400V}$. The FLP-$EBD^{G400V}\Delta NLS$ however seems to be half as active as the FLP-$EBD^{G400V}$ upon exposure to estradiol. These results await confirmation in stable clones, but seem to indicate that the amino acids involved in the nucleo-cytoplasmic cycling activity of the full length estrogen receptor do affect the properties of the FLP-EBD recombinases. It is still an open question as to whether this is due to a less stringent regulation by the EBD or to a decrease in the affinity of the fused EBDs lacking nuclear localisation signals for environmental estrogens which are presumed to be present in the culture medium, even after charcoal stripping of the fœtal calf serum.

**Effect of various androgen receptor ligands on FLP-ABD fusion proteins**

We generated a site specific recombination reporter cell line which constitutively expressed a FLP-human androgen receptor fusion protein (FLP-ABD). This cell line was subjected to a dose response experiment with 7 putative androgen receptor ligands, namely 5α-dihydrotestosterone, testosterone, RU38486, ICI 141.307, ICI 159.069, ICI 169.685 and mibolerone. The three ICI compounds were provided to us without knowledge of their structure or whether they are agonists or antagonists of the androgen receptor. Testosterone, 5α-dihydrotestosterone and mibolerone are agonists of the androgen receptor. Figure 4.12B shows a phosphoimager quantification of the Southern analysis carried out on samples from the dose response experiment. The apparent affinities for these compounds were; less than

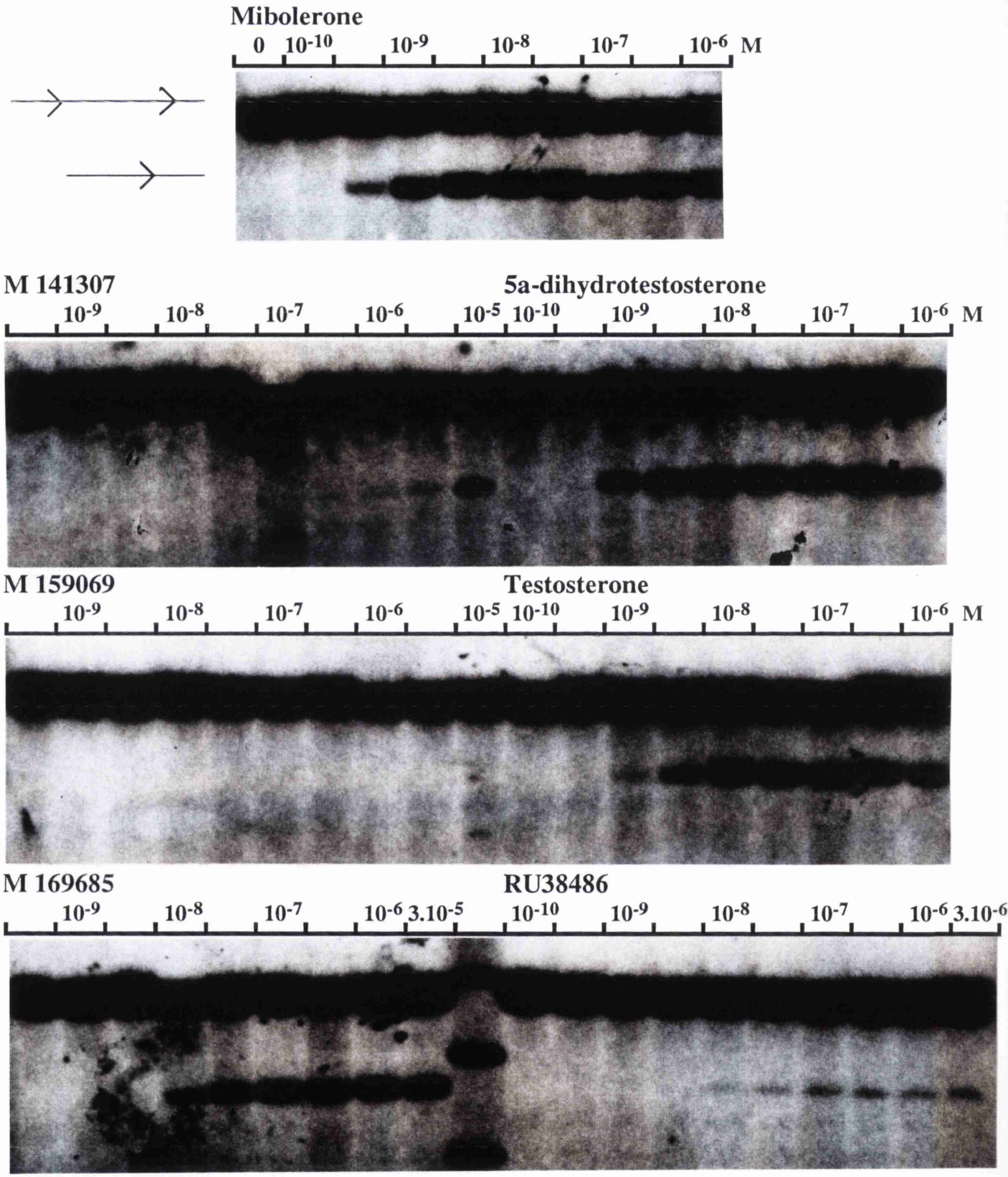



Figure 4.12A Southern blots used for the phosphorimager quantifications shown on figure 3.12B. Probe 1 (figure 3.1) was used. The indicated androgen receptor ligands were added at the indicated molar concentrations for 4 hours before genomic DNA was collected.

1nM for mibolerone, 4nM for 5α-dihydrotestosterone, 8nM for testosterone and 50nM for ICI 169.685. RU38486 caused a half maximal response at 10nM, but this induction did not lead to recombination in more than 2% of the cell population (figure 4.12 A). ICI 141.307 has a low apparent affinity for the FLP-ABD fusion protein, as a recombination signal is only visible at 3μM. ICI 159.069 did not induce any recombination at all. The levels of recombination induced by the three known androgen receptor agonists differ, indicating that recombination levels achieved by the FLP-ABD fusion protein may be influenced by ligand specific dissociation/association constants and protein stability in a similar fashion as has been described for the full length androgen receptor (Zhou *et al.*, 1995). It is also possible that the FLP moiety of the fusion protein is affected differentially by 5α testosterone, testosterone and mibolerone, resulting in the range of recombination frequencies observed.

**Effect of three glucocorticoid receptor ligands on FLP-GBD fusion proteins**

We carried out similar experiments to those described above for the estrogen and androgen receptors with a FLP-human glucocorticoid receptor fusion (FLP-GBD). The results obtained here are shown on figure 4.13. Two glucocorticoid responsive 293 recombination reporter cell lines were isolated. Recombination did not occur at the same frequency in these 2 cell lines. Time course experiments with dexamethasone, a well known synthetic glucocorticoid and with RU38486, an antagonist of the glucocorticoid receptor transcription activation function (Reik *et al.*, 1991), (Baulieu, 1989), showed a similar profile in both cell lines (figure 4.13 middle and lower panel). At saturating concentrations, dexamethasone induced recombination events almost twice as frequently as RU38486. The apparent affinities of the FLP-GBD protein was 3nM and 100nM for RU38486 and dexamethasone respectively (figure 3.13 A). A third compound tested, RU43044, did not induce recombination at all (figure 4.14 C) This is interesting because this compound is a known anti-glucocorticoid,

A

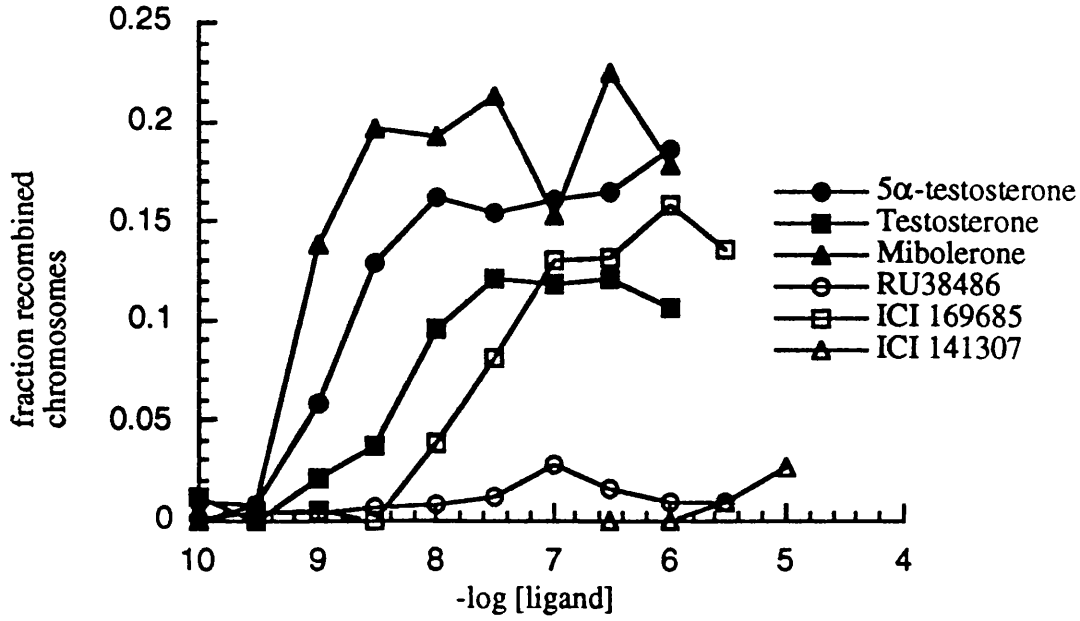



Figure 4.12B Androgen receptor ligand titrations in the 22F recombination reporter cell line. Cells were exposed to the indicated ligands for 4 hours after which genomic DNA was extracted and used for Southern analysis. The proportion of cells that had undergone recombination was determined by phosphoimager quantification of the Southern blots shown on figure 4.12A.

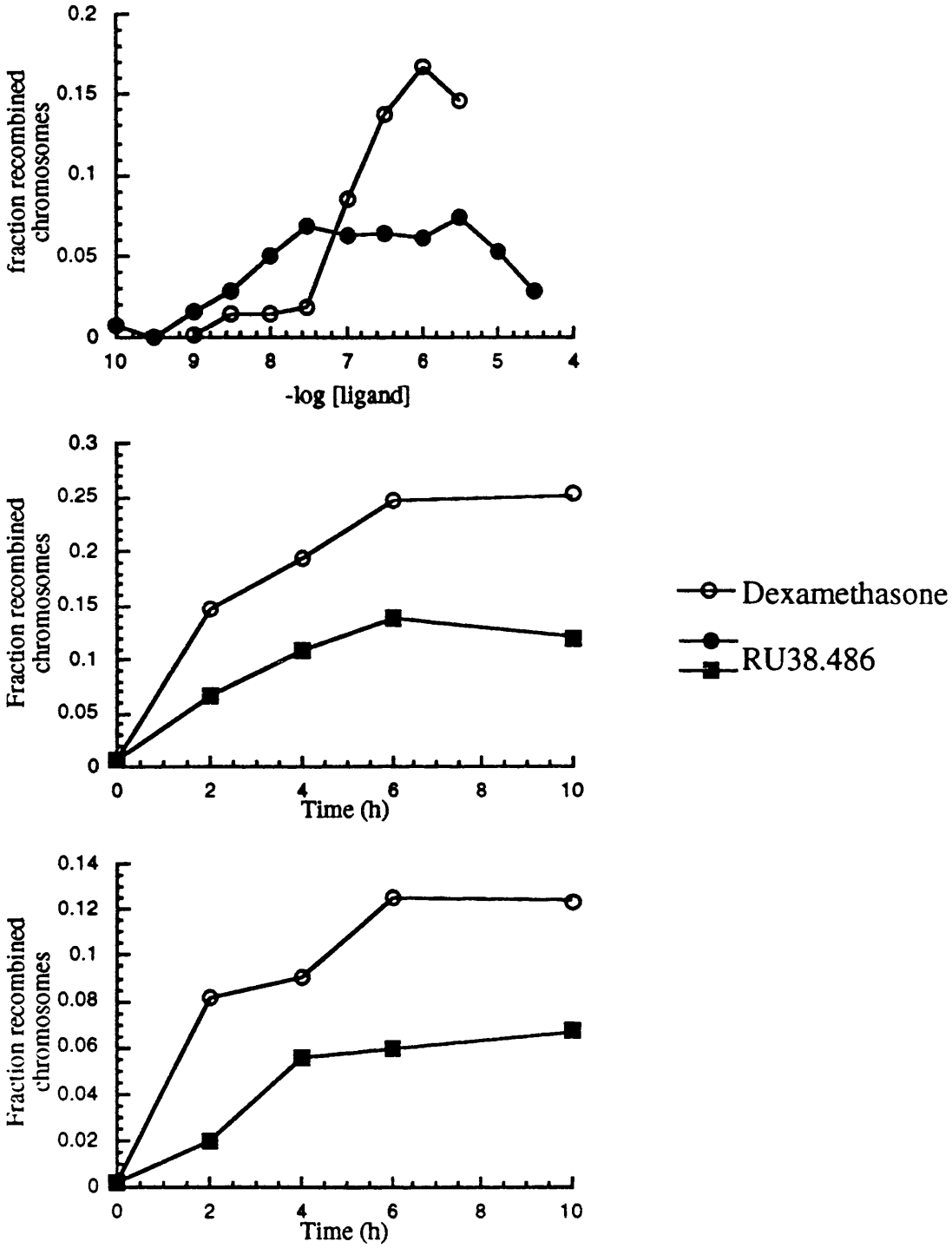



Figure 4.13 Two 293P1 derived cell lines expressing FLP-GBD protein were subjected to a time course of recombination induction with dexamethasone or RU 38.486 (lower 2 panels). The top panel shows the result of a ligand titration in the same cell line as the one used in the middle panel. The Southern blot used to generate the upper panel are shown on figure 4.14.

which is thought to antagonise glucocorticoid function by disabling its DNA binding capacity (Gronemeyer *et al.*, 1992). Whether this compound binds to the FLP-GBD protein and whether it inhibits the DNA binding activity of FLP is an open question. These questions could be answered, at least partially by treating the FLP-GBD recombination reporter cell lines with both dexamethasone and RU43044. This experiment has not been carried out to date.

## DISCUSSION

We studied the effect of 7 different estrogen receptor ligands, 3 glucocorticoid receptor ligands and 7 putative androgen receptor ligands in cell lines expressing cognate FLP-LBD fusion protein. All the estrogen receptor ligands analysed caused recombination to occur. Of the 3 glucocorticoid receptor ligands analysed; two, dexamethasone and RU38486 caused recombination to occur, while RU43044 did not. Of the 7 putative androgen receptor ligands, one, ICI 159.069, did not induce recombination while the six other did.

The activation of the FLP-EBD fusion protein by ICI 164.384, a so called pure estrogen antagonist, implies that functions encoded by the steroid receptor LBDs apart from HSP90 binding and release therefrom upon ligand binding, are disposable in order to report the presence of ligands. The inability of RU43044 to activate the FLP-GBD recombinase might indicate that some steroid antagonists are also able to antagonise FLP-LBD mediated recombination. However, antagonism by RU43044 has not yet been documented in the case of the dexamethasone activated FLP-GBD, leaving this issue open.

Southern analysis of the recombination products obtained in dose response experiments yielded apparent binding affinities which correlated well with the available ligand binding affinities of the synthetic ligands for the receptors. These experiments also indicated whether or not saturating levels of the ligands could be reached with any one compound, establishing


Figure 4.14 The 293P1GH1 cell line expressing the FLP-GBD recombinase fusion protein was exposed increasing concentrations of the indicated ligands for 4 hours upon which genomic DNA was collected. The filter was probed with probe 1 (figure 3.1). The upper and lower bands represent the unrecombined and recombined pNEOβGAL locus respectively.

that the different recombination frequencies induced by the different ligands were not due to the use of sub-saturating concentrations of ligands.

The magnitude of the recombination response, as determined by Southern analysis, was ligand specific. In the case of the estrogen receptor LBD fusions, only estrogen antagonists (ICI 164.384, ICI 182.780 and raloxifene) caused an increase in the recombination response, while agonists (hexestrol and diethylstilbestrol) and one partial antagonist (hydroxytamoxifen) induced as much recombination as 17β-estradiol. In contrast to this, saturating amounts of RU38486, a known antagonist of the glucocorticoid receptor function (Groyer *et al.*, 1987) induced lower levels of recombination than saturating amounts of dexamethasone, a well known synthetic glucocorticoid agonist (Gronemeyer *et al.*, 1992). In the case of the androgen receptor ligands, a range of responses was seen, with each ligand resulting in a specific level of recombination.

The phenomena underlying these differences can be manifold. They may be due to pharmacokinetic parameters such as alterations in the half life of the ligand bound fusion proteins or the off rate of the ligand which may cause the LBD in question to re-associate with the HSP90 repressor complex, resulting in a lower apparent activity of the recombinase fusion. Another explanation for the differences in activity would be that the ligand binding domains adopt different conformations when bound to the different ligands, resulting in a change in the intrinsic recombinase activity of the recombinase fusion proteins. Ligand specific LBD conformations may for example, result in the creation or ablation of superficial structures on the ligand binding domain leading to the appearance or disappearance of protein binding sites. Indeed, since some of the compounds studied here are known steroid hormone antagonists, it is probable that they mediate their effects by disrupting protein-protein contacts important for the function of the receptors (figure 4.1)

Ligand treatment did not affect the levels of FLP-EBD$^{wt}$ protein in the cells. This is in contradiction with the reported effect of ICI 164.384 on estrogen receptor levels in the mouse uterus (Gibson *et al.*, 1991) or in cell cultures (Dauvois *et al.*, 1992), (Dauvois *et al.*, 1993).

This may indicate that the determinants for the degradation event are not included in the present recombinase fusions or that the FLP-EBD fusions adopt a conformation that allows them to escape the ICI 164.384 induced degradation pathway. In some fusion protein contexts, ICI 164.384 has been reported to abolish estrogen induction of the fused protein's activity. In all these cases, the fused protein moiety was a transcription factor that bound DNA as a dimer (Drewes *et al.*, 1994). This may indicate that ICI 164.384 also abolished dimerisation in these contexts. Indeed, when the fused transcription factor bound DNA as a monomer, ICI 164.384 was found to be an activator (Drewes *et al.*, 1994). In the case of the c-Fos-EBD fusion, OHT did turn on transformation activity (Eilers *et al.*, 1989), experiments with ICI 164.384 were not reported in this study. A v-rel-EBD fusion protein was found to translocate to the nucleus upon treatment with estradiol, hydroxytamoxifen and ICI 164.384. The transforming potential, however, was only activated by estradiol or hydroxytamoxifen. When the DNA binding activity of v-rel-EBD was tested, it was found that only estradiol or hydroxytamoxifen treated cells contained v-rel DNA binding activity while ICI 164.384 treated cells did not (Boehmelt *et al.*, 1992). Gal4-EBD fusions act as transcription factors. Transcription activation by the Gal4-EBD transcription factor is only induced by agonists of the estrogen receptor, namely estradiol and is inhibited by both hydroxytamoxifen and ICI 164.384 (Webster *et al.*, 1988). Together these observations suggest that in certain contexts, as observed for the FLP-EBD fusion protein, hydroxytamoxifen and ICI 164.384 can act as inducers of an estrogen receptor LBD controlled factor, but that neither anti-estrogens can do so if the fused factor depends on the transcription activation function of the EBD. Furthermore, for ICI 164.384 to act as an agonist, the dimerisation function of the EBD must not be required either.

In an effort to correlate the levels of recombination induced by the different estrogen receptor ligands with the disruption of known molecular events that take place during the activation of the estrogen receptor, FLP-EBDs encoding mutations previously reported to abolish ligand induced homodimerisation (White *et al.*, 1991), (Fawell *et al.*, 1990a), (Lees

*et al.*, 1990) or ligand dependent transcriptional activation by the EBD (Danielian *et al.*, 1992) were constructed. I introduced these mutated FLP-EBD expression constructs into the genome of site specific recombination reporter cell lines. Those new cell lines were then subjected to dose response experiments. It was found that mutations reported to abolish estrogen receptor homodimerisation did not abolish the raloxifene induced increase in FLP-EBD recombinase activity relative to the estradiol induced recombinase activity. However, these mutations did abolish the difference in recombinase activities observed in the context of the wild type EBD between ICI 182.780 and the estrogen receptor agonists estradiol, diethylstilbestrol and hexestrol. This provides correlative evidence that ICI 182.780 does indeed affect estrogen receptor dimerisation as has been reported by others (Dauvois *et al.*, 1992), (Fawell *et al.*, 1990b).

In parallel experiments, it was found that mutation of residues reported to be important for the transcription activation function of the mouse EBD, greatly diminished the recombination response to all the ligands tested. This was not due to a change in protein stability since the steady state level of the FLP-EBD$^{LL->AA}$ were at least the same and probably increased over other FLPEBD protein expression levels (compare figures 4.5 and 4.8B). Moreover, estrogen treatment of these cells did not result in any significant alterations in FLP-EBD$^{LL->AA}$ levels, eliminating ligand induced protein instability as an explanation for the low induction levels observed with these recombinase fusion proteins.

One explicit aim of the above study was to generate heterologous EBDs which would no longer encode the EBD functions that are theoretically disposable in the context of the FLP-EBD fusion protein, namely dimerisation and transcription activation. The rationale being that by abolishing these functions, we could circumvent endogenous regulation that impinges on the EBD. As can be seen from figures 4.6 and 4.8B, introduced point mutations that affect dimerisation result in FLP-EBD proteins whose affinities for all ligands tested are ten fold lower than those of the EBD$^{G400V}$ domain (maximal induction reached at 300nM versus 30nM), and mutations that abolish the transcription activation function of the EBD produce

LBDs that do not fully repress the fused FLP activity and appear unable to efficiently activate the recombinase activity. Because the transcription activation mutants, $534^{stop}$ and LL539-540AA, were leaky, and since this may be due to their high affinity for ligands as is presumed in the case for the FLP-$EBD^{wt}$ (Tora *et al.*, 1989a) (cf. figure 3.3C) I combined these mutations with the G400V mutation. These plasmids produced functional, estradiol responsive recombinase fusions in transient transfection experiments (data not shown). In contrast, when these constructs were stably introduced in a recombination reporter cell line all the resulting clonal cell lines were totally unresponsive to estrogen (data not shown). No dose response experiments were carried out in the transient transfection assay, it is therefore not known whether this combination of mutations at positions 400 (G->V) and $534^{stop}$ or LL539-40AA had lowered the affinity of the EBDs for estradiol. Since a response could be seen using the wild type residue at position 400 in a transient assay with an estradiol dose that did not induce recombination in a stable context I conclude that in a transient transfection context, the bad induction property of the transcription activation mutants is not easily uncovered.

I therefore speculate that the protein domain delineated by Danielian *et al* (Danielian *et al.*, 1992) as being essential for the transcription activation function of the EBD is not only required for the transcription activation function of the EBD but also plays a role either in the maintenance of an activated state after hormone binding or in the actual release of the estrogen receptor from a repressor complex. At the molecular level, these mutations may simply affect the off rate of the ligands. This could result in a reduction in the concentration of activated fusion proteins upon ligand administration, hence leading to the poor recombination levels observed (figure 4.8B). An intracellular localisation study has been carried out on the LL->AA mutant estrogen receptor, demonstrating that it is localised to the nucleus after estrogen binding (Mahfoudi *et al.*, 1995). Thus, aberrant intracellular localisation of the FLP-$EBDLL^{539\text{-}40AA}$ is not a likely cause for the low recombination levels observed.

To gain further insight into the effect of anti-hormones on the FLP-LBD fusion system, I subjected cell lines bearing the FLP recombination reporter substrate, pNEOβGAL and expressing either a FLP-ABD or FLP-GBD fusion protein to synthetic androgen and glucocorticoid ligands. The results of these experiments were unexpected in the light of the estrogen receptor study, where saturating concentrations of agonists always induced similar levels of recombination and saturating concentrations of antagonists either induced the same or higher levels of recombination. In the case of the FLP-GBD fusion protein it was found that the synthetic agonist (dexamethasone) induced higher levels of recombination than the antagonist tested (RU38486). In the case of the FLP-ABD, a whole range of recombination levels occurred upon treatment with different agonists.

These two studies, using the FLP-GBD and the FLP-ABD fusion proteins indicated that ligand induced recombination frequencies are likely to reflect the secondary, tertiary and quaternary structures adopted by the LBDs upon ligand binding. The consequence of these structurally distinct ligand induced conformations may be to alter the capacity of the fusion proteins to interact productively during the recombination reaction. Alternatively, the different ligand bound FLP-LBDs may be recruited to varying extents into cellular protein complexes, leading to variations in the concentration of active FLP-LBD fusion proteins.

# Chapter 5 : RESULTS AND DISCUSSION

## Mutations that alter the ligand specificity of the estrogen receptor ligand binding domain

SUMMARY

The ligand binding properties of FLP-EBD fusions containing amino acid substitutions of the glycines at positions 400 and 521 of the human estrogen receptor were analysed. Two estrogen binding domains that no longer responded to estradiol but still responded to synthetic estrogen receptor ligands were generated. Different combinations of glycines, valines and arginines at positions 400 and 521 were shown to display a spectrum of ligand specificities, from sole estrogen binding loss to loss of binding by all ligands. One combination caused substantial de-repression of the fused FLP recombinase activity.

INTRODUCTION

Glycine 400 of the EBD is probably located in the single β-sheet of the otherwise α-helical ligand binding domain (Bourguet *et al.*, 1995). This β-sheet is thought to be part of the ligand binding pocket of the steroid receptors (Bourguet *et al.*, 1995). The original cDNA clone for estrogen receptor encoded a valine at this position (Green *et al.*, 1986). This was later shown to be a cloning artefact (Tora *et al.*, 1989a). The wild type and G400V receptors have a similar affinity for estradiol at 4°C but the G400V was reported to be temperature sensitive for ligand binding (Tora *et al.*, 1989a). Upon fusion of these two estrogen receptor ligand binding domains (amino acids 252-595) to FLP, I observed that the wild type EBD was not capable of fully repressing FLP activity while the G400V was. The difference in ligand 'independent' activity between the two EBDs has been reported (Tora *et al.*, 1989a)

and has been attributed to the greater affinity of the wild type receptor for residual estrogens thought to be present in the growth media of mammalian cell cultures (Berthois *et al.*, 1986), (Hammond *et al.*, 1979), (White *et al.*, 1994). The major source of steroids in mammalian cell culture media is fœtal calf serum. For all the experiments described in this work, charcoal stripped serum was used (see chapter 2), this procedure removes steroids and other lipophilic molecules from the serum. This stripping procedure may be incomplete leaving residual amounts of estradiol in the culture medium. Charcoal stripping removes or inactivates compounds which are necessary for the proper growth or differentiation of many cell types in culture. Therefore, the present FLP-EBD fusions were inadequate as ligand controlled genetic tools in many cell types. In whole animals, this problem would be even more acute since endogenously produced estrogens would activate the FLP-EBD fusion protein at undesired times.

Danielian *et al* (Danielian *et al.*, 1993) identified residues in the EBD that confer differential sensitivity to estrogen and hydroxytamoxifen. These residues are located in a stretch of amino acids extending from amino acid 514 to amino acid 531 previously identified as being important for ligand binding (Fawell *et al.*, 1990a). One mutation (G521R) attracted attention because it abolished estradiol induced transcription by the full length receptor. This mutation was combined with a glycine or valine at position 400. Furthermore, glycine 521 was also replaced by a valine, with the hope that this mutation would also change the ligand specificity of the EBD. Two combinations, 400G/521R and 400V/521V resulted in a complete loss of the estradiol induced recombination response. The 400G/521R EBD mutant only responded to antagonists and did not respond to any estrogen receptor agonists. In contrast, the 400V/521V EBD still responds to the agonists hexestrol and diethylstilbestrol but not to estradiol.

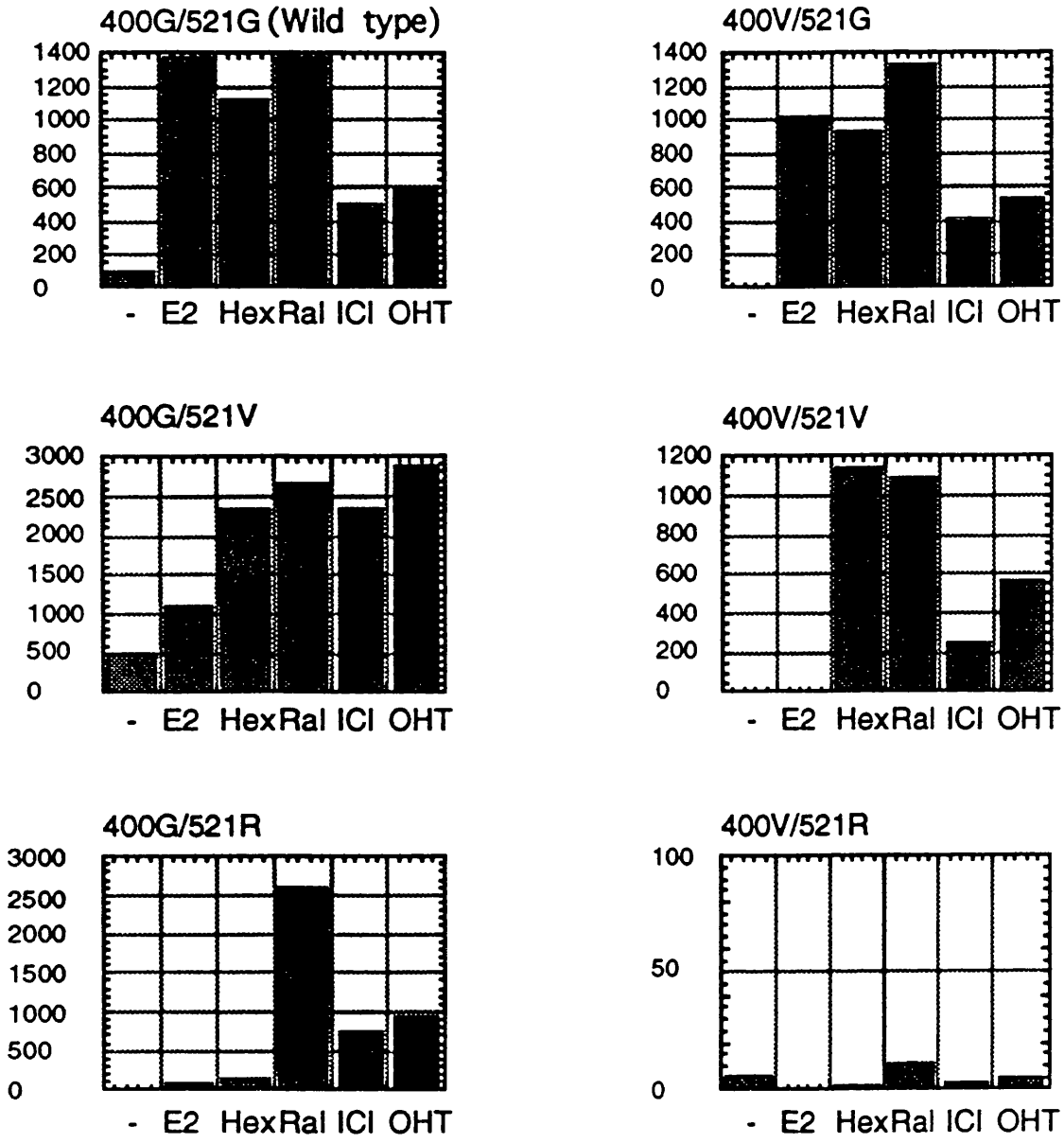



Figure 5.1 Plasmids encoding FLP-EBD fusions containing mutations at positions 400 and 521 of the human estrogen binding domain were transiently transfected into the E25B2/B2 cell line. The cells were then exposed to 1μM of ligand or ethanol vehicle for 50 hours. The cells were then stained with X-Gal and the number of blue staining cells was counted manually on a surface of 4cm2.
Ligands: -; ethanol vehicle, E2; estradiol, Hex; hexestrol, Ral; raloxifene, ICI; ICI 182.780, OHT; hydroxytamoxifen

RESULTS

Point mutations were introduced at positions 400 and 521 of the human EBD as described in chapter 2. A glycine or a valine at position 400 were combined with a glycine, a valine or an arginine at position 521. The new FLP-EBD encoding plasmids were used in transient expression experiments in the CV-1 derived recombination reporter cell line E25B/B2 (cf. chapter 3). Figure 5.1 shows the result of one such experiment. The 6 plasmids; 400G/521G (wild type), 400G/521V, 400G/521R, 400V/521G, 400V/521V and 400V/521R, were transfected in parallel into the E25B/B2 recombination reporter cell line and exposed to 1μM of either estradiol, hexestrol, raloxifene, ICI 182.780 or hydroxytamoxifen for 50 hours after which the cells were stained with X-gal and the number of blue cells on a $4cm^2$ coverslip were counted manually.

Combination of a glycine at position 400 with either a glycine or a valine at position 521 resulted in substantial basal recombinase activity. The estradiol induced levels of recombination by the 400G/521V FLP-EBD were lower than in those mediated by the 400G/521G FLP-EBD, possibly indicating that estradiol binding was reduced by the substitution of a valine for the glycine at position 521. Substitution of the glycine at position 521 for an arginine, in combination with a glycine at position 400 resulted in a dramatic loss of activity upon estradiol and hexestrol treatment. Substitution of glycine 400 for a valine while retaining the glycine at position 521, resulted in activation by all 5 ligands whilst almost no recombination occurred in the absence of added ligand. The combination of a valine at both positions resulted in a recombinase fusion which was activated by all ligands but estradiol. When an arginine is present at position 521 in combination with a valine at position 400, none of the ligands induced recombination. Thus, two of the 6 combinations abolished estradiol binding. The 400G/521R combination abolished both estradiol and hexestrol responses. The 400V/521V only abolished estradiol binding.

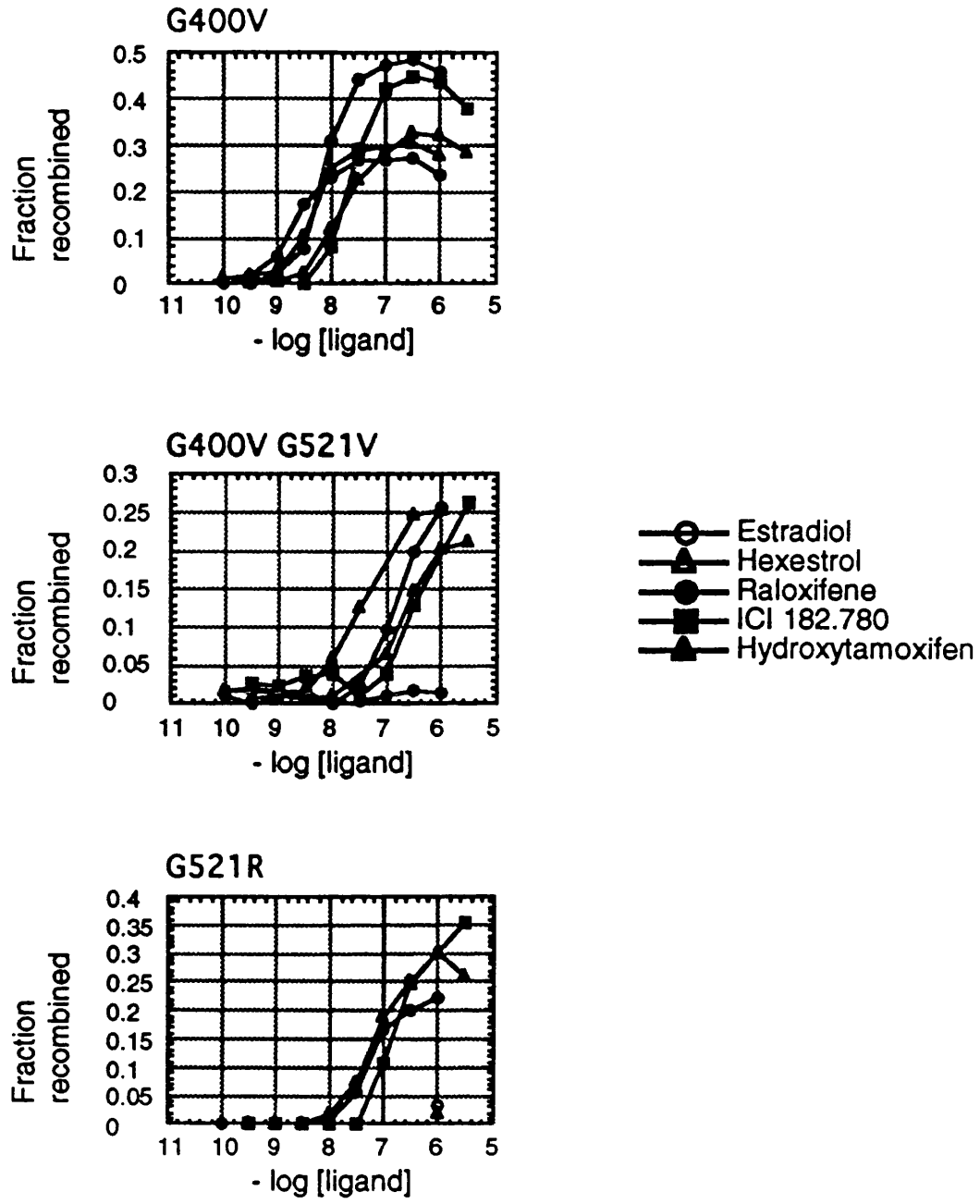



Figure 5.2 Dose response to 5 ligands in clonal derivatives of the FLP recombination reporter cell line 293R10 expressing the 400V/521G, 400V/521V or 400G/521R FLP-EBD fusion proteins. The clones were exposed to the indicated ligands for 4 hours, upon which the cells were lysed and the extracted genomic DNA was subjected to a Southern analysis in order to quantify the fraction of the cell population that had undergone FLP mediated recombination. No recombination could be detected in the absence of added ligand

Constructs encoding these two mutants were introduced into the 293R10 recombination reporter cell line and the resulting clones were subjected to a dose response experiment. The same experiment carried out on a 400V/400G clone is shown for comparison. The results shown on figure 5.2 confirm the results obtained by transient transfection experiments. The 400G/521R mutant is only activated by anti-estrogens, while the 400V/521V mutant is also activated by hexesterol but not by estradiol. The affinity of these mutants for the compounds that activate them are 100nM or above for the 400G/521R mutant and 30nM for hexesterol and 100nM or more for the other ligands in the case of the 400V/521V mutant. Saturating levels of ligand were reached with hexesterol and hydroxytamoxifen in the case of the 400V/521V-, and probably with raloxifene in the case of the 400G/521R FLP-EBDs. The 400G521R EBD does not respond to diethylstilbestrol either, indicating that this ligand binding domain is unable to respond to any of the estrogen agonists it was confronted with. The 400V/521V does respond to diethylstilbestrol (data not shown) indicating that this mutant can respond to all the ligands tested but estradiol.

The 400V/521V cell line reached a maximal level of recombination of 25% in 4 hours. The 400G/521R cell line reached 35%. To test whether these differences were clone specific, two more 400V/521V clones were treated with 1μM hexesterol for 4 hours; in one clone 19% and in the other one 28% of the cells excised the 1.3kb FRT flanked DNA segment in this time span. Therefore there are clone specific differences in the level of recombination achieved after 4 hours of ligand exposure. This does not excluded, however, the possibility that different FLP-EBD$^{mut}$ fusion proteins do have a different intrinsic recombinase activity.

## DISCUSSION

The effect of mutagenising two glycines one being located in the β-sheet that is thought to constitute part of the ligand binding pocket (Bourguet *et al.*, 1995) the other being located in a stretch of amino acids known to be important for ligand binding (Danielian *et al.*, 1993),

(Fawell *et al.*, 1990a), (Pakdel and Katzenellenbogen, 1992), was analysed combinatorially. Six constructs, encoding an EBD with a glycine or a valine at position 400 combined with a glycine, a valine or an arginine at position 521 fused to FLP were tested by transient transfection in combination with 5 known estrogen receptor ligands. Two of these did not respond to estradiol. Constructs driving the expression of these two mutants were introduced into the genome of a FLP recombination reporter cell line. These clones were subjected to increasing doses of 5 estrogen receptor ligands. One of these fusion proteins, 400G/521R, only responded to estrogen receptor antagonists. The other one 400V/521V retained the capacity to respond to both synthetic estrogen receptor agonist hexestrol and diethylstilbestrol, and responded to the 3 anti-estrogens tested. Both EBDs are therefore suitable for usage in estrogen rich environments.

The FLP-EBD$^{400G/521V}$ was as leaky as the FLP-EBD$^{wt}$ fusion protein in that recombination occurred even in the absence of added estrogens. The 400G/521V EBD did not seem to respond efficiently to estradiol. This observation, taken together with the observation that the 400V/521V does not respond to estradiol at all may mean that a valine at position 521 impairs estradiol binding. The leaky phenotype of the 400G/521V EBD taken together with its diminished capacity to respond to estradiol either means that the estrogen that activates the wild type EBD in cell culture is not estradiol, but some other estrogenic compound (Berthois *et al.*, 1986), (Hammond *et al.*, 1979), (White *et al.*, 1994) or that the weak regulative properties of the wild type EBD are caused by a natural function that can be abolished by mutations at positions 503, 507 (cf. fig 4.6), an arginine at position 521, or the combination of a valine at both position 400 and 521. The intricacy of the latter reasoning argues for the former one's validity.

Interestingly, the level of recombination induced by raloxifene in the cell line that expresses FLP-EBD$^{400V521R}$ plateaus below the level of recombination induced by both ICI 182.780 and hydroxytamoxifen (figure 5.2). Therefore a mutation that abolishes and actually reverses the increase in recombination induced by raloxifene relative to that induced by other estrogen

ligands has been isolated. This mutation does not convert hydroxytamoxifen to an agonist in the context of the full length estrogen receptor (Danielian *et al.*, 1993). It would be interesting to find out whether this is also the case for raloxifene. If this were to be the case, then it could be argued that the raloxifene effect observed in the previous chapter and the bone specific agonistic effects of raloxifene (and tamoxifen) are caused by alteration of the secondary or tertiary structures encoded by amino acids 414 to 531. Alternative explanations for the decrease in recombinase activity of the 400G/521R EBD upon exposure to raloxifene such as altered protein stability or an alteration in the off rate of the ligand, cannot be excluded until experiments addressing this issue are performed.

# Chapter 6 : RESULTS AND DISCUSSION

## Using FLP to evaluate other nuclear receptor superfamily LBDs

SUMMARY

The ligand binding domains of the human retinoid X receptor α (hRXRα), its *drosophila* homologue ultraspiracle (USP), the human retinoic acid receptor α (RARα), the human vitamin D3 receptor (VDR), the human thyroid hormone receptor β (TRβ), the *drosophila* ecdysterone receptor (EcR), the rat RZRβ receptor, the human RZRα receptor, the mouse SF-1 orphan receptor, the mouse Tlx orphan receptor or the mouse ERR2 orphan receptor were cloned by PCR behind the FLP open reading frame.

The FLP-hVDR and the FLP-hTRβ fusions repressed the activity of FLP and this repression could be relieved by addition of 1,25vitD3 and T3 respectively. The FLP-EcR fusions displayed almost no recombinase activity in the presence or absence of muristerone, an ecdysterone receptor agonist that has the capacity to enter into mammalian cells (Christopherson *et al.*, 1992), (Thomas *et al.*, 1993). The FLP-RXRα, FLP-USP and FLP-RARα as well as RZRα and RZRβ were active under all the conditions tested, as were the orphan receptor-FLP fusions FLP-ERR2, FLP-Tlx and FLP-SF-1.

Previously only the steroid receptor sub-class of the nuclear receptor superfamily were known to be sequestered away from chromatin in the absence of their ligand. These results potentially extend the list of such nuclear receptors to two members of the heterodimerising sub-class of nuclear receptors and suggest that thyroid hormone and vitamin D3 may act at the nuclear level not only by inducing the transcription activation potential of their cognate nuclear receptors but also by enabling them to compete for RXR, the co-factor of many nuclear receptors.

The results presented here indicate that the repressor function of the steroid receptor LBDs may not be a feature unique to this sub-family of the nuclear receptor superfamily but that it may, in fact, be present in other nuclear receptors.

## INTRODUCTION

As described in chapter 1, the nuclear receptor superfamily can be divided operationally into three classes, the receptors that bind steroids and homodimerise, the nuclear receptors that are activated by intracellular ligands and bind to their response elements as heterodimers with the RXR receptor and finally the orphan receptors, some of which bind to DNA as monomers and some of which heterodimerise with RXR. A further distinctive property of the steroid receptor sub-family is the recruitment of newly translated receptors into an HSP90 containing complex from which they are released upon binding ligand. A number of representatives of each class were chosen and their LBDs were fused onto the carboxyl terminus of the FLP site specific recombinase.

Previously, by fusing LBDs from the estrogen, progesterone and glucocorticoid receptors onto various transcription factors, it was functionally shown that these LBDs interact with the HSP90 complex (Mattioni *et al.*, 1994). In experiments described in chapters 3 and 4 I showed that the androgen receptor LBD confers androgen receptor ligand dependency on FLP. The members of the heterodimerising class that were chosen were RXR as well as its *drosophila* homologue USP, RARα, TRβ, VDR and EcR. For the orphan class, the putative receptors for melatonin, RZRs were chosen as well as SF-1, Tlx and ERR2.

Some of the orphan members of the nuclear receptor superfamily appear to play key roles in the determination and maintenance of organ/cell identity by affecting the expression of organ and cell type specific genes. Below I describe the tissue distribution and proposed physiological function of the nuclear receptors whose LBDs were fused to FLP.

SF-1 is involved in adrenal and gonadal development (Luo *et al.*, 1994). It has been shown to be expressed in sexually dimorphic patterns which coincide with Mullerian inhibiting substance expression patterns and Mullerian duct regression. SF-1 has been shown to bind the regulatory region of the Mullerian inhibiting substance gene (Shen *et al.*, 1994). SF-1 also regulates the expression of P450 steroid hydroxylases, enzymes that play a key role in steroidogenesis, a process that occurs in all the tissues where SF-1 is expressed (Ikeda *et al.*, 1993). A cyclic AMP response element in the human aromatase P450 has been shown to be an SF-1 binding site in human ovary cells (Michael *et al.*, 1995), arguing that SF-1 may be the target of a protein kinase A transduction cascade.

ERR2 is expressed in the kidney, testis, hypothalamus, pituitary, cerebellum and prostate. Its closest homologue, ERR1, is expressed in all tissues analysed and most highly in the cerebellum and pituitary (Giguere *et al.*, 1988). No biological role has been proposed for the estrogen related receptors, although their expression patterns suggest that like SF-1 they are involved in endocrine homeostasis.

In *drosophila*, tailless gene function is required early during embryogenesis for the proper formation of anterior and posterior structures, e.g. brain and cephalopharyngeal skeleton and the eighth abdominal segment and the posterior hindgut (Pignoni *et al.*, 1990). The mammalian homologue of tailless is expressed exclusively in the embryonic neuroepithelium (Monaghan *et al.*, 1995). The developmental defects due to ectopic expression of tailless function in *drosophila* (loss of segmentation) can be mimicked by ectopic expression of its vertebrate homologue (Yu *et al.*, 1994), providing evidence that some of the properties of these proteins are conserved between arthropods and mammalians. *Drosophila* tailless is thought to act downstream of a membrane bound tyrosine kinase, torso, because the phenotype of fly embryos expressing gain-of-function alleles of torso can be suppressed by loss of tailless activity (Pignoni *et al.*, 1990).

RZRα is expressed in many tissues but RZRβ is expressed most prominently in the neural retina, pineal gland, thalamus, hypothalamus and adrenal gland. This distribution resembles

the distribution of melatonin binding sites, the pineal gland hormone which modulates seasonal reproductive function and circadian rhythms in vertebrates. RZRβ was reported to bind melatonin (Becker *et al.*, 1994). A cell surface receptor for melatonin has been cloned and it binds melatonin with much higher affinity than RZRβ, $2.4x10^{-10}M$ versus $7x10^{-8}M$ (Reppert *et al.*, 1994), (Becker *et al.*, 1994), leaving open the question that RZRβ is a melatonin receptor. It may, however, relay or modulate the melatonin signal via an intracellular transduction pathway.

Retinoic acid has many developmental and physiological roles. One of the most interesting of these is its ability to induce the differentiation of teratocarcinoma and embryonic stem cells. This process is accompanied by the turning on of the homeobox clusters, a set of 4 groups of physically linked genes whose temporal and spatial pattern of expression is paralleled by their order within the clusters along the chromosomes, a phenomenon termed colinearity. RAR is the retinoic acid receptor.

RXR is the common co-factor for many of the members of the nuclear receptor superfamily. It is a receptor for the retinoic acid 9-cis but not all trans isoform (Levin *et al.*, 1992). RARs on the other hand bind both isoforms (Tate and Grippo, 1995). Very recently it was also shown to bind and become activated by a metabolite of methoprene acid, an analogue of the insect juvenile hormone III for which no receptor has been isolated to date (Harmon *et al.*, 1995).

The ecdysone receptor is the primary target of the ecdysterone pulses that precede moulting and metamorphosis in all invertebrates. It has been a historical paradigm of steroid hormone action because ecdysterones are hydroxylated steroid ring compounds as are all the steroid hormones. USP is the fly homologue of RXR and is the heterodimerising partner for the ecdysterone receptor (Yao *et al.*, 1992), (Thomas *et al.*, 1993).

Thyroid hormones elicit a bewildering array of actions in vertebrates, they appear to potentiate the effects of many hormones, and are often considered as permissive hormones. Thyroid hormones promote linear growth, skeletal maturation and in amphibians they

stimulate moulting and metamorphosis as well as playing a role in appendage regeneration. Thyroid receptor defects have been described in humans and usually cause generalised thyroid hormone resistance syndromes, resulting in short stature, cognitive deficits, hyperactivity, deafness, etc. In general, patients with impaired thyroid receptor function have elevated serum levels of thyroid stimulating hormone, the pituitary peptide hormone that stimulates the thyroid gland's production of thyroid hormones. The thyroid receptor acts as a heterodimer with RXR (Bugge *et al.*, 1992) although homodimer DNA binding by the thyroid receptor has been reported (Forman *et al.*, 1992)

Vitamin D is a derivative of cholesterol, like the steroid hormones. Its biogenesis involves a rupture of the steroid ring by ultraviolet light in the skin, a hydroxylation step in the liver and another hydroxylation step in the kidney. The two main targets of vitamin D3 are the gastrointestinal tract and the bone, in which it promotes calcium (and phosphate) absorption or fixation respectively. Recently, vitamin D3 has been shown to regulate growth and differentiation in the epidermis and in breast tissue, as well as having direct effects on the immune system (Bikle, 1995). The search for vitamin D3 analogues that do not have hypercalcemic effects but still do have anti-proliferative effects is a major pharmaceutical enterprise. The vitamin D3 receptor acts as a transcription factor by binding to genomic response elements as a heterodimer with RXR (Yu *et al.*, 1991).

The FLP-LBD assay reports the conditional repressor function of the LBDs of steroid receptors. The experiments described below were carried out to find out whether other members of the nuclear receptor superfamily encoded LBDs with a similar repressor function. If this were to be the case for an orphan receptor LBD, it would permit a screen to identify its ligand.

RESULTS

The above mentioned LBDs were fused to the carboxyl terminus of FLP by PCR cloning, using the primers described in chapter 2. Most of the FLP-LBD fusion proteins generated by PCR, were sub-cloned twice, from different PCR reactions, so as to minimise the possibility of analysing PCR mutation artefacts.

In the case of the heterodimerising receptor FLP-LBD fusions (RXRα, RARα, TRβ, VDR), the cognate ligand for the receptor was added to stripped serum containing culture medium.

Since no ligands are known for the orphan receptors and since most known nuclear receptor bind small lipophilic molecules that can be removed from fœtal calf serum, cells transiently expressing the fusion proteins were treated either with medium containing 10% unadulterated fœtal calf serum or with medium containing 10% charcoal stripped serum. The rationale being that if these fusion proteins' ligands were removed by stripping fœtal calf serum, and if the LBDs examined encode a conditional repressor function we would detect a difference in recombinase activity between these two experimental protocols. This strategy obviously relies on the postulate that fœtal calf serum contains the putative ligands recognised by these LBDs, and that they can be extracted by charcoal treatment.

These experiment was carried out in the CV1 derived reporter cell line E25B2/B2 and the results are shown on figures 6.1, 6.2, 6.3 and 6.4.

Of the heterodimerising LBDs, the FLP-RXRα, FLP-USP and FLP-RARα fusions were not regulated recombinases, displaying as much activity in the presence or absence of 9-cis retinoic acid or all trans-retinoic acid in the former two cases or in the latter case respectively (figure 6.1). The LBDs of the EcR, the TRβ and the VDR turned out to repress FLP function (figures 6.1 and 6.2). Addition of 1,25vitD3 or thyroid hormone activated the cognate recombinase fusions, as can be seen on figure 6.1. The first FLP-VDR subclone does not encode a repressed recombinase, sequence analysis revealed that it contained a frame shift

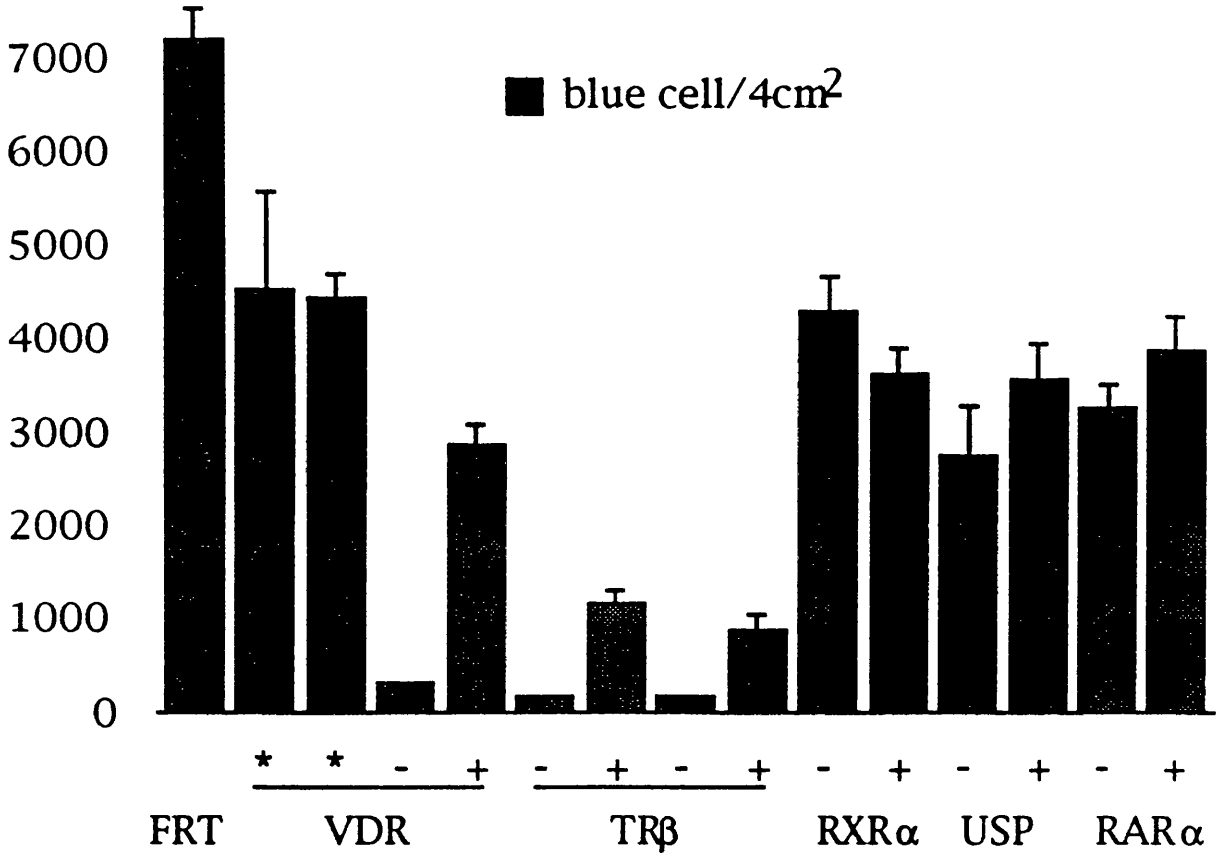



Figure 6.1 Plasmids driving the expression of FLP fused to the LBD of the indicated heterodimerising nuclear receptor were transfected into the E25B2/B2 recombination reporter cell line and incubated for 50 hours in medium containing 10% fœtal calf serum stripped with charcoal and either ethanol or DMSO vehicle or 1mM 1,25OHvitD3 (VDR), 100nM thyroid hormone (TRβ), 100nM 9-cis retinoic acid (RXRα and USP) or 100nM all trans retinoic acid (RARα). Two independent subclones were transfected for VDR and TRβ, only one clone for RXRα and USP since these LBDs were subcloned without using the PCR technique and only one clone of RARα since only on PCR subclone of this LBD was recovered. * indicates that this subclone encoded a stop codon resulting in premature translation termination. After staining for X-Gal, the number of blue cells was determined in 5 randomly chosen ocular field. The total number of blue cells was extrapolated to obtain the total number of blue cells on a $4cm^2$ surface area. Error bars represent the standard error from the five measurments

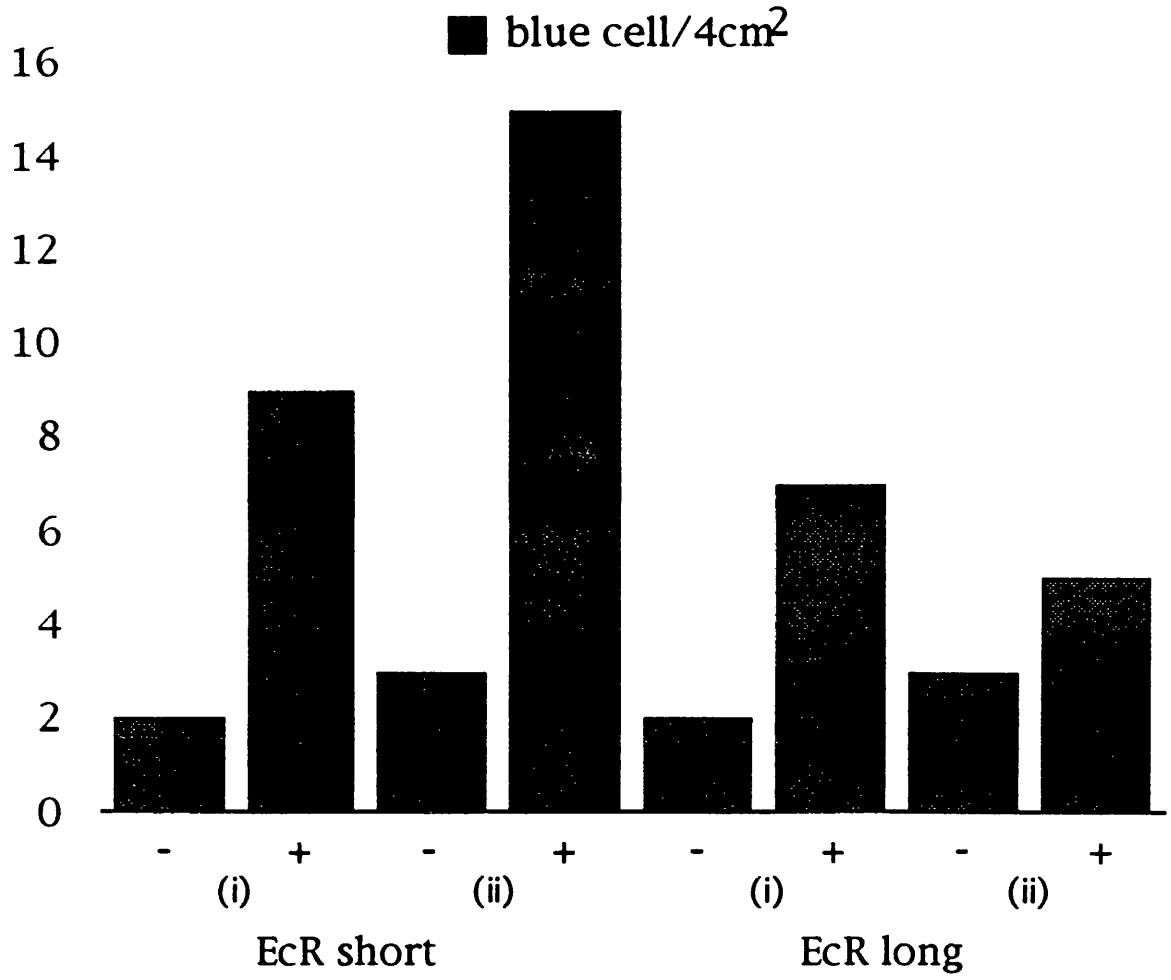



Figure 6.2 Plasmids driving the expression of FLP fused to EcR LBD were transfected into the E25B2/B2 recombination reporter cell line and incubated for 50 hours in medium containing 10% fœtal calf serum stripped with charcoal and ethanol vehicle (-) or 1μM muristerone (+). Blue cell numbers were counted manually on a surface of $4cm^2$ containing approximately 30,000 cells. Note the very low number of blue cells.

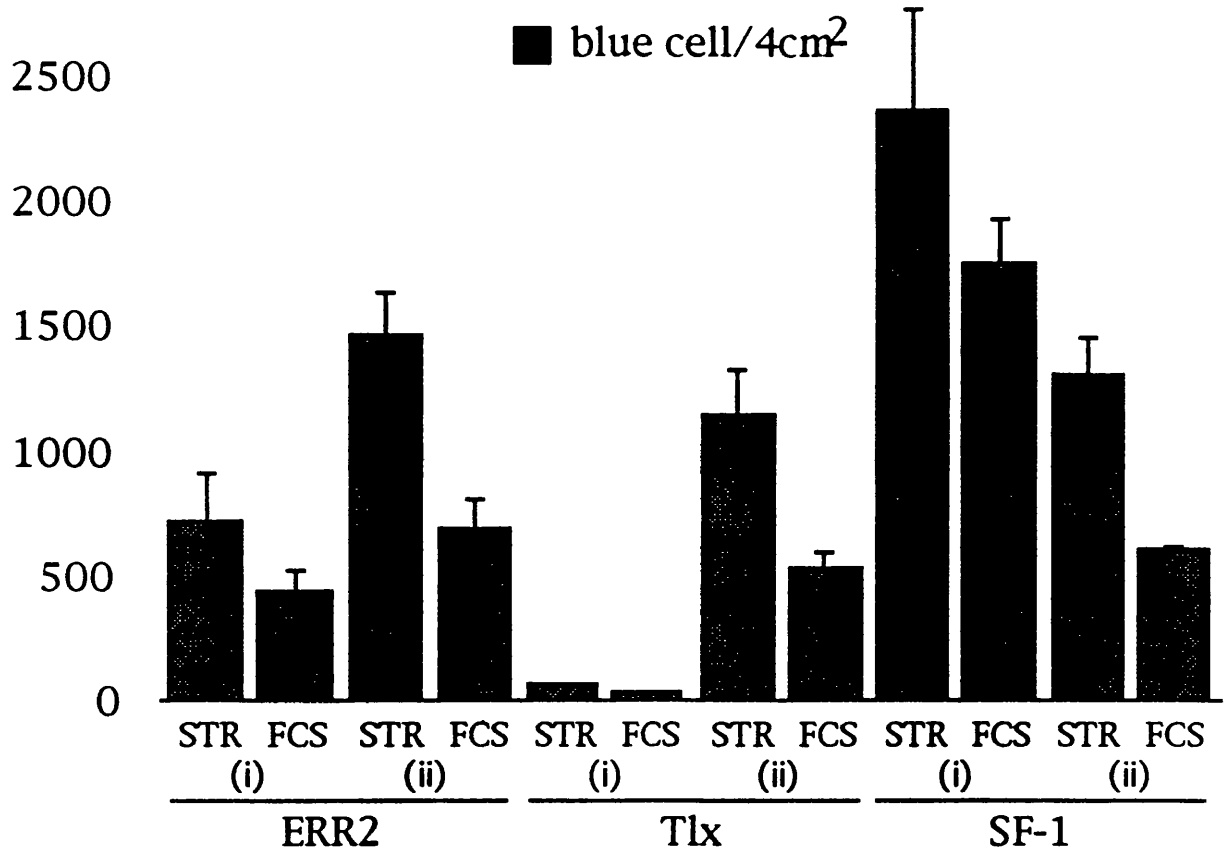



Figure 6.3 Plasmids driving the expression of FLP fused to the LBD of the indicated orphan receptor were transfected into the E25B2/B2 recombination reporter cell line and incubated for 50 hours in medium containing 10% fœtal calf serum (FCS) or 10% fœtal calf serum stripped with charcoal (STR). Blue cell numbers were determined as described in the legend to figure 6.1

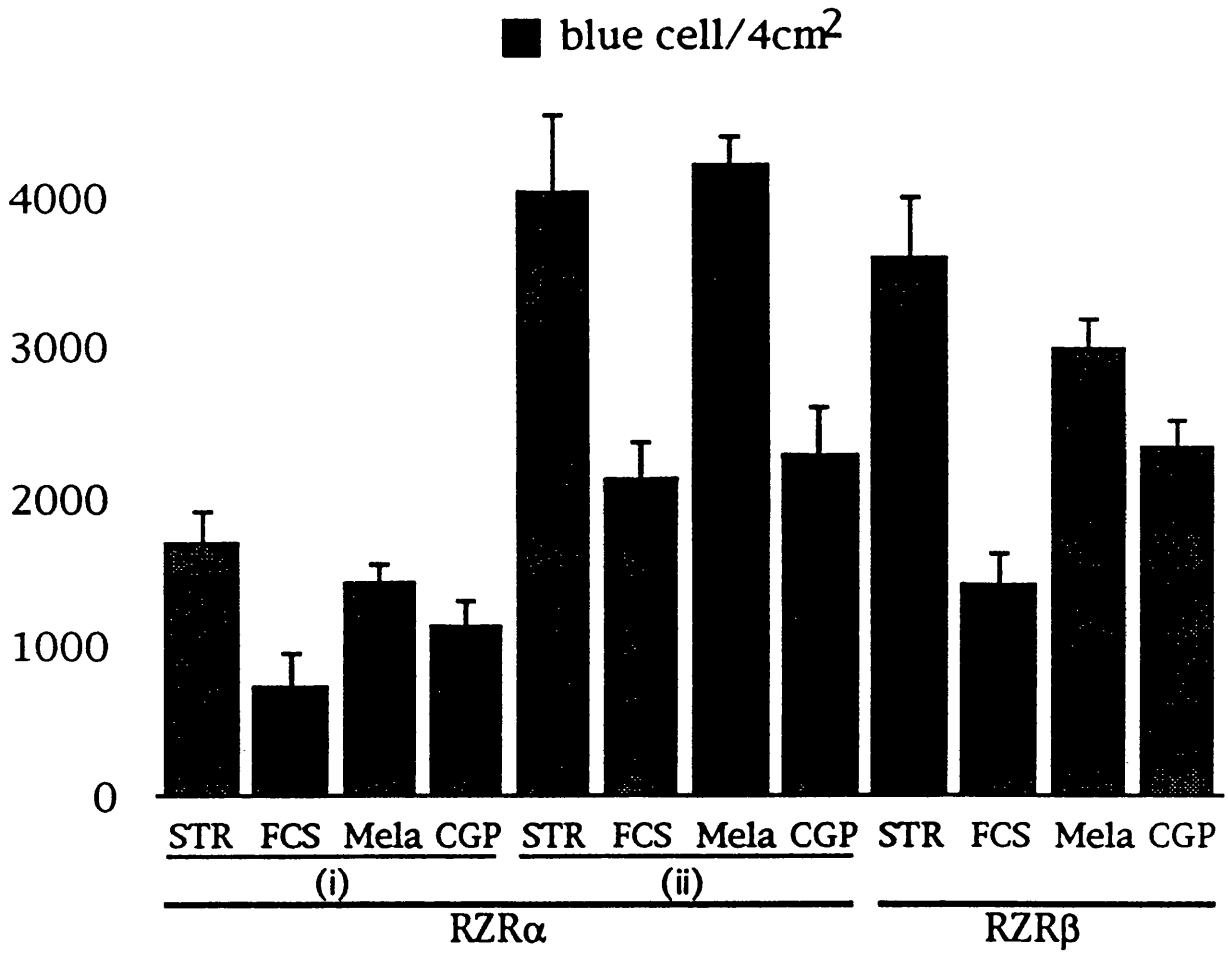



Figure 6.4 Plasmids driving the expression of FLP fused to the indicated LBD were transfected into the E25B2/B2 recombination reporter cell line and incubated for 50 hours in medium containing 10% fœtal calf serum (FCS) or 10% fœtal calf serum stripped with charcoal (STR) or 10% fœtal calf serum stripped with charcoal containing 1μM melatonin or 1μM CGP52608. Blue cell numbers were determined as described in the legend to figure 6.1

resulting in premature translational termination of this fusion protein. It was included as a positive control in the experiment shown on figure 6.1 as it represents an unregulated recombinase. Addition of muristerone had a very marginal but reproducible effect with all the tested FLP-EcR fusions (figure 6.2). Two FLP-EcR fusion were generated, one coded for the full length EcR LBD and the other one for a shorter EcR LBD in which the carboxyl terminal 200 amino acids were deleted (EcRshort), this did not affect the repressor property of the EcR LBD but also did not result in significantly better ligand activation.

The LBDs of SF-1 and ERR2 as well as those of the RZR sub-family, were unable to confer regulation onto FLP (figure 6.3). Stripping the serum reproducibly resulted in a higher recombinase activity (figures 6.3 and 6.4), this may be due to a difference in lipofection efficiency under these two culturing conditions or to some other unknown experimental parameter. The putative ligand for the RZR receptors, melatonin, did not affect recombination. CGP 52608 is a putative RZR ligand isolated and kindly provided to us by Ciba-Geigy, Basel Switzerland. It did not affect the FLP-RZR fusion's activity either (figure 6.4). One of the Tlx fusions behaved like the other orphan receptor LBDs in that it did not repress FLP activity substantially. The other subclone however encoded a recombinase fusion which was inactive whether the cells were treated with serum that had been stripped with charcoal or not. Complete sequencing of both clones or shuttling the Tlx LBD into another vector may provide an explanation for this observation.

## DISCUSSION

The ligand binding domains of the general heterodimerising partner of the nuclear receptor superfamily, namely the retinoid X receptor α (RXRα) and that of its *drosophila* homologue ultraspiracle (USP) were fused to FLP. These two fusion proteins were constitutive recombinases and the presence of the ligand for RXR, 9-cis retinoic acid, did not affect the activity of either of them. This is not a surprising result since one would expect that RXR and

USP have to be available for their partners even in the absence of their own ligand(s) and therefore should not be sequestered in a repressor complex.

The ligand binding domain of RARα also failed to repress FLP activity. This is in agreement with experimental results obtained by fusing the VP16 transcription activation domain to RARα, the resulting chimeric receptor induced transcription of its target promoters in teratocarcinoma cells even in the absence of ligand (Underhill *et al.*, 1994) demonstrating that, contrary to the steroid receptors, RAR does not encode a repressor function.

The LBDs of the TRβ and VDR do encode a conditional repressor function, although the FLP-TRβ fusion protein does exhibit substantial basal activity, a phenomenon that is quantitatively analogous to that observed with the wild type estrogen receptor LBD (see chapters 3 and 4). This might be due to inefficient removal of thyroid hormone from fœtal calf serum by the charcoal stripping procedure. Other explanations such as a weak repressor function cannot be excluded. The conditional repressor function of the TRβ LBD is somewhat in contradiction with the observations that the TRβ is not recruited in an HSP90 complex after its translation in rabbit reticulocyte extracts (Dalman *et al.*, 1990). This experimental discrepancy can be rationalised in a number of ways. For example, it could be that isolation of the TRβ LBD from it natural context results in an alteration of its HSP90 binding properties. It may also be the case that the cell culture media used in the above studies and rabbit reticulocyte lysate contain sufficient amounts of thyroid hormone to dissociate the receptor from the HSP90 complex. Finally it is possible that the hormone effects observed in this study are not mediated by the same repressor function as that of the steroid receptors. It has been demonstrated that the ligand free thyroid receptor is able to repress transcription gene linked to a thyroid hormone response element (Damm *et al.*, 1989). Lately, the silencing activity of the thyroid receptor α and β have been demonstrated *in vitro*. The thyroid receptors appear to inhibit transcription at an early step during transcription pre-initiation complex assembly and thyroid hormone relieves this inhibition (Fondell *et al.*, 1993), (Tong *et al.*, 1995). The above transcription repressor function has

been mapped to the LBD of the thyroid receptors (Baniahmad *et al.*, 1992). It is therefore possible that the repression imposed by the TRβ LBD onto FLP is caused by recruitment of the fusion protein into 'frozen' transcription complexes rather than by recruitment of the fusion proteins in the HSP90 complex described in chapter 1. This last contention could be examined by assaying the activity of a FLP-TRβ in yeast strains expressing the YDJ1 allele that encodes the G531D mutation, since this mutation leads to the de-repression of the glucocorticoid and estrogen receptors (Kimura *et al.*, 1995). A similar experimental approach would confirm that the regulative property of the VDR LBD is mediated by the same cellular protein complex as the one that acts on the steroid receptors.

The discovery that the TRβ and VDR repress FLP activity conditionally, suggests that ligand binding may not only control the transcription function of these receptors but also their capacity to compete for RXR. This may be an important mode of action for these hormones as their presence may not only activate their cognate receptors but may also reduce in the availability of RXR to other signalling pathways.

The EcR LBD fusion proteins present an interesting puzzle since they result in the inactivation of the fused recombinase moiety whether or not a cognate ligand was added to the culture medium. A very small but reproducible, ligand induced, activation could be detected (4 out of four experiments using 4 independent PCR clones)(figure 6.2). It may be that EcR requires an additional activity that is not present in CV1 or 293 cells (data not shown) to productively bind to its ligand. At the present time, I cannot exclude that the lack of recombinase activity seen here is due to a lack of expression of the fusion protein. Western blot analysis is required to address this point. Christopherson *et al.* (1992) reported that transiently expressed VP16-LexA-EcR LBD or a glucocorticoid-EcR fusion protein encoding the amino terminal and DNA binding domains of the glucocorticoid receptor and the complete LBD of the EcR were inducing transcription approximately 100 fold from appropriate reporter plasmids in 293 cells. The levels of hormone independent transcription level was approximately 5 fold above that seen by co-transfecting the receptor plasmid with an 'empty'

expression plasmid. Thus hormone addition resulted in a 20 fold activation. This may have been due to transcription activation by the ligand activated EcR LBD rather than to a release from a repressor complex. The capacity of the EcR LBD to mediate conditional repression is therefore still an open issue.

None of the orphan receptor LBDs tested seemed capable of repressing FLP recombinase in this experimental context. It is therefore likely that these receptors do not encode a conditional repressor function. This statement must be qualified since it is possible that the cell culture media used contain their cognate ligands and that the charcoal stripping procedure does not remove them.

The RZRβ has been reported to bind melatonin. No effect was detected with this hormone nor with CGP 52608, another compound thought to bind RZRβ, however, it was not proven that these compounds penetrated the cells in the assays.

In conclusion, this experimental strategy shows great promise for the isolation of natural or synthetic compounds and protein factors that activate nuclear receptors LBDs if those LBDs have the capacity to inactivate the FLP recombinase. Cell lines expressing FLP-LBD fusions could be screened either with many compounds or a cDNA library. Such screens would have the advantage over other screens that the effect of recombinase activation results in a genotypic alteration, enabling amplification of low levels of signal by clonal expansion.

# Summary

Site specific recombination and the enzymes that mediate this reaction represent a major biological resource for genetic engineers in that they should allow precise and predictable modifications to be introduced into any living genome. Attention has been focused on site specific conservative DNA recombinases that encode all the necessary functions for the recombination reaction within one polypeptide. Examples of these enzymes include the phage P1 and yeast 2 μ circle encoded recombinases, CRE and FLP.

The work presented in this thesis describes how the site specific recombination activity of FLP recombinase from the *S.cerevisiae* 2μ circle can be controlled in mammalian cells with steroid ligands by virtue of fusion to steroid receptors ligand binding domains. Changes in genotype were induced that resulted in rapid changes in cell phenotype, such as the appearance of β-galactosidase activity or the disappearance of a drug resistance marker, using a ligand regulated FLP recombinase.

Plasmids or DNA segments borne on plasmids were integrated into pre-existing recombination targets in mammalian chromosomes at frequencies equivalent to those of random integration using cells expressing the estrogen modulated FLP recombinase.

To broaden the range of experimental systems amenable to ligand activated FLP recombination, mutations were introduced into the estrogen receptor ligand binding domain that abolish estradiol binding but still allow activation by synthetic estrogen ligands. Thus, the FLP-EBD fusion proteins may be used in estrogen rich environments such as the mouse.

Steroid receptors have been the focus of much research in the last few decades. This sustained attention is due to the medical importance of steroid receptors which are the targets of some of the most powerful hormones and drugs (glucocorticoids as immunosuppressants, estrogens, progestins, anti-estrogens and anti-progestins as contraceptives, anti-cancer or abortion drugs, androgens as anabolic drugs). Thanks to this pharmaceutical impetus, much is known about the role of steroids and their receptors.

FLP-steroid receptor ligand binding domain fusion proteins represent a new means to study steroid receptor activation. The main novelty of this system is that it quantitatively reports the transient event of ligand binding as changes in chromosome DNA. This assay differs from previously available steroid receptor assays in that all the previous assays relied on measurements of the transcriptional activity while the DNA recombination assay does not. Synthetic compounds that inhibit the transcription activation function of the steroid receptors can be analysed directly with the FLP-LBD system. By combining dose response experiments with site directed mutagenesis of the estrogen receptor ligand binding domain, insight was sought into the molecular mode of action of the estrogen receptor and the mechanisms by which anti-estrogens inhibit its transcriptional activity.

Steroid receptors form a sub-family within the nuclear receptor superfamily of transcription factors. When this work was started, only three steroid receptors (the estrogen, glucocorticoid and progesterone receptors) were known to encode a conditional repressor function that could control the activity of fused on proteins (Mattioni *et al.*, 1994). I showed that this property is also shared by the androgen and vitamin D3 receptors.

Fusion of the thyroid ligand binding domain onto FLP resulted in ligand dependent site specific recombination activity. It is still an open issue as to whether, in the FLP-fusion context, the thyroid and vitamin D3 receptor receptor ligand binding domains operate in the same fashion as the steroid receptor ligand binding domains.

Other nuclear receptor superfamily members were put through the assay. The *drosophila* ecdysterone receptor LBD inhibited FLP activity under all conditions tested. In contrast, the ligand binding domains of the retinoic acid receptor α, retinoic X receptor α, of the ultraspiracle gene product, and of the mammalian orphan receptors Tlx, ERR2, RZRα, RZRβ and SF-1 did not repress FLP.

Although this screen was not exhaustive and was conducted in a single mammalian cell type, it indicates that conditional sequestration is not a general feature of the nuclear receptor superfamily but rather that is limited to a subset of its members. To date, all the receptors that

display the repressor function are activated by natural ligands that are derived from cholesterol.

The applications of the FLP-ligand binding domain fusion proteins include the temporal control of recombinase activity in living cells by ligand exposure, the discovery of new ligands for ligand binding domains (that encode a repressor function) and the functional dissection of the intra- and inter protein contacts made by nuclear receptor ligand binding domains upon binding to their ligands.

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