The role of the oestrogen receptor in antioestrogen resistant breast cancer

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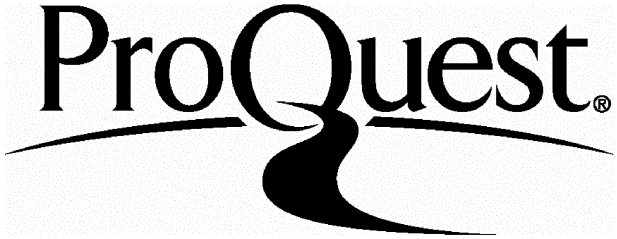

Dedicated to

Becky

William, Edward, and Sean

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Abstract

Breast cancers are often treated with antioestrogens, response rates being highest in cancers expressing oestrogen receptors, responsive tumours will however eventually become resistant. This thesis examines the role of altered oestrogen receptors in the development of antioestrogen resistance. Oestrogen receptors are nuclear hormone receptors that on binding oestradiol bind to specific sequences on DNA and are able to modify the rate of transcription of target genes. Antioestrogens competitively bind to the oestrogen receptor displacing oestradiol but are unable to fully activate the receptor. Oestrogen receptors with aberrant properties may lead to antioestrogen resistance and could arise from mutation of the gene or through alternate splicing of exons. Variant oestrogen receptor mRNA arising from alternative splicing of coding exons has been reported in breast cancers. One splice variant lacking exon 5 encodes a truncated receptor, which has constitutive activity in yeast. To explore the role of this variant in antioestrogen resistant breast cancer, stable cell lines expressing the variant on a conditional promoter have been isolated. These clones do not appear to have altered proliferative response to oestrogen or antioestrogens and there is no constitutive induction of oestrogen responsive genes in the presence of splice variant receptor, however modest activity of some reporter constructs is seen.

A short region of the hormone binding domain of the receptor has been identified as being important in determining the response of the receptor to ligand, specific mutations leading to loss of oestradiol activation but leading to stimulation of transcription by antioestrogens in HeLa cells. The activity of such mutant receptors has been examined in breast cancer cell lines. Modest agonist activity is seen in transient expression systems and attempts to establish stable expression of mutant receptors are described. A system for the identification of mutations in this region has been used to screen for mutations in human breast cancers.

Mutations that alter the ligand binding properties of the receptor have been reported. In order to identify mutations that are unable to bind tamoxifen but are still activated by oestrogen, a yeast screening system was used to screen randomly mutated oestrogen receptors. The properties of mutations identified from this system are described.

CONTENTS

Figures

Tables

Chapter 1

Introduction

Introduction

The treatment and prevention of breast cancer is an increasingly complex area of medicine. Hormonal manipulation in the form of antioestrogens, such as the triphenylethylene compound tamoxifen, is used in all stages of breast cancer treatment. Not all breast cancers respond to tamoxifen and even those that do respond eventually become refractory to this drug. Tamoxifen resistance is therefore a major issue in breast cancer management. This chapter reviews our knowledge of the role of oestrogens in the aetiology and progression of breast cancer, the mechanisms of oestrogen action mediated through the oestrogen receptor and the mechanism of antioestrogen action. The process by which breast cancers become resistant to antioestrogens and refractory to hormonal influence will be discussed with particular reference to the role of the oestrogen receptor in this process. Subsequent chapters will describe experiments designed to improve our understanding of the role of the oestrogen receptor in the process of antioestrogen resistance. The final chapter will discuss the relevance of these and other experiments to our understanding of oestrogen receptor function and antioestrogen resistance.

Human Breast Cancer

Breast Cancer; a major clinical issue

Breast cancer is the most common cancer among women in the western world. It is a devastating illness giving rise to both physical and psychological morbidity and eventually causing the death of hundreds of thousands of women annually throughout the world. Estimated world-wide incidence for the year 2000 is 859,300 (Parklin *et al.* 1984). In England and Wales annual incidence for 1991 was 34,500 and there were 15,180 breast cancer deaths in 1990 (CRC 1996). One in twelve UK women will develop breast cancer. While incidence has been steadily increasing since the 1950s it is encouraging to note that in the last few years mortality from breast cancer has been falling in many developed countries (UK deaths in 1995 was 14,080). This fall is largely attributed to the wide-scale adoption of adjuvant therapies after primary surgery most notably the use of adjuvant tamoxifen

and to a lesser extent adjuvant chemotherapy (Beral *et al.* 1995; Berrino *et al.* 1995). There may also have been an impact as a result of the National breast cancer screening programme.

Synopsis of breast cancer management

Breast cancer deaths are nearly all attributable to the development of distant metastatic spread. However, most breast cancer patients present with clinically early stage disease. Clinically localised disease is treated surgically by removal of the primary tumour with a margin of uninvolved tissue. The extent of this operation is determined by a wide variety of factors, most importantly the size and site of disease. Modern surgical management is aimed at providing the maximal chance of cure and minimal morbidity using breast-conserving techniques, where possible. At definitive surgery, it is now customary to remove tissue from the ipsilateral axilla as both a therapeutic procedure and to obtain prognostic information to guide additional treatment. This usually means that complete macroscopic clearance of early breast cancer is achieved. Despite this only about half of clinically early stage disease is cured by surgery. This is believed to be primarily due to the development of clinically occult micrometastatic disease at an early stage in the natural history of breast cancer. It is possible to demonstrate breast cancer cells in the bone marrow of many patients undergoing primary breast surgery (Dearnaley *et al.* 1981) although this is not an automatic marker for the development of subsequent overt metastatic disease (Diel *et al.* 1996)

Following primary surgery a large number of independent prognostic features can be analysed such as tumour size, histological grade, oestrogen receptor or specific oncogene expression such as erb b2 (Dhingra *et al.* 1996) However the most important single prognostic factor after primary breast surgery is the presence or absence of axillary lymph node metastases, with increasingly poor prognosis with increasing number of involved nodes. These factors are used clinically in determining the likely benefit from additional adjuvant therapies aimed at reducing chances of relapse. Currently these adjuvant treatments consist of hormonal therapy, cytotoxic chemotherapy and loco regional radiotherapy. While these modalities are of undoubted benefit in reducing relapse rates and increasing survival (Bentley *et al.* 1992), research is constantly refining their use. Despite many advances

in the treatment of early breast cancer approximately half of these patients eventually develop metastatic disease.

In patients where overt distant metastases exist at the time of diagnosis or who subsequently develop metastases the disease is considered incurable by conventional treatment. Therapeutic goals of standard treatments in these circumstances are aimed primarily at disease control, with the palliation of symptoms and enhancement of quality of life the primary focus of therapeutic intervention. There are limited expectations in terms of prolongation of life. In addition to the therapeutic modalities described above a complex program of management involving many specialist health care professionals is often needed to provide optimum individualised care for patients with metastatic breast cancer.

Epidemiology and risk factors for breast cancer

Widespread geographical variation in breast cancer incidence points to the importance of environmental factors with age standardised incidence ranging from 12.1(per 100,000) in Japan to 79.9 in the USA (Los Angeles white population) (Waterhouse 1976). Studies of Japanese migrants to the USA have shown a tendency for breast cancer incidence to reach local levels within two generations. There is a demographic variation in incidence with higher incidence in more affluent sections of the community.

Dietary factors are likely to be important but are difficult to separate from confounding variables. National breast cancer incidence correlates with national total and saturated fat intake, with low fat intake subgroups within affluent nations demonstrating a lower incidence of breast cancer (Armstrong *et al.* 1975). In animal studies of dietary influences on breast cancer, high fat intake in rats leads to an increase in DMBA induced mammary tumours (Caroll *et al.* 1979). The influence of fat intake may be more important in early years as fat intake in latter life has little influence on breast cancer risk (Phillips 1980). Increased alcohol consumption is reported to increase breast cancer risk and smoking is also likely to influence risk, with recent studies showing a significant increase in risk to smokers compared to non smokers unexposed to passive cigarette smoking. (Morabia *et al.* 1996). Radiation exposure is also a recognised factor increasing breast cancer risk.

The hormonal environment is undoubtedly an important consideration in the aetiology of breast cancer, with a number of observations indicating

alterations in hormonal environment influence breast cancer risk (see Table 1). Early menarche and late menopause are both associated with increased risk of breast cancer, (Trichopoulos *et al.* 1972; Kampert *et al.* 1988) suggesting that the total number of ovarian cycles has implications for breast cancer risk. Pregnancy has a protective effect with highest incidence in nuliparous women and lowest incidence with multiparity (Mustacchi *et al.* 1961). Age at first birth is also influential, an increasing breast cancer incidence with increasing age at first birth has been established (MacMahon 1970). Artificial hormonal interventions affect breast cancer risk, for instance surgical oophorectomy under the age of forty years of age reduces risk (Lillenfield 1956). The risk associated with hormone replacement therapy has been extensively studied but earlier results have been controversial. Recently larger studies and overviews have demonstrated an increased breast cancer risk, but this is only apparent after prolonged exposure. After five years continued use, an increased breast cancer risk is detectable. There is a tendency for risk to rise with increasing duration of exposure. After 8 - 15 years hormone replacement therapy relative risk increases to 1.3 (Steinberg *et al.* 1991; Sillero-Arenas *et al.* 1992)also reviewed in (Ewertz 1996). Most of the studies of long term hormone replacement have been on women taking unopposed oestrogens. Therefore there remains uncertainty concerning any risk associated with combined oestrogen and progestin replacement.

The influence of exogenous oestrogens in the form of oral contraceptives in breast cancer has been more controversial with increased risk reported in some but not all studies (Veronesi *et al.* 1995). Recently an overview of 54 epidemiological studies has reported a small but definite increase in breast cancer incidence in users of the combined oral contraceptive, relative risk 1.24 (Collaborative group on hormonal factors in breast cancer 1996). This risk is still detectable five years after stopping treatment but is absent after ten years. This observation is consistent with the view that oestrogen is not a genotoxic agent and the role of oestrogens in breast cancer is in tumour promotion.

Genetic factors

The genetic influence on breast cancer risk was first described more than fifty years ago (Wassink 1935) where increased risk amongst first degree relatives of breast cancer cases was described. This risk was particularly strong for relatives of patients with bilateral breast cancer (Bucalossi *et al.* 1954) or where the index case was pre menopausal at diagnosis (Anderson 1972; Anderson 1976) Molecular genetic studies of families with particularly high numbers of breast cancers has led to the identification of mutations within tumour suppresser genes such as p53, BRCA1 and BRCA2. Heterozygous inheritance of mutations in these genes results in high to near certain life time risk of developing breast cancer. (Szabo *et al.* 1995) However inherited mutations in these known genes probably account for only 2-5-% of all breast cancers. (Peto *et al.* 1996) with BRCA 1 alone accounting for 5-10% of breast cancer under the age of forty. Clearly there are other genetic factors producing less well defined patterns of genetic inheritance. It is of interest to note that while mutations in BRCA1 in sporadic cancers are not seen, the expression of BRCA1 transcript is reduced in sporadic breast tumours compared to normal breast. tissue (Thompson *et al.* 1995).

Table 1.1 Reproductive / hormonal risk factors for breast cancer

Risk factor	Comparator	Risk category	Relative risk
Age at menarche	16 yrs	13 yrs	1.3
Age at birth of first child	Before age 20	›30yrs	1.9
		nuliparous	1.9
Age at menopause	45-54 yrs	›55 yrs	1.5
		‹45 yrs	0.7
Oral contraceptive	Never used	current use	1.24
		past use	1.0
Hormone replacement therapy	Never used	10 yrs use	1.3

Modified from Harris *et al* NEJM 1992 327 5 319-328

Malignant transformation is believed to occur mainly in the terminal ductal/lobular unit of breast tissue. Benign hyperplasia is common and carries little risk of invasive cancer but atypical hyperplasia is associated with a five fold risk of invasive disease. *In situ* carcinoma is a condition in which morphologically neoplastic cells are seen within ducts (DCIS) or lobules (LCIS) but there is no invasive component. *In situ* carcinoma is associated with high incidence of invasive carcinoma. Invasive breast cancer may develop within *in situ* carcinoma and may take place at an early stage in the situation where at the time of diagnosis small micro invasive disease is found with little surrounding *in situ* disease. In some carcinomas it is not possible to find evidence of any *in situ* disease. Breast cancers may arise in a clonogenic fashion from a single cell but because of genetic instability they inevitably become heterogeneous and the behaviour of a tumour changes with time as cell populations with different properties outgrow the previous dominant cell population that characterised initial behaviour. It is presumably the heterogeneous nature of breast cancer that forms the basis for the development of cancer that is resistant to any single therapy.

Oestrogen action in normal and malignant breast tissue

From epidemiology and animal work oestrogenic stimulation is an important aspect of breast cancer pathogenesis. Oestrogen is required for normal breast formation at puberty. Oestrogen and progesterone replacement in hypogonadal deficiency states such as Turners syndrome where ovarian development does not occur, can restore breast development. However gonadotrophins are also required since in hypogonadotrophic conditions oestrogen replacement is not sufficient (Laron *et al.* 1989) . Therefore a number of mammotrophic hormones functioning in concert are required to control breast development and maintenance.

Oestrogens have been implicated in the pathogenesis of breast cancer not only through epidemiological studies but also in animal models. DMBA induced mammary carcinomas are more frequent in ovariectomised animals supplemented with oestrogen (Lyons 1958; Lieberman *et al.* 1978). Oestrogen stimulates the formation of terminal end buds in the developing rat mammary gland. It is at the stage of maximal terminal end bud

formation (at the beginning of first oestrus), that maximal DMBA induced tumour formation occurs (Huggins *et al.* 1961; Russo *et al.* 1983). Thus experimental evidence suggests that oestrogens act as tumour promoters through their mitogenic action by producing increased target cells with shorter growth cycles exposed to carcinogenic risk. Epithelial cells with early changes remain under oestrogenic stimulation increasing the risk of developing fully invasive disease. Study of human breast cancer cell lines and clinical observation of breast cancer response to oestrogen deprivation clearly demonstrate that many breast cancers show either oestrogen dependence or oestrogen stimulated growth, reviewed in (Dabre *et al.* 1989; Clarke *et al.* 1991). Oestrogen is also required for effective implantation in xenograft tumour formation for several human breast cancer cell lines, (Soule *et al.* 1980) Reviewed in (Welsch 1982), which may have implications for implantation of distant metastases in humans.

Endocrine aspects in the management of breast cancer.

While there are numerous aetiological factors associated with the development of breast cancer the strongest influence in the development of sporadic cancer is hormonal. The sensitivity of established cancers to hormonal manipulation has attracted substantial research efforts to understand the mechanisms involved in hormone action in breast cancer and to identify differences that characterise hormone sensitive and insensitive breast cancer.

The importance of endocrine influence in the practical treatment was first described over 100 years ago when Beatson showed responses in advanced breast cancer after surgical oophorectomy (Beatson 1896). This landmark description laid the foundation for a variety of hormonal manipulations such as adrenalectomy and hypophysetomy. In the early 1970s the triphenylethylene compound tamoxifen was introduced for the treatment of advanced breast cancer (Cole *et al.* 1971). This compound initially developed as a contraceptive was found to have potent antioestrogenic effects and was used with success in the treatment of advanced breast cancer. At the same time oestrogen receptor (ER) binding assays were applied to breast tumour samples. A correlation was made between ER content of primary breast cancer and prognosis (McGuire 1975) and that ER negativity was a predictor of early relapse (Knight *et al.* 1977).

This then raised the possibility of predicting response to hormone therapy using ER assays (Horwitz *et al.* 1975). It has become firmly established that the presence of ER predicts for response, not only to tamoxifen but also for all other active hormonal therapies, see (Veronesi *et al.* 1995) for review. The prediction of response to tamoxifen according to ER content is relatively crude with only 50% of ER positive breast cancers responding to treatment. The absence of ER expression is however a more accurate predictor of treatment failure with only 10% of ER negative tumours responding to endocrine therapy. This prediction of response can be refined by the measurement of progesterone receptors (PR). The presence of both ER and PR increases the likelihood of response to tamoxifen to 70% . PR is induced by oestrogens and it is likely therefore that tumours expressing PR have a functional hormonal response pathway and are therefore more likely to be sensitive to antioestrogens. While the response rate for ER positive PR negative tumours is lower, it is far from insignificant at around 30%. Finally the phenotype ER negative PR positive is encountered and here the response rate is around 50%

The definition of hormone receptor negativity is dependent on the technique used and has for most older studies been defined as less than 10 fmol/mg of tumour tissue. This is largely a pragmatic cut off based on assay sensitivity and may explain why antioestrogen responses are seen in a proportion of ER 'negative' tumours. With current hormone receptor assays based on sensitive immunohistochemical studies it may be possible to be more accurate and certainly weak hormone receptor staining is prognostically favourable compared to no staining (Elledge *et al.* 1997).*

The ER negative PR positive phenotype is somewhat anomalous since ER is required to stimulate the expression of PR. Analysis of this phenotype resulted in the identification of variant ER mRNA generated by alternate splicing. This will be discussed in detail later in this chapter.

*Reference is not listed in alphabetical order see page 195, also see Clark 1997 page 195

Table 1.2 ***Response to Endocrine therapy***

Steroid receptor content	Objective Response Rate %
ER- /PR-	9
ER-/PR+	53
ER+/PR-	32
ER+/PR+	71

From McGuire *et al* Seminars in Oncology (1983)

Adjuvant endocrine therapy

The success of endocrine therapy for inoperable or advanced disease prompted the investigation of the use of hormonal therapy as adjuvant therapy after primary surgery. Adjuvant therapy has the theoretical advantage of influencing the behaviour of micrometastatic disease where the disease burden is low and presents an opportunity of inhibiting breast cancer progression before the emergence of resistant clones. Adjuvant treatment therefore introduces the possibility of cure for some patients who would otherwise die from metastatic disease. Application of adjuvant therapy exposes all patients to the side effects and toxicity of treatment but does not provide benefit to all patients since some patients have been cured by surgical treatment and others relapse despite adjuvant therapy. The benefit of adjuvant therapy can only be described as a population effect rather than a benefit to an individual patient. Many trials of adjuvant ovarian ablation and adjuvant tamoxifen have demonstrated a significant benefit to adjuvant hormone therapy but the magnitude of these benefits was demonstrated with extraordinary statistical accuracy by the Early Breast Cancer Trialist's Collaborative Group's overview (Bentley *et al.* 1992). In pre menopausal patients there is a clear benefit to ovarian ablation with a 30% reduction in annual odds of recurrence. Adjuvant treatment with tamoxifen is beneficial in terms of reduced relapse free and overall survival rates for both pre and postmenopausal age groups, with an overall reduction in annual odds of recurrence of 25%. While the reduction in annual odds is similar in node

negative and node positive patients, in terms of absolute benefit there is a larger effect on axillary node positive patients since prognosis is substantially worse, a similar percentage reduction in annual odds translates into many fewer events. The pattern of improvement after adjuvant therapy in terms of survival is similar in all groups to the relapse free survival data for that group.

Hormonal Prevention of breast cancer

Analysis of data from adjuvant studies of tamoxifen shows a 40% reduction in the incidence of contralateral breast cancers in the treated population (Bentley *et al.* 1992). Many patients at substantially increased risk of developing breast cancer such as those with strong family histories may benefit from preventative drug therapy (Cuzick *et al.* 1986). However, this further raises the threshold of concern about exposure to toxicity of drug intervention. Large scale clinical trials are evaluating the role of tamoxifen in this situation (Jordan 1997).

The mitogenic response to oestrogen.

Oestrogen treatment results in a variety of cellular responses in different tissues. These responses are the result of regulation of transcription from specific genes. A prerequisite for an oestrogenic response is thought to be the presence of the oestrogen receptor. This section will discuss the process by which oestrogens influence mitogenesis.

Hormone response elements and oestrogen responsive genes

The ER is known to bind to specific DNA sequences termed oestrogen response elements (EREs) located in the vicinity of oestrogen responsive genes. These were first proposed by comparison of upstream regions of identified oestrogen responsive genes (Walker *et al.* 1984) allowing a "consensus " ERE to be described consisting of an inverted repeat of six nucleotides separated by a three base pair central region, AGGTCANNNTGACCT . Functional analysis of oestrogen responsive genes has confirmed that this sequence is sufficient for high affinity DNA binding,.see (Gronemeyer 1991; Martinez *et al.* 1991) for reviews. Many oestrogen response elements are not perfect palindromes and when placed in front of hormone insensitive reporter genes are weakly active, however when

arranged closely together they co-operate to become strongly induced by oestrogen. For instance the Xenopus vitelogenin promoter, which contains two imperfect EREs is strongly activated by oestrogen (Klein-Hitpass *et al.* 1986). Thus individual EREs can form a larger entity, the hormone response unit (HRU) (Martinez and Wahli 1991). HRUs can comprise several different steroid response elements leading to sensitivity to a number of different steroid hormones (Martinez and Wahli 1991). Oestrogen responsive genes can also be influenced by other transcription factors controlled through other signalling pathways. For instance the c-fos promoter contains an ERE and AP1 site (Weisz *et al.* 1990). The ER has also been shown to affect transcription by interaction with AP1 sites (Philips *et al.* 1993; Webb *et al.* 1995) discussed in more detail later. This results in complex regulation of genes under the influence of a number of different stimuli with the potential for synergism and competition.

Cell cycle control by oestrogens in breast cancer

Oestrogen treatment of breast cancer cells results in increased growth and morphological changes (Dabre and Daly 1989). The G1 phase of the cell cycle is shortened and non cycling cells are recruited into G1(Brunner *et al.* 1989)). Cell cycle control genes such as myc and fos are implicated as targets for oestradiol stimulated mitogenesis (Wilding *et al.* 1988; Watts *et al.* 1994) followed by induction of cyclin D1. Cyclin D1 is an important regulator of early G1 progression and in breast cancer cells artificially overexpressing D1 on an inducible promoter the growth inhibitory effects of antioestrogens can be overcome (Musgrove *et al.* 1993; Musgrove *et al.* 1994; Watts *et al.* 1994) reviewed in (Sutherland *et al.* 1995).

The relationship of oestrogen and cytokines in growth control of breast cancer

Proliferation in breast cancers remains to a greater or lesser extent under regulation by a complex set of endocrine paracrine or autocrine influences that control the growth and development of normal mammary gland. Many growth factors or cytokines play a role in regulating the growth of breast cancers. The mitogenic response to oestrogens in cell culture using serum free medium may require additional factors such as insulin (Sutherland *et al.* 1992) but this is not universal (DeFriend *et al.* 1994). Some

cytokines implicated in growth regulation of breast cancers form part of the oestrogenic pathway of growth regulation. TGFα is a polypeptide cytokine that is secreted by breast cancer cells in response to oestrogenic stimulation (Bates *et al.* 1988).

TGFα promotes mitogenesis through the activation of the plasma membrane bound EGF receptor tyrosine kinase. It can cause growth stimulation of breast cancers through autocrine paracrine and endocrine effects (Clarke *et al.* 1991), but it is not the sole mediator of oestrogen stimulated growth. While oestrogen induced mitogenesis is less marked when exogenous TGFα is present, there is a detectable mitogenic effect of adding oestrogen to TGFα stimulated breast cancer cell lines (Vonderhaar 1988) and they remain partially sensitive to antioestrogens (Koga *et al.* 1987). Further more, growth stimulation of tumours by TGFα is blocked by neutralising antibodies to TGFα but this does not abolish oestradiol growth stimulation (Clarke *et al.* 1989). Overexpression of TGFα does not confer oestrogen independence (Clarke *et al.* 1989) and TGFα can not replace oestrogen as a requirement for xenograft implantation (Clarke *et al.* 1989) but does enhance implantation rates when used simultaneously with oestradiol (Dickson *et al.* 1986).

Oestrogen stimulated proliferation is therefore a complex process involving multiple pathways. Another important cytokine pertinent to the oestrogenic pathway is TGFβ. This is generally a growth inhibitory cytokine in breast cancers. Synthesis and secretion is reduced during oestrogen stimulation and is increased during antioestrogen treatment (Knabbe *et al.* 1987). TGFβ can be secreted by stromal fibroblasts and this may occur in response to antioestrogens in an ER independent fashion. (Butta *et al.* 1992). This is an example of where the cellular environment can influence tumour behaviour. Many other cytokines have been implicated in the regulation of proliferation in breast cancers including EGF, IGF I, IGF II, FGFs and PDGF reviewed in (Lippman *et al.* 1989; Dickson *et al.* 1991; Murphy 1994 1203: Clarke, 1991 ; .

The role of growth factor receptors is also of undoubted importance in the regulation of breast cancer proliferation. Of particular interest is the regulation of EGF-R and related receptors. EGF-R and IGF-R expression can be induced by oestrogen, interestingly overexpression of EGF-R may form the basis for the development of oestrogen independence in some

breast cancers and there is in general an inverse relationship between ER and EGF-R expression in breast cancers. It is now becoming increasingly apparent that there is extensive cross-coupling between different signalling pathways such that oestrogens and antioestrogens can affect the response through several signalling pathways and that other signalling pathways affect the oestrogenic pathway. Thus receptor cross-coupling adds a further dimension to cellular responses to an environment of multiple growth signalling systems. (This aspect of the oestrogen response will be covered later.) To complicate the overall situation further there are as described above with regard to TGFβ complex interactions between tumour and supporting stromal elements. It is therefore always necessary to interpret experimental *in vitro* or tumour Xenograft systems with caution before extrapolating to predict events in patients. There are considerable differences between different breast tumours in their cytokine production, cytokine receptor content and response to individual hormones and cytokines This gives rise to heterogeneous behaviour of breast cancers to growth regulating factors (Kern *et al.* 1990).

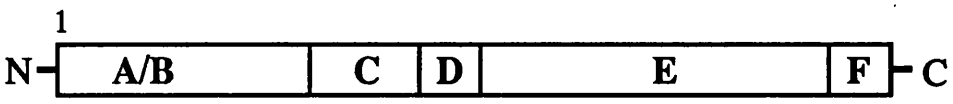
Oestrogen receptor structure and function

The human ER is a 595 amino acid protein. It can be divided into six regions A-F based upon homology with other steroid hormone receptors (Krust *et al.* 1986) (Fig 1.1). Study of proteolytic fragments or artificially expressed mutant forms of the receptor including deletions and mutants created by site directed mutagenesis, has led to the description of functional domains (Fig 1.1) and the identification of small regions of the protein critical to specific functions of the molecule. The crystal structure of the DNA binding domain of the ER has been determined. Crystal structures for the ligand binding domains for RAR RXR and most recently the ER have also been described. The ER from several other species have been cloned including the mouse oestrogen receptor (MOR) (White *et al.* 1987). This shows 89% homology with the human receptor. There is absolute conservation of the entire DNA binding domain across species and many areas of absolute conservation in sequences associated with specific functions (White *et al.* 1987; Fawell *et al.* 1990; Green *et al.* 1991; Danielian *et al.* 1992). This section will review the identification, analysis the structure and mechanism of action of the ER.

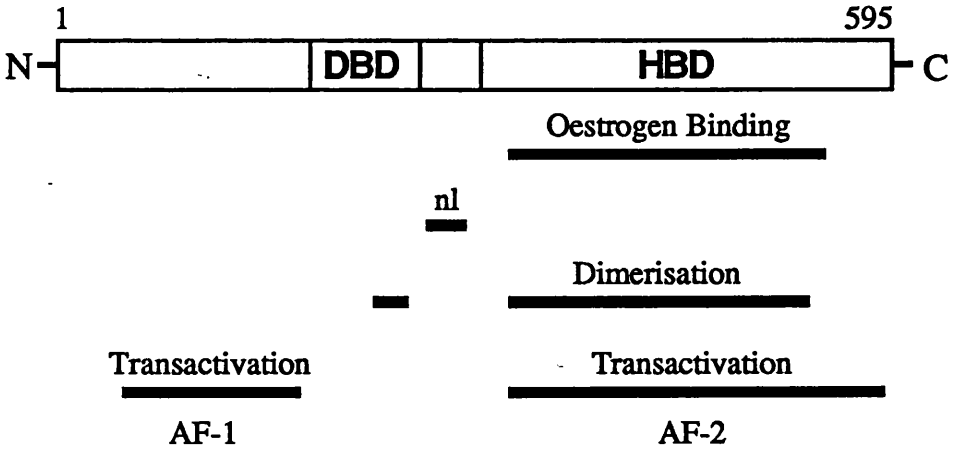
The simplistic model for ER function is that in the absence of ligand the receptor is bound as an inactive complex within the nucleus and upon binding ligand it is released from chaperone proteins and forms homodimers. These dimers bind to DNA at specific oestrogen response elements within the promoter regions of target genes. Through a direct or indirect interaction between ER and the basic transcriptional machinery, the rate of gene transcription is altered. While there is good evidence that this model is essentially correct, this is a complex process and it is now becoming apparent that this is only one of several ways in which the ER can influence gene transcription. Other signalling pathways can influence ER activity either by modulating response to oestradiol or through ligand independent mechanisms. Therefore a detailed examination of our knowledge of structure and function is of value in understanding the role of ER and the responses to antioestrogens.

Fig 1.1

Homology Domains in the Oestrogen Receptor



Functional Domains in the Oestrogen Receptor



Identification of oestrogen receptors and the nuclear receptor super family

The "discovery of the oestrogen receptor" is said to have been made when oestrogen responsive tissue was first noted to retain radio labelled oestradiol against a concentration gradient (Jensen 1962). Early work identified the binding of steroid in the "cytoplasmic fraction" in the absence of ligand and in the "nuclear" fraction after addition of oestradiol. Sedimentation studies revealed the cytoplasmic fraction had a sedimentation coefficient of 9.5S and the nuclear fraction 5S (Toft *et al.* 1966; Gorski *et al.* 1968; Jensen *et al.* 1968). Thus a two step hypothesis was proposed in which oestradiol binding led to nuclear translocation coupled to a structural transition to an active form that could interact with DNA (Jensen *et al.* 1968). This sedimentation change can be induced by high salt or heat treatment and correlates with the loss of associated proteins. The most important of these are the heat shock proteins particularly hsp90 but many other proteins are bound in a complex including hsp70, hsp59, p54, p50 and p23, reviewed in (Pratt 1993; Smith *et al.* 1993). The binding of hsp90 is thought to maintain the receptor as an inactive complex. On binding to ligand the receptor dissociates from these chaperone proteins and becomes free to interact with target genes. Ligand binding is associated with a presumed conformational change of the receptor demonstrated by an altered pattern of proteolysis in the presence of ligand (Kumar *et al.* 1988) and altered mobility on band shift assay (Lees *et al.* 1989).

The oestrogen receptor gene was cloned from a cDNA library from MCF-7 human breast cancer cells and the primary amino acid sequence has been derived from this (Green *et al.* 1986). Subsequent to the cloning of the oestrogen receptor a large and expanding number of related receptors including all the recognised steroid hormone receptors have been cloned and collectively termed the nuclear receptor super-family. Members of this family of nuclear receptors share structural homology and many aspects of the mechanism of action of these proteins are shared. This family of proteins function as transcription factors modulating the rate of transcription of target genes in response to ligand activation. The family consists not only of steroid hormone receptors but also thyroid, vitamin D, farnesoid, retinoic acid and retinoid X receptors. There are also a number of so called orphan receptors. which currently make up the majority of the family. These are

proteins for which there is as yet no identified ligand, alternatively they may function as unliganded transcription factors, reviewed in (Evans 1988; Green *et al.* 1988; Beato 1989; Ham *et al.* 1989; Parker 1993).

A second oestrogen receptor : ERβ

Recently a second oestrogen receptor has been cloned from rat and human DNA (Kuiper *et al.* 1996; Mosselman *et al.* 1996). The human gene has 96% homology with the DBD of the original ER (ERα) and 58% homology with the HBD, but a truncated N-terminus. The pattern of expression is relatively restricted but it has been detected in breast tumours and breast cancer cell lines (Dotzlaw *et al.* 1997). This receptor is functional as an oestradiol dependent transcriptional activator on oestrogen responsive promoters. It forms heterodimers with ERα (Cowley *et al.* 1997) but its biological significance has not been established.

Hormone binding

The hormone binding region of the ER was found to be present within region E of the receptor (residues 302-552). This is the most functionally complex region as it also contains regions responsible for receptor dimerisation and ligand dependent activation.

Ligand binding activity was identified within domain E by demonstration of a C terminal proteolytic fragment that bound ligand (Katzenellenbogen *et al.* 1987). Using either ketononesterol aziridine or tamoxifen aziridine (Harlow *et al.* 1989) cysteine 530 was labelled thus identifying more accurately the region of importance for ligand binding. However this individual residue is not critical as substitution of cysteine 530 does not affect ligand binding (Fawell *et al.* 1990). This implies that cysteine 530 is close to but does not form part of the ligand binding pocket. Ligand binding is lost after C terminal deletion to residue 539 in the MOR (equivalent to 535 for hER) (Fawell *et al.* 1990). Critical point mutations within this region can lead to interesting effects on ligand binding. Mutation at MOR 518 (hER 514) abolishes all ligand binding. Substitution of glycine at position MOR 525 (hER 521) with arginine also abolishes oestradiol binding but it does not prevent activation of transcription by hydroxytamoxifen implying that hydroxytamoxifen still binds this mutant (Danielian *et al.* 1993). This demonstrates that while the ligand binding

pockets for oestrogen and antioestrogens are in a similar region, small changes to the receptor can alter the ability of the receptor to discriminate between different ligands. A further point of interest is that the original description of the hER cloned sequence contains a presumed cloning error, resulting in a codon for valine at position 400 rather than the true wild type glycine. The valine substituted mutant receptor produces a temperature sensitive alteration in ligand binding affinity. The true wild type receptor (HEGO) binds oestradiol with high affinity at both high and low temperature but the valine mutant (HEO) binds with wild type affinity only at low temperature (4°C) with reduced affinity and stability at 25°C (Tora *et al.* 1989). It has also been found that the partial agonist effect of tamoxifen through HEO is greater than HEGO when these receptors are expressed in an ER negative breast cancer cell line (Jiang *et al.* 1992). In this situation rather than simply a reflection of altered binding specificity accounting for differences seen there appears to be a qualitative change in the nature of the response.

DNA Binding and dimerisation

The DNA binding domain corresponds to the 66 amino acid region C of the receptor and is the most highly conserved part of the receptor superfamily containing two "zinc finger" motifs which comprise four cysteine residues that are able to complex with a zinc atom. (Freedman *et al.* 1988) The zinc finger motif is characteristic of several DNA binding proteins. The C region was confirmed as responsible for DNA binding and ERE specificity by showing that swapping the C domains of ER with that of the GR allowed the hybrid receptor to bind to a GRE (Green *et al.* 1987)

The palindromic nature of the response element suggested that ER would bind as a dimer. This was confirmed by gel shift analysis of full length and an N terminal truncated ER demonstrating an intermediate band when mixed representing a heterodimer of the two different sized proteins (Kumar and Chambon 1988). Further analysis showed that full length ER could not bind a half site ERE. The major dimerisation interface was found to be located within the hormone binding domain since a truncated receptor lacking the HBD could not heterodimerise with full length receptor. However a weak secondary dimerisation interface exists within the DNA binding domain since homodimers of HBD truncated mutants are able to

bind to an ERE (Kumar and Chambon 1988). Further detailed analysis has shown a two amino acid region between the third and fourth cysteines of the N terminal zinc finger and a third amino acid residue between the zinc fingers as critical in determining ERE recognition, permitting transcriptional activity and forms the 'P box', reviewed in (Green and Chambon 1991). A third region, the D box, is important in determining the specificity for the spacing between half sites of the response element.
Crystallographic and NMR analysis has shown that the DBD is monomeric in solution and that on binding DNA a homodimer is formed that co-operatively binds to DNA interacting with the major grove of the double helix. The DBD dimerisation interface is only formed on binding to DNA, the dimerisation interface strengthens the interaction with DNA by contacts with the phosphate backbone of the DNA (Hard *et al.* 1990; Schwabe *et al.* 1990; Schwabe *et al.* 1993) also see (Zilliacus *et al.* 1995).

The major dimerisation interface within the HBD appears to overlap with the ligand binding region (Fawell *et al.* 1990). DNA binding in the absence of the HBD can be detected but with10 fold lower affinity than with the full-length protein. The full length protein is capable of binding DNA in vitro in the absence of ligand although in some circumstances DNA binding is ligand dependent (Metzger *et al.* 1988). Transcriptional interference assays have suggested that ligand is required for DNA binding *in vivo* (Pham *et al.* 1991) unless excess receptor is used in which case DNA binding occurs in the absence of ligand. However, other experiments suggest DNA binding can occur in the absence of ligand (Zhuang *et al.* 1995).

Subcellular localisation

Early studies in which ER was isolated from cytosolic fractions have been misleading. Under steady state conditions the ER s predominantly located in the nucleus (Greene *et al.* 1984; King *et al.* 1984). It does however move between compartments being taken up by the nucleus in an energy dependent manner which requires the presence of nuclear localisation signals located in both the D region and the HBD (Ylikomi *et al.* 1992; Dauvois *et al.* 1993). The ER can exit the nucleus in a passive process. The ER is therefore subject to a nucloecytoplasmic shuttling process. The equilibrium of this process is such that the great majority of ER is located within the nucleus, reviewed in (Defranco *et al.* 1995).

Transcriptional activation

The ER contains two separable activation regions, termed, AF1 located in the N terminal A/B region ,and AF2, located within the HBD. The functional importance of these two regions is variable depending on the context in which they are tested being both cell type and promoter dependent, reviewed in (Green and Chambon 1991).

AF1 functions in 'ligand independent' fashion in that deletion mutants lacking the HBD show constitutive activity, however the activity relative to the full length receptor varies depending on both the cell type and target gene. In many cell types for instance HeLa cells or Cos cells activity is 5% or less of the full length ER. In chicken embryo fibroblasts AF1 contributes up to 70% of full-length receptor activity (Kumar *et al.* 1987; Lees *et al.* 1989; Lees *et al.* 1989; Danielian *et al.* 1992). The promoter system used to test for AF1 activity is also important in determining the relative contribution of AF1 activity. Thus removal of the N terminal domain of the receptor results in little reduction in oestradiol stimulated transcription in HeLa cells from a promoter containing the vitelogenin ERE upstream of the tyrosine kinase promoter. When the promoter for the pS2 gene is used the N-terminal truncation shows only 20% activity of the full length receptor (Kumar *et al.* 1987). The constitutive nature of a truncated receptor lacking the HBD indicates that despite the lower affinity for DNA binding, in transient transfection experiments at least, this receptor is able to bind DNA. There is now accumulating evidence that the N terminal region may also be a target for activation by other signalling pathways particularly EGF and this may be mediated by phosphorylation of the N terminal region at serine 118. The effect of various growth factors on ER function will be discussed later.

AF2 is in most circumstances ligand dependent. Deletion of the C-terminal portion of the MOR identified sequences between 538 and 552 that was required for hormone dependent activation but did not affect the ability of the receptor to bind ligand (Lees *et al.* 1989). Further analysis of this region identified an amino acid sequence in which the residues were consistent with the formation of an amphipathic α helix. This sequence is highly conserved amongst the nuclear receptors. Mutation of pairs of hydrophobic residues within this region virtually abolished ligand induced transcription in mouse 3T3 cells (Danielian *et al.* 1992), without affecting

ligand binding or DNA binding. This confirmed that the structure of this region is critical to the activity of AF2 and has been confirmed by others using similar mutations (Ince *et al.* 1993; Tzukerman *et al.* 1994). Recently it has been shown that mutation of Tyrosine 537 which is also highly conserved, or the equivalent MOR 541 results in strong constitutive activity (Weis *et al.* 1996; White *et al.* 1997). Other mutations within the N terminal portion of the HBD have been shown to affect transcriptional activity without affecting ligand binding affinity (Pakdel *et al.* 1992).

In some situations the integrity of both AF1 and AF2 are required for full activity thus in 3T3 or HepG2 cells there is negligible or weak activity attributable to AF1 alone but deletion of AF1 markedly reduces ligand dependent activation. Some mutations that abolish AF2 activity in the absence of AF1 have partial activity restored if AF1 is present (Danielian *et al.* 1992; Tzukerman *et al.* 1994). AF1 and AF2 therefore show cooperativity. A third activation region was identified in the N-terminal region of the HBD using a yeast screen. A deletion mutant containing residues 179- 339 showed strong constitutive activity in yeast. The activation region was mapped between residues 302 and 339. (Pierrat *et al.* 1994) This was inactive in mammalian cells and therefore it's significance is unclear, however this is the region bound by a transcription cofactor $TAF_{II}30$ and corresponds to a known activation region for the GR.

Crystal structure of the HBD

The crystal structure has been determined for the HBD of unliganded RXRα, the liganded RARα, and TRα (Bourguet *et al.* 1995; Renaud *et al.* 1995; Wagner *et al.* 1995). Most recently the HBD structure for the ER bound to both oestradiol and raloxifene has also been reported (Brzozwski *et al.* 1997) These studies demonstrate a similar overall structure for the ligand binding domain of nuclear receptors .The HBD is composed of 12 α helical regions termed H1-12. However, in some receptors such as ER, H5 and H6 form a continuous helix. These helices are arranged in three antiparallel layers, H5/6, H9 and H10 are sandwiched between two other layers composed of H1-4 and H7, H8 and H11. This scaffold forms a wedge shaped structure with a ligand binding pocket at the narrow end of the wedge. Surrounding the ligand binding region there are 2 antiparallel β strands S1 and S2. The ligand binding region comprises a hydrophobic core

formed by H3, H6, H11, S1 and S2. When bound oestradiol lies enclosed within this compartment. The A ring hydroxyl group of oestradiol forms hydrogen bonds with Glu353 and Arg 394 and the 17α hydroxyl group of the D ring forms hydrogen bonds with His 524. There are a number of non polar contacts with other residues that allow specific recognition of oestradiol. H12 which contains the conserved amphipathic α helix lies across the external surface of the HBD and makes contacts with H3, H5/6 and H11, and forms a 'lid' over the ligand binding pocket but does not contact ligand directly. Structural analysis in the presence of raloxifene shows that this antioestrogen is held in the same ligand binding region and forms analogous hydrogen bonds with Glu 353, Arg 394 and His 524 but this leaves a side chain free which protrudes from the ligand binding cavity. A crucial consequence of this is that the alignment of H12 is disrupted and is unable to form contacts with H5/6 and H11. The positioning of H12 in the raloxifene bound ER is however different to the H12 position reported for unliganded RXRα, where H12 points away from the body of the LBD structure and may be held in this position by H7. In the raloxifene bound ER, H12 lies along a groove formed between H3, H4, and H5/6. It appears therefore, that upon binding ligand, there is a structural alteration causing H12 to swing across the ligand binding pocket locking the ligand in position. The realignement of H12 results in contacts with other regions of the HBD and formation of new surface available for interaction with cofactors important in mediating AF2 activity. Antioestrogens appear to prevent correct alignment of H12 and may as a result interfere with the ability of the ER to interact with cofactors.

Transcriptional regulation via coactivators of the oestrogen receptor

Eucaryotic gene transcription by RNA polymerase II is a highly complex process involving the assembly of an initiation complex comprising multiple components, in association with RNA polymerase. A key component of the complex is TBP (TATA binding protein which forms part of a larger assembly, TFIID), this binds to a TATA box element upstream of the transcription start site. The complex can then respond to stimulation by regulatory transcription factors reviewed in (Sheldon *et al.* 1995). In addition TBP is associated with another set of intermediary molecules which are thought to bridge the interaction of transcription factors and the pre

initiation complex called TAFs, reviewed in (Hernandez 1993; Zawel *et al.* 1993). ER can bind to TBP but it is unlikely that this association is sufficient to mediate transcriptional activation, as it is not abolished by inactivation mutants of AF2. Overexpression of ER leads to squelching of activation suggesting that intermediary proteins are involved in mediating the transcriptional effects of ER (Brou *et al.* 1993). A number of proteins have been isolated that interact with ER in a ligand dependent manner and are candidates for the mediation of ER induced transcriptional regulation. Probably the most significant of these are the p160 family of proteins which include SRC-1/p160, TIF2/GRIP-1, p/CIP/AIBI and the p300/CBP family. These have been demonstrated to enhance ligand induced activation of several nuclear hormones including ER (Onate *et al.* 1995; Kamei *et al.* 1996; Voegel *et al.* 1996; Torchia *et al.* 1997). SRC1 binding is abolished by mutations in H3 and H4 of the HBD known to be important in AF2 activity (Onate *et al.* 1995). Of interest is a recent finding that an SRC-1 like protein AIBI, is amplified and mRNA is overexpressed in some ER positive breast and ovarian cancers (Anzik *et al.* 1997). A number of other proteins have been isolated that interact with nuclear receptors in a ligand dependent manner. These include RIP 140, TIF-1, TRIP-1/SUG-1 (Cavaillès *et al.* 1994; vom Baur *et al.* 1996) The effect of these proteins is less clear since the ability to enhance nuclear receptor mediated transcription is less marked (Cavailles *et al.* 1995). An increasing number of interacting proteins are being identified that interact with different subsets of nuclear hormones some of which appear to act not as coactivators, but as repressors of receptor induced activation. The field of nuclear receptor interacting proteins is still very new and further refinements in our understanding of the true physiological role of these factors can be expected, reviewed in (Horwitz *et al.* 1996; Heery *et al.* 1997). Clearly however the existence of a number of different proteins that have ligand dependent affinity for the ER and can affect the performance of the receptors provides a potential explanation for the heterogeneous responses to oestrogens and other ER ligands.

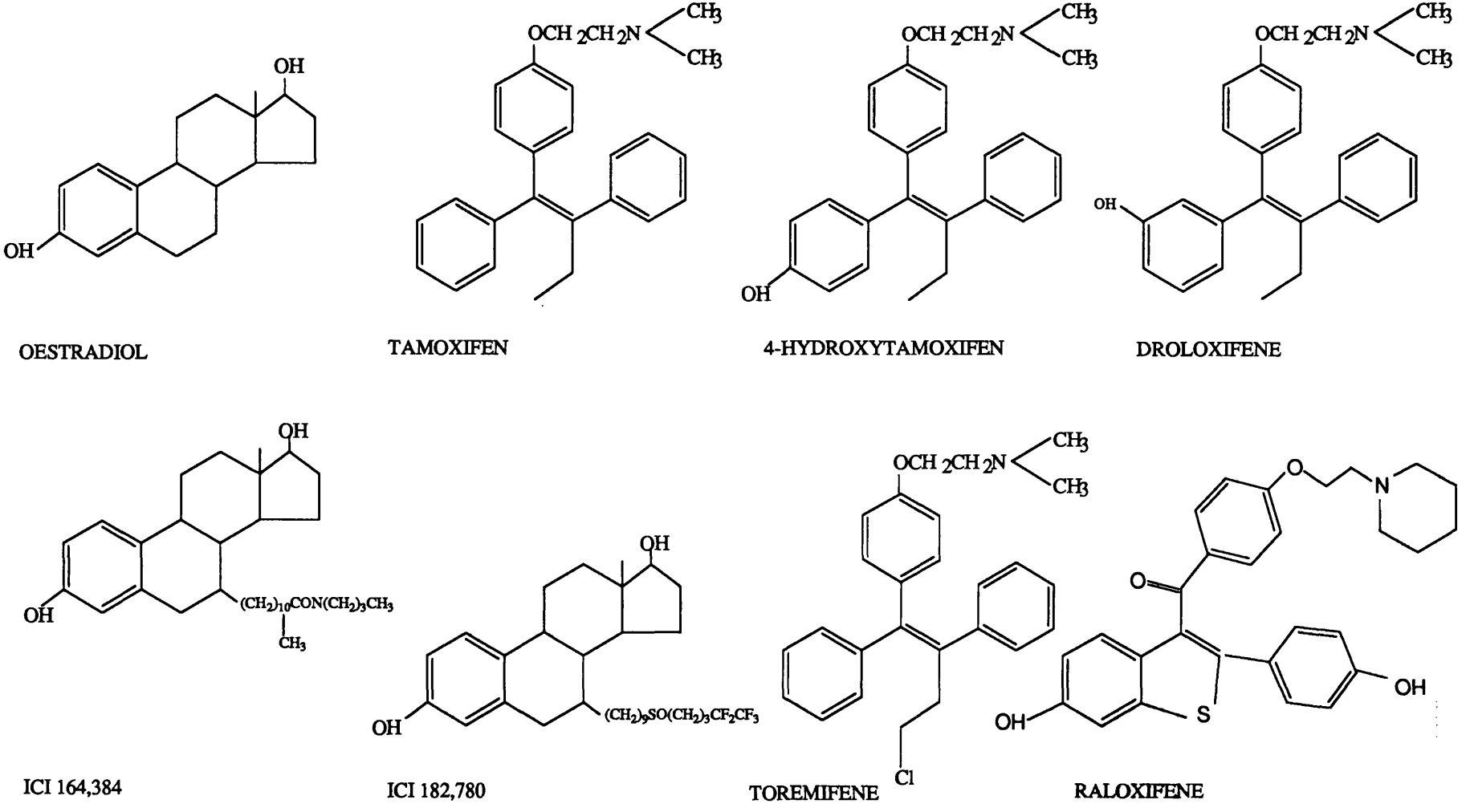
Mechanism of action of Antioestrogens

Antioestrogens are compounds that compete with oestrogens for binding to the ER but fail to elicit the normal agonist effects of oestradiol in effector cells or organs. A large number of such compounds have been developed, many of which are structurally related. Few of these compounds are in routine clinical use but a number of antioestrogens are undergoing clinical evaluation in a variety of clinical applications (Fig 1.2). Antioestrogens can be conveniently divided into the triphenylethelene derivatives or nonsteroidal antioestrogens of which tamoxifen is the principal compound in routine clinical usage. These are often referred to as type I antioestrogens. The other distinct category of antioestrogens, the type II, steroidal or 'pure antioestrogens' are structurally distinct and display different properties. Depending on the system chosen to demonstrate oestrogen antagonism by antioestrogens it is often possible to demonstrate a degree of agonist activity. The triphenylethelene antioestrogens are considered as partial agonists with the degree of agonist activity dependent on the physiological response used to assess activity. Different triphenylethyline antioestrogens show different patterns of partial agonism.

Tamoxifen and related antioestrogens

Tamoxifen or ICI 46,474 a triphenylethylene derivative was originally developed as a potentially less toxic derivative of the first antioestrogen MER 25 (ethamoxytriphetol). This clinically unusable compound was a weak but active antioestrogen with antifertility properties. Tamoxifen was also not clinically useful as originally intended as a post coital contraceptive but the antioestrogenic properties led to development as hormonal therapy for breast cancer.

Fig 1.2 Chemical structures of triphenylethylene and steroidal antioestrogens



OESTRADIOL

TAMOXIFEN

4-HYDROXYTAMOXIFEN

DROLOXIFENE

ICI 164,384

ICI 182,780

TOREMIFENE

RALOXIFENE

Tamoxifen metabolism is complex, for reviews see (Furr *et al.* 1984; Jordan 1984). It is extensively protein bound and has a long biological half life. It is converted in the liver to a variety of related compounds one of which is 4-hydroxytamoxifen. This metabolite has approximately ten fold higher affinity for ER which is comparable to that of oestradiol (Jordan *et al.* 1977). The half life of 4-hyroxytamoxifen is however comparatively short, it is therefore unclear if the metabolite or the parent compound is the most important contributor *in vivo*. Tamoxifen can be isomerised from trans to cis form, the cis form shows a greater degree of agonist activity. Other metabolites such as metabolite E also display more potent agonist properties. Tamoxifen and metabolites are excreted in bile after conjugation.

The partial agonist activity of tamoxifen results in clinically important effects notably the lowering of plasma lipid and cholesterol levels in postmenopausal women and the preservation of bone mineral density (Love *et al.* 1988; Fentiman *et al.* 1989; Turken *et al.* 1989; Love *et al.* 1992; Love *et al.* 1994). These oestrogenic effects may have clinical benefits such as of reduction in cardiovascular disease (McDonald *et al.* 1991; Constantino *et al.* 1997), and reduced osteoporotic complications. Tamoxifen has significant agonist activity in the uterus, in both rats and humans. Uterine weight in the rat is a commonly used assay of oestrogenic activity. In ovariectomised rats oestrogen replacement causes uterine weight gain. This can be reduced in a dose dependent fashion by co-administration of tamoxifen but tamoxifen can not block all uterine weight gain. Tamoxifen alone causes an increase in uterine weight in the ovariectomised rat uterus demonstrating the properties of a partial agonist.

The rat uterine weight assay can be used to compare the relative partial agonist activity of different compounds. Derivatives of tamoxifen such as raloxifene and idoxifene show reduced partial agonist activity and are being evaluated as drugs for the treatment of breast cancer and also as agents for treatment or prevention of osteoporosis.

Tamoxifen competes for ER binding and promotes DNA binding but does not induce the same conformational change as oestradiol. The protease digestion pattern seen in the presence of oestrogen or antioestrogen is different (Allan *et al.* 1992; Tzukerman *et al.* 1994). There is a difference in mobility of the DNA-ER complex seen in band shift assays in the presence of

oestradiol or hydroxytamoxifen. (Kumar and Chambon 1988; Lees *et al.* 1989; Sabbah *et al.* 1991; Emmas *et al.* 1992). The structural basis of this conformational difference has been discussed above with respect to the crystal structure of the HBD. Transient transfection experiments using different reporter constructs and different cell types demonstrate that tamoxifen can stimulate gene transcription but that the relative strength of transcription is highly variable. The degree of agonism seen with hydroxytamoxifen approximates to the contribution of AF1 to the overall activity (Berry *et al.* 1990). The importance of AF1 as a mediator of tamoxifen agonist activity is further demonstrated by the use of truncated ER mutants lacking AF1. In this situation hydroxytamoxifen has no agonist activity but oestradiol retains all or some agonist activity depending on the cell type and reporter construct used (Berry *et al.* 1990), giving rise to the concept that hydroxytamoxifen agonism is mediated solely through AF1. In situations where AF1 makes a significant contribution to the ER mediated transcription there will therefore be an agonist effect from tamoxifen but not in situations where AF1 contributes little. The complexity of the response element may play a role in the degree of partial agonism seen with hydroxytamoxifen since where oestrogen response elements are multimerised the degree of agonist effect seen with hydroxytamoxifen increases (Tonetti *et al.* 1995). There are also differences in the binding affinity for response elements between agonist and antagonist occupied receptor depending on the flanking sequences and spacing between response elements (Anolik *et al.* 1993).

Antioestrogens and growth factor secretion

One puzzling aspect of tamoxifen therapy is the observation that some apparently ER negative tumours respond to tamoxifen therapy and adjuvant tamoxifen is of benefit in ER negative subgroups, particularly postmenopausal women (Bentley *et al.* 1992). This may be in part due to false negative assays or may be because ER negativity is a relative term with an arbitrary definition based initially on the limits of assay techniques. An alternative explanation may be that tamoxifen can act through ER independent mechanisms, as previously mentioned, tamoxifen has been shown to stimulate the secretion of TGFβ not only from breast cancer cells but from stromal fibroblasts (Butta *et al.* 1992). TGFβ secretion is stimulated

by tamoxifen in both ER positive and ER negative breast tumours. A further possibility is that ER negative tumours may contain sufficient ERβ to mediate an antioestrogen response. Tumours expressing only ERβ would be antigenically ERα negative although they would be ER positive by ligand binding methods and therefore clearly can not be a sole explanation since ligand binding assays have been widely used in the past.

Steroidal pure antioestrogens

The steroidal antioestrogens form a separate class of oestrogen antagonist in being almost devoid of any agonist properties. There are important differences in the mechanism of action of these antagonists in comparison to the triphenylethylene antioestrogens, however the data concerning the mechanism of action is in some respects contradictory. This class of antioestrogens are structurally identical to oestradiol with the exeption of a side chain at the 7α position (Fig 1.3). The length of this side chain is important in determining the antagonist properties of the molecule (Bowler *et al.* 1989). Affinity for the ER is similar to that of oestradiol and can competitively inhibit oestradiol binding. ICI 164,384 was demonstrated to have no agonist effect on the uterus (Wakeling *et al.* 1987; Wakeling *et al.* 1988) or transcription (Berry *et al.* 1990; Fawell *et al.* 1990) and to be a powerful antagonist of oestradiol induced MCF-7 cell proliferation (Thompson *et al.* 1989; Wakeling *et al.* 1989)

An unusual effect on receptor content and localisation is seen with pure antioestrogens, with a dramatic down regulation of steady state receptor content in Cos-1 cells (Dauvois *et al.* 1992) mouse uterus (Gibson *et al.* 1991) and primary human breast tumours (DeFriend *et al.* 1994) after exposure to pure antioestrogen compounds. This is associated with a marked increase in receptor turnover and decreased half life for the receptor. This down regulation is accompanied by a subcellular shift of receptor from the nucleus to cytoplasm and is associated with interference of normal nuclear uptake of receptor (Dauvois *et al.* 1992; Dauvois *et al.* 1993). The effect of pure antioestrogens on DNA seems to depend on the experimental system used DNA binding is inhibited *in vitro* in oestrogen receptors overexpressed in insect cells and Cos-1 cells but not from breast cancer cells (Lees *et al.* 1989; Fawell *et al.* 1990; Arbuckle *et al.* 1992). This inhibition of DNA binding can be rescued by co-incubation with the ER antibody MP16. MP16 has the

ability to rescue DNA binding ability in dimerisation deficient mutant ER. The restoration of DNA binding of pure antioestrogen occupied ER by MP16 suggests that the pure antioestrogens inhibit dimerisation (Fawell *et al.* 1990). Other studies have reported no effect of ICI 164,384 on the ability of ER from a variety of sources to bind ERE *in vitro* (Sabbah *et al.* 1991; Metzger *et al.* 1995). However a reduction in receptor stability at 37° C in the presence of ICI 164,384 compared with other ligands has been observed (Metzger *et al.* 1995). It seems therefore that the conditions used to study the effects of DNA binding are critical. *In vivo* evidence using a transcriptional interference assay suggests that these compounds can promote DNA binding. However there is no agonist effect indicating that the pure antioestrogens fail to generate a transcriptionally competent form of the receptor (Pham *et al.* 1991). A possible explanation is that preformed dimers are not disrupted by pure antioestrogens but dimer formation is inhibited. A proposed mechanism of action for the pure antioestrogens is that dimerisation of monomers is inhibited as a consequence of steric interference of the 7α side chain but that the disruption of preformed dimers is less efficient (Arbuckle *et al.* 1992). The reduced receptor content is a result of inhibition of nuclear uptake, perhaps as a result of impaired dimerisation and subsequent degradation (Dauvois *et al.* 1993). In addition binding of pure antioestrogen to any remaining DNA bound ER still leaves the complex in a conformational state that is incapableof activating both AF1 and AF2. This combination of effects results in highly effective antagonism. The relative importance of these mechanisms to the overall effect is not known.

The clinical utility of pure antioestrogens is being evaluated. Because of difficulties with solubility the only compound tested to date is ICI 182,780 administered by intramuscular depot injection. A small study in women with tamoxifen resistant metastatic breast cancer has demonstrated clinical activity (Howell *et al.* 1995). Of interest is the apparent long duration of response in a number of the patients that have responded to treatment (Howell *et al.* 1996)

Effect of mutant oestrogen receptor on antagonist activity

A number of mutant oestrogen receptors have been artificially constructed or isolated from breast cancer cell lines that display altered sensitivity to antioestrogens, stimulating interest in the possibility that ER mutants may result in antioestrogen resistant breast cancer.
A point mutant MOR has been constructed with a mutation in the region known to be important in ligand binding (G-525R.). This receptor is unable to bind oestradiol but retains the ability to bind hydroxytamoxifen and displays normal agonist activity on exposure to hydroxytamoxifen when expressed in CEF cells (Danielian *et al.* 1993). A further point mutation in the hER was identified from a tamoxifen resistant clone derived from MCF-7 breast cancer cells (Wolf *et al.* 1994). This mutation (A-351T) has been expressed in ER negative breast cancer cells in transient and stable expression systems and shows an agonist response to a fixed ring hydroxytamoxifen analogue in transfection experiments (Catherino *et al.* 1995)In stable systems it causes paradoxical growth inhibition. This is similar to the effect of oestradiol when wild type receptor is expressed in these cells. Oestradiol causes a paradoxical growth inhibition but stimulates transiently expressed reporters. (Catherino *et al.* 1995). The interpretation of the mutant receptor experiments is that the antioestrogen has an agonist effect. Further study of antioestrogen effects on MOR mutants has produced unexpected observations. Double mutants of highly conserved H12 hydrophobic residues (L-543A/L-544A and M-547A/L-548A), have been found to function as strong agonists under the influence of both triphenylethylene and pure antioestrogens (Mahfoudi *et al.* 1995). These mutants with defective AF2 activity under oestradiol stimulation were originally used to demonstrate the importance of the C terminal amphipathic α helix. They require the presence of AF1 for this antioestrogen stimulated agonist activity. It is not clear why tamoxifen is able to stimulate strong AF1 dependent activity through these mutants, while being unable to stimulate through the wild type receptor. Interestingly the down regulation of wild type ER with ICI 164,384 is not seen with these mutations. Similar antagonist to agonist switching has now been shown in hER mutations in the analogous region to the MOR mutations. Here the effect has been shown to

be cell type dependent suggesting a role for cofactors in determining the activity of antioestrogens (Montano *et al.* 1996).

Cross coupling of ER signalling with other signalling pathways

In addition to the classical steroid hormone receptor signalling pathway described for the ER, there is increasing evidence that other signalling systems can influence ER responses either by modifying ligand induced responses or inducing ligand independent responses mediated through the ER. The ER has also been shown to affect other signalling pathways. The physiological significance of these observations is not clear but some of these effects may be important aspects of antioestrogen responses.

Signalling that affects ER responses

One of the first observations that ER was able to respond to alternative signals was that the neurotransmitter dopamine was able to induce activation of ER expressed in HeLa cells in the absence of oestrogenic ligand (Power *et al.* 1991). This activation was inhibited by ICI 164,384 but not by hydroxytamoxifen (Smith *et al.* 1993). Stimulation of DNA synthesis induced by the growth factor EGF in rat uterus was blocked by co-administration of ICI 164,384 (Ignar-Trowbridge *et al.* 1992). The stimulation of an ERE reporter gene in uterine cells by EGF, was similarly blocked by ICI 164,384. This EGF effect appears to be mediated through the N terminus of the ER since it is not seen with N terminal deleted ER (Ignar-Trowbridge *et al.* 1993). cAMP and activators of protein kinase C have also been reported as independent activators of ER mediated transcription (Aronica *et al.* 1993; Cho *et al.* 1993; Aronica *et al.* 1994). IGF-1 has been implicated in the modification of response to oestradiol resulting in enhancement of the response to oestradiol but not behaving as an independent activator (Aronica and Katzenellenbogen 1993). In addition to the ligand independent effects the response to antioestrogens is modified. In the presence of IGF or activators of PKA, such as 8 bromocyclic AMP, there is a greater partial agonist response to hydroxytamoxifen (Ince *et al.* 1994). Transcriptionally inactive mutant ER can be 'rescued' by raising intracellular cAMP restoring response of the mutant receptor to oestradiol (Ince *et al.* 1994). Many of these effects may be associated with ER phosphorylation. An important

phosphorylation site is serine 118 of hER, this is phosphorylated during oestradiol treatment (Ali *et al.* 1993), also after stimulation with phorbol esters (Joel *et al.* 1995) and after activation of MAP kinase (Kato *et al.* 1995). EGF induced activation of MAP kinase has been shown to directly phosphorylate serine 118. Substitution of serine 118 by alanine abolishes the ligand independent activation of ER by EGF (Bunone *et al.* 1996). EGF or E2 are still able to phosphorylate this mutant receptor, indicating that other phosphorylation events take place. These further phosphorylation sites are functionally important since mimicking serine 118 phosphorylation by substitution with glutamic acid, which mimics constitutive phosphorylation, does not result in ligand independent activation. EGF is able to activate this mutant producing ligand dependent activity, suggesting that while serine 118 phosphorylation is critical it is not the only phosphorylation site required. While EGF activity is dependent on serine 118 phosphorylation the activation of ER by cAMP is independent of serine 118. It is however, blocked by inhibitors of cAMP activated protein kinase A (El-Tanani *et al.* 1997).

ER influence on other signalling pathways

A number of steroid hormone receptors have been shown to have an influence on the activating protein-1 complex (AP-1). AP-1 refers to a transcription factor complex, which regulates transcription through AP-1 response elements located within a number of genes important in mediating the mitogenic responses to many growth factors. Members of the Fos and Jun families of transcription factors form the principal components of the AP-1 transcription family forming homo or heterodimers that control transcription from AP1 sites. ER has been demonstrated to influence AP-1 responses. In MCF-7 breast cancer cells, IGF-1 or EGF induced stimulation of AP-1 is increased in the presence of oestradiol. When antioestrogens (tamoxifen or ICI164,384) were used there was an inhibitory effect on growth factor stimulated AP1 activity (Philips *et al.* 1993). The AP1 effects are mediated through ER since the effect is magnified by increasing ER expression (Gaub *et al.* 1990; Philips *et al.* 1993). Oestradiol enhancement of AP-1 does not appear to involve DNA binding of the ER since removal of the DBD does not abolish AP-1 enhancement (Webb *et al.* 1995). ER is able to make protein-protein contact with Jun (Webb *et al.* 1995), therefore ER influence on AP1 activity may occur to some extent through DNA

independent contact with AP1 members. The interaction is complex and not fully understood. Forinstance, it has also been observed that after prolonged exposure (4 days), basal AP-1 activity is suppressed by tamoxifen, but the growth factor stimulated AP-1 activity is enhanced by tamoxifen (Astruc *et al.* 1995). In other cell lines such as endometrial or HeLa cells both oestradiol and hydroxytamoxifen activate transcription through the AP1 response element of the **Collagenase**promoter (Webb *et al.* 1995).
In contrast to the oestradiol agonist effect, the tamoxifen agonist effect seen in some cells appears to be dependent on the presence of the ER DNA binding domain (Webb *et al.* 1995). In breast cancer cells there is strong oestradiol induced AP-1 activation but hydroxytamoxifen has no independent stimulatory action. While the data on ER influence is inconsistent and controversial, it is apparent that the influence of ER on AP-1 activity is complex and a potentially important mechanism through which oestrogens and antioestrogens can influence proliferative activity in cells. However, the biological significance of these effects is not clear.

Antioestrogen resistance in breast cancer

Despite the expression of oestrogen receptors in the majority of breast cancers and the clinical utility of oestrogen receptor expression in predicting response to endocrine therapy not all ER positive tumours respond to first line hormone therapy with tamoxifen and are regarded as having innate or de novo resistance. In the context of metastatic breast cancer the development of tamoxifen resistance is regarded as inevitable even after response with a median duration of remission of 12-18 months before this occurs. The mechanisms through which previously hormone dependent tamoxifen sensitive tumours escape hormonal regulation and acquire antioestrogen resistance has been the subject of extensive research but remain to a large extent unknown. Tumours demonstrating tamoxifen resistance can be divided clinically into tumours that respond to second line hormonal manipulation and those that are refractory to further endocrine manipulation. A number of factors can be used to make statistical predictions as to the likely response to second line therapy. The best predictor of response is a history of response to previous endocrine therapy (Henderson 1990; Howell *et al.* 1995). Intuitively, it is the tumours retaining second line sensitivity where we might best be able to identify disturbances in

the specific response to tamoxifen, with other tumours being more likely to show alterations downstream of the hormonal axis or stimulation of proliferative responses through separate mitogenic pathways. However it is arguably more important to identify these mechanisms since we have no effective alternative treatment in this situation. These observations do however clearly demonstrate that tamoxifen resistance is not a single entity and there are likely to be multiple mechanisms.

Oestrogen receptor expression

Since ER negative tumours are largely unresponsive to antioestrogens it has been speculated that antioestrogen resistance may result as a consequence of loss of ER expression. This is unlikely as a mechanistic explanation since isolated loss of ER expression would result in growth arrest in hormonally dependent tissue but may be a marker of escape from hormonal control. ER expression has been compared in primary and secondary tumours and in paired samples where acquired resistance has been observed to develop between sampling time points. While a proportion of tumours do lose ER expression the majority of tumours retain ER expression (Encarnacion *et al.* 1993; Johnston *et al.* 1995). Acquired tamoxifen resistance generated in cell lines is seldom associated with loss of ER expression (Westley *et al.* 1995; Lykkesfeldt 1996)

Tamoxifen pharmacology

No evidence of altered systemic tamoxifen metabolism in tamoxifen resistance has been demonstrated in animal or clinical situations. Tamoxifen and metabolites within tumours have been studied producing interesting results. When MCF-7 xenografts grown to visible size in ovariectomised nude mice are subsequently treated with tamoxifen the tumours initially respond, but after prolonged exposure resistant tumours eventually emerge. Not only are these tumours refractory to the cytostatic effects of tamoxifen, they become stimulated by tamoxifen and are dependent on continued administration of tamoxifen (or oestradiol) for viability. In these resistant tumours the intra tumour tamoxifen concentration is frequently lower (up to 10 fold) than in sensitive tumours. The level of 4-hydroxytamoxifen is also lower and the ratio of the cis and trans isomers of hydroxytamoxifen was altered such that relatively more cis isomer was found (Osborne *et al.* 1991).

Since the cis isomer is a more potent agonist this may account for the resistance. These findings do not explain the tamoxifen dependence of these tumours since it was found that non isomerisable fixed ring analogues of tamoxifen were still able to stimulate growth as were a wide variety of other triphenylethelene antioestrogens (Wolf *et al.* 1993; Osborne *et al.* 1994). In human tumours there are two studies that show a reduced tamoxifen content in some tamoxifen resistant breast cancer samples compared to sensitive tumours (Osborne *et al.* 1992; Johnston *et al.* 1993). The mechanism involved in reduced tamoxifen concentration is not known and an efflux pump mechanism has been postulated. Although it has been noted that tamoxifen resistance is associated with increased expression of P-glycoprotein (Keen *et al.* 1994), tamoxifen is not a substrate for this efflux mechanism. Interest has also been expressed in the intracellular binding capacity for tamoxifen, which in cell extracts is higher than the binding for oestrogens. These so called antioestrogen binding sites, which have not been characterised, are increased in a tamoxifen resistant cell line compared to the parent cell line (Pavlik *et al.* 1992). While antioestrogen binding sites may represent a sink that sequesters tamoxifen it is difficult to see that this would result in a reduced free intracellular tamoxifen level in the steady state unless the bound tamoxifen were eliminated and fresh unbound antioestrogen binding sites synthesised.

Tamoxifen resistance models

In vitro cell culture has been employed extensively to study the acquisition of hormone independence and subsequently antioestrogen resistance. The most commonly used cell line is the MCF-7 breast cancer cell line, although other oestrogen dependent lines such as ZR-75 and T47-D have been used in similar experiments (See Table 3). The MCF-7 cell line will growth arrest, or grow only very slowly in tissue culture after rigorous elimination of oestrogenic stimulus. Exposure to oestrogens results in increased proliferation. MCF-7 cells inoculated into ovariectomised nude mice require oestradiol supplementation in order to form viable tumours, reviewed by (Brunner *et al.* 1990). However cell culture and xenograft techniques have been used to isolate clonal lines derived from hormone sensitive breast cancer cell lines by long term culture in the absence of oestrogen. Initial models of oestrogen dependence were developed by long

term culture in steroid depleted conditions and resulted in clonal selection of cells able to proliferate in the absence of oestradiol. This independence was reversible in some cases. Oestrogen independence did not automatically equate with antioestrogen resistance (Daly *et al.* 1990)

Table 3 Cell line models of aquired Antioestrogen resistance

Name	Origin	Anti-oestrogen	Method	ER	PR	pS2	Comment	Reference
R27	MCF-7	TAM	Agar/tam	wt	wt	wt		Nawata 1981
R3	MCF-7	TAM	Agar/tam	wt	↓	wt		Nawata 1981
RTx6	MCF-7	TAM	Mono/tam	wt	wt	wt		Faye 1983
LY2	MCF-7	LY117018	Mono/tam + soft agar	↓	0	wt		Bronzert 1985
MCF7/TAMR	MCF-7	TAM	Mono/tam	↓	0	R	tam supression of42 Kd Protien lost ICI sensitive	Lykkesfeldt 1986
ZR-75-9a2	ZR-75	TAM	Mono/tam	0	0	-		Van den Berg 1989
RL3	MCF-7	TAM	Mono/tam	wt	-	wt	IGF sensitivity increased IGFR-1 increased pNR-25 decreased	Wiseman 1993
MCF-7 LCC2	MCF-7	TAM	2 step xenograft + Mono/tam	wt	↓	wt	ICI sensitive	Brunner 1993
MCF-7 TOT	MCF-7	TAM	Mono/tam	↓		R		Herman 1994
MCF-7 clone 9	MCF-7	TAM	cDNA library/tam	wt	wt	wt		Toi 1993
Unnamed	MCF-7	TAM	Retrovirus /tam	↓	-	-	Multiple clones (80)	Dorssers 1993
MCF-5-23	MCF-7	TAM	no selection	wt	-	-	ICI sensitive	Hu 1993
MCF-7 164R1	MCF-7	ICI164384	Mono/ICI164	↓	0	-		Lykkesfeldt 1995
MCF-7 182R1	MCF-7	ICI182780	Mono/ICI182	↓	↓	-	tamoxifen sensitive	Lykkesfeldt 1995

A progressive model of acquired oestrogen independence and tamoxifen resistance

In one example a serial collection of cell lines have been developed using xenograft and cell culture techniques to model serial acquisition of first oestrogen independence and then hydroxytamoxifen resistance. The MCF-7 variant MIII was isolated from a tumour xenograft after withdrawal of oestrogen (Clarke *et al.* 1989). This line could establish new tumours in xenografts and could be maintained in cell culture independent of oestradiol. Further xenograft passage resulted in isolation of LCC-1 (Clarke *et al.* 1989), which is able to produce tumours in ovariectomised mice much faster than MIII but proliferation in both lines was still increased in response to oestradiol. LCC-1 was then used to generate a hydroxytamoxifen resistant line by long term culture exposed to hydroxytamoxifen (Brunner *et al.* 1993). This line continues to retain partial oestrogen sensitivity and is growth inhibited by pure antioestrogens. As expected ER expression is maintained in these lines but there is a parallel increase in the invasive and metastatic potential of these derived clones (Thompson *et al.* 1993), reviewed in (Brunner *et al.* 1995). The progression to antioestrogen resistance is also associated with increased constitutive expression of oestrogen responsive genes such as cathepsin D and PR and also marker genes associated with increased invasive/metastatic potential such as mta-1 (Toh *et al.* 1994).

Single step models of antioestrogen resistance

Most antioestrogen cell lines have been produced by prolonged and graded exposure to antioestrogens in tissue culture with derived clones achieving antioestrogen resistance as a 'one step' process. While differences in the expression of oestrogen regulated genes have been demonstrated in many of these cell lines (table 3), the mechanisms generating these changes in gene expression have remained elusive. Nearly all these clones retain ER expression although the level of expression is often different. A further common finding is that virtually all the clones selected for triphenylethylene antioestrogen resistance remain sensitive to pure antioestrogens. More unexpected is the observation that after selection for pure antioestrogen resistance, tamoxifen sensitivity is retained (Lykkesfeldt *et al.* 1995). If this is true also of clinical breast cancer then this may be important since tamoxifen

could then be used as second line treatment after primary treatment with pure antioestrogens.

A further approach to identification of genes involved in development of antioestrogen resistance has been the introduction of cDNA libraries into the breast cancer cells. This approach has to be combined with antioestrogen selection to demonstrate antioestrogen resistance (Toi *et al.* 1993) and therefore it is not clear if isolated clones are "naturally occurring" antioestrogen resistant clones or generated as a result of specific gene inserts. Unfortunately it has not proved possible to recover the putative genes responsible, which could then be reintroduced to demonstrate a causal link. A similar approach, using defective retrovirus integration to increase gene expression has reported success in producing antioestrogen resistance and has identified a common site for viral integration in antioestrogen resistant clones (Dorssers *et al.* 1993). The mechanism of antioestrogen resistance here may be different to the conventionally generated resistant cell lines since these resistant clones do not express oestrogen receptor. It remains to be demonstrated whether altered expression of specific genes in the vicinity of this integration site can result in antioestrogen resistance or whether this is a mechanism that occurs in clinical breast cancers.

Antioestrogen stimulation of breast cancers

A clinical observation that some tumours that have developed tamoxifen resistance regress when tamoxifen is discontinued is well recognised (Legault-poisson *et al.* 1979; Howell *et al.* 1992), occurring in up to 20% of cases in one series (Howell *et al.* 1992). Tamoxifen stimulation and dependence in xenograft models has been described earlier. Tumour cells reimplanted in a second mouse requires the presence of either tamoxifen or oestradiol (Gottardis *et al.* 1988; Osborne *et al.* 1991). These observations clearly demonstrate the ability of breast cancer cells to respond to tamoxifen as a mitogenic agonist and suggests that changes have occurred within the ER signalling system that result in tamoxifen agonism. Mutated ER is clearly a candidate for this type of alteration and has resulted in the previously cited identification of an ER point mutation from one such tumour (Wolf and Jordan 1994). The molecular basis for the development of tamoxifen stimulated growth in most cases remains unknown but since several tissues respond to tamoxifen as an agonist it is not difficult to conceive that changes

in the expression of cofactors or modulators of the oestrogenic signalling pathway to resemble the situation in for instance uterine tissue could produce this type of change.

Abnormal oestrogen receptor species and function in breast cancer

The ER is clearly a crucial element in the generation of oestrogenic responses and in the mediation of the action of antioestrogens. Alteration of ER function is clearly a candidate for the generation of antioestrogen resistance in breast cancer. Site directed mutations and a single example from an in vitro human breast cancer cell line demonstrate the potential for ER mutations to change breast cancer responses. The ER from breast tumours has therefore been the subject of detailed study by several groups over a number of years

Mutations in the ER gene in breast cancer cell lines

While the majority of examinations of ER from breast cancer cell lines have revealed wild type coding sequence a DNA library from one T47D subline known to be genetically highly unstable was found to contain a large variety of ER mutants. cDNA clones coding for a variety of truncated ER species and some containing unique sequences have been isolated from this subline. Perhaps the most interesting of these contains a point deletion within exon 5 that results in a premature stop codon, and is predicted to encode a truncated receptor containing 417 amino acids with a unique 7 amino acid carboxyl tail (Graham *et al.* 1990). The function of this mutant receptor has not been studied. The T47Dco subline shows marked heterogeneity in PR expression and it is possible that mutant ER is responsible for constitutive triggering of PR expression. The concern with this model is that since the genetic diversity is so marked the ER mutants may simply be a marker of genetic instability, rather than functionally important in themselves.

Amplification of ER in breast cancer

Breast cancers have been examined for alterations in the ER gene at both DNA and RNA level There is little evidence for substantial ER gene amplification or gross gene disruption with one study showing no amplification or gross rearrangement of the gene (Koh *et al* 1989), and

another study demonstrating at maximum a three fold amplification in some tumours (Nembrot *et al.* 1990).

Mutations in the ER gene in breast tumours

A restriction fragment polymorphism RFLP was identified in breast cancers (Garcia *et al.* 1988) and shown to result from a single base pair change at nucleotide 257 resulting in a C-T substitution at amino acid position 86. (Garcia,1989). Although this is now regarded as a polymorphism having been isolated in germ lines it has been linked to an apparent increased incidence of spontaneous abortion (Lehrer et al 1990). Further studies of ER from breast cancers have used single strand conformational polymorphism analysis to identify mutant ER. Karnik and co-workers analysed 40 tumour samples consisting of equal numbers of hormone sensitive and hormone resistant tumours (some of these were available as pre and post tamoxifen pairs). They used RT-PCR followed by single strand chain polymorphism (SSCP), to isolate mutant receptor sequences. They found in one resistant tumour a rearrangement in exon 6 where 47 nucleotides were replaced by 42 nucleotides from exon 5 which is predicted to produce a truncated protein 454 amino acids in length (premature stop codon). This mutant has not been functionally analysed but it may behave in a similar way to the exon 5 deletion variant. The mutation was heterozygous since wild type sequence was also present and the tumour was ER positive by immunoassay (H222 antibody). A single base pair deletion at codon 432 was identified in the resistant metastasis of a further tumour sample but not the corresponding primary tumour material. This mutation produces a premature termination sequence at amino acid 437. A third mutant, a point mutation in a sensitive tumour leading to a Glu to Val substitution at amino acid 352 was identified. In addition a number of silent mutations were also noted (Karnik *et al.* 1994). Roodi and co-workers analysed 118 ER positive and 70 ER negative primary tumours, only one of which carried a potentially functional change. They identified two mutations in codons 69 (Asn to Lys) and 396 (Met to Val) from the same tumour. Silent mutations or polymorphisms were also identified. A silent polymorphism at codon 325 was found to be present in 28% of patients and presence of this polymorphism correlated with a family history of breast cancer (Roodi *et al.* 1995).

These studies reported during the course of the work presented here show that ER mutations do occur in breast cancers but with low frequency. Although mutations are unlikely to represent a common mechanism for hormone resistance the methods used to screen for mutations will inevitably miss some mutations and if these occurred at specific hotspots they might conceivably be more common than anticipated. It was therefore appropriate to attempt to identify specific mutations that have the potential to produce antioestrogen resistance and then direct more comprehensive mutational analysis towards areas of specific interest. It is on this basis that breast cancer samples were subjected to rigorous examination for mutations within the C- terminal portion of the hormone binding domain. These experiments are described in chapter 4. Experiments to identify tamoxifen stimulated ER mutants using a yeast screening system were performed as described in chapter 5.

Splice Variant oestrogen receptor mRNA

The human oestrogen receptor gene transcript contains 8 coding exons, which give rise to a 595 amino acid protein (See Fig 3.1). The hER mRNA is a 6.5 Kb in size. Shorter mRNA fragments have been isolated from breast tumours (Murphy *et al.* 1989). These transcripts were found to contain 5' ER sequence spliced to non ER sequence and in one instance to an unrelated sequence derived from a different chromosome (Dotzlaw *et al.* 1992). ER mRNA from breast cancers have been extensively studied and a number of variants have been described lacking internal coding sequences. RNA isolated initially from the paradoxical ER negative PR positive phenotype breast cancers has been analysed by RT-PCR and revealed transcripts containing deletions of entire coding exons. The first of these identified a cDNA clone containing exon four sequence fused to exon six sequence eliminating the exon 5 coding region (Fuqua *et al.* 1991). Subsequently an increasing number of splice variant ER mRNA species have been isolated from a variety of both normal and neoplastic tissue that lack one of the internal coding exons 2-7. In addition variants lacking multiple internal exons have been described (Gotteland *et al.* 1995; Pfeffer *et al.* 1995). Little is known about the mechanisms controlling the appearance of splice variant ER mRNA and also little is known about their physiological significance. The splice variant mRNAs are predicted to result in the

generation of truncated oestrogen receptor proteins. These are of two types, firstly proteins with internal deletions corresponding to the missing exon sequence as in exon 3 and exon 4 deletions and secondly proteins that contain C-terminal truncations, these arise because the new splice junctions introduce a reading frame shift in the coding sequence. The frame shift results in the generation of a premature stop codon preceded by a short unique sequence of amino acids. The splice variants therefore are all predicted to encode incomplete proteins but because of the nature of the truncations produced they lead to proteins which are functionally distinct.

The Exon 5 splice variant ER

The exon 5 variant is perhaps the most interesting of the splice variant ER species. It contains the coding region up to amino acid 366 and due to a reading frame shift a further 5 codons that are unrelated to full length sequence, followed by a stop codon. This receptor retains the N-terminal activation region AF-1, the entire DBD, the nuclear localisation signal and the first 64 amino acids of the HBD. It lacks regions essential to AF2 activity, ligand binding and the major dimerisation interface within the HBD. It was first isolated from a human breast tumour with an ER negative PR positive phenotype (Fuqua *et al.* 1991). These receptor assays were based on ligand binding. The variant receptor was shown to have constitutive transcriptional activity in a yeast transcription assay. This was unaffected by addition of full length receptor thus it is described as a dominant positive.

The protein products of each of the commonly reported splice variants are summarised in Fig 3.1 and are discussed further in the discussion chapter.

The identification of the exon 5 variant from an ER negative PR positive tumour immediately suggested the possibility that the variant receptor was inducing the expression of PR, explaining PR expression in the apparent absence of ER. Furthermore this raised the possibility that a constitutively active ER variant could contribute to the progression of breast cancer towards an oestrogen independent state stimulating growth in the absence of oestrogen. This possibility is further extended to suggest that the expression of dominant positive ER of this type that could not bind tamoxifen could result in antioestrogen resistance. The expression of this variant has been studied in detail and it was noted that although isolated

from an apparently ER negative tumour wild type mRNA was still detectable by PCR (Fuqua *et al.* 1991). In fact in all cases where the exon 5 variant has been detected it has been in association with full length mRNA. The exon 5 variant has been detected in tumours of all ER and PR receptor phenotypes reflecting the increased sensitivity of PCR over immunoassay or ligand binding assay. The variant has not only been isolated from breast cancers and breast cancer cell lines but has been isolated from normal uterus and normal breast epithelium (Fuqua *et al.* 1991; Gotteland *et al.* 1995; Pfeffer *et al.* 1995).

Clearly the role of the exon 5 variant in the growth of breast cancer is an important issue which may shed light on the development of oestrogen independence. If involved in the generation of antioestrogen resistance, it has important implications regarding the development of new pharmacological agents for the treatment of antioestrogen resistant breast cancer. Experiments to examine the functional significance of the exon 5 variant ER have been performed and are described in chapter 3.

Oestrogen receptor DNA binding in breast cancer

In an attempt to study functional aspects of ER from breast cancers the DNA binding of ER has been examined. DNA binding activity did not always correlate with the ER level determined by immunoassay. Western blotting showed truncated ER protein bands of around 50 KD (Scott *et al.* 1991). This and similar studies are difficult to interpret since ex vivo proteolysis (despite appropriate handling) may occur. The high degree of non concordance of DNA binding and immunoassay in tumour samples is not seen in analysis of resistant cell lines does lead to concern over the validity of these observations.

Chapter 2

Materials and Methods

MATERIALS.

Chemicals.

All general chemicals and solvents were of analytical grade and obtained from either Sigma Chemicals Ltd Poole, UK, BDH Chemicals Ltd, Poole UK. or FSA Laboratory Supplies, Loughborough UK. Additional reagents are listed.

Absolute alcohol	Hayman Ltd Witham UK.
Acrylamide	Boehringer Mannheim UK.
Agarose	Flowgen.
Ammonium persulphate	Bio-Rad, UK.
Ampicillin	Beechams Research Laboratories UK.
Bromophenol blue	Bio-Rad.
Casamino Acids	Difco Laboratories
Dithiothreitol	Bio-Rad.
Dextran T-70	Pharmacia Biotech UK.
Hygromycin B	Calbiochem - Novabiochem Corporation California USA.
TEMED	Bio-rad
Tween-20	Bio-rad
SDS	Serva Feinbiochemica GMBH & Co.
Yeast nitrogen base w/o amino acids	Difco
X - gal	Calbiochem -Novabiochem,

Radiochemicals

All supplied by Amersham International PLC (Amersham UK)

[1 ^{14}C] acetyl-coenzyme A	50-60 mCi/mmol.
[γ-^{32}P] ATP	3000 Ci/mmol
[α^{32}P] dCTP	3000 Ci/mmol
[α^{35}S] dATP	400 Ci/ mmol
[2, 4, 6 ,7,-^{3}H] oestradiol	96 Ci/ mmol

Enzymes	
Restriction endonucleases,	New England Biolabs, USA
Calf intestinal phosphatase,	Boehringer Mannheim
T4 DNA ligase	Gibco BRL, UK.
T4 Polynucleotide kinase	Pharmacia Biotech
Taq polymerase	ICRF
Membranes	
Hybond N	Amersham
Hybond N+	
NA 45 DEAE	Schliecher and Schuell, Germany.
Nitrocelulose	Schliecher and Schuell,
Plasmids	
pSG5	Stratagene
pMEP4	Invitrogen
pSG5 HEGO	P Chambon Strasbourg France
ERE PBL CAT	Molecular Endocrinology
ERE2 TATA CAT	ICRF
pJ3 MOR	
pJ3 MOR MUTANTS	
Yeast Expression Plasmids	
pCup hER Mt1	Ian Purvis (Glaxo research)
Yeast reporters	
pRLΔ21U3ERE	P Chambon
pYCpEREIacZ	Ian Purvis
Miscellaneous	
Cyanogen bromide activated sepharose	Pharmacia
CloneAmp™ system.	Life Technologies Inc.
Dried skimmed milk powder	Boots Plc, UK
Film	
RX	Fuji

XAR	KODAK
Filtration units	Nalgene Co Rochester USA.
Galactolight β-galactosidase assay kit	TROPIX Inc. Massachusetts, USA.
T7 Sequenase sequencing kit (version 2.0)	U.S. BiochemicalCorporation, USA.
ECL detection reagent kit	Amersham.
Ready To Go DNA labelling kit	Pharmacia
Nick columns	Pharmacia
Wizard Maxiprep DNA purification system	Promega.
Cycle sequencing dye terminator kit	Perkin Elmer Corporation USA

Buffers. and stock solutions

All solutions were prepared using quartz distilled and deionised water and were stored at room temperature except where stated otherwise.

CIP buffer (10x)	0.5 M Tris-HCl, 1 mM EDTA pH8.5 (stored at 4°C)
DCC suspension	0.025% (w/v) dextran, 0.25% (w/v) charcoal. in 10 mM Tris-HCl pH 7.4, 1 mM EDTA
T4 DNA ligase buffer (5x)	0.25 M Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG # 8000 (stored at -20°C)
Digestion buffer	100 mM NaCl, 10 mM Tris-HCl pH7.4, 25 mM EDTA, 0.5% (w/v) SDS
DNA loading buffer (5x)	0.25% bromophenol blue, 5x TBE 25% (v/v) glycerol
Galacto-light reaction buffer diluent	100 mM sodium phosphate pH 8.0, 1 mM $MgCl_2$(stored at 4°C)
HBS (2x)	40 mM HEPES, 275 mM NaCl pH 7.1, (filter sterilised and stored at 4°C)
Kinase buffer (10x)	0.5 M Tris-HCl pH 7.4, 100mM $MgCl_2$, 1 mM EDTA pH 8.0
Ligand binding buffer	10 mM Tris-HCl pH7.4, 1 mM EDTA, 0.1% (w/v) ovalbumin.

Luciferase/CAT/β-gal EDTA lysis buffer	0.65% NP40, 10 mM Tris-HCl pH 8.0, 1mM EDTA 150 mM NaCl,
Luciferase reaction buffer	25 mM glycylglycine pH7.8, 5 mM ATP pH 8.0, 15 mM $MgSO_4$ (stored at -20°C)
Mineral Stock (1000x)	2 mM FeCl3, 0.8M $MgSO_4$
NTE buffer (1x)	0.1 M NaCl, 10 mM-Tris-HCl pH 7.5, 1 mM EDTA pH 8.0
PBSA	140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2 PO_4
Phosphate buffer for X-gal Plates (10x)	1M KH_2PO4, 0.15M $(NH4)_2SO_4$, 0.75M KOH
Protein loading buffer (2x)	4% (w/v) SDS, 125 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 0.05% bromophenol blue, 2% mercaptoethanol
Repair buffer (10x)	0.5 M Tris-HCl pH 7.4, 70 mM $MgCl_2$, 10 mM DTT, (stored at -20°C)
Restriction enzyme buffers (stored at -20°C)	
low salt (10x)	100mM Tris-HCl, 100mM Mg Cl_2, 10 mM DTT, pH 7.9
medium salt (10x)	As above with 0.5 M NaCl.
high salt (10x)	As above with 1.0 M NaCl.
RIPA (2x)	50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate 0.1% SDS, (stored at 4°C)
Running Buffer for column purification	TBS with 0.1% NP40
SDS-PAGE buffer	25 mM Tris base, 190 mM glycine, 0.1% (w/v) SDS,
STET buffer	8% (w/v) sucrose, 0.5% Triton X-100 (v/v), 50mM EDTA, 10 mM Tris-HCl pH 8.0
TBE (10x)	0.89 M Tris base, 0.89 M boric acid, 25 mM EDTA
TBS	25 mM Tris-HCl pH8.2, 144 mMNaCl
TE (1x)	10 mM Tris-HCl pH 8.0, 1mM EDTA
TEL buffer	100 mM Lithium acetate in TE, pH 8.0
Tfb1	30 mM potassium acetate, 100 mM rubidium

(filter sterilised)	chloride, 10 mM $CaCl_2$, 50 mM Manganese chloride 15% glycerol (v/v) pH 5.8 (with acetic acid and stored at 4°C)
Tfb2	10 mM MOPS, 75mM $CaCl_2$ 10 mM rubidium
(filter sterilised)	chloride, 15% glycerol (v/v), pH 6.5 (with KOH, stored at 4°C)
Vitamin stock (100x)	0.04 mg/ml Thiamine, 2 µg/ml biotin, 0.04 mg/ml pyridoxine, 0.2 mg/ml inositol, 0.04 mg/ml pantothenic acid (filter sterilised and stored at 4°C)
Western blot	192 mM glycine , 25 mM Tris base,
Transfer buffer	20% methanol
Whole cell extract	0.4 M KCl, 20 mM HEPES pH 7.4, 1 mM DTT
buffer	20% (v/v) glycerol, 0.5 mg/ml bacitracin, 40 µg/ml PMSF, 5µg/ml pepstatin A 5 µg/ml leupeptin. 10 µg/ml aprotinin (stored at 4°C, DTT and protease inhibitors added immediately prior to use).
Yeast lysis buffer	50 mM HEPES pH 7.9, 5mM EDTA, 5 mM
EGTA,	
for protein analysis	0.5 mg/ml bacitracin , 40 µg/ml PMSF, 5µg/ml pepstatin A, 5µg/ml leupeptin,. 10 µg/ml aprotinin 1mM DTT (stored at 4°C, DTT and protease inhibitors added immediately prior to use).
Yeast lysis buffer	100mM Tris-HCl pH 7.5 0.05% (v/v) Triton X-100
Z buffer	60 mM Na_2HPO4, 40 mM NaH_2 PO4 10 mM KCl, 1mM $MgSO_4$ pH 7.0

Bacterial Media and Plates.

L-broth	1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5%(w/v) NaCl, 0.1% (w/v) glucose.
L plates	L-broth with 1.5 (w/v) bactoagar,
Ψ broth	2% (w/v) bactotryptone 0.5% (w/v) yeast extract, 0.5% (w/v) $MgSO_4$ (pH 7.6 with KOH)
Ψ plates	Ψ broth with 1.4% bactoagar.

SOC	2% (w/v) bactotryptone, 0.5% (w/v) yeast extract 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose

Yeast Media and Plates.

YP media	2.2% (w/v) bactopeptone 1.1% (w/v) yeast extract, 0.0055% Adenine sulphate. autoclaved and then made up to 2% glucose from 20% (w/v) glucose stock
YP plates	YP medium with 2.2% (w/v) bactoagar

Selective medium

Selective medium lacking essential amino acids are prepared by adding casaminoacids deficient in uracil and tryptophan or by adding a mix of all required amino acids except those for which selection is desired (dropout mix).,described in Rose et al (1990).

Minus uracil minus tryptophan mg/ml medium (-ut)	1.1% (w/v) Casaminoacids, 0.8% (w/v) Yeast nitrogen base (w/o amino acids) 0.1 tyrosine, 0.16 mg/ml adenine, 2% glucose.
Minus uracil medium (-u)	For uracil selection tryptophan is added to -ut medium at a concentration of 0.1 mg/ml by adding from a 5 mg/ml stock (filter sterilised)
Minus leucine medium (-l)	0.2% (w/v) Dropout mix (-leucine), 0.8% (w/v) Yeast nitrogen base (w/o amino acids), 0.1 mg/ml tyrosine, 0.16 mg/ml adenine, 2% (w/v)glucose.
Minus leucine-minus uracil medium (-ul)	As -l medium using dropout mix without uracil and leucine
Selective plates	Composed of selective medium and 2.2% agar
X-gal indicator plates	Composed of 2% agar 1x phosphate buffer 1x mineral stock, 1x vitamin stock, 0.02% (w/v) dropout mix (minus uracil and leucine)

50 μM X-gal, 50μM $CuSO_4$

Cell culture materials and media.

Chick serum	Sigma
Dulbecco's modified	ICRF central services media supplies
Eagles medium	(suplied with and without phenol red)
Foetal calf serum	Gibco BRL, UK.
Trypsin	0.01% (w/v) trypsin 0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.01% (w/v) Na_2HPO_4 0.01% (w/v) dextrose, 0.3% Tris-HCl pH 7.7, 0.01% streptomycin, 100U/ml penicillin
Versene	0.02% (w/v) EDTA in PBSA with phenol red.
Tissue culture flasks	Falcon
Tissue culture plates	Nunc

Archival human tumour sample

Breast cancer samples were provided by Diana Barnes (Guy's Hospital) and M Dowsett and S Johnston (Royal Marsden Hospital)

METHODS.

Bacterial Transformation.

preparation of competent DH5α strain Escherichia *coli*

(Scott and Simanis unpublished)

DH5α bacterial colonies were streaked out and grown to 2 mm on Ψ plates. Single colonies were inoculated into 5 ml Ψ broth, grown at 37°C with continuous agitation to an OD595 0.3. Bacteria were added to 100 ml prewarmed Ψ broth and grown to an OD595 of 0.48. The culture was chilled on ice and bacteria pelleted by centrifugation at 3000 rpm at 4°C. The pellet was re suspended in 40 ml cold TfbI and incubated on ice for 5 minutes. the bacteria were pelleted again by centrifugation at 4°C, re suspended in 4 ml TB 2 and the cells snap frozen on dry ice in 0.3 ml aliquots. and stored at -70°C.

Transformation of DH5α strain Escherichia *coli*

Competent cells were thawed on ice and 25-100 µl of cells added to 10-25 µl of ice cold DNA sample or ligation mix. Bacteria were incubated with DNA for 30 minutes on ice followed by heat shock for 90 seconds at 37°C, bacteria were returned to ice for 30 seconds. Following this 4 volumes of L-broth were added and bacteria were incubated at 37°C. Bacteria were then spread onto L-broth plates containing 100 µg/ml Ampicillin and grown at 37°C overnight.

Preparation of competent SCS-1 *Escherichia coli*

(Modified from Young and Goodbourne, unpublished)

SCS-1 strain *Escherichia coli* were streaked onto LB plates and grown overnight. Three colonies were inoculated into 20 ml prewarmed L-broth and grown at 37°C with continuous agitation to an OD595 0.2, the culture was added to 80 ml warm L-broth and grown to OD595 0.2. The culture was added to 250 ml L-broth and grown to an OD595 0.4. The culture was cooled on ice for 5 minutes and bacteria pelleted by centrifugation at 2500 rpm for 10 minutes. Cells were re suspended in 200 ml cold water pelleted again by centrifugation for 10 minutes at 4°C re suspended in 100 ml cold water repelleted by centrifugation. Bacteria were re suspended in 20 ml

water with 10%v/v glycerol. After re pelleting bacteria were re suspended in 1/2 the pellet volume with water and 10% (v/v)glycerol. 100 µl aliquots were snap frozen on dry ice and stored at -70°C.

Transformation of competent SCS-1 *Escherichia coli*

Bacteria were thawed on ice mixed and 40 µl added to 4 ml DNA prepared from yeast in a precooled 0.1 cm electroporation cuvette. Bacteria were electroporated using 1.8 kV, 200 Ohms and 25 mF settings of a Bio-Rad Gene-Pulsar electroporation apparatus., producing a time constant of 4.5 - 4.9 milliseconds. Immediately after electroporation 1 ml SOC was added and the bacteria incubated for 60 minutes at 37°C. Bacteria were then spread onto L-broth plates and incubated at 37°C overnight.

Preparation of Plasmid DNA

Plasmid DNA was prepared by both small and large scale methods depending on the quantities required.

Small scale preparation of DNA (miniprep)

Bacterial colonies or glycerol stocks of transformed bacteria were inoculated into 5 ml L-broth with 80 µg/ml ampicillin. Cultures were grown overnight and 1 ml culture centrifuged in a microfuge. the bacterial pellet was re suspended in 0.35 ml STET buffer with 0.5 mg /ml lysozyme. The bacteria were placed in a boiling water bath for 40 seconds and then centrifuged in a microfuge for 10 minutes. The pellet was carefully removed with a toothpick and DNA precipitated by the addition of 40 µl 3M sodium acetate pH 7.0 and 420 µl isopropanol and freezing on dry ice for 30 minutes. The DNA was pelleted by centrifugation for 10 minutes in a microfuge , liquid removed by aspiration and the pellet air dried for 30 minutes. DNA was then dissolved in 50 µl distilled water.

Large scale plasmid preparation

Caesium chloride density gradient method(modified from D Ish-Horowitz unpublished ICRF)

400 ml L-broth containing 80 µg ml ampicillin was inoculated from a colony or glycerol stock and grown overnight at 37°C. Bacteria were harvested by centrifugation at 6,000g for 10 minutes. The pellet was

resuspended in 20 ml TE containing 0.05M glucose. 100 mg lysozyme was added and cells lysed at room temperature for 10 minutes. 30 ml 0.2 M NaOH, 1% SDS was added mixed and incubated for 5 minutes on ice, after which 25 ml 5M potassium acetate pH 4.8 was added and incubated for a further 15 minutes on ice. The solution was centrifuged at 6,000g for 10 minutes and filtered through sterile muslin gauze. Plasmid DNA was precipitated by the addition of 52 ml isopropanol and centrifugation at 7,000g for 10 minutes. Liquid was poured off and the pellet air dried. The pellet was re suspended in 8.5 ml TE and 0.7 ml 0.5M EDTA pH 8.0 and 240 µl 1M Tris base pH 8.0 added, to this 10.5g caesium chloride was added with 100 ml ethidium bromide (5 mg/ml). After dissolving the caesium chloride the solution was used to fill a Beckman 5/8 x3" quick- seal centrifuge . before heat sealing heat sealing tubes were balanced and the centrifuged at 64,000 rpm for 24 hours in a Beckman L3 50 ultracentrifuge. Plasmid DNA was visualised under uv illumination and super coiled plasmid DNA removed with a19 gauge needle and syringe. The aspirated sample was made up to 5 ml with water and 5 ml isobutanol added. The organic phase was removed and the aqueous phase extracted twice more with isobutanol. The DNA was then precipitated by addition of 2.5 volumes of ethanol and incubation at -20^{0}C for 20 minutes. DNA was recovered by centrifugation at 10,000g for 10 minutes. liquid poured off and the pellet washed with 70% ice cold ethanol. Briefly centrifuged at 10.000g, the ethanol poured off and the pellet air dried. the pellet was then re suspended in 500 µl TE or water. DNA was quantified by measurement of OD_{260} and OD_{280} of an appropriately diluted aliquot. Concentration was calculated taking an OD_{260} of 1.0 = 50 µg/ml the ratio of OD_{260} to OD_{280} was required to be 2.0 for adequate purity. DNA was also visualised on agarose gel for quality.

Promega Wizard Maxiprep Method

This commercial kit supplied by Promega was used for latter work. 400 ml of bacterial culture obtained as above was centrifuged at 5,000 g for 10 minutes and the bacterial pellet re suspended in 15 ml 50 mM Tris base pH 7.5 10mM EDTA 100 µg/ml RNase A. Bacteria were lysed by addition of 15 ml 0.2 M NaOH 1% SDS. after 5 minutes intermittent mixing at room temperature the mixture was neutralised with 15 ml 1.32 M potassium acetate pH 4,8. The solution was centrifuged at 14.000g for 10 minutes at

4°C strained through sterile muslin gauze and 0.6 volumes isopropanol added to precipitate the DNA. The DNA was collected by centrifugation at 14,000g for 10 minutes at 4°C, the pellet re suspended in 2 ml TE. The DNA was purified by adding 10 ml purification resin to the DNA mixed and poured into a Maxicolumn on a vacuum manifold This was washed twice with 12 ml ethanol based column wash followed by 5 ml 80% ethanol. The resin was dried on the vacuum manifold and DNA eluted by addition of 1.5 ml distilled water at 68°C. The DNA was collected by centrifugation of the maxicolumn at 1,300g. The DNA was re precipitated by the addition of 30µl 5M NaCl and 2.5 volumes of ethanol freezing on dry ice and centrifuging at 10,000g for 10 minutes at 4°C. followed by washing with 70% ethanol. The DNA was air dried and re suspended in 200 µl water, concentration and purity determined by measurement of OD_{260} and OD_{280} Quality was determined by visualisation on agarose gel.

Isolation of genomic DNA from breast cancer samples

Tissue samples were recovered from storage at -70°C and were broken to a fine powder in a dismembranator. Samples were kept frozen by immersing the dismembranator chambers in liquid nitrogen. DNA was released by overnight incubation in 200 µl digestion buffer containing 0.5 mg/ml protienase K. Samples were centrifuged for 10 minutes to remove insoluble debris and DNA precipitated from the supernatant

DNA manipulation and cloning.

Restriction endonuclease digestion.

Restriction enzyme digests were performed using buffers recommended and supplied by the manufacturer. Double enzyme digests were performed using buffers recommended by New England Biolabs. DNA was digested at 37°C using a 5 fold excess of enzyme in a volume not less than 20 µl. Enzyme reactions were terminated by the addition of DNA loading buffer or by extraction with an equal volume of phenol/chloroform, followed by ethanol precipitation. This was performed by the addition of 5M NaCl to a concentration of 100 mM and 2.5 volumes 100% ethanol, the mixture frozen on dry ice for 30 minutes and centrifuged in a microfuge for 10 minutes. The liquid was removed and the DNA washed with 70% ice cold ethanol re centrifuged and air dried prior to re suspension.

Agarose gel electrophoresis of DNA

Agarose gels of 1-2% were prepared by boiling agarose in 1xTBE in a microwave oven. The solution was cooled to approximately 60°C prior to the addition of ethidium bromide to a concentration of 1µg/ml. Agarose solution was poured onto casting a casting tray and slot formers inserted. Once set, the gel was immersed in 1xTBE and DNA samples loaded and run at 2- 7.5V/cm until DNA fragments were separated. DNA was visualised by uv illumination. Fragment size was assessed by running DNA size markers with the DNA samples Such as Lambda phage DNA digested with Hind III, or Hind III and EcoRI. or pSP65 digested with Hinf I.

Purification of DNA fragments

Restriction enzyme digests were run on fresh agarose gels with fresh buffer. When well separated fragments were either directly cut out of the gel or transferred to NA-45 DEAE membrane by cutting the gel in front of and behind the band and inserting a membrane either side of the band. The gel was run until the fragment had moved onto the membrane. The membrane was removed from the gel and DNA eluted in 250 µl 1M NaCl at 70°C for 20 minutes. The membrane was removed and DNA was recovered by ethanol precipitation. Fragments cut from the gel were recovered using Gene Clean (Bio101 Inc.). In this method the manufacturers instructions were followed. The agarose was dissolved at 55°C in a solution of potassium iodide and TBE modifier solution, followed by the addition of Glassmilk and incubated on ice for 15 minutes to bind DNA. The mixture was briefly centrifuged in a microfuge and liquid removed. The glassmilk pellet was washed twice with "NewWash" and once with 80% ethanol. The DNA was then recovered by adding 25 µl of water and incubating at 55°C for 10 minutes The sample was centrifuged and the liquid removed and stored in a new tube. Adequate recovery of purified fragments were confirmed by gel electrophoresis of an aliquot.

Preparation of vectors for cloning

2 µg of plasmid DNA was cut with the required restriction endonucleases. which were then removed by phenol chloroform extraction followed by ethanol precipitation of the cut DNA. The DNA was re

suspended in phosphatase buffer and 20 units calf intestinal phosphatase added followed by incubation at 37°C for 30 minutes to remove the 5' terminal phosphate thus preventing re ligation. The sample was re extracted with phenol /chloroform, ethanol precipitated and re suspended in water at 20 ng/µl

Oligonucleotide kinasing and annealing

To construct the exon 5 deletion coding sequence complementary oligonucleotides were synthesised. These were kinased together prior to annealing by mixing 100 ng of each oligonucleotide in 20 µl kinase buffer containing 1mM dATP and 5 mM DTT 10 units of T4 polynucleotide kinase was added and incubated for 60 minutes at 37°C. 10 µl NTE was added and volume made up to 100 µl with water. The sample was annealed by heating to 80°C and cooling slowly to room temperature.

Ligations

Most ligations were carried out using 20 ng of prepared vector and variable amounts of insert DNA ranging from equimolar to 10x excess insert. consisting of PCR products gel purified DNA fragments or annealed oligonucleotides. The ligation was performed in 20 µl 1x ligase buffer with 1-2 units of T4 DNA ligase , Ligations were incubated for 4-15 hours .

pAMP Ligations

PCR amplification products from genomic DNA were subcloned into pAMP vector suplied in the CloneAmp™ system. The CloneAmp™ system requires PCR product generated with primers containing 4 dUMP sequences spaced within the 5' ends. The primer pair used is illustrated

5'-CAUCAUCAUCAUCAAAGGCATGGAGCATCTGTAC-3'

3'-CCGAGATGAAGTAGCGTAAGGAAUCAUCAUCAUC-5'

The uracil residues are removed by digestion with uracil DNA glycosylase leading to exposed 3' overhangs on the complementary strand, these overhangs are complementary to 3' overhangs in the linearised pAMP vector supplied and will spontaneously anneal to each other during combined digestion annealing reaction . Ligation was achieved by using 2µl PCR product and 25 ng pAMP vector DNA incubated with 1 U uracil DNA glycosylase in 20 µl annealing buffer, incubated for 30 minutes at 37°C. 10

µl the ligation reaction was used to transform 100 µl of DH5α competent bacteria generating approximately 1000 -2000 colonies.

Automated colony picking

The screening of tumour DNA for low frequency mutations requires the analysis of multiple colonies from the above PCR subcloning process (384 from each tumour) therefore an automated bacterial colony picking system was used. This system has been developed by the ICRF genome analysis laboratory.(ref) The instrument identifys bacterial colonies on 24x24 cm plates picks a sample from each colony onto a 96 pin picking head and transfers the bacteria into 384 well microtitre plates containing L-broth with 10% glycerol. The bacteria are grown overnight at 37°C. Further copies of the plates are made by inoculating a fresh plate using a sterile disposable 384 point comb. The plates can then be stored at 4°C for up to one week or indefinately at -70°C.

Automated spotting of reamplified DNA to Nylon membranes

The automated picking system was used for transfering PCR product from 384 well plates onto Nylon membrane. The PCR reactions were opened by removing the heat sealed film and the robot programmed to spot the PCR product onto Hybond N^+ as a gridded array using a 96 point spotting head with acurately machined 0.25 mm diameter tips. These pins transfer approximately 0,2 µl of PCR product to the membrane after each contact It was empirically determined that each PCR product required 5 individual contacts to transfer an optimum ammount of DNA to the membrane. The robot was programmed to spot each individual PCR sample from 15 different tumours into a 4x4mm array The final position within the 4x4 array was reserved for an ink spot to allow orientation of the membrane. Using this pattern it was possible to transfer the PCR products from 5700 individual PCR reactions onto a membrane measuring 12 x 8 cm. The robot was programmed to produce multiple copies of each filter.

Hybridisation to decamer oligonucleotides.

The membranes spotted with PCR products were allowed to air dry and were then wetted in Sarcosl hybridisation buffer.and placed into htbridsation bottles. 5 ml sarcosyl buffer containing 100 ng end labeled

decamer oligonucleotide was added and incubated at 4°C for 4 hours with continuous rotation. After hybridisation the filters were washed once in sarcosyl buffer and transfered to 200 ml fresh buffer and mixed gently for 15 minutes at 4°C Filters were wrapped in cling film and hybridisation detected by exposure to Kodack XAR film at 4°C without the use of intensification screens,

Polymerase chain reactions

PCR was used to amplify oestrogen receptor sequences from genomic DNA derived from breast tumours, to amplify the subcloned plasmids derived from these PCR products and to introduce random mutations into the hormone binding domain of ER.

Amplification of sequences of oestrogen receptor hormone binding domain

PCR was performed in 0.5 ml microtubes containing 100 µl 1xTaq buffer with 1.5 mM $MgCl_2$, 20 µM dNTPs (20 µM each of, dATP, dCTP, dGTP, dTTP) 200 ng of each primer 0.5 µg of genomic DNA and 5 U Taq polymerase..These mixtures were overlaid with mineral oil and incubated in a thermal cycler set to heat to 94°C for 5 minutes to fully denature the template. Thermal cycling then proceeded with annealing at 50°C for 1 minute extention at 72°C for 1 minute and denaturation at 94°C for 1 minute., repeated for 30 cycles, and finally extended at 72°C for 5 minutes. Satisfactory yield was determined by gel electrophoresis of 10 µl of product. The PCR product was subcloned into pAMP (see ligations)

Re amplification of pAMP subcloned PCR fragments

Re amplification of pAmp clones was performed in 384 well polypropylene microtitre wells 80 µl of 1x Taq buffer with 20 µM dNTPs, 1.5 mM $MgCl_2$, and 200 ng each primer (complementary to pAmp sequences). Template was added by innoculating the reactions with a small amount of transformed bacteria using a 384 point innoculating comb. The reactions were heat sealed with plastic film and subjected to thermal cycling in a custom built large scale thermal cycling robot (Genome analysis laboratory ICRF). using a 2 temperature cycle holding at 92°C for 30 seconds and 72°C for 300 seconds.

Degenerate PCR mutagenesis of oestrogen receptor hormone binding domain

PCR reactions were performed using conditions permiting degenerate replication introducing point mutations approximately every 1000 nucleotides in the final PCR product. 100 µl of 1x Taq buffer with 250 µM dNTPs, 7.5 mM $MgCl_2$, 20 ng M13 hER template, and 20 picomoles of each primer. and 5 U Taq polymerase. The reaction was overlaid with mineral oil and subjected to 30 amplification cycles consisting 1 minute denaturation at 94°C, 30 seconds annealing at 55°C and 2 minutes elongation at 72°C

DNA Sequencing.

Denaturation of DNA for sequencing with dideoxynucleotides

Approximately 2 µg of plasmid DNA was denatured in 20 µl 0.2M NaOH at 70°C for 20 minutes. This was neutralised by the addition of 8 µl 5M ammonium acetate pH 5.4 and precipitated by addition of 120µl ethanol and stored on dry ice for 10 minutes. DNA was recovered by centrifugation and washed with 70% ethanol. After drying the DNA was re suspended in 7µl water and 2 µl 5x sequenase buffer 2.5 ng (1.5 pmol) oligonucleotide sequencing primer was added in 1µl and placed in a water bath at 80°C and allowed to cool to 37°C over 30 minutes.

Sequencing reactions with Sequenase II

The Sequenase (USB) protocol was followed Annealed primer/DNA was added to a mixture containing the appropriate dilutions of dNTPs {$^{35}S\alpha$} dATP and sequenase II enzyme for sequencing within the required distance from the primer. The reaction incubated for 1- 10 minutes following which the reaction mixture was divided equally into 4 tubes containing 2.5 µl of one of 4 dideoxynucleotide solutions. These tube s were incubated at 37°C for 5 minutes and the reaction stopped with the addition of 4 µl Stop solution.

Electrophoresis of sequencing reactions

Denaturing gels were prepared from 75 ml 6% Easigel pre made acrylamide/urea/TBE solution by the addition of 400 µl 10% ammonium persulphate and 80 µl TEMED poured between 24x30 cm sequencing plates with 0.25 mm spacers. After washing the top of the gel with 1/2 TBE the spacer comb was inserted and the gel pre run for 20 minutes prior to the

addition of 3 µl of sequencing reaction per lane. Gels were run at 45-50 milliamps for 1-2 hours. Gels were fixed in 10% methanol 10% acetic acid, transferred to Whatman 3MM paper and dried under vacuum.. sequences were visualised by auto radiography using Kodak XAR film.

Fluorescent sequencing

ABI PRISM ™ Dye Terminator cycle sequencing was used for multiple sample sequencing, using reagents supplied by Perkin Elmer Corporation.

0.5-1 µg of plasmid DNA and 3.2 pmol sequencing primer was added to 8 µl of dye terminator mix containing. A-Dye terminator, C-Dye terminator, G-Dye terminator, T-Dye terminator, dITP, dATP, dCTP, dTTP Tris-HCl pH 9.0, $MgCl_2$, thermal stable pyrophosphatase and Ampli-Taq DNA polymerase. The sample made up to 20 µl and subjected to 25 thermal cycles consisting 96°C for 10 seconds ,50°C for 5 seconds, 60°C for 4 minute.s. Samples were cooled to 4°C and DNA precipitated with the addition of 2 µl 3M sodium acetate pH 4.6 and 50 µl ethanol . Samples were then incubated on ice for 15 minutes and centrifuged in a microfuge for 30 minutes. Samples were washed with 70% ethanol and air dried. Samples were resuspended in deionised formamide/25 mM EDTA pH 8.0 with blue dextran, Ratio 5:1 and loaded onto sequencing gels run in an ABI 373 automated sequencing apparatus. Sequences are interpreted from histograms of florescence intensity.

Radiolabelling of DNA probes

End labelling of oligonucleotides

Synthetic oligonucleotides were end labelled with [^{32}P γ] ATP.

100 ng of oligonucleotide was incubated with 30µCi of [^{32}P γ ATP and 2U T4 polynucleotide kinase in 30 µl 1x phosphorylation buffer, incubated at 37°C for 30 minutes and the reaction stopped with the addition of 1µl 0.5 M EDTA.

Adequate incorporation of label was confirmed by Thin layer chromatography of 1µl of the reaction. The probes were used freshly made and added to 5 ml of hybridisation solution at 4°C.

Random primer probes for northern blot analysis

Sequence specific probes were generated by random priming. Plasmid DNA was cut with appropriate restriction enzymes and fragments separated by gel electrophoresis and recovered with "Gene Clean". 25 ng of DNA in 45 µl water was heated to 95°C for 2 minutes cooled then added to a "Ready to Go" reaction tube containing lyophilised polymerase, random nonomers and nucleotide mix. 50 µ Ci [^{32}Pα] dCTP was added and the reaction incubated at 37°C for 30 minutes. The incorporated nucleotides were purified by eluting on a "NICK" column. 1µl of the eluted probe was counted in a scintillation counter to determine specific activity (typically 5x 10^8 dpm per µg DNA).

ERE oligonucleotides

Complementary oligonucleotides forming 5' overhangs were annealed and labelled by filling in the overhangs. 200 ng of annealed oligonucleotide was incubated in a total of 20 µl containing 1x repair buffer with 0.1 mM dATP, dGTP dTTP, and 8 µl [α-^{32}P] dCTP(10 mCi/ml, 3000 Ci/mmol) and 2 U Klenow. for 30 minutes at room temperature. The reaction was extracted twice with phenol chloroform and the DNA precipitated by the addition of 20 µl 3M sodium acetate 1µl glycogen and 100µl ethanol. The sample was dissolved in water, re precipitated, washed with 70% ethanol air dried and resuspended in 200 µl water. 1 ng labelled oligonucleotide was used in each band shift reaction.

Mammalian and avian cell culture.

Growth of cell lines

All cell lines were routinely maintained as exponentially growing stocks in Falcon tissue culture flasks at 37°C in a humidified atmosphere supplemented with 10% CO_2. All breast cancer cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v Foetal calf serum (FCS). Chicken embryo fibroblasts were grown in DMEM with 10% FCS and 1% Chick serum (CS). Medium was changed at least every 5 days. Prior to reaching confluence cell cultures were subcultured into fresh flasks. Medium was removed and cells washed once with PBSA 3-5 ml trypsin/versene (1:5) was added and cells incubated until they started to

detatch from the surface. 5-10 ml of serum containing medium was added and cells transferred to a polystyrene universal container and centrifuged at 1500 rpm in an MSE benchtop centrifuge. The supernatant was removed, cells resuspended in fresh medium, and 1/20 th to 1/5 th of the volume used to seed a fresh flask.

Storage of cell lines

Subconfluent cells were recovered by trypsinisation as above, after centrifugation and removal of the supernatant, cells were resuspended in FCS containing 10% v/v DMSO approximately $3x10^6$ cells in 1 ml of serum were placed in a 2 ml Nunc freezing tube and placed at -20°C for 1 hour , then at -70°C overnight and then immersed in liquid nitrogen for long-term storage. Cells were recovered by thawing rapidly at 37°C and adding the whole tube of cells to a 75 cm flask, the medium was changed the following day to remove dead cells and residual DMSO.

Charcoal treatment of serum

Foetal calf serum contains endogenous steroids and phenol red acts as a weak oestrogen. Most experiments were performed in medium lacking phenol red and containing dextran charcoal stripped serum (DCC. FCS). Dextran charcoal was prepared by adding 1 g dextran T 70 and 10 g activated charcoal to 10 ml 1M Tris HCl pH 7.4. This was made up to 1L with dislilled water, divided into four 250 ml disposable Corning centrifuge tubes and centrifuged at 2000 g for 10 minutes. The supernatant was aspirated and half the charcoal resuspended into 1L of FCS. This was incubated at 55°C with agitation for 30 minutes in a 2 L flask, the serum centrifuged as above , the serum transferred to a second flask and the remainder of the charcoal added. The serum was incubated at 55°C for a further 30 minutes and re centrifuged as above. The serum was then filtered through a 0.4 µm Nalgene filter and then through a 0. 22 µm Nalgene filter and stored at -20°C in 50 ml aliquots.

Transient Transfections.

Chicken embryo fibroblast transfections

CEF cells were transfected by the HBS calcium phosphate coprecipitation method modified from (Graham and Van Der Erb,1973).

Cells growing in log phase were harvested by trypsinisation and seeded into 6 cm dishes at a density of $2x10^5$ cells in 4 ml phenol red free medium with 10% DCC. FCS. and 1% DCC chick serum. The following day the medium was changed and calcium phosphate/DNA precipitate containing 10 µg DNA per dish applied. Transfections were always performed in duplicate, the calcium phosphate/ DNA precipitate was prepared by mixing freshly prepared solutions A and B to form sufficient precipitate to treat the required number of dishes.

For 2 dishes Solution A

500 µl 2x HBS pH 7.1

5 µl 70 mM NaH_2PO_4, 5 µl 70 mM Na_2HPO_4,

Solution B

500 µl distilled water

60 µl 2M $CaCl_2$, 20 µg supercoiled plasmid DNA

Solution B was added dropwise to solution A while mixing continuously by bubbling air through the solution with a glass pipette.. The mixed solutions were left for 20 minutes to form a visible cloudy precipitate and a mock transfection solution containing no DNA was prepared at the same time. 500µl of precipitate was applied to each dish of CEF cells and the cells incubated in an atmosphere of 5% CO_2 for 15 hours. Following this the cells were washed with serum free medium up to 4 times incubating for 10 minutes between changes of medium until all visible precipitate had dispersed. Fresh medium was added and carrier ethanol or ligands in ethanol added. Ethanol concentration was adjusted were necessary to 0.1% for all dishes. Cells were incubated at 37°C in 10% CO_2 in the presence of ligand for 24 hours prior to harvesting.

MCF-7 cell transfections

MCF-7 cells were transfected by BBS calcium phosphate coprecipitation method (Chen *et al.* 1987). Cells were plated into 24 well or 6 well plates at 70% density in phenol red free medium containing 10% DCC. FCS. 24-48 hours after plating the medium was changed and DNA applied. Transfections were always performed as duplicates. To transfect 2 wells the DNA was prepared by diluting a total of 2µg supercoiled DNA into 90µl distilled water , adding 10 µl 2.5M $CaCl_2$ and 100µl 2xBBS. This was

mixed gently and left to stand for 15 minutes at room temperature. This was mixed again and 100 µl applied to each well. For experiments requiring more than 2 wells the quantities were scaled up appropriately. The precipitate was allowed to form overnight while incubating in 5% CO_2 at 37°C. The following day the precipitate was removed by washing with up to 4 changes of serum free medium and finally medium containing 10% DCC FCS was applied and additional ligands or ethanol carrier added. The cells were incubated for a further 24 hours in the presence of ligands in 10% CO_2 at 37°C prior to harvesting. For 6 cm dish transfections a total of 5 µg DNA was used per dish and a final volume of 200 µl precipitate prepared for each dish using the same proportions as for 24 well plates.

Electroporation

Cos-1 cells were transfected by electroporation. Cells were grown to 70% confluency in 175 cm flasks, trypsinised and after centrifugation resuspended in 3 ml PBSA 0.8 ml cells were added to 20 µg supercoiled plasmid DNA in a 0.4 cm electroporation cuvette. incubated on ice for 10 minutes and electroporated using a Bio-rad gene pulser electroporation apparatus, at 450 V and 250 µF, with a time constant of 4.5-5.0 msec. Following electroporation cells were incubated on ice for a further 10 minutes and then resuspended in 10 ml medium and plated onto 9 cm dishes. The following day medium was changed and cells grown for a further 48 hours prior to harvesting.

Harvesting cells from transient transfections for luciferase β-galactosidase and CAT assay

Media was removed from cells which were washed twice with PBSA. the final wash was removed as completely as possible and 50-100 µl lysis buffer added to each dish on a flat surface. After 2 minutes cell lysis was confirmed by microscopic examination with only nuclei visible. The lysate was recovered into ependorf tubes and placed on ice. The lysate was cleared of cell debris by centrifugation in a microfuge for 2 minutes and the supernatant transferred to a new tube. Luciferase assay was performed within 2 hours but lysate could be stored at -20°C prior to CAT or β-galactosidase assay.

Luciferase assay

Luciferase assay was performed according to (De Wet *et al.* 1987). Between 5 and 20 µl extract was added to 350 µl of luciferase reaction buffer in a luminometer cuvette. Samples were loaded into an LKB luminometer. 33 µl of 3 mM luciferin was injected into each sample, mixed and the peak light emission recorded. Extracts from mock transfections were used to obtain background recordings

β–galactosidase assay

β-galactosidase activity was assayed using Galacto-light kit TROPIX Inc. Galacton substrate was diluted 1:100 with Galactol-light reaction buffer. 5-10 µl extract was added to 100 µl diluted galacton in a luminometer cuvette and incubated at room temperature for 1 hour . Samples were then loaded into an LKB luminometer and 165 µl of Galacto-light accelerator injected with continuous mixing, peak light emission was recorded for each sample. Extracts from mock transfections were used to obtain background recordings and these were subtracted from the values obtained from the experimental samples.

Chloramphenicol acetyl transferase assay CAT assay

The method used is described by (Sleigh 1986). Initially 20 µl extract was heated to 65°C for 5 minutes to inactivate endogenous CAT activity and added to a mix composed of 20 µl 8 mM chloramphenicol, 10µl lysis buffer, 30 µl 250 mM Tris HCl pH 7.8, and 20 µl acetyl coenzyme A mix containing 0.5 mM cold acetyl coenzyme A and 0.1 µCi[1- ^{14}C] acetyl coenzyme A. This was incubated at 37°C for 1 hour and placed on ice. The reaction was stopped and acetylated chloramphenicol extracted by the addition of 100 µl ice cold ethyl acetate. Samples were vortexed and centrifuged. 80 µl of the upper organic phase was transferred to a scintillation vial containing 5 ml scintillation fluid. A further 100 µl ethyl acetate was added to the sample which was re extracted and a further 100 µl organic phase added to the scintillation vial. Samples were counted in a scintillation counter. Counts above 50,000 cpm were above the linear range of the assay and re assayed using less extract. Mock transfection extracts were used to make up residual sample volumes and counts obtained from mock extract assays were subtracted from experimental samples.

Isolation of stable cell lines

To isolate stable cell lines the inducible expression vector pMEP4 was used. MCF-7 cells in 9 cm plates growing in normal medium were transfected using the calcium phosphate coprecipitation method described for transient transfections of MCF-7 cells. A total of 1 ml of precipitate suspension containing 25 μg of DNA was applied to each plate. After incubation overnight the precipitate was washed off and the cells allowed to recover for 24 hours. Cells were trypsinised and plated down at a 20% confluency. After a further 24 hours medium was replaced with medium containing 200U/ml Hygromycin B. Medium was replaced every 3 days and after approximately 4 weeks colonies of resistant cells were well established and most non resistant cells had been removed during media changes. Entire dishes of cells were trypsinised and plated into 25 cm flasks to grow stocks of pooled clones. Other plates were used to isolate individual clones using 5 mm diameter steel rings coated in vacuum grease. These were autoclaved and used to encircle individual clones approximately 2 mm in diameter.. 100μl of trypsin/versene was applied to the ringed clone, after incubation for 2-3 minutes 100 μl medium was added and the clone dispersed by repeated pipeting. Each dispersed clone was placed in 1 ml of medium in a 24 well plate. The clones were allowed to reach confluence and then subcultured to 6 well dishes and finally into 75 cm flasks. When 80% confluent the cells were trypsinised and frozen stocks made. The remaining cells were screened for the presence of inducible ER by western blotting. Positive clones were grown further and more stocks of early passage cells stored.

Indirect Immunoflouresence

Washed cover slips were autoclaved and placed in 6 well dishes. incubated overnight with 0.5 mg /ml polylysine in PBSA and then washed with PBSA. Cos-1 cells were transfected by electroporation and plated onto the coverslips and allowed to grow for 24- 48 hours. then washed three times with with PBSA.(PBSA x3). Cells were fixed with 4% paraformaldehyde in PBSA for 20 minutes, then washed with PBSA x3 and the fixative quenched for 2 minutes with with 50 mM ammonium chloride in PBSA. Cells were washed with PBSA and permeabalised in 0.2% triton X100 in PBSA for 4

minutes. Cells were washed three times in PBSA with 1% BSA (BSA/PBS) Coverslips were then lifted and inverted onto 40 µl antibody solution placed on parafilm. Antibody solution consisted 10 ng/ml antibody (H222, H226, MP40) or neat N-ter hybridoma culture conditioned medium. Cells were incubated with antibody for 1 hour and then washed with BSA/PBS x3 and incubated for 1 hour using the parafilm tecnique with an FITC or Texas red conjugated antibody against the primary antibody species. Cells were again washed in BSA/PBS x3 and mounted on microscope slides in citiflour. Slides were examined with a confocal microscope or with Zeiss Axiphot flouresence microscope.

Protein Isolation and analysis

Whole cell protein extract from cultured mammalian cells

Cells were grown to 70-80% confluence on 9 cm dishes. The monolayer was washed twice with PBSA, 5 ml PBSA added and the cells scraped off the dish with a "rubber policeman", the dish was rinsed with a further 5 ml PBSA to collect all cells in a universal container. The cells were recovered by centrifugation at 1500 rpm in an MSE benchtop centrifuge, the supernatant removed and the cell pellet frozen on dry ice . At this stage cells could be stored at -70°C. Cells were thawed on ice after the addition of 10 volumes of whole cell extract buffer containing freshly added protease inhibitors and DTT. After re suspension the cells were further lysed by passing 5 times through a 25 gauge needle. Debris and insoluble material was removed by centrifugation at 50,000 g for 15 minutes at 4°C and the soluble fraction stored in aliquots at -70°C.

Determination of protein concentration

Protein concentration was determined by the dye binding assay described by (Bradford,1976). Dye concentrate supplied by Biorad was used. Samples to be assayed were diluted into 800 µl water, and 200 µl dye concentrate added. Samples were mixed and left to stand for 10 minutes after which OD595 was recorded in a disposable spectrophotometer cuvette . A set of known standards prepared from BSA was used to construct a nomogram from which the protein concentration of test samples could be extrapolated. As albumin binds approximately twice as much dye as most

proteins adjustment was also made to allow for this in determining protein concentration.

SDS polyacrylamide gel electrophoresis

Proteins were separated by discontinuous polyacrylamide gels using Atto corporation AE-6220 dual slab chamber electrophoresis apparatus. Gel plates (14x16 cm) with 1.5 mm spacers were used to prepare gels . The resolving phase of the gel was made up from 10% acrylamide (30% acrylamide 0.8% bisacrylamide stock, 375 mM Tris HCl pH 8.8 and 1% SDS). Gels were polymerised by the addition of ammonium persulphate 0.1% and TEMED 0.1%. After pouring the resolving gel to within 3 cm of the sample wells the acrylamide was overlaid with water saturated isobutanol and allowed to set. The isobutanol was poured off and the gel rinsed with stacking buffer. The stacking gel was prepared from 4% acrylamide 125 mM Tris HCl pH 6.8 and 1% SDS. The gels were polymerised by addition of ammonium sulphate and TEMED as above and poured, the sample comb inserted and the gel allowed to set. The comb was removed and the slots washed with 1x SDS PAGE buffer. The gel was loaded into the gel tank and filled with 1xSDS PAGE buffer. Protein samples consisting of up to 200 µg total protein were loaded in a maximum volume of 100 µl protein loading buffer. Samples were boiled for 2 minutes immediately prior to loading. Samples were always run with a lane containing Rainbow molecular weight markers. Gels were run at 250 volts.until appropriate separation was achieved.

Western Blotting

Proteins resolved by SDS PAGE were transferred by wet blot technique (Sambrook *et al.* 1989). The gel was placed as a "sandwich" in a cassette with nitro-cellulose membrane carefully applied directly onto the gel ensuring no trapping of air bubbles with 2 layers of Whatman 3MM paper either side this was surrounded by fibrous pads. The assembly was performed while components were submerged in blotting buffer. The cassette was placed in a Biorad electrophoretic blotting tank filled with blotting buffer and run at 30 V for 15 hours followed by 2 hours at 70 V. The sandwich was dismantled and the nitro-cellulose stained with 2% tricarboxylic acid 0.2% Ponceau S for 1 minute . The membrane was washed in PBSA and transfer

of proteins inspected visually. The membrane was placed in blocking agent for 1 hour with gentle agitation. The membrane was then incubated with primary antibody diluted appropriately in blocking agent inside a heat sealed polythene envelope with vigorous agitation for 1 hour at room temperature. The membrane was then washed for 30 minutes with 5 changes of blocking agent and then incubated as above with horse radish peroxidase conjugated secondary antibody diluted in blocking agent. The membrane was then washed for 30 minutes with 5 changes of PBSA with 0.1% Tween 20. Antibody/receptor complexes were detected using the Amersham enhanced chemoluminescence system (ECL). Equal volumes of reagent A and B were mixed and applied to the nitrocellulose for 1 minute, the excess liquid drained off before wrapping the nitrocellulose in cling film and detecting light emission using Fuji RX film.

Gel retardation assay

The ability of wild type and mutant oestrogen receptors to bind to DNA was assessed by gel retardation or band shift assay.

1-10 μg whole cell extract was pre incubated for 10 minutes in 18 μl 1x band shift buffer containing 1μg poly (dI-dC).(dI-dC), 0.1 mg BSA, with or without preimmune or specific anti ER antisera MP16. Ligands or carrier were also added prior to incubation . 1 ng of labelled double stranded oligonucleotide probe was added and incubated for a further 20 minutes at room temperature. Samples were applied to a non denaturing gel prepared from 6% acrylamide, 0.5% TBE and set up as described for SDS PAGE gel electrophoresis. Gels were pre run at 250 V for 10 minutes and samples run at the same voltage for 50 minutes. Gels were fixed in 10% acetic acid 30% methanol and dried under vacuum. Retarded bands were detected by auto radiography.

Ligand binding assay

Cos-1 whole cell extract from cells transfected with wild type or mutant ER were incubated in 50μl ligand binding buffer with different concentrations of {3,4,6,7-^{3}H} oestradiol at 25^{o}C for 2 hours. Following incubation samples were placed on ice and 50 μl DCC added and incubated for 5 minutes. Samples were centrifuged at 5000g for 5 minutes and the supernatant fraction counted in a scintillation counter as bound and the

pellet fraction as free steroid. Non specific binding was determined by the inclusion of 500 fold excess cold oestradiol. Controls using extract from cells transfected with empty vector were performed, All assays were performed in duplicate for each data point.

Competition assays

Competition assays were performed by incubating Cos-1 extracts as above, but in addition to labelled oestradiol, a range of concentrations of unlabelled 4-hydroxytamoxifen or oestradiol were added.

Antibody isolation

Generation of polyclonal antisera

The peptide R-V-P-G-T-R-E-N-V Corresponding to the last 9 amino acids of the exon 5 variant protein sequence were synthesised by N O'Reilly (ICRF) using a model 430A Applied Biosystems Solid phase Synthesiser. The peptide was analysed by reverse phase HPLC and mass spectroscopy. The peptide was coupled to a carrier protein Keyhole limpet haemocyanin (KLH) G Evan (ICRF unpublished). 6.25 mg KLH and 6.25 mg peptide were dissolved in 3 ml 0.1 M $NaHCO_3$. Glutaraldehyde was added to a concentration of 0.05% and mixed overnight at room temperature. The pH was adjusted to 8.4 with NaOH and 1M glycine ethyl ester added to a concentration of 0.1 M, the solution was incubated for a further 30 minutes with mixing. The coupled conjugate was precipitated with 5 volumes of cold acetone and placed on dry ice for 30 minutes. The conjugate was pelleted by centrifugation at 10,000g for 10 minutes and resuspended in saline. The concentration was adjusted to 1 mg/ml. The conjugate was used to immunise 2 rabbits (D Watling ICRF Central Services animal unit) using the animal unit standard immunisation schedule consisting seven immunising doses of 0.6 ml each administered intradermally initially as a 1:1 emulsion with Freunds complete adjuvant into 4 separate sites. Further immunising injections were performed at 2 weekly intervals using Freunds incomplete adjuvant. Preimmune serum samples were obtained and bleedouts performed after 16 weeks. Serum samples obtained were stored at 4°C with 0.1 % sodium azide.

Affinity purification of antisera

Immunoglobulin was precipitated by adding 12.3 ml saturated ammonium sulphate to 15 ml antiserum containing 5 mM EDTA, stiring and standing for 15 minutes, then centrifuging for 10 minutes at 10,000g at 4°C. The pellet was resuspended in 15 ml running buffer.

Cyanogen bromide separose was washed and hydrated by adding 2 g to 200 ml 1 M HCl, mixed and left to stand for 15 minutes. The sepharose was collected on a sinter funnel and mixed with 7 ml PBSA containing 700 µg of the immunising peptide. The mixture was rocked gently for 2 hours, poured onto a sinter funnel and washed with 30 ml PBSA, 30 ml 100 mM sodium acetate pH 4.0, 2M NaCl in TBS. The separose was suspended in TBS and packed into a Pharmacia reversible column. Using a peristatic pump. The column was washed with 100 ml running buffer. The antibody was then run into the column at a rate of 1 ml / minute. The column was washed with 100 ml running buffer . The column was reversed and washed again with running buffer. The afinity purified antibody was eluted with 40 ml 100mM sodium citrate pH 2.5 run in over 10 minutes. The eluate was imediately neutralised to pH 6.0 with 2M Tris base. The antibody was precipitated with 1 volume saturated ammonium sulphate., centrifuged at 10,000g for 10 miutes at 4°C, resuspended in 0.5 ml water and dialysed against TBS with 0.1% azide. The affinity purified antibody was stored at -20°C (Hancock *et al.* 1992).

RNA analysis

Isolation of RNA from cultured cells

Glassware baked at 180°C for 2 hours was used and RNAse free water used to prepare solutions. Cells were grown in 15 cm dishes to 80% confluence , washed three times with cold PBSA and 10 ml PBSA added to the plates. The cells were scraped off with a "rubber policeman" and the dish washed with a further 10 ml cold PBSA. Cells were pelleted in an MSE benchtop centrifuge and resuspended in 2.5 ml NTE (10) to which 250 µl of (v/v) NP40 was added, then transferred to a glass "Corex" tube. All subsequent manipulations were performed on ice , cells were left for 10 minutes prior to centrifugation at 10,000g in a sorval SS34 rotor at 4°C. The supernatant was transferred to a fresh tube and 1/10 volume of 10% SDS added. An equal volume of phenol/chloroform (1:1) was added and the

mixture vortexed to mix thoroughly. After centrifugation for 2 minutes the organic lower layer was removed leaving the interface behind. The organic extraction was repeated as above twice and a 4th extraction with chloroform alone performed. The aqueous layer was then removed and transferred to a fresh tube. RNA was precipitated by the addition of 2.5 volumes of ethanol and incubating at -20°C for 2-15 hours. RNA was recovered by centrifugation at 10,000g for 10 minutes as above. The pellet was washed in ice cold 70% ethanol and air dried at room temperature for 15 minutes. RNA was resuspended in 100 µl water and stored at -70°C in aliquots. Yield and purity was determined by spectrophotometry of a diluted aliquot at 260 and 280 nm .

Gel electrophoresis of RNA

RNA was separated by electrophoresis in denaturing 1.25% agarose. 2.5 g agarose was dissolved in 144 ml water and 20 ml 10x running buffer, by boiling in a microwave oven. 36 ml of 38% (w/v) formaldehyde was added and the gel poured in a fume hood. 20 µg total RNA for each track was precipitated with ethanol and after drying resuspended in 4.5 µl water 16 µl RNA sample buffer added and the RNA denatured by heating to 65°C for 2 minutes. 8 µl loading dye was added and the sample loaded onto the gel which was immersed in 1x running buffer in an electrophoresis apparatus. Electrophoresis was run at 5 V/cm for 5 hours. After electrophoresis RNA was stained by immersion in running buffer containing 200 ng/L ethidium bromide. for 20 minutes followed by destining for several hours in running buffer. RNA was visualised by uv illumination to confirm adequate separation and quality of RNA judged by the sharpness of 18S and 28S ribosomal RNA bands. Gels were photograped by polaroid film or videocamera.

Northern blotting and hybridisation

RNA was transferred to Hybond N+ nylon membrane by capillary elution overnight using 20 x SSC (Sambrook *et al.* 1989). After transfer RNA was crosslinked to the membrane by uv fixation. The membrane was pre hybridised at 65°C in Church and Gilbert hybridisation buffer for 3 hours after which the membrane was hybridised at 65oC with specific DNA probes for 15 hours. Membranes were then washed in Church and Gilbert wash buffer three times for 30 minutes each. Membranes were wrapped in

saran wrap and hybridisation detected by auto radiography using XAR film. Blots were also analysed by phosphoimmaging to obtain quantitative data. After autorads were obtained the membranes were stripped by immersion in boiling 0.1% SDS for 2 minutes. following which membranes were rinsed in hybridisation solution and re probed with a second specific probe for γactin mRNA to confirm aproximately equal loading.

Yeast transformation and mutant screening

The protease deficient *Saccaromyces cerevisiae* strain BJ 5458 (from Berkeley Stock centre) was used for expression and functional screening of ER

Transformation

Modified from (Ito *et al* ,1983)

Exponentially growing cells at a density of $2x10^7$cells/ml (OD_{600} of 0.6-0.8) were centrifuged at 1500 rpm in an MSE benchtop centrifuge and resuspended in water, re centrifuged resuspended in TEL buffer pelleted and resuspended in TEL at 10^9 cells/ml. 100 µl cells were added to 1 µg of supercoiled DNA and 15 µg of salmon sperm DNA in 50 µl water, incubated at 30°C for 30 minutes, then 700 µl 40% PEG_{4000} in TEL was added and incubated for a further 60 minutes. The cells were subjected to a heat shock of 42°C for 5 minutes, centrifuged in a microfuge at 6,500 rpm for 10 seconds, washed twice with selective medium and then plated out onto minimal selective plates. Plates were then incubated at 30°C until colonies appeared. Transformation efficiency is variable but up to 10^5 colonies per microgram of DNA can be obtained.

Phenotypic screening of mutated oestrogen receptors in yeast

Unpublished method of I Purvis Glaxo Research.

Mutant hER was introduced into *Saccaromyces cerevisiae* strain BJ 5458 which had previously been transformed with the oestrogen responsive reporter YCp ERE lacZ.

The hER expression plasmid pCuphERMt1 was cut with NcoI and BglI.

gel purified and recovered with "Geneclean". 1 µg of cut vector was used with 5 µg of degenerate PCR product to transform yeast carrying YCp ERElacZ The transformed yeast was plated onto 20 double selective plates (-U/-L) and incubated at 30°C until colonies were 1 mm in size. The plates were then replica plated using Whatman filter discs and a felt pad onto X gal plates containing 50µM $CuSO_4$ with or without added ligand. The colonies were incubated at 30°C for up to 5 days. Plates were examined daily for the development of blue coloration . Colonies conforming to the required phenotype were picked and re streaked onto X-gal plates to confirm the phenotype.

DNA recovery from yeast.

Described in (Rose *et al* ,1990)

Yeast was grown in a 5 ml culture overnight with appropriate selection. 1.5 ml of culture was centrifuged briefly to pellet cells . the supernatant removed and 200 µl of 2% triton X100, 1% SDS 100 mM NaCl, 10 mM Tris HCl pH 8.0, 1 mM EDTA added. The pellet vortexed into suspension and 200 µl of phenol:chloroform:isoamyl alcohol added. (25:24:1). The mixture was vortexed for 2 minutes with intermittent cooling after the addition of 0.3 g of acid washed glass beads. The tube was centrifuged for 5 minutes in a microfuge and the aqueous portion ethanol precipitated. The pellet was resuspended in 5 µl of water and 1-2 µl used to transform SCS-1 strain bacteria by electroporation.

Protein extraction from yeast

Yeast colonies were inoculated and grown at 30oC in 50 ml selective medium to a OD_{660} of 0.8 Cells were pelleted by centrifugation and washed in ice cold yeast lysis buffer and subsequently maintained at 4°C. Cells were transferred to a 1.5 ml ependorf tube, repelleted and resuspended in 350 µl lysis buffer containing freshly added protease inhibitors. An equal volume of glass beads were added and the tube vortexed with intermittent cooling for 5 minutes. 200 µl of RIPA buffer with protease inhibitors was added the tube mixed and centrifuged. The supernatant was removed and a further 200 µl of RIPA added and after mixing left on ice for 10 minutes. The tube was recentrifuged and the supernatant added to the first extraction. The protein extract contains an oily layer therefore the aqueous layer is carefully

removed to a fresh tube. Protein concentration was determined and the sample stored in aliquots at -70°C.

Liquid β-galactosidase assay for yeast

(modified from B Amati unpublished ICRF)

Yeast with expression vector and reporter were inoculated into 4 ml selective medium at a density of 0.2 OD 600 and grown for 15 hours with shaking at 30°C in the presence of 50 μM CuSO4 and ligand or ethanol carrier alone. Cells were harvested by centrifugation, washed with water twice and transferred to a 1.5 ml ependorf tube. The pellet was resuspended in 200 μl lysis buffer and frozen on dry ice. The cells were thawed on ice fully resuspended and OD_{600} of an aliquot recorded, Tubes containing 0.5 ml Z buffer with freshly added β-mercaptoethanol to 50 mM and 0,1 ml 4 mg/ml ONPG were prepared and warmed to 37°C To these tubes 100 μl of cell suspension was added and incubated at 37°C. The time taken for a pale yellow colour to appear was recorded and the reaction stopped by the addition of 250 μl 1M Na_2CO_3. Samples were cleared by centrifugation and OD_{420} recorded using a lysis buffer only sample as a blank . Where the absorbance was outside the linear range of the assay (0.2-0.4) the assay was repeated using a shorter incubation time or using a smaller volume of cells, making up to full volume with lysis buffer.

Results were normalised for cell density and incubation time to give comparable values in arbitrary units..

Chapter 3

Analysis of the exon 5 variant oestrogen receptor

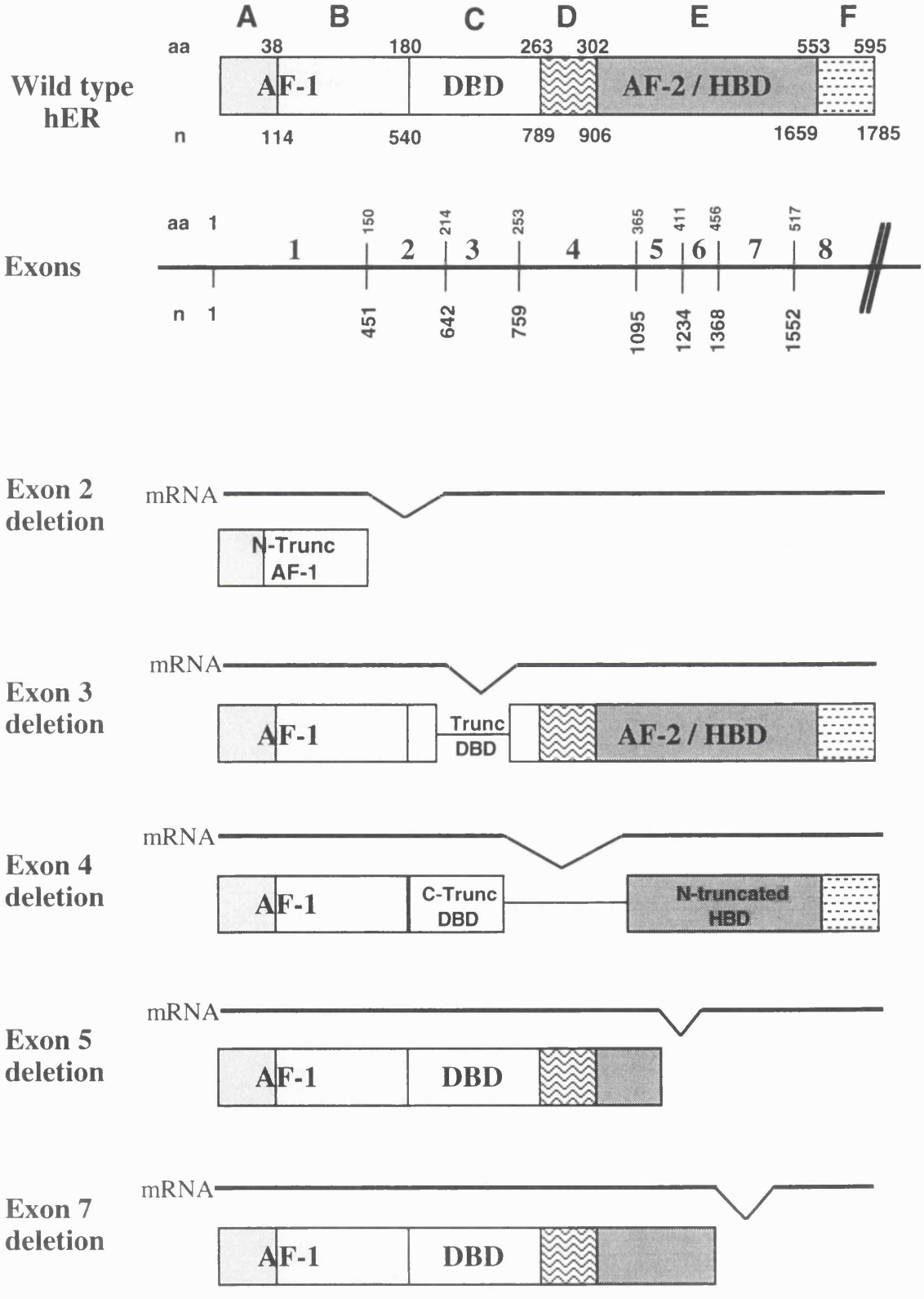
Introduction

The identification and initial analysis of splice variant ER has been described in chapter 1. This chapter describes a detailed analysis of the function of the exon 5 variant oestrogen receptor. The variant ER cDNA was cloned into two expression vectors to allow protein expression in transient transfections and to permit the development of stable cell lines expressing variant protein through an inducible promoter. The expression of variant expression has been confirmed by the raising of a specific antiserum that recognises only the variant protein. The transcriptional activity of the variant receptor has been analysed in both these systems. Finally the effect of the variant on cell proliferation has been investigated.

Construction of an expression vector for the exon 5 variant ER

To create an expression vector for the exon 5 variant ER the full length cDNA of pSG5HEGO was modified to encode the truncated protein. This was achieved by replacing the sequences 3' to the HindIII site at position 1017 with a synthetic oligonucleotide pair that formed the exon 4/6 junction and sequences as far as the premature stop codon (see appendix). The modified cDNA was therefore not a true splice variant but contained all the necessary coding sequence to form the 371 amino acid protein identical to that coded by the true splice variant mRNA. The expression of the variant protein was established by transient transfection of Cos-1 cells and western blotting of the extract using the oestrogen receptor monoclonal antibody H226 as a secondary antibody. H22 recognises an epitope in the N-terminal part of the full length receptor close to the junction with the DNA binding domain, this epitope is retained in the exon 5 variant receptor and in the N-terminal truncated receptor 121-595, this truncation does not contain the first 121 residues of ER but still contains a small portion of region B including the H226 epitope. This receptor and full length proteins were used as controls of receptor expression and stability. The western blot detected a band of approximately 42 kD corresponding to the predicted size of the exon 5 variant ER (see Fig 3.2). Full-length receptor was expressed and as expected a band of 65 kD was detected and the 121-595 truncation appears as an intermediate band. The two proteins are detected in approximately equal amounts indicating that there is no major difference in the stability of the two proteins. It is already known that the truncated MOR

Oestrogen Receptor Variants



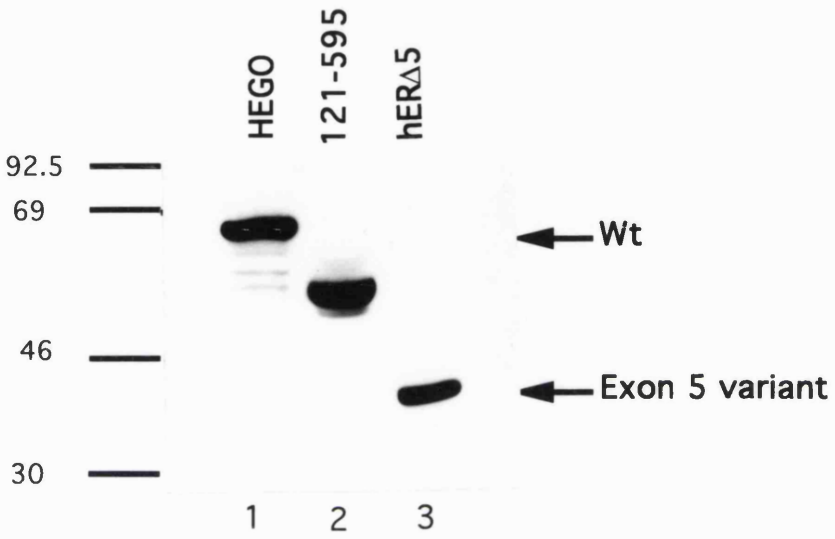


Fig 3.2

Western blot of full length and exon 5 deleted oestrogen receptor expressed in Cos-1 cells

Oestrogen receptors were expressed by transient transfection in Cos-1 cells 20 µg protein extract was loaded in each lane and oestrogen receptor was detected using H226 as secondary antibody. Full length oestrogen receptor (lane 1) N-terminal truncation 121-595 (lane2) Exon 5 deletion construct (lane3)

containing residues 1-339 has a similar half life to full length MOR expressed in Cos-1 cells (Dauvois 1992).

Transcriptional activity of the variant in chicken embryo fibroblasts

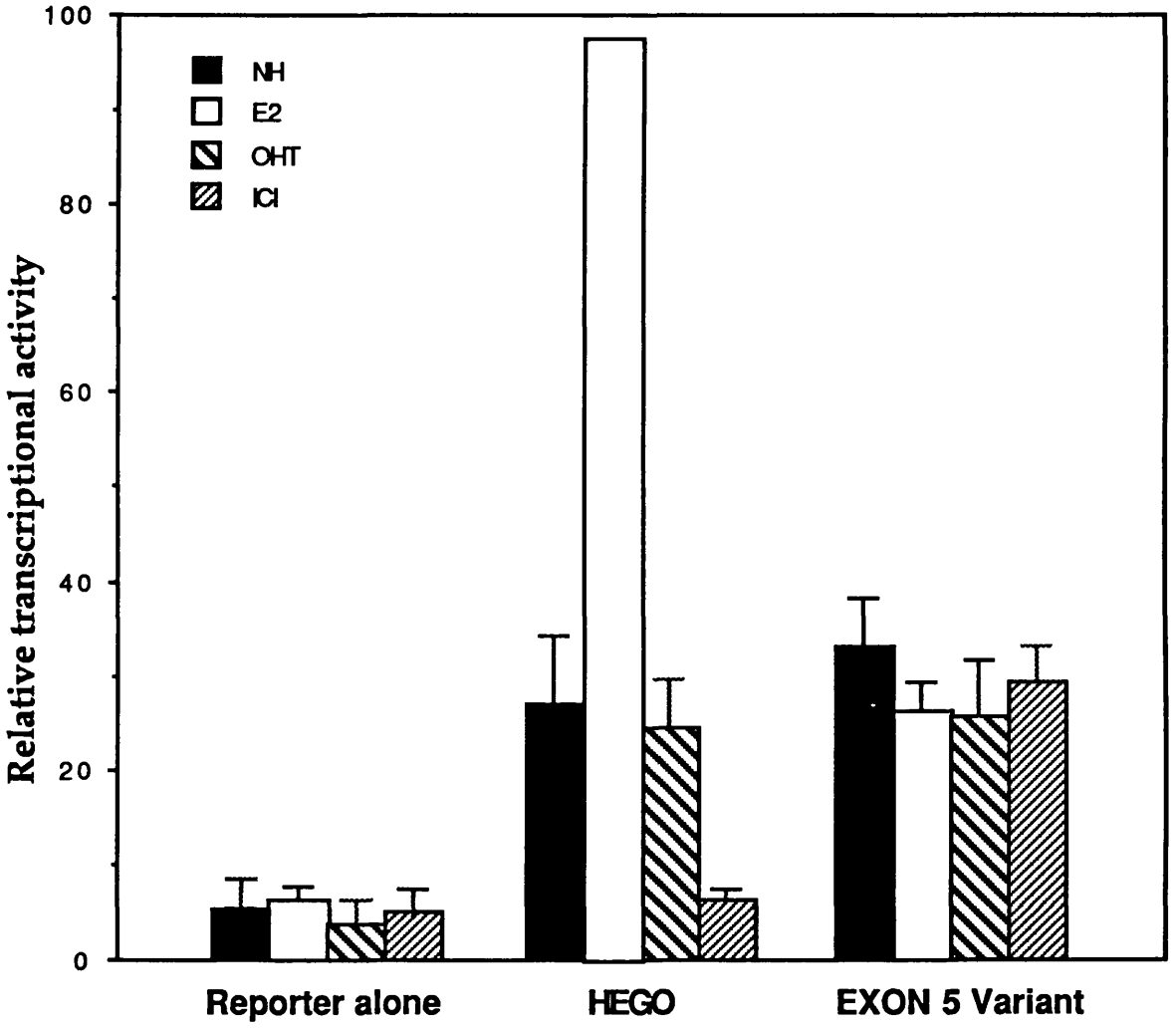
To determine the ability of the variant to induce transcription of an oestrogen responsive reporter in animal cells we chose chicken embryo fibroblasts because these cells are known to respond well to stimulation mediated by AF1. The transcriptional activity of the exon 5 variant was compared to full length receptor activity in transient transfections. The oestrogen responsive reporter construct ERE tk luciferase was cotransfected with receptor. This reporter consists of the vitellogenin ERE upstream of the thymidine kinase promoter driving expression of luciferase. The effect of E2, and antioestrogens on transcriptional activity is shown in Fig 3.3. The exon 5 variant receptor is able to stimulate transcription 5 to 6 fold above that observed in the presence of the reporter alone. This activation is completely unaffected by the addition of E2, OHT or ICI 182,780 indicating that the exon 5 variant is indeed constitutively active in this situation and that the stimulation can not be influenced by ligand. Full-length receptor showed a 5 fold increase in activity in the absence of added ligand and this was further increased to 20 fold on the addition of E2. In the presence of OHT there was a 5 fold stimulation, similar to that seen in the absence of added ligand but in the presence of the pure antioestrogen ICI 182780 there was no significant stimulation of the reporter. In this system OHT is able to stimulate the reporter to a moderate degree but the pure antioestrogen has no stimulatory activity and suppresses the background activity seen in the absence of added ligand. The absence of basal activity in the presence of ICI 182,780 is a reflection of complete ER blockade mediated through multiple mechanisms.

The moderate activity seen in the presence of no added ligand is thought to be caused by residual oestrogenic ligands contained in the stripped serum in which the experiments are performed. Support for this interpretation is gained by noting that the mutant MORG525-R which is unable to bind E2 has negligible activity in the absence of ligand but is stimulated by OHT (Danielian1992). It is interesting to note that the sub maximal stimulation seen with the exon 5 variant is similar to the stimulation of full length receptor by OHT.

Fig 3.3

Transient transfection of chicken embryo fibroblasts with full length and Exon 5 variant oestrogen receptor.

Chicken embryo fibroblasts were transfected with 1μg PJ7lacZ internal control plasmid 5μg of EREtk lucferase reporter and 0.5 μg pSG5 expression vector. DNA was made up to a total of 10μg with pSG5. After transfection cells were treated for 24 hours with ethanol carrier alone (■), 10^{-8}M 17-β oestradiol (□), 10^{-6}M 4-hydroxytamoxifen (▨), 10^{-6}M ICI 182780 (▨). Cells were then harvested and analysed for luciferase and β-galactosidase. Luciferase values were firstly normalised for transfection efficiency using β-galactosidase. and then expressed as a percentage of the value obtained with full length receptor stimulated with oestradiol. The results shown represent the mean of three independent experiments performed in duplicate with standard errors indicated by error bars.


100
80
60
40
20
0
Relative transcriptional activity
NH
E2
OHT
ICI
Reporter alone
HEGO
EXON 5 Variant

Generation of a specific antibody to the exon 5 variant receptor.

One of the difficulties in assessing the significance of the exon 5 variant receptor has been the absence of positive evidence that the protein is expressed. A specific antibody to the variant ER is a potentially useful tool to use to study protein expression. Since the exon 5 variant contains 5 C-terminal amino acids that are not present in the full length receptor this may produce a unique epitope that would allow specific antigenic distinction of exon5 variant receptor. A peptide was synthesised containing the last 9 amino acids of the exon 5 variant sequence. This length was selected empirically with the expectation that a peptide of this length would contain the appropriate epitope without sufficient wild type sequence to form a wild type epitope. This peptide was used as a conjugate with Keyhole limpet haemocyanin to immunise rabbits. The resulting antiserum (MP40) was tested on Western blots of Cos-1 extracts overexpressing the wild type and variant receptors. While the antiserum did not recognise full length receptor, a band of 42 kD was more apparent in the lane containing exon 5 variant extract. A background band of indistinguishable size was however seen in the full length receptor track. This is probably not a degradation product since it was not picked up with other ER antibodies (data not shown), and is thought to be a contaminating non specific band. The antiserum was therefore purified on an affinity column using the immunising peptide to trap the specific antibodies. The ability of this antibody to recognise ER was then compared to H226 in western blots of Cos-1 cell extracts (Fig 3.4). Here it can be seen as previously that H226 recognises both full length and exon 5 variant receptors. MP40 fails to recognise the full length receptor but does recognise the exon 5 variant protein. The antibody specificity was further demonstrated by competing off the specific band by the addition of excess native immunising peptide during incubation with primary antibody.

Stable expression of exon 5 variant receptor in MCF-7 cells

To study the effect of the exon 5 variant ER on proliferation of breast cancer cell lines it is necessary to establish stable expression of the variant protein and it is desirable to be able to induce receptor expression. This was achieved using an episomally maintained expression vector pMEP4

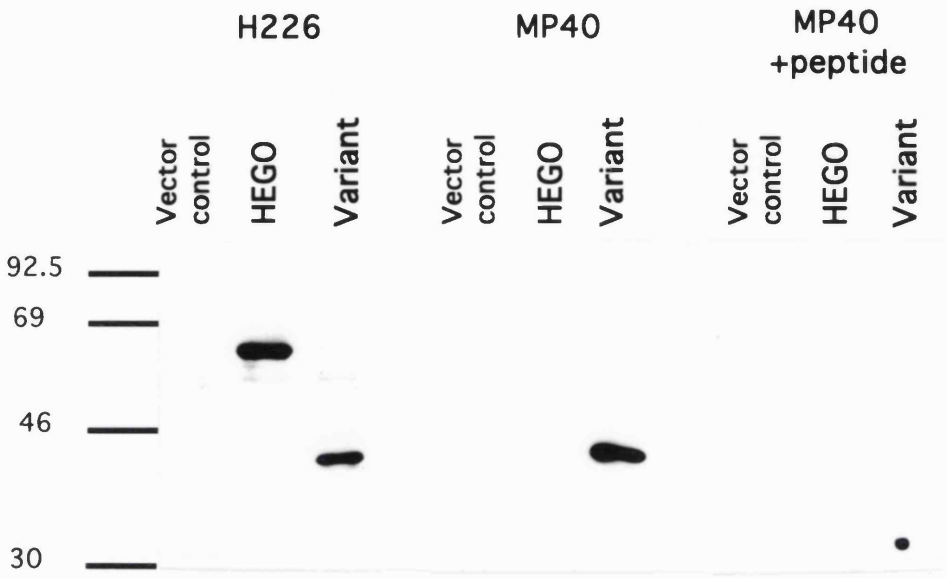


Fig 3.4

Detection of Exon 5 variant with a specific rabbit antiserum MP40.

To establish the specificity of the MP40 rabbit antiserum, the exon 5 variant (hERΔ5), the wild-type receptor (HEGO) and the expression vector pSG5 (vector control) were over-expressed in Cos-1 cells .10 µg of extract was separated by SDS PAGE. Receptors were then detected after Western blotting with either H226, MP40 or MP40 in the presence of an excess of the peptide used to generate MP40 antiserum.

expressing the variant under the control of the human metallothionein IIA promoter. This promoter is responsive to heavy metals and can be used to induce the expression of variant in a dose dependent manner. The vector carries the hygromycin B gene conferring hygromycin resistance to transfected mammalian cells. The plasmid contains the Epstein Barr virus origin of replication and is able to replicate extra chromosomally and does not rely on chromosomal integration for stable maintenance of resistance and for expression of the foreign gene. We used the Breast cancer cell line MCF-7 because it is a well characterised ER positive cell line that shows a positive mitogenic response to E2. ER negative lines that have been transfected with ER show a paradoxical negative growth response to oestrogens and in this artificial situation it is difficult to interpret any changes seen in proliferation. In addition, since the exon 5 variant mRNA is only found in association with of full length ER mRNA, we wanted to study the growth effects of the exon 5 deletion in the context of wild type receptor expression.

Isolation and characterisation of pooled clones expressing exon 5 variant receptor

The cDNA for full length and the exon 5 variant ER were transferred into pMEP4 and hygromycin resistant clones isolated from parental MCF-7 cells transfected with pMEP4, pMEP4HEGO and pMEP4hERΔ5. Firstly transfected cells were exposed to hygromycin and resistant populations from each plasmid transfection were established on 9 cm dishes. Each dish produced approximately 100 individual colonies. At first passage, each dish was maintained as a continuously growing culture comprising multiple clones which were merged during the first passage procedure and are refered to as pooled clones. These were used to demonstrate the ability to induce expression of either exon 5 variant protein or additional wild type receptor when exposed to cadmium. Expression of receptors was monitored by western blotting using H226 as secondary antibody (Fig 3.5). Here it can be seen that the parental MCF-7 and the cells transfected with pMEP4 vector alone both express a similar amount of wild type receptor. The cells transfected with pMEP4HEGO also show similar levels of receptor in the absence of induction but when exposed to cadmium the amount of receptor increases. In the two control cells the amount of receptor is decreased in the

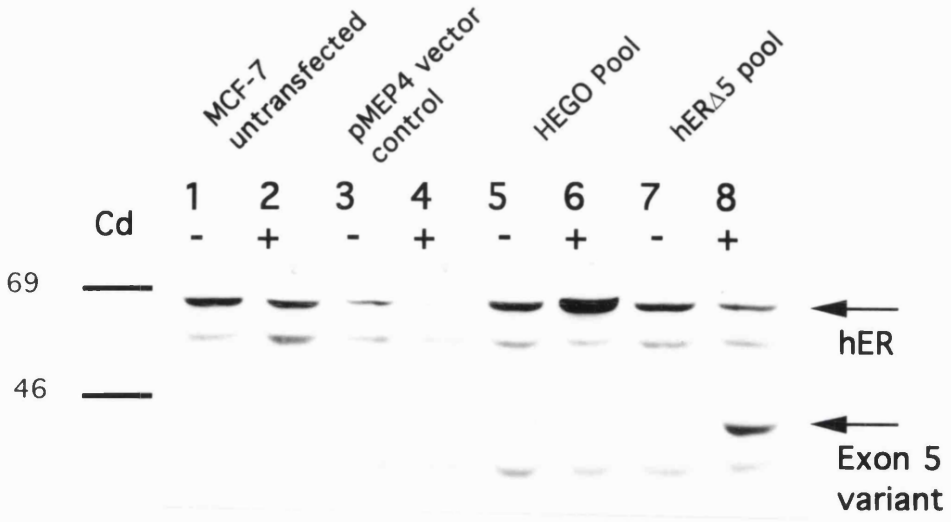


Fig 3.5

Western blot of MCF-7 cells stably transfected with full length and exon 5 variant receptor.
Hygromycin resistant cells were isolated after transfection with empty pMEP4, or vector containing full length hER or exon 5 variant. Individual colonies were pooled together. Cells were maintaned in steroid depleted medium and extracts prepared from untreated cells (uneven lane numbers) and cells induced with 5 μM cadmium chloride (even lane numbers). Receptors were detected using H226 anti ER antibody. Untransfected MCF-7 cells are shown as a control (lanes 1and2), empty vector control is shown (lanes 3 and4), full length hER (lanes 5 and6) and exon 5 variant (lanes 7 and 8).

presence of cadmium. This observation has been made by others who report that cadmium causes down regulation of ER, and has oestrogenic type effects on MCF-7 cells including stimulation of oestrogen responsive reporters and an increase in cell growth (Garcia-Morales *et al* 1994). In the cells transfected with the pMEP4hERΔ5, normal levels of endogenous receptor are seen but on induction with cadmium a band of approximately 42 kD was detected corresponding to variant ER expression. This band is not apparent in the other cell lines but can be seen faintly in the uninduced lane indicating that the metallothionein promoter is weakly active in the absence of added heavy metals.

Use of different heavy metals to induce receptor expression.

Since cadmium but not zinc have been shown to exibit oestrogenic like activity (Garcia-Morales *et al* 1994) we used zinc chloride in most subsequent work, especially in assessing the activity of the variant on cell growth and gene activation. In preliminary experiments we found that zinc chloride was tolerated in doses up to 200 μM with no apparent effect on cell growth or morphology. Cadmium however was only tolerated up to 1 μM. At higher concentrations it was noted to retard cell growth and at concentrations above 5 μM it was toxic causing cells to detach from the culture dishes.

Isolation and characterisation of clonal cell lines expressing exon 5 variant ER

Individual colonies of hygromycin resistant cells obtained after transfection with pMEP4 vectors were isolated by ring cloning and screened for the expression of full length and variant receptor. All of the clones analysed showed similar levels of full length receptor but most did not express variant receptor (data not shown). Three clones expressing variant receptor in an inducible manner were isolated which we named Var 1-3. The amount of ER expressed before and after stimulation with cadmium or zinc was analysed by Western blotting and is shown (fig 3.6). Semi quantitative estimates of relative abundance of receptor were made using multiple photographic exposures of the blots. Var-1 cells express detectable variant receptor in the absence of induction at a level comparable to the full length receptor. On induction with 200μM zinc chloride there is a dramatic rise in the level of variant receptor expression such that it exceeds the

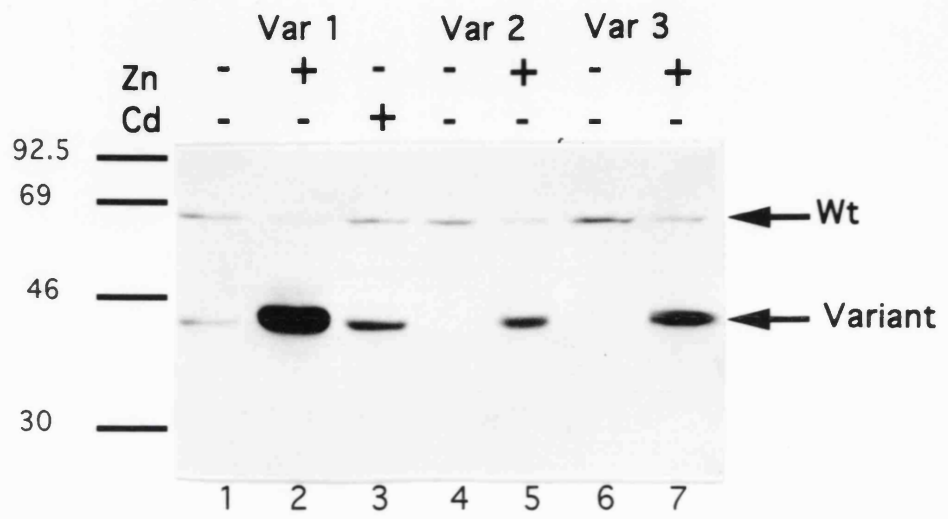


Fig 3.6

Induction of exon 5 variant expression in stable MCF-7 clones

The amount of full length and exon 5 variant receptor is demonstrated by western blot analysis of extracts prepared from three separate clones stably transfected with exon 5 variant ER. 100 µg of extract was separated by SDS PAGE. Blots were probed with H226. Lanes 1-3 show the cell line **var-1** in the uninduced state (lane1) after 24 hours induction with 5 µM cadmium chloride (lane 2) and after 24 hours induction with 200 µM zinc chloride. Lanes 4 and 5 show cell line **var-2** ,uninduced (lane 4) and with 200 µM zinc chloride (lane 5). Lanes 6 and 7 show cell line **var-3** ,uninduced (lane 6) and with 200 µM zinc chloride (lane 7).

amount of full length receptor by approximately 10 fold. It was noted that at the maximum tolerated doses, zinc chloride was more potent as an inducer of variant expression than cadmium chloride, which is reported as the most potent inducer of the metalothionien promoter. Var-2 cells do not express detectable levels of variant in the absence of induction but on induction express variant receptor at 2-3 times the level of endogenous receptor. Similarly Var-3 cells do not express variant receptor until exposed to heavy metals when zinc chloride can induce variant expression to a level 3-4 times the endogenous receptor level.

The specific antibody MP40 was used to confirm that the 42 kD H226 immunoreactive band seen in transfected cells was indeed genuine variant receptor by analysing Var-1 cells treated with increasing doses of zinc chloride. Extracts were analysed by western blotting using with MP40 and H226 as primary antibody (Fig3.7). Here it can be seen that with increasing doses of zinc chloride an increasing amount of variant receptor is detected. Doses above 200 μM were not used, as these would have been in the toxic range

Duration of Exon 5 receptor expression.

In order to assess the effect of exon 5 variant expression on cell growth it is necessary to have prolonged expression of the receptor we therefore analysed the duration of exon 5 variant receptor expression in Var-1 cells during prolonged exposure to 200μM zinc chloride. Cells were grown for up to 6 days in the presence of zinc chloride and analysed by western blotting. (Fig 8) Maximal expression is seen after 1 day but the variant remains expressed at substantially higher levels than the wild type for 6 days. In this experiment we also note that the level of the endogenous receptor is decreased at 1 day but that over the time course of the experiment the endogenous receptor recovers towards the uninduced level.

Effect of ligands on the expression of variant receptor

The expression of endogenous and exon 5 variant receptor in Var-1 cells was examined in the presence of E2 and antioestrogens, both in the absence and presence of zinc chloride (Fig 3.9). In the uninduced state the endogenous receptor is seen to respond to ligands as expected with a reduction in ER after exposure to E2. There is little effect of OHT and

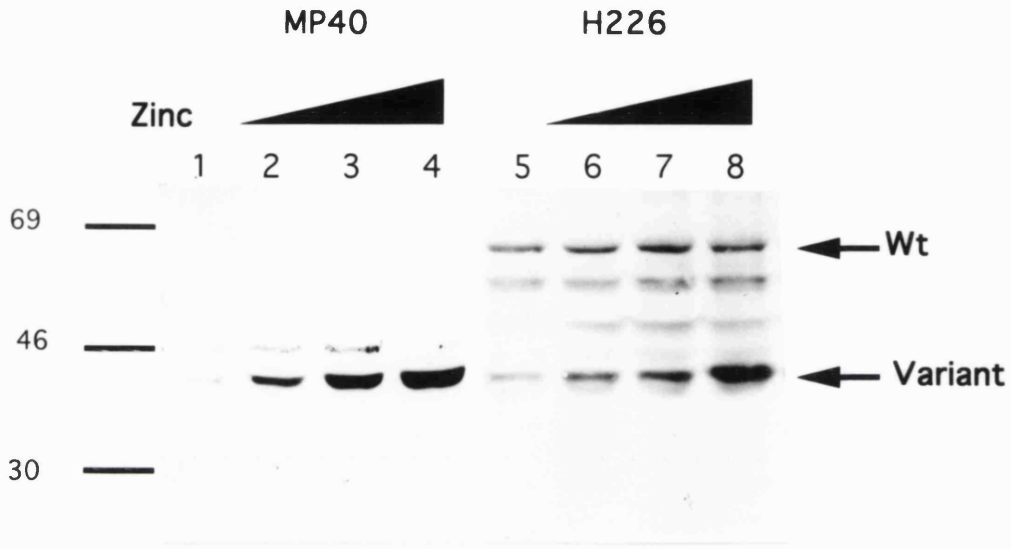


Fig 3.7

Dose dependency of exon 5 variant oestrogen receptor induction detected by a specific and non specific antibody

To examine the dose dependency of variant induction **var-1** cells were induced with increasing concentrations of zinc chloride. Duplicate samples were separated by SDS PAGE Western blots were probed using exon 5 specific antibody MP40 (lanes 1-4), or H226 (lanes 5-8). Lanes 1and 5 are control lanes with no inducer present, Lanes 2 and 6 were induced with 50 μM zinc chloride. Lanes 3 and 7, 100 μM zinc chloride. Lanes 5 and 8, 200 μM zinc chloride.

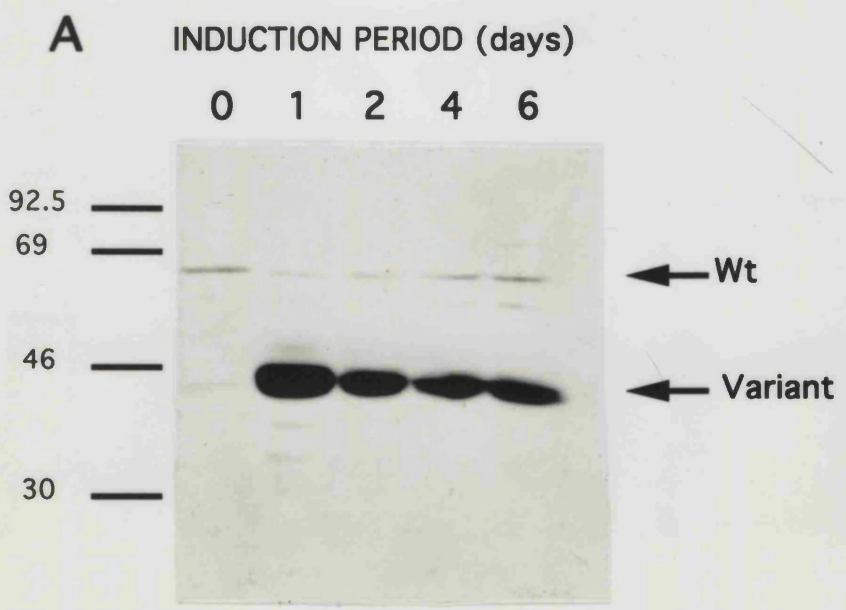


Fig 3.8

Duration of induction of exon 5 variant oestrogen receptor

To determine the duration of induced expression of variant receptor **var-1** cells were grown in the pressence of zinc chloride for up to 6 days. Variant receptor expression was determined by western blot analysis of cell extracts using H226. The induction period is indicated over each lane

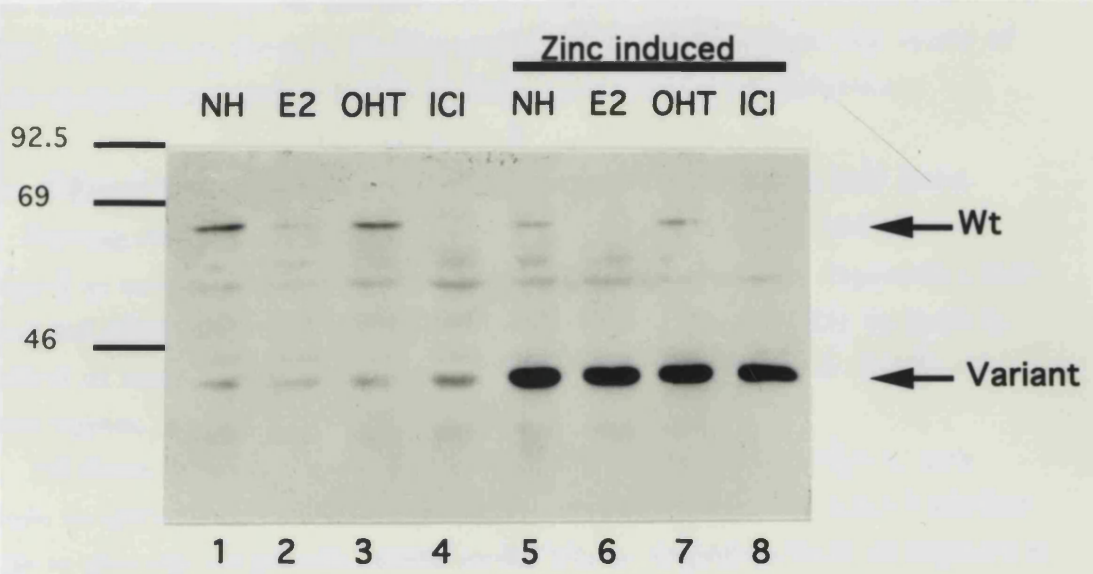


Fig 3.9

The effect of ligands on the expression of wild type and variant receptors in Var-1 cells

The effect of ligands on receptor levels was assessed by treating uninduced (lanes 1-4) and induced cells (lanes 5-8) for 15 hours with no ligand (NH, lanes 1 and 5); 10^{-9} M 17-β estradiol (E2, lanes 2 and 5); 10^{-6} M 4 hyroxytamoxifen (OHT, lanes 3 and 7) or 10^{-6} M ICI 182,780 (ICI, lanes 4 and 8). Receptors were then detected by Western blotting using H226.

complete disappearance full length ER in the presence of ICI 182780. The fall in endogenous receptor is again seen with the addition of zinc chloride. This is further reduced by addition of E2 and is undetectable with ICI 182,780. In contrast there is no discernible effect of ligand on the levels of variant receptor expression in the absence or presence of induction.

Effect of Exon 5 variant ER expression on growth of stable cell lines

Having established a system where the exon 5 variant could be expressed at several different ratios and established that this expression was maintained after induction for more than 5 days it was possible to analyse the effect of variant expression on cell growth in the presence of E2 or antioestrogens.

All three cell lines were grown in the absence or presence of zinc chloride to alter the amount of variant expressed and the effects of adding ligands to the rate of growth monitored. These responses were compared to MCF-7 cells transfected with the parental pMEP4 vector (Vector control). All clones were seen to grow at similar rates in the absence of ligand. Increasing cell number by 4-5 fold after 7 days (Fig10). The effect of E2 on variant expressing clones was similar in all cases to that of the control cells, increasing the growth rate such that cell numbers increased 15-20 fold after 7 days. In the presence of OHT there was a small decrease in cell growth compared to cells grown in the absence of hormone. The pure antioestrogen ICI 182780 had a more pronounced effect reducing the growth of all clones further. Although there was slight variability in the degree of growth inhibitory action of the antioestrogens between the three variant expressing cell lines, these differences were comparatively small. In all cases there was no discernible effect of inducing expression of exon 5 variant ER. The control cells were unaffected by the addition of zinc chloride.

Effect of exon 5 variant ER on the ability of antioestrogens to antagonise the growth stimulation of E2

The ability of antioestrogens to block the growth stimulatory effect of oestrogens is an important aspect of their clinical efficacy, therefore the ability of the variant to influence this action was tested in Var-1 cells. In this experiment cell growth was followed over a 15 day period. E2 stimulated

FIG 3.10

Effect of inducing exon 5 variant ER expression on the proliferative response to oestrogen and antioestrogens

Three variant expressing clones (Var-1-3) are compared to cells transfected with empty vector (Vector control). Cells were plated at equal densities and increase in cell number determined after 7 days growth. Each cell line was grown in the absence of the inducer zinc chloride (-) or with 200 μM zinc chloride (+). In addition cells were exposed to ethanol carrier alone (■), 10^{-9}M 17-β oestradiol (□), 10^{-6}M 4-hydroxytamoxifen (▧), 10^{-6}M ICI 182780 (▨). Results are expressed as fold increase in cell number above the number of cells present at the time treatments were applied. The results shown represent the mean values of three independent experiments performed in duplicate, error bars indicate the standard error.

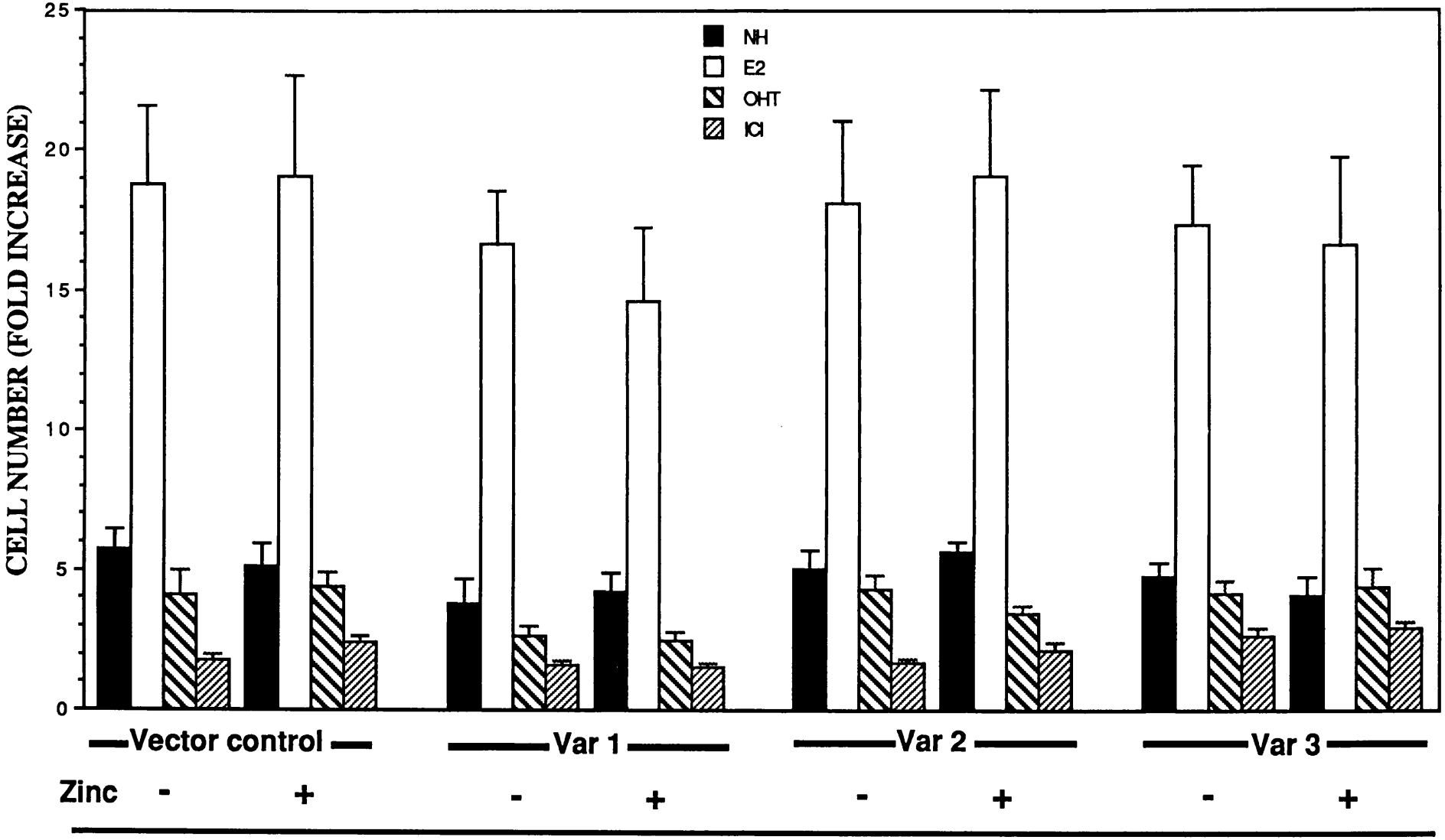
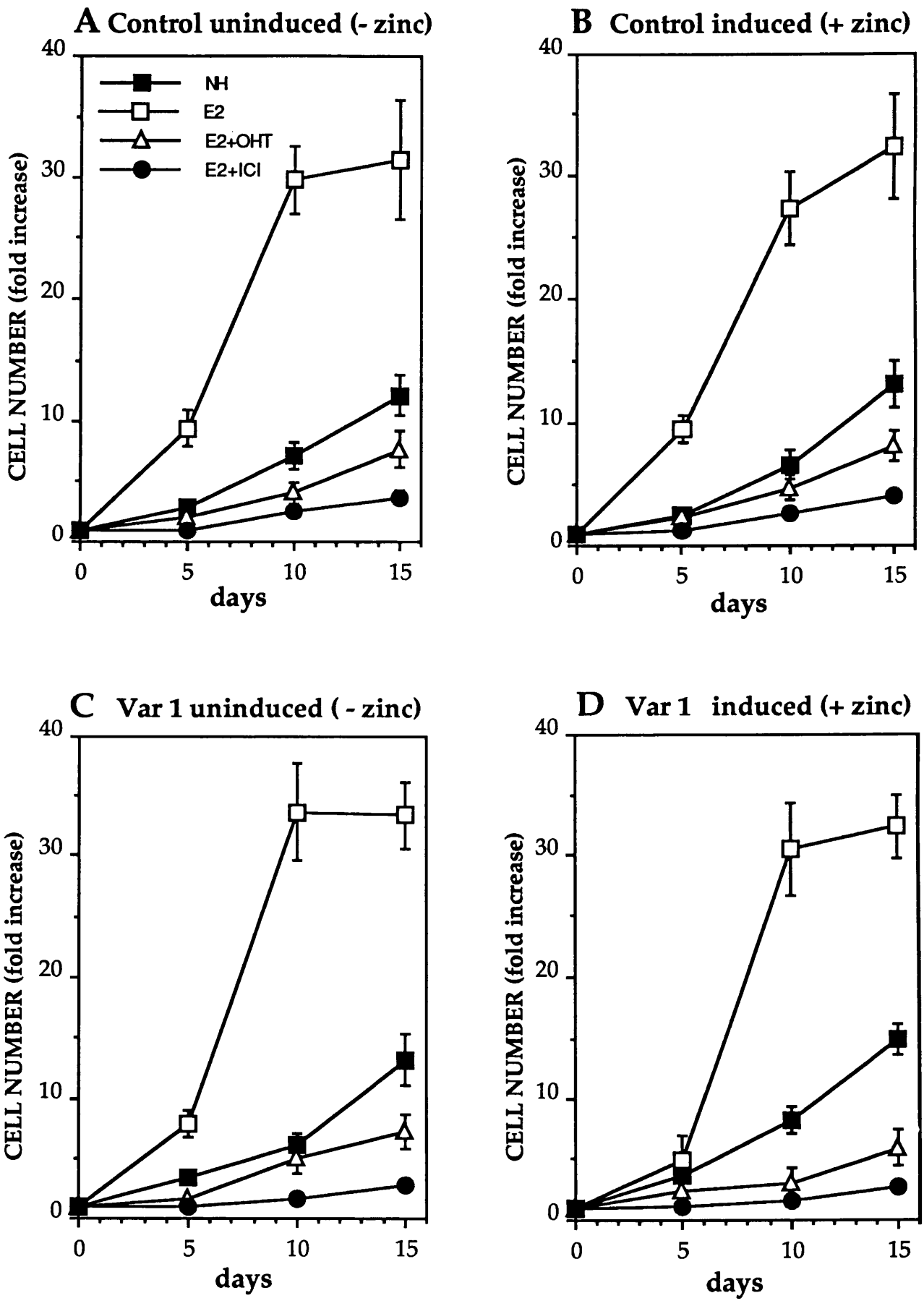

CELL NUMBER (FOLD INCREASE)
25
20
15
10
5
0
NH
E2
OHT
ICI
Vector control
Var 1
Var 2
Var 3
Zinc
-
+
-
+
-
+
-
+

Fig 3.11

Antagonism of oestrogen stimulated growth by antioestrogens

Vector control and Var 1 cells were plated out and left to recover for two days and then treated in the absence or presence of 200 μM zinc chloride (-/+zinc) for periods up to 15 days with ethanol carrier alone (■), or in the presence of 10^{-8}M 17-β oestradiol (□), 10^{-8}M 17-β oestradiol plus 10^{-6} M 4 hydroxytamoxifen (Δ), or 10^{-8}M 17-β oestradiol plus 10^{-6} M ICI 182,780 (●). Results are plotted as fold increase compared to cell number at the time of adding ligand. Data shown represents the mean of three separate experiments performed in duplicate, standard error is indicated by error bars.


A Control uninduced (- zinc)
B Control induced (+ zinc)
C Var 1 uninduced (- zinc)
D Var 1 induced (+ zinc)
NH
E2
E2+OHT
E2+ICI
CELL NUMBER (fold increase)
days
0
5
10
15
20
30
40

cells were compared to cells grown with both E2 and excess antioestrogens. The effect of inducing variant expression on these responses was monitored. In both control cells and variant expressing cells the growth stimulation seen in the presence of E2 was completely reversed by both OHT and ICI 182780 (Fig 3.11). The rate of growth was suppressed below that seen in the absence of added hormone, ICI 182780 showing more potent growth retardation than OHT. The antagonistic action of both antioestrogens was unaffected by induction of variant with zinc chloride which also had no effect on the responses in the control cells.

These experiments demonstrate that despite the presence of AF-1 (which was shown to be constitutively active in chicken embryo fibroblasts), the exon 5 variant ER is not capable of significantly stimulating the growth of MCF-7 breast cancer cells in the absence of ER ligand. It is also not capable of influencing growth stimulation response to oestradiol or the antagonistic action of the partial agonist hydroxytamoxifen or the pure antioestrogen ICI 182,780. It appears therefore that in isolation, expression of exon 5 variant receptor even in large excess is not sufficient for antioestrogen resistance in MCF-7 cells.

Activity of the Exon 5 variant ER on gene activation in MCF-7 cells

Although there was no effect of the exon 5 variant on cell growth it may have an effect on individual gene expression. Therefore the action of the variant on 2 reporter genes and 2 endogenous oestrogen responsive genes was analysed.

Transient transfection of variant expressing cells with an ERE tk luciferase reporter

The effect of variant expression on gene transcription in breast cancer cells was examined by transient transfection of Var-1 cells using the ERE tk luciferase reporter described earlier. Comparisons were made between induced and uninduced cells. It was noted that the addition of 200 μM zinc chloride increased the expression of the internal control reporter plasmid pJ7 lacZ consistently by 3 fold. It was therefore not possible to use the β-galactosidase assay to correct for transfection efficiency unless an adjustment was made to allow for this effect. Therefore the results of each well of transfected cells were normalised for protein concentration. In general the

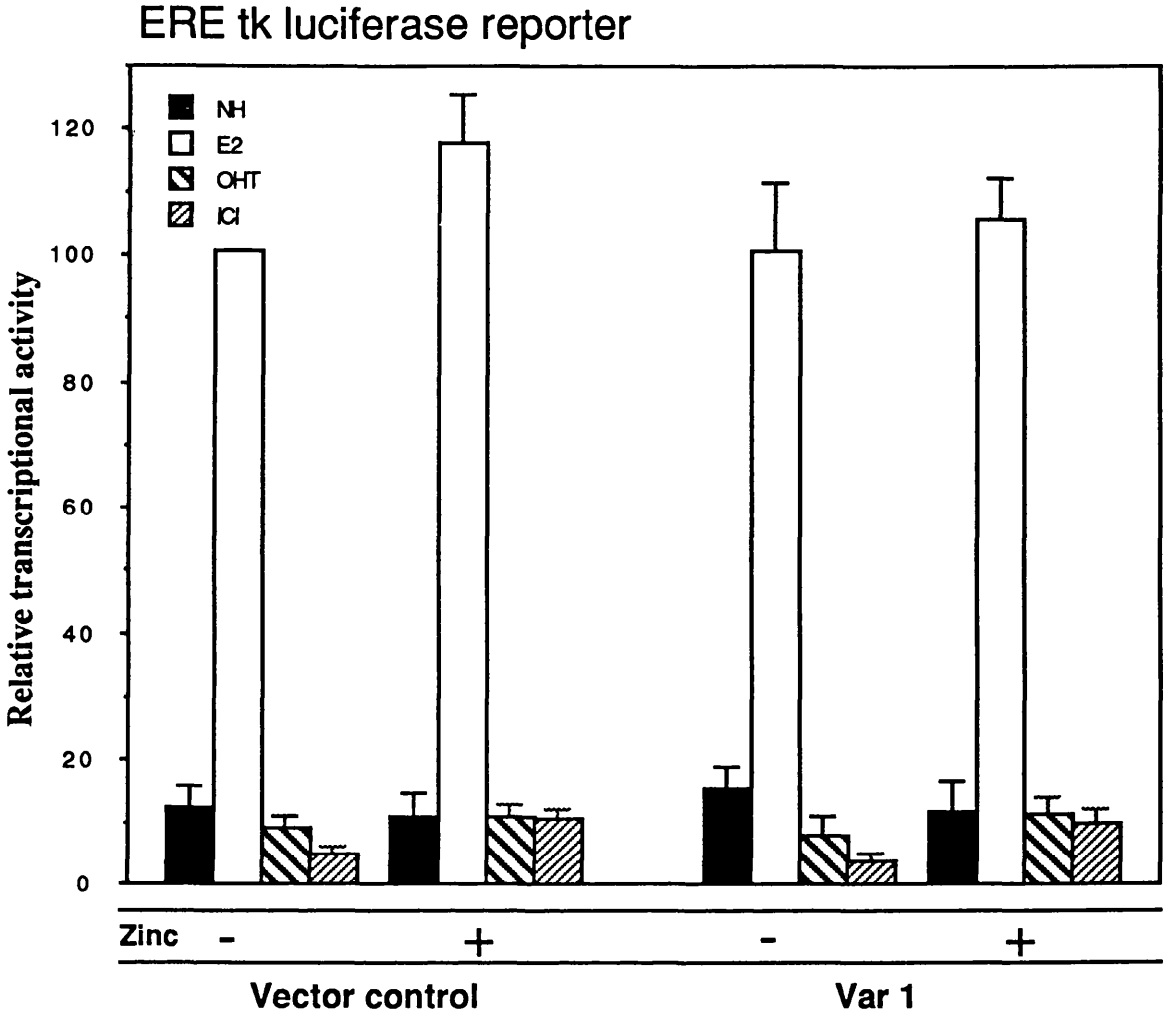


Fig 3.12

Transient transfection of exon 5 variant expressing cells with an ERE tk luciferase reporter. Control cells and variant expressing cells were transiently transfected in 24 well plates with 0.5 μg ERE tk luciferase and 0.1 μg pJ7 β-galactosidase reporters in a total of 1 μg DNA . After transfection cells were incubated for 24 hours in the absence or presence of 200 μM zinc chloride with ethanol carrier alone (■), 10^{-8} M oestradiol (□), 10^{-6}M 4-hydroxytamoxifen (▧), or 10^{-6}M ICI 182,780 (▨). Cells were harvested and assayed for luciferase activity. Protein concentration was determined and β-galactosidase activity was monitored. Luciferase activity was corrected for protein concentration and has been expressed as the percentage of the activity seen in the control cells stimulated by oestradiol in the absence of induction by zinc chloride. Transfections were performed in duplicate and the results shown represent the mean of three independent experiments. Standard error is indicated by error bars.

variation in protein concentration between wells was less than 10% and these differences paralleled the β galactosidase results.

Transfection results are summarised in Fig 3.12. In both Var-1 and vector control cells in the absence of inducer there is low basal activity in the absence of added ligand this Stimulation of approximately 8 fold is seen on treatment with E2. Treatment with OHT has a minimal suppressive effect on basal activity and ICI 182,780 suppresses basal activity substantially. When the cells are induced with zinc chloride there is no observable increase in basal activity in Var-1 cells or control cells. The response to E2 is unchanged but there is no significant suppression of basal activity in the presence of either antioestrogen. Since this loss of suppression is seen in both control and var-1 cells it is presumably an effect of zinc chloride and not the induced variant ER. Zinc chloride therefore may have some weak stimulatory effect on this reporter but this is only apparent when the basal ER stimulation is suppressed. It is likely that this stimulation occurs in an ER independent manner since the pure antioestrogens result in receptor depletion. Similar results were obtained when Var-2 cells were transiently transfected with ERE tk luciferase. The important conclusion from this experiment is that the exon 5 variant ER is unable to stimulate gene transcription and it does not affect the ability of the full length receptor to stimulate in response to E2. Thus as with the growth assays it has not been possible to demonstrate significant functional activity of the variant receptor.

Transient transfection of variant expressing cells with an ERE2TATA CAT reporter.

The effect of the variant on a simple promoter was investigated with a further set of transient transfection experiments using a reporter construct consisting of two xenopus vitelogenin EREs upstream of the adenovirus major late TATA box linked to CAT. In these experiments we increased the number of cells in each transfection scaling up the transfection from 24 to 6 well plates because the activity of this promoter is weak and more cells are required for an accurate assay. As before the transfections were normalised for protein concentration but a similar result was obtained if a correction in β galactosidase activity is made in zinc treated transfections. The results of these experiments are summarised in Fig 3.13. With this reporter the basal activity is low and both control and Var-1 cells show a 50 fold stimulation of

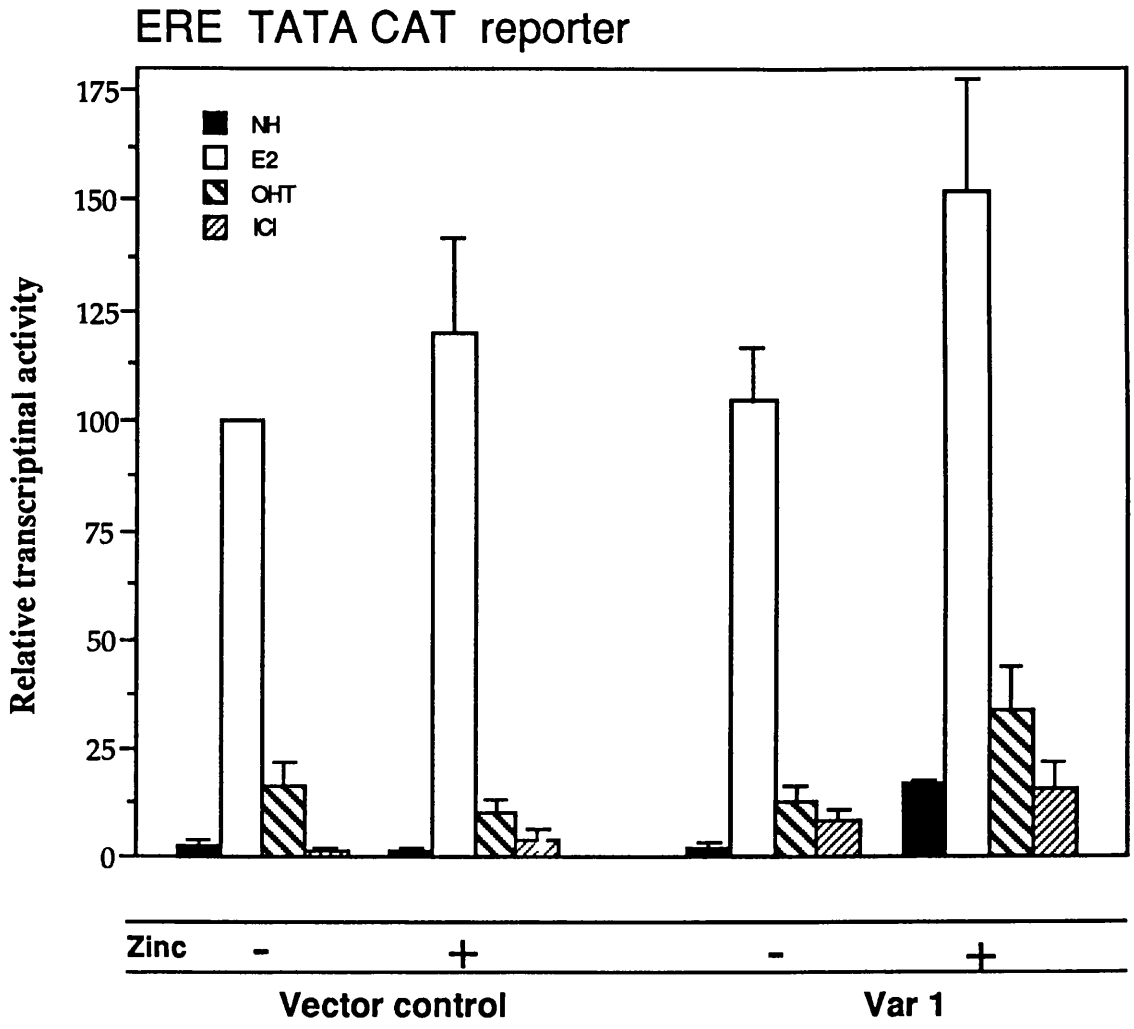


Fig 3.13

Transient transfection of exon 5 variant expressing cells with an ERE TATA CAT reporter. Control cells and variant expressing cells were transiently transfected in 6 well plates with 2.0 µg ERETATACAT and 0.5 µg pJ7 β-galactosidase reporters in a total of 5 µg DNA . After transfection cells were incubated for 24 hours in the absence or presence of 200 µM zinc chloride with ethanol carrier alone (■), 10^{-8}M oestradiol (□), 10^{-6}M 4-hydroxytamoxifen (▧), or 10^{-6} M ICI 182,780 (▨). Cells were harvested and assayed for CAT activity. Protein concentration was determined and β-galactosidase activity was monitored. Luciferase activity was corrected for protein concentration and has been expressed as the percentage of the activity seen in the control cells stimulated by oestradiol in the absence of induction by zinc chloride. Transfections were performed in duplicate and the results shown represent the mean of three independent experiments. Standard error is indicated by error bars.

CAT activity with E2 (without zinc induction.). This is unaffected by zinc chloride in the control cells but var-1 cells show a significant 5 fold increase in ligand independent activity on addition of zinc chloride suggesting that this reporter is constitutively stimulated by increasing the amount of exon 5 variant ER. The reporter remains fully sensitive to stimulation by E2. There is a modest stimulation of this reporter with the addition of OHT in both cell lines of 3-4 fold. This stimulation is enhanced in var-1 cells induced with zinc, although since basal activity is already elevated there is a reduction in fold increase compared to basal activity. The response to ICI 182,780 is also dependent on the expression of the variant. In control cells there is a suppression of basal activity by ICI 182,780 but after zinc induction this suppression is not seen (a similar effect was seen with the tk promoter). In the Var-1 cells there is a paradoxical increase in activity of uninduced cells when ICI 182,780 is added although this rise is small and of uncertain significance. In the presence of zinc induction ICI 182,780 stimulation increases to a similar level to that seen with no added hormone.

It therefore seems that this reporter is weakly responsive to stimulation by Exon 5 variant receptor and this stimulation is not suppressed by antioestrogens. There is a tendency for the ligand independent stimulation to increase in the presence of the pure antioestrogen. The response to stimulation by E2 is unaffected.

Analysis of pS2 expression by Northern blot analysis

To examine the effect of the exon 5 variant on natural oestrogen responsive genes total RNA was extracted from Var 1 and control cells before and after induction with zinc chloride or cadmium chloride. Cells were also treated with E2. RNA from these samples was analysed by probing a northern blot with DNA probes for pS2 and with human γ actin The signal from these two probes was quantitated by phosphoimager, to quantify the induction of pS2, Fig 3.14. The Northern blot analysis shows that in both control and var-1 cells pS2 is induced as expected by addition of E2 by 7 and 4 fold respectively. There is however no significant effect of zinc chloride and only a minor increase in pS2 with cadmium chloride 1.3 and 1.5 fold respectively which is of doubtful significance. This evidence suggests that the exon 5 variant is not capable of inducing pS2 expression.

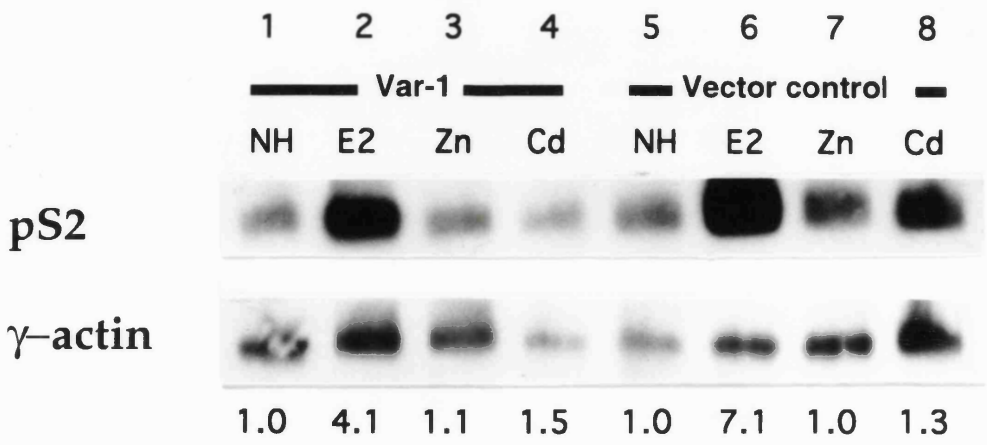


Fig 3.14

Northern blot analysis of pS2 expression in variant expressing cells

Total RNA from exon 5 variant oestrogen receptor expressing var-1 cells (lanes 1-4) and control cells (lanes 5-8) was analysed for pS2 expression after 24 hours treatment with no added ligand (lanes 1 and 5), 17β–oestradiol 10^{-8}M (lanes 2 and 6), zinc chloride 200 μM (lanes 3 and 7), or cadmium chloride 5 μM (lanes 4 and 8). The blot was stripped and reprobed for γ-actin expression. Signal intensity was quantified by phosphoimager. Numbers below each lane indicate the fold induction of pS2 signal compared to the no ligand lanes for each cell line. after correction for γ–actin signal intensity.

Expression of progesterone receptor protein

The ability of the exon 5 variant to influence expression of progesterone receptors was examined by analysing cell extracts from control and variant expressing cells treated with E2 or zinc chloride for progesterone receptors using a commercial immunoassay kit (assays were kindly performed by Diana Barnes and William Harris ICRF oncology unit Guys Hospital).

The results are indicated in table 3.1. In the absence of hormone PR levels were similar in the two cell lines. E2 induced the expression of PR 15 fold in control cells and 6 fold in var-1 cells. Induction with zinc chloride resulted in a modest rise in PR content of both cell lines 2.3 and 1.7 fold respectively. Clearly there is an effect of zinc causing a rise in PR expression but since this occurs in the control cells it is an effect of the inducing agent and not due to increased exon 5 ER expression.

Table 3.1 ***Expression of progesterone receptors***

	Progesterone receptor levels (fmol/mg protein)	
Cell line	**Vector control**	**Var-1**
Treatment		
No Hormone	**48**	**60**
Oestradiol (10^{-8}M)	**762**	**375**
Zinc chloride (200μM)	**111**	**102**

Cellular localisation of the exon 5 variant.

The variant contains the nuclear localisation signals contained within region D of the receptor and is therefore predicted to be nuclear but since ligand also has an influence on nuclear localisation and the variant can not

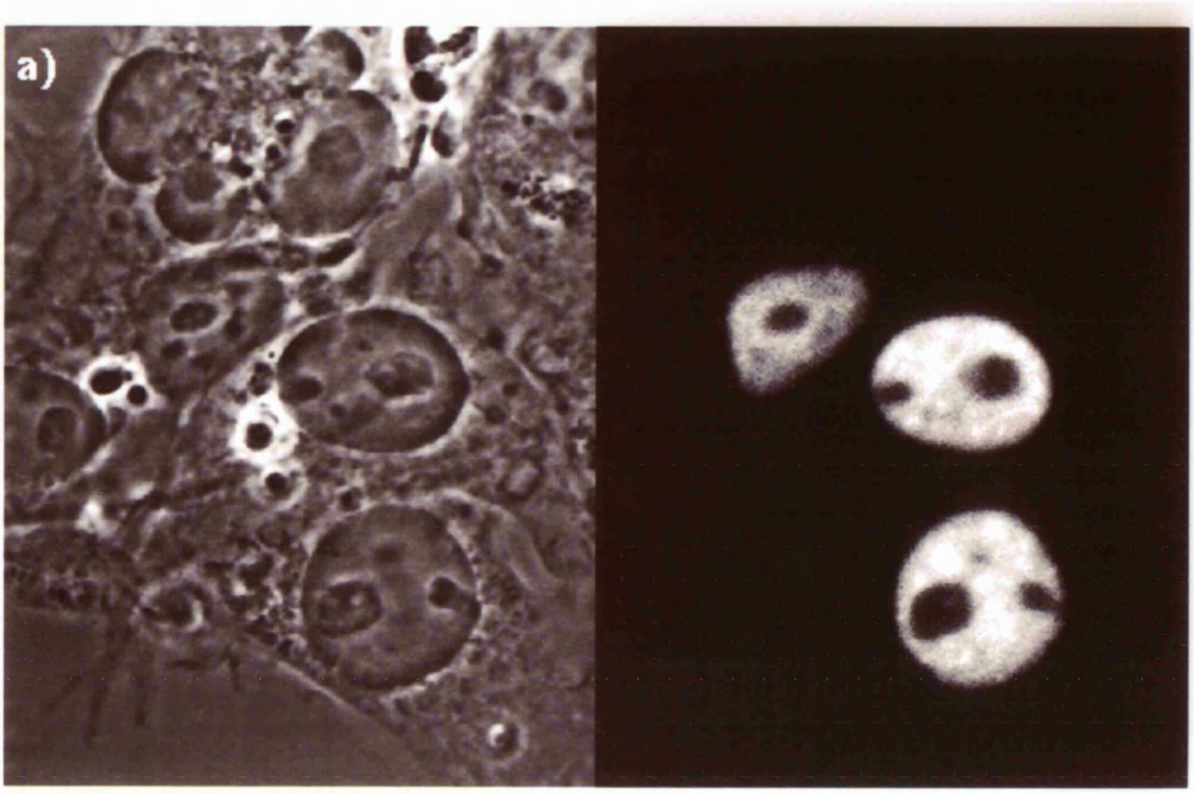
bind ligand indirect immunoflouresence studies were performed. Cos-1 cells were transiently transfected with variant or wild type receptor and detected by indirect immunoflouresence using MP40, N-ter, (a monoclonal antibody against the N terminus of the ER {Dauvois unpublished but similar to MP15}) or H222. Fig 3.15 a; shows images generated by confocal microscopy of Exon 5 variant receptor expressed in Cos-1 cells and detected with MP40 and FITC conjugated anti rabbit antibody. The receptor is clearly detectable only in the nucleus of the cells. The distribution is identical to full length receptor detected with N-ter Fig3.15 b. This experiment demonstrates that the MP40 antibody is effective as a reagent for detecting exon 5 variant histologically. To confirm the specificity of the antibody for the variant receptor Cos-1 cells were independently transfected with either variant or full length receptor, after eletroporation and recovery on ice the two transfected populations were mixed together before plating onto coverslips. These cells were then incubated with a mixture of two primary antibodies prepared from different species and two secondary antibodies, one to each of these species. The secondary antibodies were conjugated to different fluorescent markers responding to different wavelengths of light. Fig 3.16 shows images from this experiment, in 3.16A, exon 5 variant receptor is detected by MP40 (using an antirabbit FITC conjugate). In 3.16 B, both full length and variant receptors are detected by using N-ter (and Texas red conjugated antimouse antibody). This experiment clearly demonstrates the specificity of MP40 in indirect immunoflouresence studies. This specificity is further demonstrated in fig 3.17 where Cos-1 cells were transfected with full length and variant receptor, mixed as described above and stained using a mixture of both MP40 and H222 followed by secondary staining with anti rabbit FITC conjugate anti rat Texas red conjugate. H222 reacts to a C-terminal epitope in the full length receptor but will not detect the exon 5 variant. Hence exon 5 variant cells are detected only as green fluorescence and full length receptors as red fluorescence. In this experiment the full length receptors have produced a weak signal because the Texas red conjugate used had deteriorated in quality. This has resulted in poor definition of the nuclei stained with Texas red, which does not accurately reproduce the naked eye appearance seen with dark adapted vision in which weak intensity but well defined nuclei can be seen.

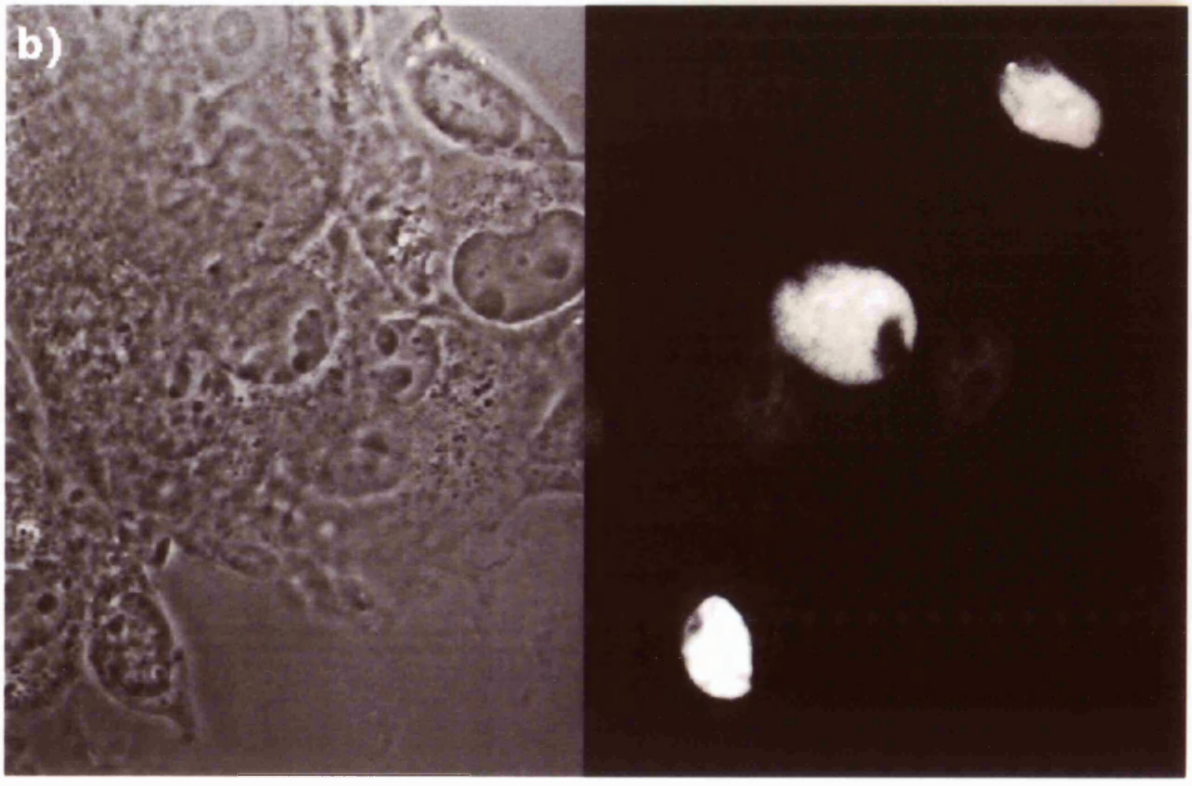
3.15

Indirect immunoflouresence of exon 5 variant and full length receptor expressed in Cos-1 cells

a) Cos-1 cells transfected with exon 5 variant receptor were detected using MP40 and FITC conjugated anti rabbit immunoglobulin. The left hand panel shows a phase contrast immage and the right hand panel shows the flouresence image showing nuclear staining of transfected cells

b) Cos-1 cells transfected with full length oestrogen receptor detected with N-Ter anti ER antibody and FITC conjugated anti mouse antibody. The left hand panel shows a phase contrast immage and the right pannel shows the flouresence image showing nuclear staining.


a)

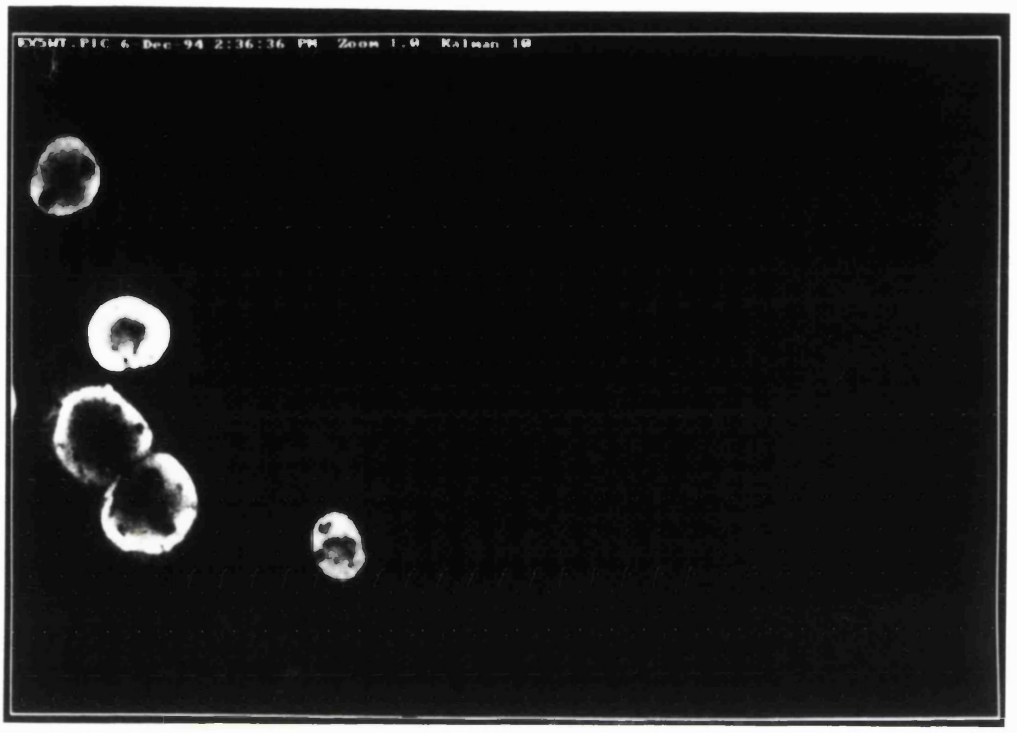

b)

3.16

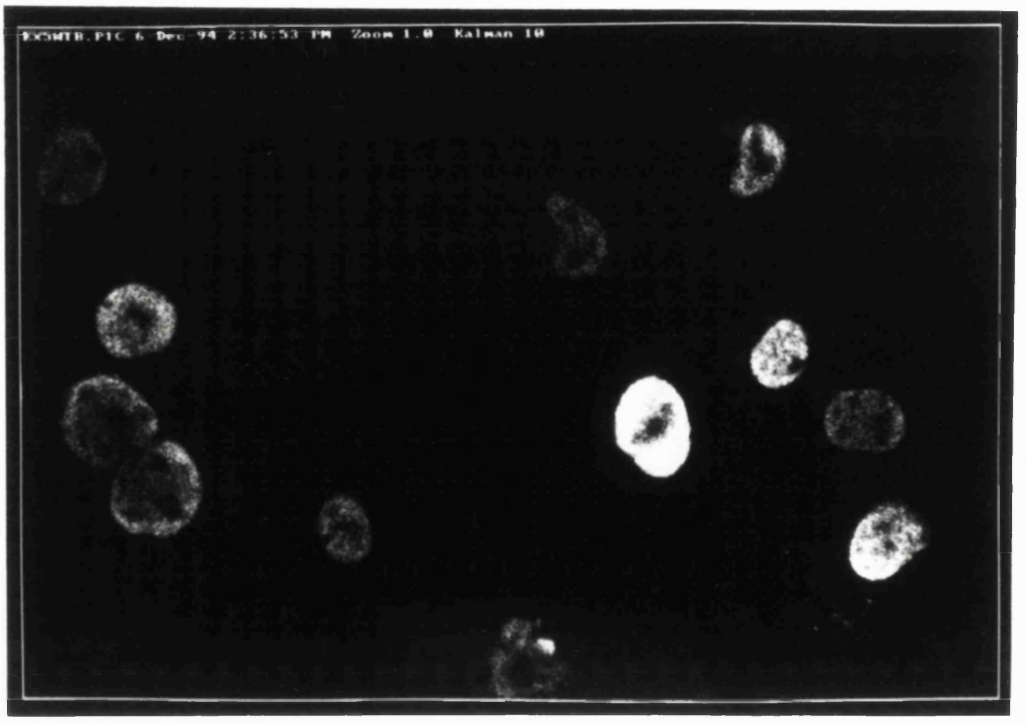
Indirect immunoflouresence of mixed exon 5 variant and full length receptor expressed in Cos-1 cells.

Cos-1 cells transfected with full length and variant receptor were mixed together and stained simultaneously with MP40 and N-Ter. The antibodies were detected with FITC conjugated anti-rabbit immunoglobulin (MP40) and Texas red conjugated anti-mouse immunoglobulin(N-Ter). a) shows the FITC flouresence image detecting only nuclei transfected with exon 5 variant.
b) shows the Texas red flouresence immage detecting both exon 5 variant receptor and full length receptor.

A


EXSWT.PIC 6 Dec 94 2:36:36 PM Zoom 1.0 Kalman 10

B


EXSWTB.PIC 6 Dec 94 2:36:53 PM Zoom 1.0 Kalman 10

3.17

Indirect immunoflouresence of mixed exon 5 variant and full length receptor expressed in Cos-1 cells.

Cos-1 cells transfected with full length and variant receptor were mixed together and stained simultaneously with MP40 and H222. The antibodies were detected with FITC conjugated anti-rabbit immunoglobulin (MP40) and Texas red conjugated anti-rat immunoglobulin(H222). This image shows the green FITC flouresence image detecting a single nucleus from a cell transfected with exon 5 variant and superimposed is the texas red image showing 2 nuclei from cells transfected with full length receptor.

Detection of exon 5 variant in cell lines and tumour samples

The exon 5 variant is readily detectable in var-1 cells with MP40 (Fig 3.17) however using both western blot and immunoprecipitation. It has not been possible to detect exon 5 variant receptor in normal MCF-7 cells or the ER positive ZR 75 and T47D cells. ER negative BT20 and MDA231 cells also show no detectable variant ER (Data not shown). In a preliminary immunohistochemical study performed by Diana Barnes and William Harris the MP 40 antibody has not revealed the presence of exon 5 variant ER in histological sections of ER positive human tumours.

Further studies on tumour material have been performed by S Johnston on tamoxifen resistant material using immunohistochemical staining. The staining technique has been validated using paraffin embedded blocks prepared from pelleted control and var-1 cells. No specific MP40 staining has been seen in tumour blocks. This includes blocks from tumours that are known to have a high exon 5 variant to wild type ratio.

Summary and conclusions

The exon 5 variant ER mRNA codes for a truncated ER retaining AF-1 and the DNA binding domain of the full length receptor. This protein has been shown to activate transcription in transfected animal cells where AF-1 is known to have strong activity, this activity is unaffected by ER ligands. The Exon 5 variant receptor has been stably introduced into MCF-7 cells under the control of the metalothionien promoter. Variant receptor can be induced and expression maintained for long periods with zinc chloride. In three independent cell lines examined no apparent effect on proliferation was seen when the variant receptor was induced. There was no change in the proliferative response to oestrogen, the partial agonist 4-hydroxytamoxifen or the pure antioestrogen ICI 182780. There was no effect on the ability of the oestrogen antagonists to reverse the proliferative stimulus of oestradiol. The effect of variant expression on gene transcription was studied, there was a modest constitutive stimulation of a simple promoter when variant receptor was induced but this effect was not seen on a more complex tk promoter. The stimulation seen was not blocked by addition of ICI 182780. The stimulation seen with variant receptor did not affect the response to oestradiol and appeared to be additive to the modest stimulation seen on this

promoter with 4-hydroxytamoxifen. In natural oestrogen responsive genes no increase in pS2 expression was seen after induction of the variant receptor and progesterone levels are not affected by the variant receptor. It is therefore concluded that although the expression of some oestrogen responsive genes may be affected by the exon 5 variant ER it does not elicit a positive proliferative response in MCF-7 breast cancer cells and is not able to modify response to antioestrogens. It is therefore unlikely that oestrogen independence and tamoxifen resistance in breast cancer arises solely from increased expression of splice variant ER.

Chapter 4

Activity of mutant oestrogen receptor in breast cancer cells and screening for mutations of the oestrogen receptor in breast cancer

Introduction

This chapter describes experiments in which oestrogen receptors containing mutations which affect AF2 function are transiently expressed in breast cancer cells. The transcriptional behaviour of these mutants are investigated in a cell type in which oestrogens are an important mitogen. These experiments model the effects that could occur in breast cancer cells as a result of acquired mutations in AF2. Following this a system for the identification of mutations in the ER present at low frequency within a tumour sample is described. This novel approach to identification of mutations was used with the aim of identifying mutations that may be present in only a small proportion of the tumour cells particularly at the primary tumour stage. If expression of ER mutations resulted in insensitivity to the growth inhibitory action of antioestrogens then clones of resistant cells may arise from a single cell carrying an antioestrogen resistant ER mutation and may potentially give rise to metastatic clones dominated by a functionally important mutation. The system selected permits analysis of tumour DNA that consists of DNA derived from both stromal elements and malignant breast epithelial cells. The results of screening both untreated primary breast cancers and tumour tissue known to have developed acquired tamoxifen resistance are presented. This chapter also describes attempts to establish stable expression of AF2 mutant receptors in breast cancer cell lines intended to further evaluate the potential role of mutant AF2 ER in the proliferative responses of breast cancer cells in response to antioestrogens and establish whether these mutations are a potential causes of antioestrogen resistance.

Transient expression of mutant oestrogen receptors in MCF-7 breast cancer cells

The effect of introducing mutant oestrogen receptor or additional wild type receptor into MCF-7 cells was studied in transient transfection experiments. Using ERE tk luciferase as reporter and a constitutive pJ7 lac Z reporter as an internal control, the effect of oestrogen and antioestrogens on reporter activity in the presence of artificially introduced ER was examined. Three mutants (L-543A/L-544A, M-547A/L-548A and Δ 540-552) were introduced two of these had mutations in the amphipathic α-helix located at the C-terminal part of the ligand binding domain, in which

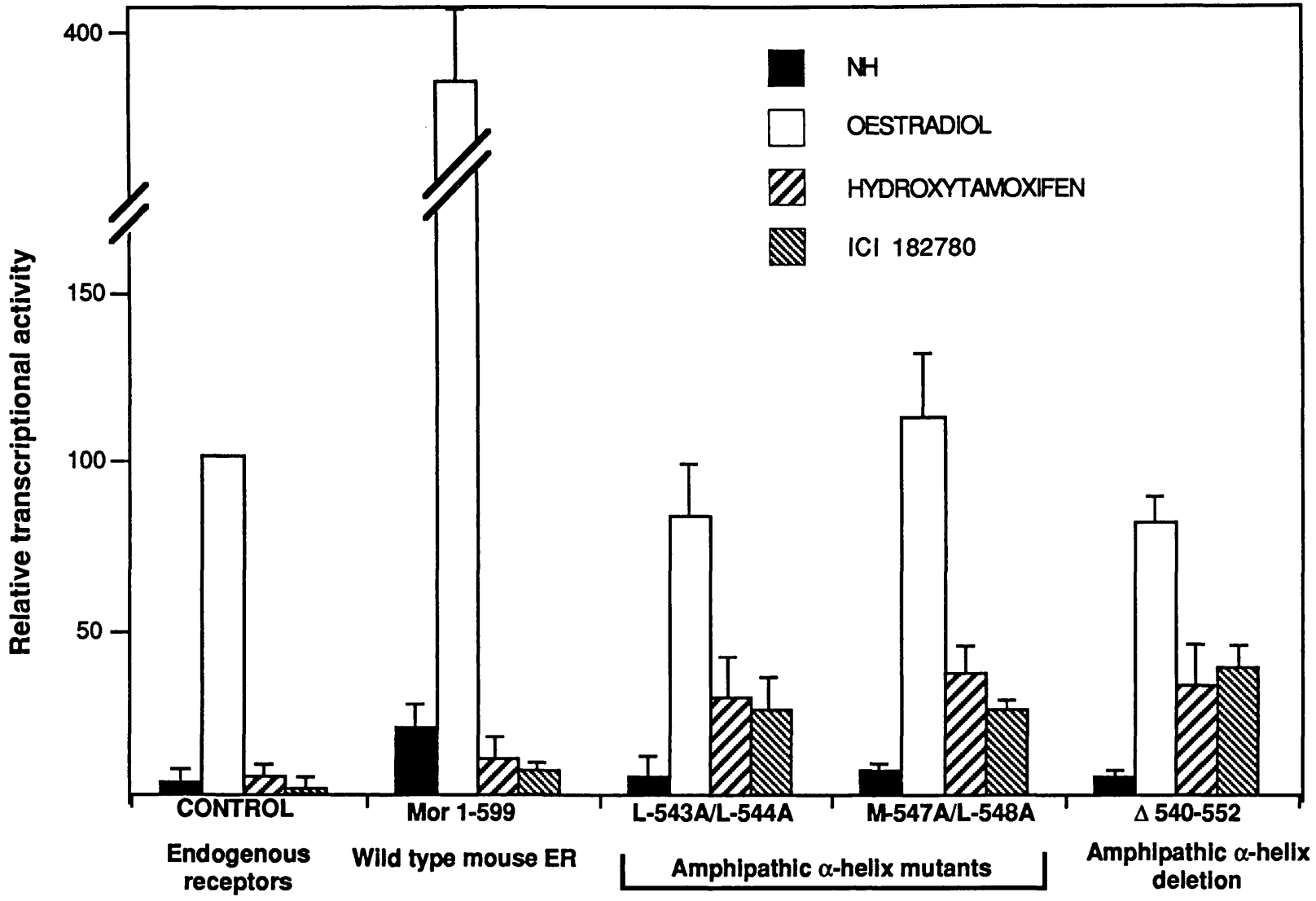
adjacent pairs of hydrophobic amino acids were replaced by alanines (L-543A/L-544A and M-547A/L-548A). The third mutant had the entire conserved region between amino acids 540 and 552 deleted (Δ 540-552). As discussed in chapter 1, this region is critical for E2 stimulated AF2 activity. The first two mutant receptors used in the following experiments not only abolish E2 dependent AF2 activation but are known to alter the pharmacology of antioestrogens resulting in agonist activity in the presence of both OHT and ICI 164,384 in HeLa cells (see Chapter 1). In addition to the mutants the wild type sequence was also used as a control.

The results of transfecting MCF-7 cells with these receptors is summarised in Fig 4.1. In this figure, as described in the previous chapter, E2 stimulates transcription and while OHT has little effect compared to basal activity, ICI 182,780 causes suppression of basal activity. When wild type MOR is added there is a 5 fold increase in basal activity and a 4 fold increase in E2 stimulated activity indicating that the cells were expressing transfected receptor substantially in excess of the endogenous hER. OHT can be seen to reduce basal activity and ICI 182,780 suppresses activity still further. When the mutants are transfected, a different pattern is observed. The basal activity is not significantly increased and there is no apparent effect on the magnitude of induction by E2. This is a surprising result, as these mutants would be expected to behave as dominant negative receptors since they are known to bind ligand and DNA but AF2 can not be activated by E2. In other systems similar mutants have been described as showing dominant negative activity and similar mutations of the thyroid hormone receptor also appear to show dominant negative effects (see discussion). The response to antioestrogens is similar for all three mutant receptors. There is a 4 to 5 fold stimulation above basal activity in the presence of OHT or ICI 182,780. In comparison to the stimulation seen when additional wild type MOR is stimulated by E2 this increase is small but the importance of this small stimulation in terms of mitogenic response to antioestrogens is unknown. There is unfortunately no practical method of determining the relative amount of each receptor expressed in these experiments since they are of identical size and not antigenicllly distinguishable. It is assumed but not certain that the mutant MOR receptors are being expressed at the same level as the wild type MOR. It is also not known if the stability and intracellular localisation of the mutant receptors in the presence of pure antioestrogen is

Fig 4.1

Transient transfection of mutant and wild type receptor in MCF-7 breast cancer cells

MCF-7 breast cancer cells were tranfected in 24 well plates with 0.5 μg ERE tk luciferase reporter plasmid 0.1 μg pJ7 lacZ internal control plasmid and 0.1 μg pJ3 receptor expression plasmid. The pJ3 vector contained either no insert (CONTROL), wild type mouse oestrogen receptor (Mor 1-599), mouse oestrogen receptor with mutations of pairs of hydrophobic residues in the AF2 amphipathic α-helix (L-543A/L-544A) or (M-547A/L-548A), or a deletion of the amphipathic α-helix (Δ 540-552). Total DNA was made up to 1 μg with additional empty pJ3 plasmid. The transfected cells were treated for 24 hours with ethanol carrier alone (■), 10^{-8}M oestradiol (□), 10^{-6}M 4-hydroxytamoxifen (▨) or 10^{-6}M ICI 182780 (▩) Cells were harvested and β-galactosidase and luciferase assays performed. The β-galactosidase values were used to correct luciferase values for transfection efficiancy. Results are expressed as a percentage of the activity seen with oestradiol in the absence of any added receptor (ERE only) Results represent the mean of three independent experiments performed as duplicates.


Relative transcriptional activity
400
150
100
50
NH
OESTRADIOL
HYDROXYTAMOXIFEN
ICI 182780
CONTROL
Mor 1-599
L-543A/L-544A
M-547A/L-548A
Δ 540-552
Endogenous receptors
Wild type mouse ER
Amphipathic α-helix mutants
Amphipathic α-helix deletion

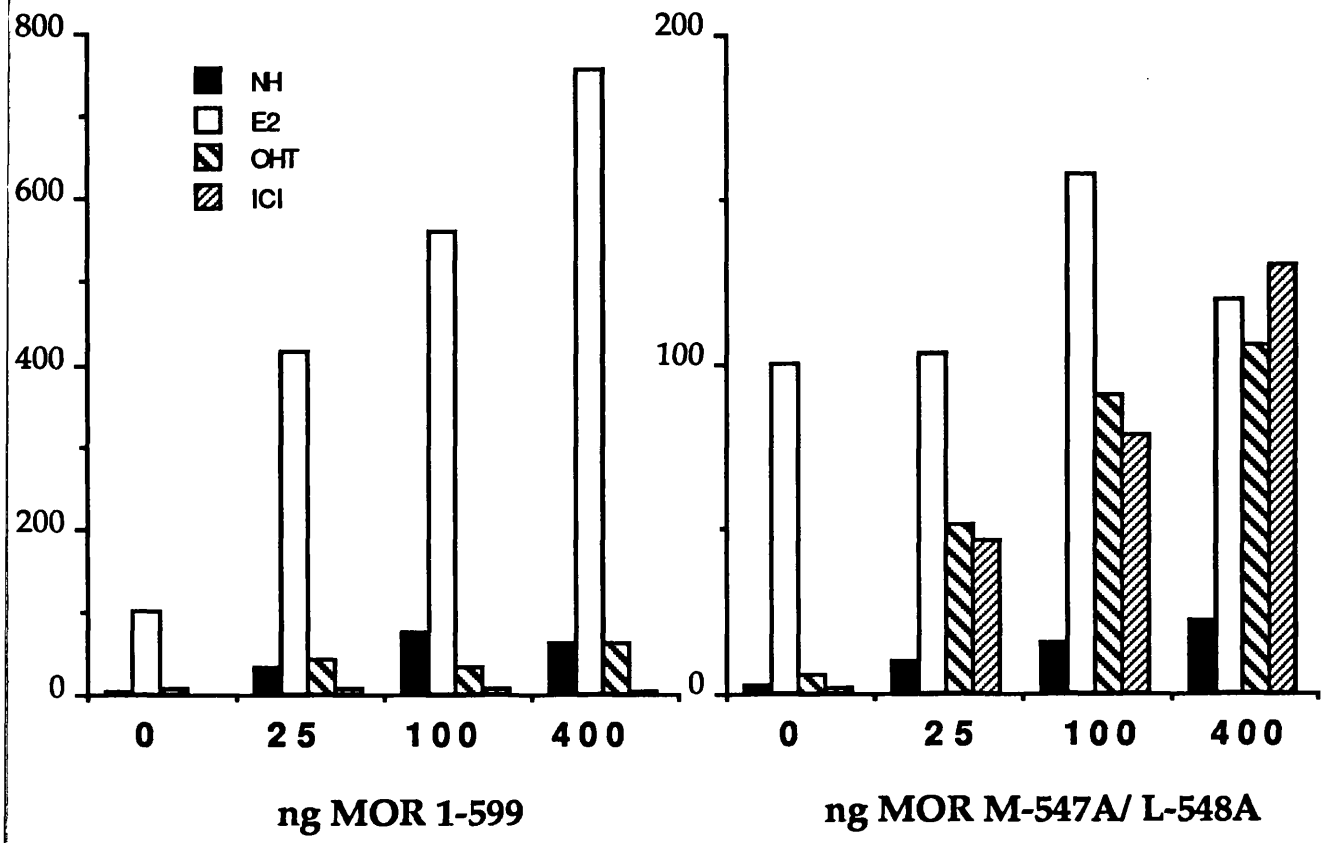


Fig 4.2

Dose response of wild type and mutant mouse oestrogen receptor transiently expressed in MCF-7 cells.

MCF-7 cells were transiently transfected with 0.5 µg ERE tk luciferase 0.1µg pJ7 lacZ and increasing amounts of wild type mouse oestrogen receptor (MOR1-599) or mutant receptor (MOR M-547A/L-548A). Transfected cells were exposedfor 24 hours to ethanol carrier alone (■), 10^{-8} M oestradiol (□),10^{-6} M 4-hydroxytamoxifen (▧) or 10^{-6} M ICI 182,780 (▨).Luciferase values were normalised for transfection efficiency using β-galactosidase and are expressed relative to the activity seen in cells tranfected with reporter alone and stimulated with oestradiol. The results shown represent the mean value from duplicate samples. Note that the Y axis scales are different in the two plots shown.

affected in the same way as is seen in Cos-1 cells, where unlike wild type receptor, the mutant receptors remain nuclear during ICI182, 780 treatment. An experiment in which increasing amounts of wild type or mutant receptor were transfected into MCF-7 cells is shown in Fig 4.2. In this figure the antioestrogen induction is seen to be dependent on the amount of mutant receptor transfected but the E2 response is unaffected by the amount of mutant receptor. In contrast the wild type receptor shows a dose (amount of transfected MOR) dependant rise in E2 induced activity.

These experiments demonstrate that introducing mutant ER into breast cancer cells can result in agonist responses to antioestrogens. These responses are dependent on the amount of transfected mutant ER DNA and by inference the amount of mutant ER protein expressed. The magnitude of these agonist responses is small in comparison to the ability of E2 to stimulate the reporter in the presence of transfected wild type receptor. In addition there does not appear to be any effect of the mutant receptor on E2 induced transcription.

Examination of tumours for mutations in the oestrogen receptor

The observation that these mutant receptors can lead to stimulation of oestrogen responsive reporter genes in the presence of both partial agonist antioestrogens and the pure antioestrogens suggests that mutations of this type could potentially result in antioestrogen resistance in breast cancers. Human breast tumour material was therefore examined for the presence of genomic DNA mutations within the conserved region identified as critical to AF2 function.

Tumour samples

Two groups of tumour samples were examined. The first group examined was obtained from the Guys Hospital ICRF oncology unit tumour bank courtesy of Professor R D Rubens and Diana Barnes. All these samples were from primary breast tumours removed during initial surgical management. At the time of operation patients had received no prior treatment but the subsequent clinical course of each patient was known. Oestrogen receptor positive tumour samples were selected from patients that relapsed within 2 years of primary therapy and had been treated on relapse with tamoxifen but had developed progression of disease within 6 months of

commencing tamoxifen. This group therefore represented primary tumour material from patients who are known to subsequently develop tamoxifen resistant metastatic breast cancer. The antioestrogen sensitivity of the tumour at the time of biopsy is unknown.

The second group of tumour samples were obtained from The Royal Marsden Hospital Fulham Road (courtesy of Professor M Dowsett and S. Johnston). These oestrogen receptor positive tumour samples were obtained from patients who had developed acquired resistance to tamoxifen prior to the removal of tumour material. These samples consisted of both primary tumours (treated with neoadjuvant hormone therapy), and biopsy material from metastatic tumour deposit

Oligonucleotide hybridisation

Genomic DNA was isolated from the tumour samples and analysed for the presence of mutations within the conserved C-terminal region of the hormone binding domain. Mutations can be detected by identification of amplified DNA fragments that fail to hybridise to specific decamer oligonucleotides complementary to the wild type sequence. This technique allows the identification of point mutations or deletions within a short region of DNA and has the potential to detect mutations present at low frequency within a population of cells. This is therefore an appropriate technique to apply to primary tumour material, which may contain a sub population of cells carrying a mutation within the ER gene. The principal of mutation detection is illustrated in figure 4.3 and the steps in the process required are illustrated in the flow diagram in fig 4.4. Primers were designed to amplify a 174 base pair fragment of exon 8 encompassing the coding sequence for the 13 amino acid conserved region of the hormone binding domain. The PCR product from each tumour was subcloned into the pAMP cloning vector and the plasmids containing the PCR derived fragments were used to transform bacteria, which were plated onto large agar plates. Bacterial colonies from these plates were recovered using an automated system developed by the ICRF Genome analysis laboratory. This equipment uses a video camera and computerised image analysis system that is able to recognise and accurately locate individual bacterial colonies. A picking head consisting of 96 separate picking pins is then used to pick colonies and transfer them to 384 well microtitre dishes. The colonies are grown

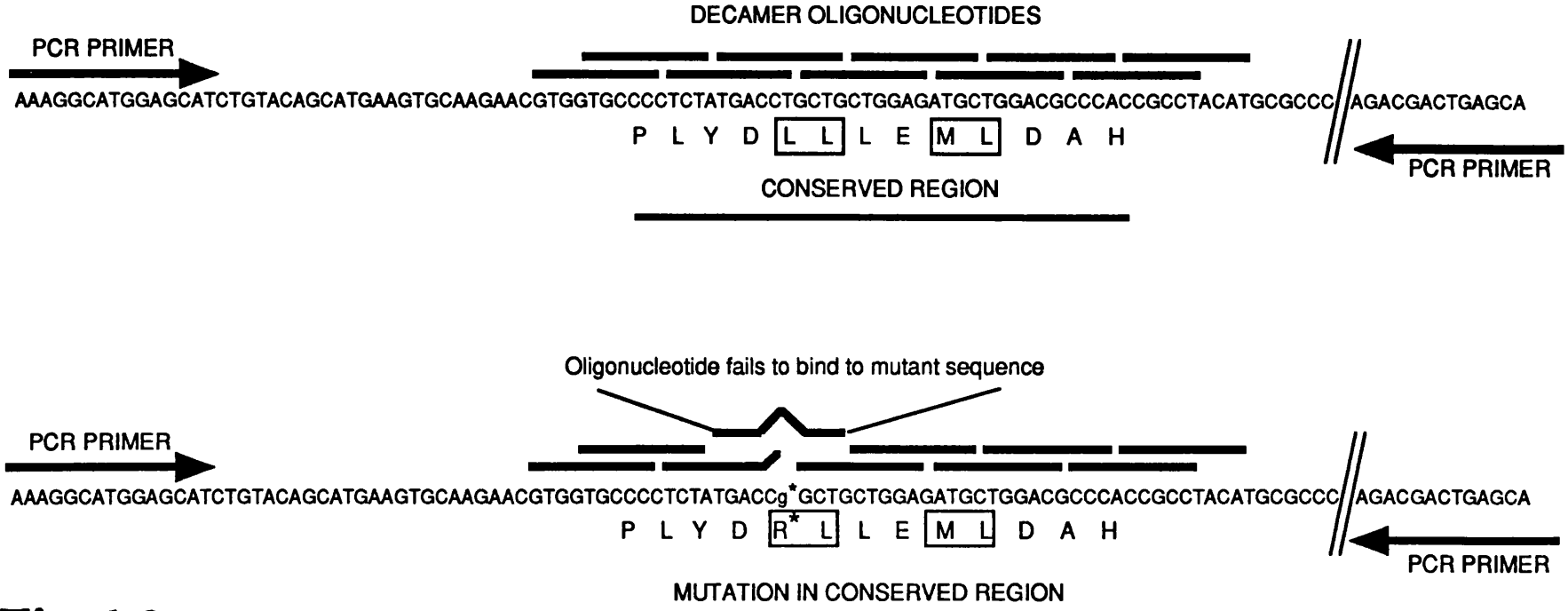
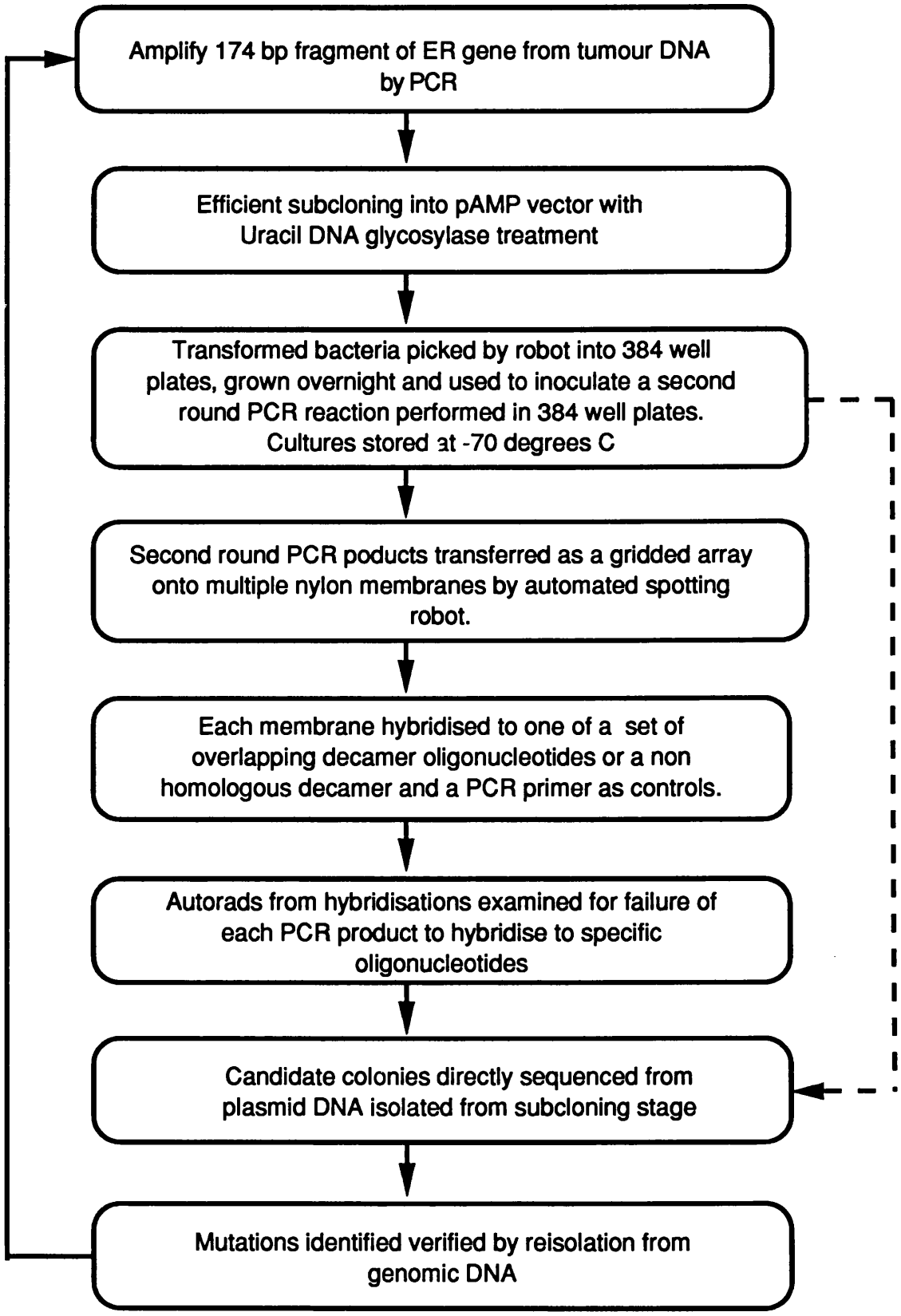


Fig 4.3

Ilustration of the principal of mutation detection by oligonucleotide hybridisation.
Single point mutations result in failure to hybridise with oligonucleotides complementary to the normal sequence if the mismatch is within the middle six bases. Therefore all point mutations are covered by a set of decamer oligonucleotides overlapping by 5 base pairs each. 10 oligonucleotides will cover a 50 base pair region. Oligonucleotides are chosen that are not complementary to sequences within the PCR product.

Fig 4.4

Stages in analysis of tumour DNA for mutations in the hormone binding domain of the oestrogen receptor



overnight and stored indefinitely. To generate sufficient DNA sequence to perform several dot hybridisations the bacteria were used as a source of template for a second PCR amplification. Because of the high number of reactions required to identify possible infrequent mutations this amplification was performed in 384 well microtitre plates identical to those used to grow the bacterial colonies. It was thus feasible to analyse 384 separate clones derived from each tumour sample. Inoculation of PCR reactions was performed automatically using a 384-pointed inoculating comb to transfer a few bacteria to the PCR reaction mixture. The plasmid DNA contained in the bacteria is sufficient to act as template for the PCR Primers. The resulting PCR products were then spotted onto membranes in an ordered array again using automated spotting equipment. The spotting process was repeated 12 times to produce 12 identical filters. Each filter was used in a hybridisation reaction to one of a set of 10 overlapping radiolabeled decamer oligonucleotides complementary to the 50 nucleotides from 1597 to 1646 of the human ER cDNA. These nucleotides code for hER amino acids 543 to 548. Under the hybridisation conditions used the oligonucleotides will hybridise to the wild type sequence producing a small dot on the autoradiograph(Meir-Ewart 1994). The presence of a point mutation in the central portion (middle six nucleotides Meir-Ewart unpublished information) of an oligonucleotide sequence will result in the failure of hybridisation. This results in a blank spot on the autorad exposed to the nitrocelulose filter. The use of oligonucleotides overlapping by 5 base pairs ensures that all single base pair mismatches can be detected. Deletions within the region covered by the oligonucleotides would also be revealed by this technique as non hybridising blank spots on the filter. The oligonucleotides were selected to ensure they were not complementary to other sequences within the PCR product including the primer sequences.

Screening primary breast tumour samples

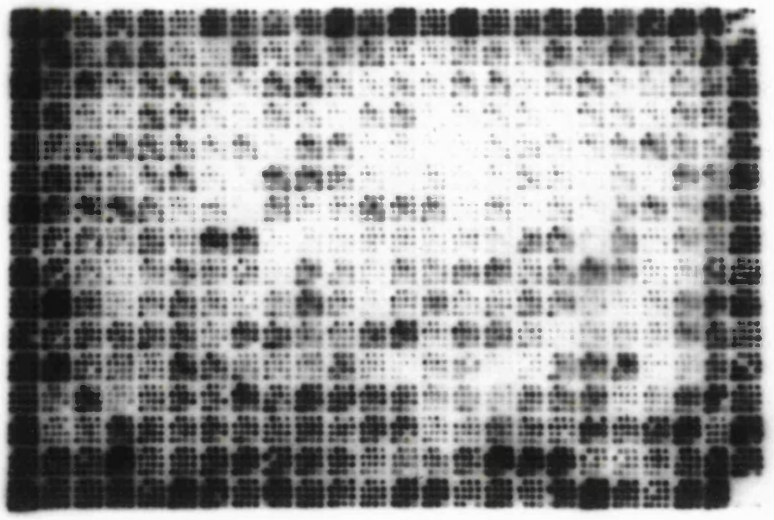
DNA from 15 primary breast tumours were analysed. Two of the second round PCR products from each tumour were analysed by gel electrophoresis and these confirmed that a PCR product of approximately 280 bp was generated. The autoradiographs generated from three filters are shown in Fig 4.5. From panel A of this figure it can be observed that the filter consists of 383 panels of spots in a 4x4 array in which the top left spot

Fig 4.5

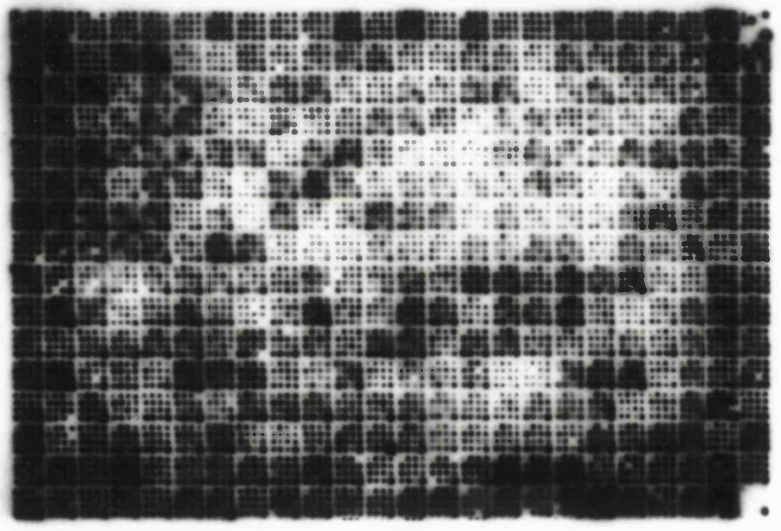
Hybridisation of oligonucleotides to ER sequence PCR products to detect mutations in genomic DNA.

Subcloned and reamplified PCR products from breast tumour DNA were spotted onto Hybond N+ nylon membrane. Multiple copies of each filter were made and each filter hybridised to a different ^{32}P end labeled decamer oligonucleotide. Panel A shows a filter hybridised to a decamer homologous to part of the amphipathic α-helix spanning hER residues 539-541. (nucleotides 1617-1626). Panel B shows a duplicate filter hybridised to the 5' pAMP PCR primer used in the second round amplification stage. Panel C shows a third duplicate filter hybridised to a non homologous decamer sequence. Note that this autorad has deliberately been over exposed compared to the filters above to reveal residual background binding.

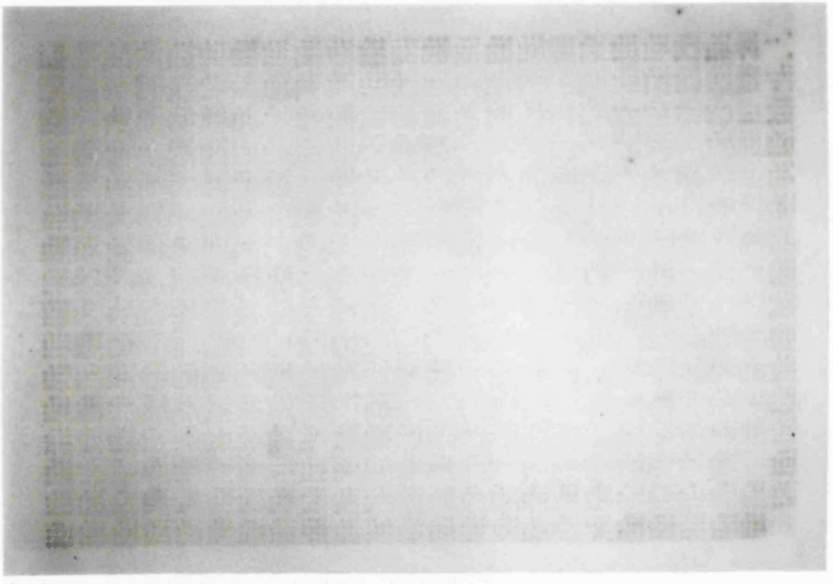
A



B



C



does not hybridise, this position is an ink mark for orientation. The other 15 positions in each 4x4 array represent the position of the 15 different tumours tested and each of the 383 arrays represents a single PCR product from each tumour. Panel A shows the autoradiograph from a hybridisation to a complementary oligonucleotide and panel B shows an autoradiograph from hybridisation to the PCR primer. A few gaps are apparent in the PCR hybridisation and these gaps are all reproduced in panel A indicating that this position does not contain a PCR product This is a result of a failure of the automated system at any of the sequential steps such as a picking error at the stage of bacterial colony picking (This could be seen visually in the microtitre wells some of which failed to grow bacteria and remained as clear culture broth) or failure of an individual PCR reaction. Also approximately 1% of the cloneamp product is expected to recombine without any PCR product present. In the single exposure illustrated is difficult to see all PCR hybridisations but using several different exposures and a strong light source better definition is obtained and allows virtually all the positions on each filter to be assessed for the degree of hybridisation. It is possible to identify some positions where there is a positive signal in the PCR control but no signal in the corresponding position in the test hybridisation. This is illustrated in an enlargement of part of panel A and B see Fig 4.6 and figure legend. In panel C of Fig 4.6 a negative control is illustrated. This filter has been hybridised to a non homologous decamer and requires heavy overexposure as illustrated to demonstrate a minimal background signal.

A total of 22 mismatched sequences were identified 4 of which were derived from a single tumour. The subcloned first round PCR products were sequenced but were all found to comprise wild type sequence only. No mutant sequences could be identified.
These false positives could arise from a number of possibilities and is discussed in Chapter 6.

Screening tamoxifen resistant tumours

Tamoxifen resistant tumours were analysed in the same manner as the primary tumours. In this group of tumours, 30 'mismatch hybridisations' were identified and sequenced directly. All these sequences were wild type further demonstrating that this technique is associated with a significant false

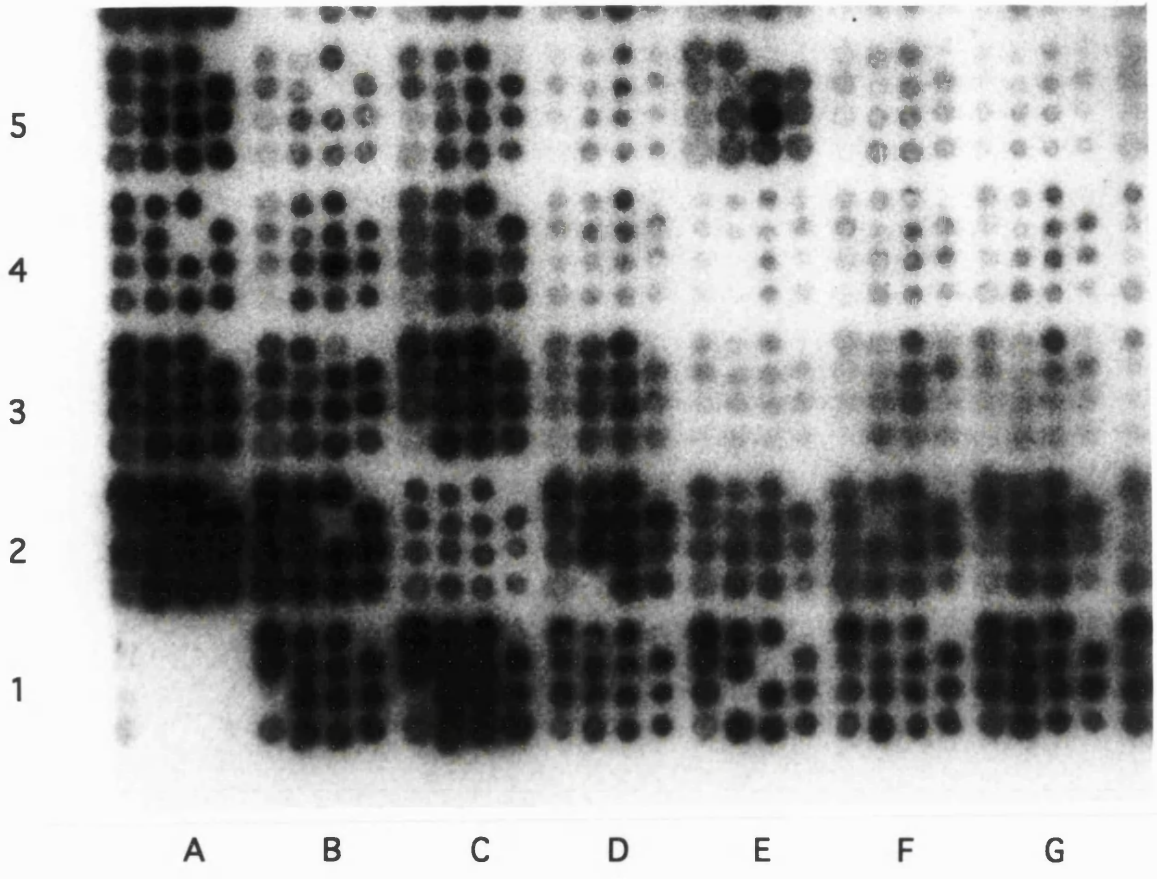
Fig 4.6

Enlargement of hybridised filters shown in Fig 4.5

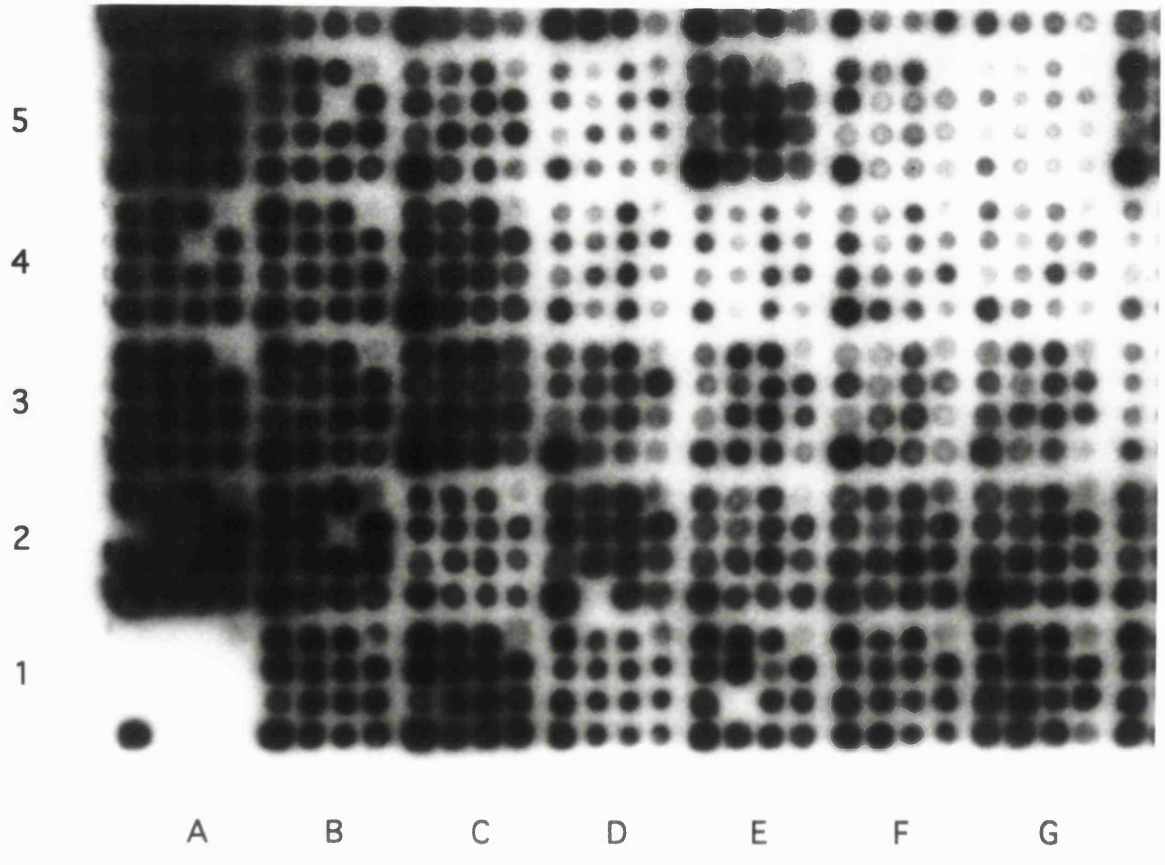
Part of each of panel A and panel B from figure 4.3 are shown as enlargements. Again panel A is hybridised to a homologous decamer oligonucleotide and panel B to the PCR primer as positive control. Comparing block4B in each panel it can be seen that bottom left spotting position fails to give any signal in Panel A but there is a strong signal in panel B in the corresponding position. This clone was selected for direct sequencing.

In block 1E there is no signal from two spotting positions. In the corresponding control block one position fails to give any signal and in the other position the signal is weak. This clone was also selected for direct sequencing.

A



B



number of false positive artefacts. No mutant sequences were identified suggesting that the tumour samples contain only wild type ER sequence.

Since no mutations were identified using this unconventional approach it was considered necessary to use an alternative method to confirm that the tumours did not contain any mutations in this region. The tamoxifen resistant tumours were considered more likely to contain mutations therefore five of the PCR clones from each of the 15 tamoxifen resistant tumours were analysed by direct sequencing using ABI fluorescent sequencing. Of 75 sequencing reactions 65 produced readable sequence information 62 contained wild type oestrogen receptor sequence and 3 clones contained vector sequences only and are presumed to represent background from uncut or religated vector. The failure to identify mutations of the C-terminal part of the hormone binding domain of the ER in both primary breast cancers and tamoxifen resistant tumours indicates that this is not a common site for mutation and there is no selection for mutations in this region in tamoxifen resistant breast cancer.

Stable expression of mutant mouse oestrogen receptor in MCF-7 cells.

Despite (or perhaps because of) the lack of evidence for mutations in the conserved C- terminus of the HBD in human breast tumours it is of interest study the effects of mutations in this region on the growth of breast cancers in response to antioestrogens. It would be interesting to know if the agonist activity seen in transient transfection experiments is mirrored by a positive mitogenic response in breast cancer cells. If this were not seen it may perhaps explain why we do not see these mutations in resistant tumours and if they are able to generate antioestrogen resistant or stimulated tumours the question of why this is not mimicked in the natural situation needs to be explained. Therefore an attempt to establish MCF-7 cells stably expressing the MOR as either wild type or with mutations in this region was undertaken. Since it was not necessary to have an inducible system we attempted to introduce the oestrogen receptor under the control of a constitutively active promoter using the pJ3 vector expressing MOR under the control of the SV40 early promoter. MCF-7 cells were therefore transfected with empty pJ3, pJ3 MOR (1-599), or pJ3 MOR (1-599 L-543A/L-544A) and pSV hygromycin plasmids in a ratio of 20:1. Hygromycin resistant cells were then pooled or individually ring cloned. It was noted that

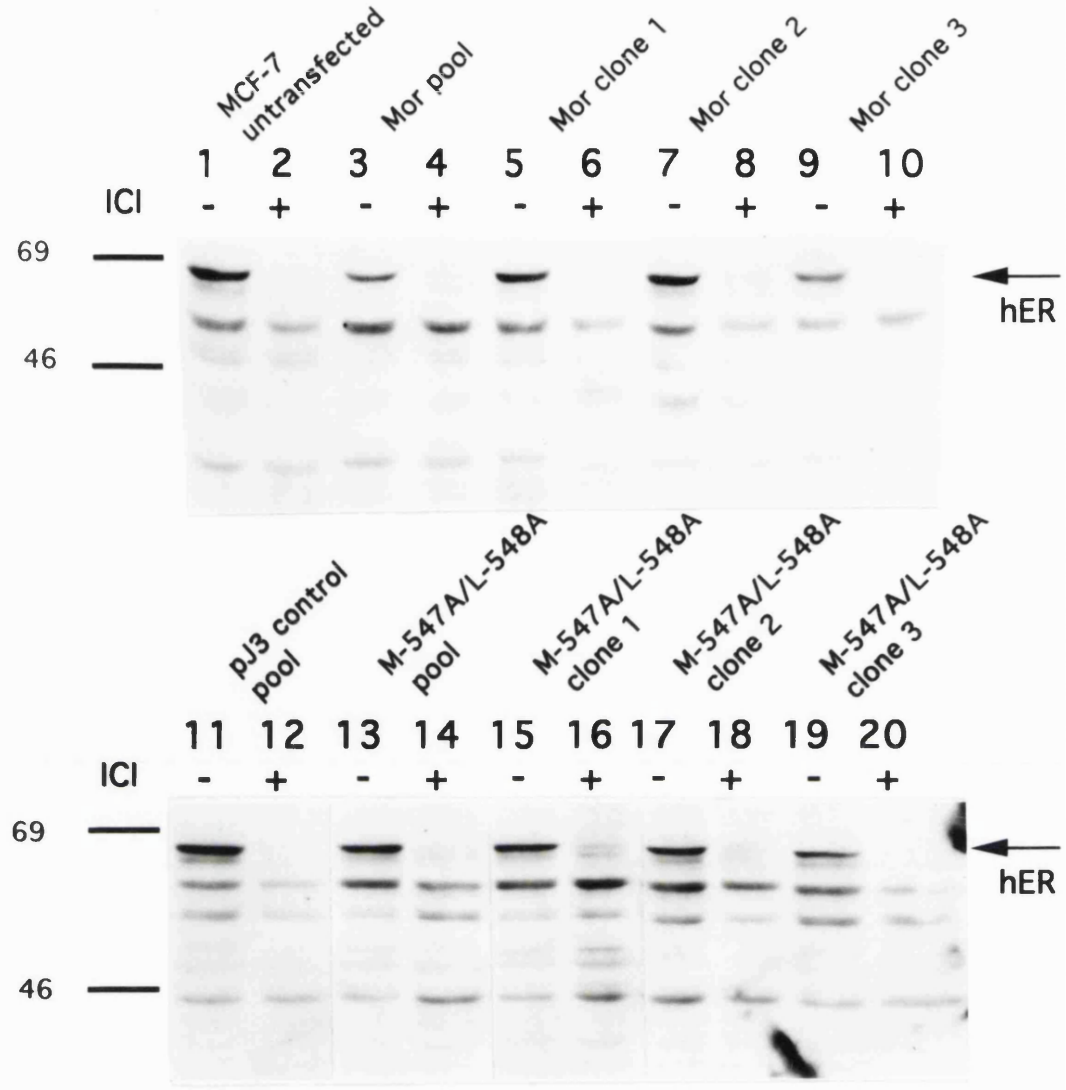


Fig 4.7

Western blots of stable cell lines transfected with mutant and wild type mouse oestrogen receptors

MCF-7 cells were transfected with pSV hygromycin resistance plasmid and with empty pJ3 plasmid. or pJ3 containing Mor (1-599) wild type, or mutant Mor(1-599 M-547A/L-548A). Stable cell populations were pooled or isolated as individual clones.

Extracts were prepared from each cell line after growing in steriod depleted medium(odd numbered lanes) or after 15 hours incubation with 10^{-6} M ICI 182780 (even numbered lanes)

Lanes 3-10 show examples of Mor1-599 transfected cells and lanes 13-20 show examples of cell lines transfected with mutant receptor. Receptors were detected with H222 monoclonal anti ER

compared with control cells transfected with empty pJ3, cells transfected with both mutant and wild type MOR gave rise to 5- 10 fold fewer colonies. Pooled cells and ring cloned colonies were analysed by western blotting for the level of ER expression. Since the level of mutant MOR transiently expressed in Cos-1 cells is stable under the influence of ICI 182,780 we tested the effect of ICI 182,780 on the level of ER in the stable clones arguing that mutant receptor expression would be maintained but wild type receptor would be down-regulated. This would allow us to identify stable cell lines expressing mutant receptor.

In addition to the analysis of pooled cells derived from 25 -50 colonies we analysed 6 separate clones transfected with wild type MOR and 11 separate clones transfected with mutant MOR Examples of this analysis are shown in Fig 4.7. It can be observed that the introduction of empty pJ3 has no apparent effect on the level of receptor compared with the parental MCF-7 cell line and that ICI 182,780 reduces the amount of wild type hER below detectable limits. It can also be observed that in neither the wild type or mutant MOR stable transfectants is there any significant increase in detectable ER and that ER present in the absence of ligand is always absent after ICI 182,780 treatment. The interpretation is therefore that none of the stable clones or pools of clones was expressing any additional receptor (wild type MOR or mutant MOR) It appears therefore that this system is unsuitable for the expression of MOR in MCF-7 cells. The reduction in the number of colonies obtained when MOR is introduced suggests that there may be a toxic effect of overexpressing both wild type and mutant receptors under the control of the SV40 early promoter.

Summary and conclusions

Addition of wild type MOR into MCF-7 breast cancers resulted in an increase in basal reporter activity and an enhanced response to oestradiol, the response to antioestrogens is unchanged. Transfection of MORs with specific mutations of the hormone binding domain which are known to abolish AF2 stimulation by oestradiol but are stimulated by antioestrogens was also examined. In this situation where both transfected mutant and endogenous wild type receptors are expressed in the same cells, the response to oestradiol is unaffected but stimulation by both OHT and ICI 182,780 is observed. However the magnitude of this stimulation is small in comparison to the

enhanced oestradiol response seen with wild type MOR. It is concluded therefore that mutant ER in breast cancer cells can alter the response to antioestrogens.

We have screened 15 primary tumours and 15 tamoxifen resistant tumours using a system to identify mutations within a short region of the ER important in AF2 activation. None of these tumours carried mutations within this highly conserved region. To study the effects of AF2 mutations on cell growth, attempts to express AF2 mutant ER stably in MCF-7 cells were made using a constitutive promoter. These attempts were unsuccessful. There is some evidence to suggest that MCF-7 cells are unable to tolerate overexpression of receptor using this system.

Chapter 5

Identification of tamoxifen insensitivity by phenotypic screening in yeast

Introduction

Mutations in the hormone binding domain of the oestrogen receptor that abolish oestradiol binding but permit tamoxifen binding have been described. The opposite phenotype where oestradiol binds normally but tamoxifen is unable to bind, would have the potential to cause tamoxifen resistance in breast cancer. To identify mutations with such a phenotype a yeast screen was utilised that allowed screening of random mutations of the oestrogen receptor hormone binding domain. While in mammalian systems tamoxifen can be used as an antagonist of oestradiol mediated stimulation, in yeast it is not possible to see antagonism of oestradiol with tamoxifen. This is not only because tamoxifen functions as an agonist in yeast, but partly as a result of relatively poor penetration of tamoxifen compared with oestradiol. Therefore the screening strategy used was to compare oestradiol and tamoxifen as agonists.

Establishing a screening system in yeast

A mutant receptor with enhanced activity in yeast.

As the aim of the screening system was to identify mutants with altered binding specificity, it was possible to increase the sensitivity of the screen by the use of a mutant oestrogen receptor producing a strong response in yeast. This receptor was provided by I Purvis (unpublished), it contains a point mutation at position 362 such that lysine is replaced by glutamic acid (hER K-362E). This mutation which was itself identified by mutational screening in yeast, produces a higher ligand dependent signal in yeast compared with wild type ER stimulated with either oestradiol or 4-hydroxytamoxifen. This makes screening for loss of activity in response to a weak agonist possible (compare wild type and mutant receptor responses in Fig 5. 3).

Yeast strains, expression vectors and reporters

The protease deficient *Saccharomyces cerevisiae* strain BJ 5458 was used in all experiments. Receptor activity was monitored by using a reporter plasmid containing two xenopus vitelogenin EREs upstream of the PGK promoter linked to LacZ. (YcP ERE lacZ). The yeast expression vector pCup 1 in which expression is inducible with copper sulphate was used to express ER.

Yeast initially transformed with the reporter construct was transformed with a yeast expression vector containing the mutated receptor (pCup 1 hER K-362E) Initial experiments showed that yeast colonies containing expression vector and reporter produced a ligand dependent colour change when grown for 3-4 days on Xgal plates containing 50 μM copper sulphate. Concentrations of ligand producing optimum colour change were selected for the screening procedure, 10^{-6}M 4-hydroxytamoxifen and 10^{-9} M oestradiol were used.

Generation of yeast colonies containing random mutations in the hormone binding domain of the ER

Random mutations were introduced into the HBD of the oestrogen receptor by degenerate PCR, using high magnesium and high nucleotide concentrations in the PCR reaction. An 800 bp fragment was generated using primers located in the D region and F regions of the receptor. M13 phage DNA containing the entire hER cDNA was used as template. The PCR products were incorporated into the yeast ER expression vector by transforming yeast with a mixture of PCR product and ER expression vector that had been cut with two restriction enzymes which removed an 84 bp fragment from the centre of the HBD. The cut vector is unable to replicate unless it undergoes homologous recombination with the PCR product to bridge the gap in the plasmid (Fig 5.1). This homologous recombination occurs efficiently in yeast and a single transformation was found to produce 1000-2000 colonies.

Identification and characterisation of a mutant receptor with aberrant response to 4-hydroxytamoxifen in yeast

The screening system described above was used to identify colonies that produced a normal colour development in the presence of E2 but failed to produce a colour change with OHT.

Isolation of phenotypically abnormal mutant ER

From two separate transformations 6 initial positive clones were identified that retained the phenotype when retested on fresh Xgal plates. Of these only 3 were successfully recovered and grown as bacterialy replicated plasmids. The reason for the failure to isolate plasmid DNA from the

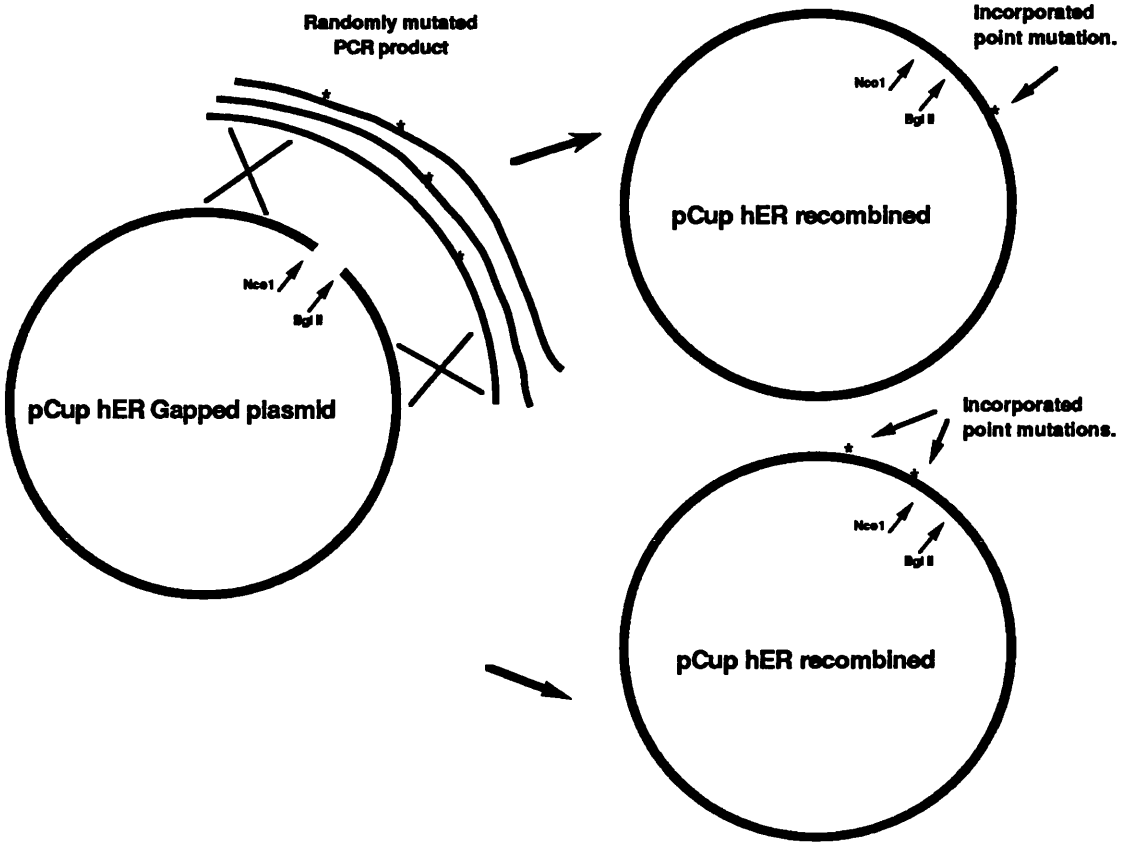


Fig 5.1

Ilustration of Homologous recombination of mutated PCR fragments into an incomplete plasmid

Plasmid DNA containing oestrogen receptor cDNA is cut with Bgl II and Nco I creating incompatible overhangs. The purified cut plasmid is mixed with a stoichiometric excess of PCR product produced by degenerate amplification of the ER hormone binding domain . The mixture is used to transform *sachcaromyces cerevisiae.* The PCR product is incorporated into the plasmid sequence by homologous recombination generating replicating cicular plasmid DNA . Transformed yeast colonies can then be examined for abnormal phenotype resulting from mutation of the ER hormone binding domain.

remaining clones has not been established but it is likely to represent a technical failure since even where recovery was successful this was the result of many repeated attempts. It was found necessary to use electroporation of bacteria to recover the plasmids as no plasmids were recovered using the standard cation prepared competent bacteria. All three plasmids were sequenced across the hormone binding domain and were all found to contain at least one mutation. The mutations identified are indicated in Fig 5.2. It is interesting to note that in two of the clones the same mutation has been isolated. These clones were isolated from the same screening run and it is likely that this position was mutated at an early point during the PCR reaction.

The recovered clones were used to retransform yeast and retested on X-gal plates. Only one mutant receptor hER K-362E/A-382V was found to retain the phenotype, the other two mutants were found to respond to E2 and OHT normally. It is not clear what the reason for losing the phenotype is but it may be because the original yeast clones contained more than one mutant receptor, and that on both occasions, the receptor recovered was not responsible for determining the original phenotype.

Dose response of mutant ER in Yeast

To further characterise the effect of the mutation on ligand induced activity a dose response analysis was performed using a liquid β-galactosidase assay. The responses of the original mutation hER K-362E and the tamoxifen insensitive mutation hER K-362E/A-382V were compared to HEGO. The data from these experiments is summarised in Fig 5.3. It is clear from this data that hER K-362E/A382V is completely insensitive to OHT but remains responsive to E2.

Effect of ligand on receptor stability in yeast

The possibility that the insensitivity to OHT was a result of ligand induced changes in receptor stability was tested by preparing extracts from yeast expressing wild type or mutant ER and analysing for receptor content by western blotting. It can be observed in Fig 5.4 that there is no significant effect of ligand on the amount of receptor detected after exposure to E2 or OHT and that there is equal expression of both wild type and mutant receptor.

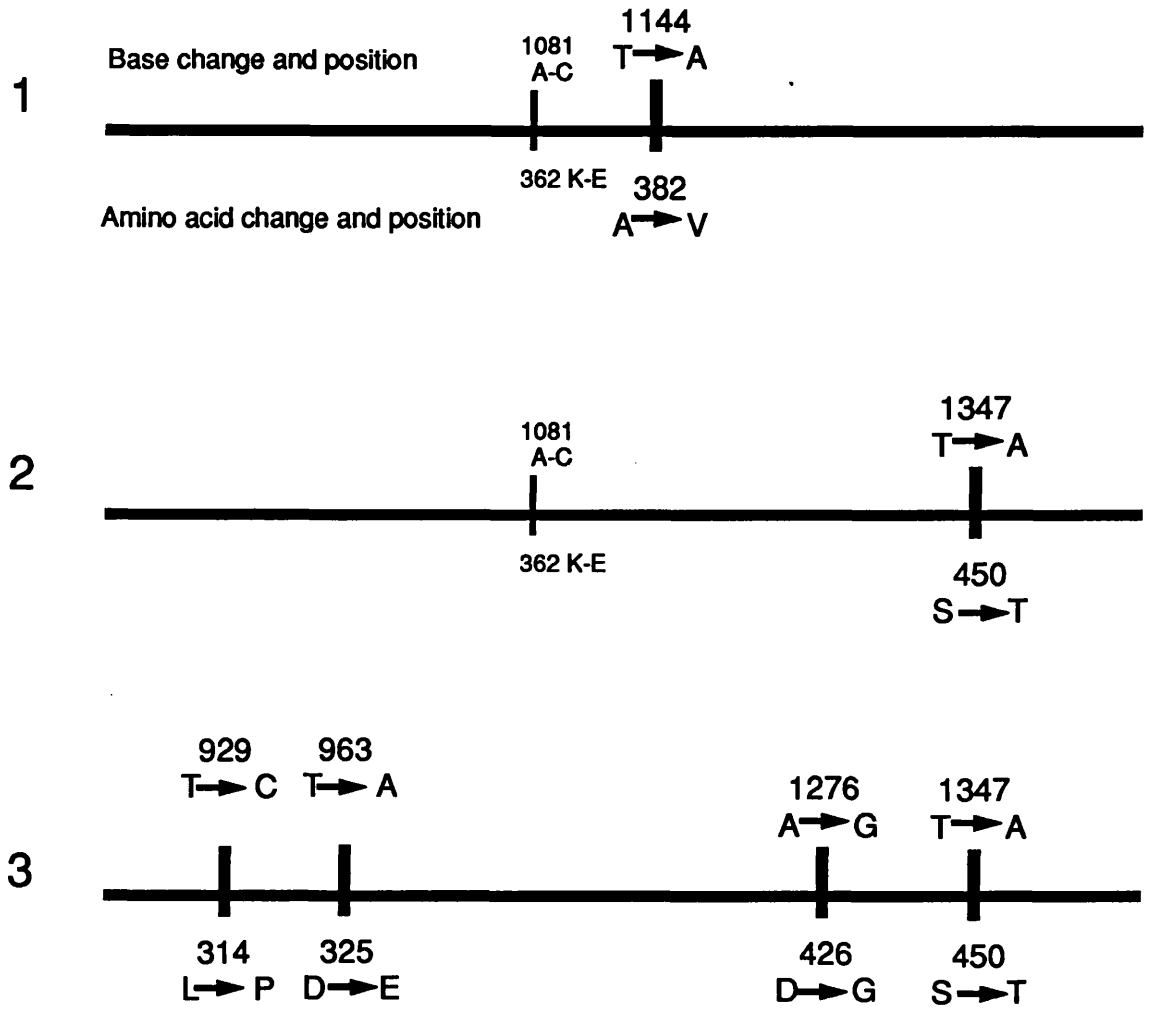


Fig 5.2

Position of mutations present in the hormone binding domain in plasmids recovered in yeast phenotypic screen

Plasmids recovered from the yeast screen that responded normally to oestradiol but were unresponsive to 4-hydroxytamoxifen were sequenced across the entire hormone binding domain. All three recovered plasmids contained missense mutations, the positions of these are indicated. Only the first sequence shown (1) containing a mutation at nucleotide 1144 causing a substitution of valine for alanine at position 382 retained the phenotype after reintroducing into yeast. The first two mutant sequences retained the mutation present (at nucleotide 1081) in the original mutant receptor used as starting material. This mutation was absent in the third sequence. Nucleotide number starts from the first nucleotide of coding sequence. Amino acid number also starts from the N-terminal amino acid.

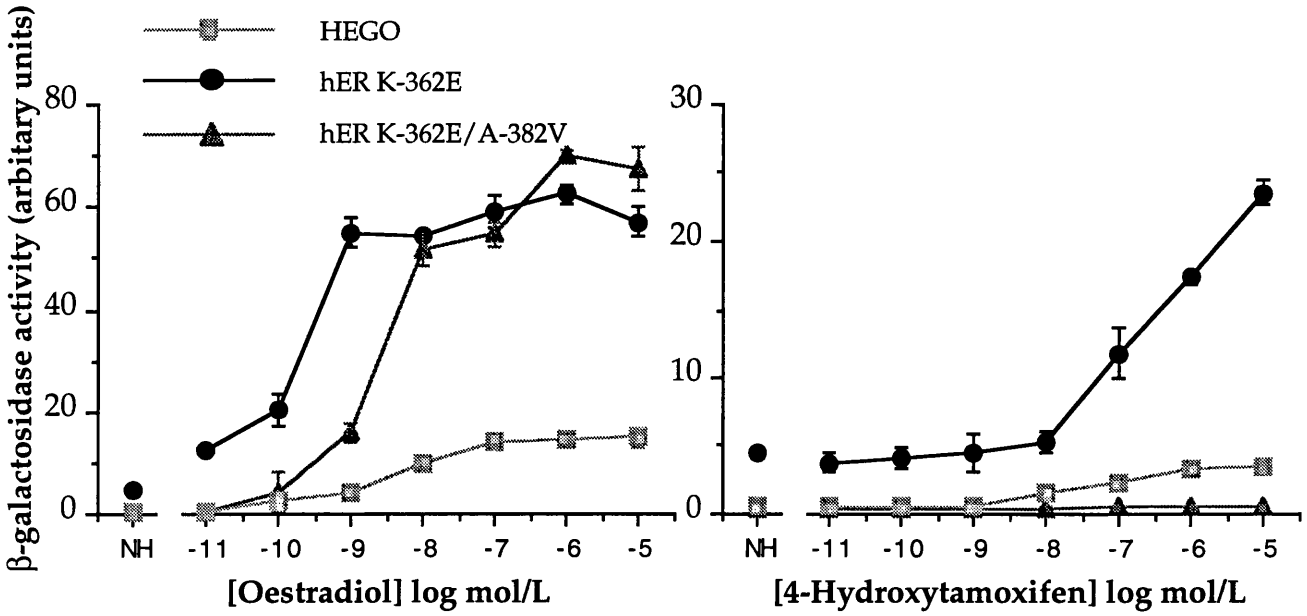


Fig 5.3

Transcriptional activity of mutant and wild type oestrogen receptors expressed in *saccharomyces cerrevisiae*

The dose dependent effect of oestradiol and 4-hydroxytamoxifen on the transcriptional activity wild type and mutant oestrogen receptors was monitored in *saccharomyces cerrevisiae* transformed with an ERE PGK lacZ reporter and a copper inducible expression vector for the mutant hER K-362E (●) andhER K-362E/A-382V (▲)or wild type receptor (■). Cells grown in double selective medium were induced with 50 μM copper sulphate and increasing concentrations of oestradiol or 4-hydroxytamoxifen were added. Cells were incubated for 15 hours and β-galactosidase activity determined. The data shown represents the mean of three independent experiments performed in duplicate. The error bars where visible represent the standard error.

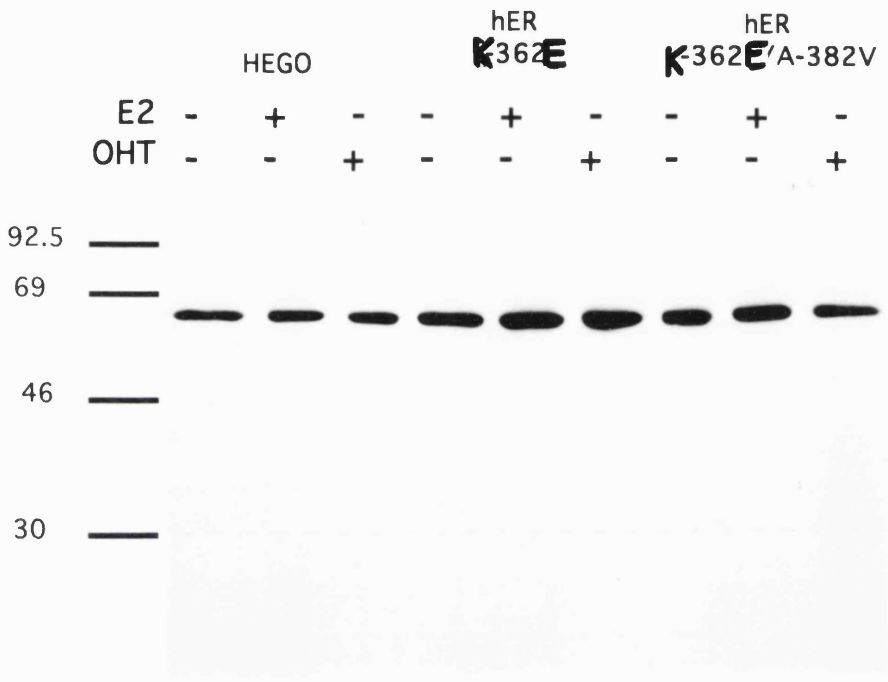


Fig 5.4

Western blot analysis of wild type and mutant oestrogen receptor in yeast

Yeast transformed with expression vectors for wild type or mutant oestrogen receptors were grown in selective medium for 15 hours in the presence of 50 µM copper sulphate with ethanol carrier or oestradiol 10^{-8} M or 10^{-6} M 4-hydroxytamoxifen. 100 µg of protien was separated by SDS PAGE, transfered to nitrocelulose and oestrogen receptor detected with H222.

Ligand binding properties of mutant oestrogen receptors

The Ligand binding properties of the mutant receptors was examined. Firstly the receptor cDNAs were transferred into the mammalian expression vector pSG5 and then expressed transiently in Cos-1 cells. Whole cell extracts were prepared and used to perform a Scatchard analysis using [^{3}H] labelled oestradiol. Ligand binding curves and Scatchard plots are shown in Fig 5.5.

It can be observed that there is little difference in the affinity of all three receptors hER K-362E/A382V has a minimally reduced affinity Kd .99 nM Vs 0.65 nM for HEGO and 0.66 for hER K-362E. The Kd obtained for HEGO of 0.65 nM is higher but in a comparable range to the figure of 0.35 nM reported for HEGO expressed in HeLa cells. (Tora 1989)

Competition assay of 4-hydroxytamoxifen binding to mutant oestrogen receptor

Preliminary attempts to determine affinity of the receptors to OHT by Scatchard analysis proved unsuccessful because high non specific binding of OHT to the plastic tubes used to incubate the receptors led to inconsistent results. Therefore OHT binding was determined indirectly by competition assay with labelled [^{3}H] labelled oestradiol. An example of a competition assay is shown in Fig 5.6. It can be observed that OHT is able to displace E2 from the wild type and both mutant receptors. The Relative binding affinity was calculated from a regression analysis of the data and while the RBA of OHT for the tamoxifen insensitive hER K-362E/A382V is lower than for HEGO 0.91 Vs 0.66 this difference is minor and is not able to account for the total absence of tamoxifen stimulation in yeast by hER K-362E/A-382V

Analysis of the transactivation properties of mutant ER in chicken embryo fibroblasts.

The mutant receptors hER K-362E and hER K-362E/A-382V clearly show a difference in the maximal response in yeast and have different dose response characteristics that would not have been predicted from their ligand binding characteristics. To examine if these differences were specific to the yeast model or were more generally applicable the transcriptional activity of the mutants was examined in chicken embryo fibroblasts. These cells were

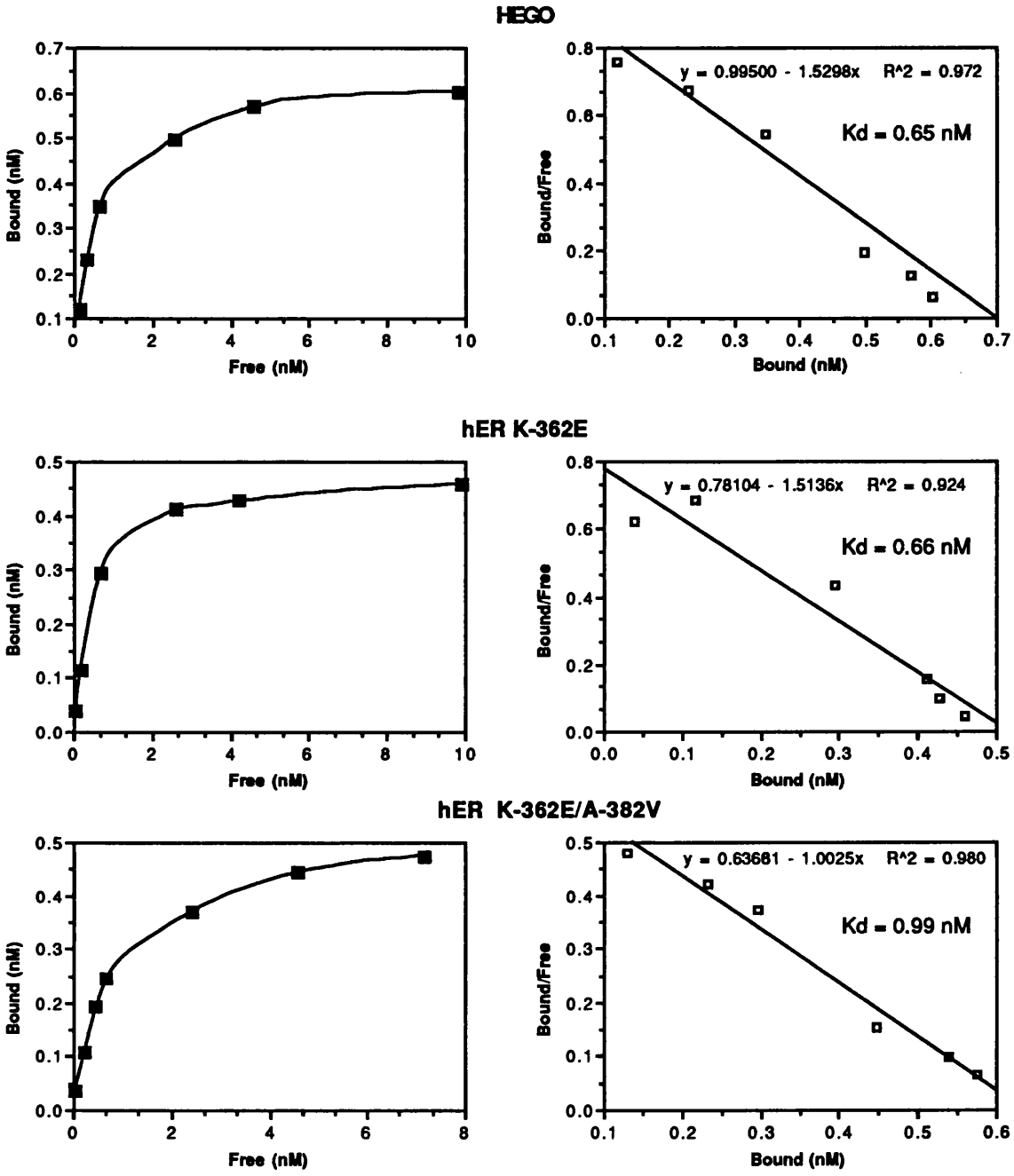


Fig 5.5

Scatchard analysis of mutant oestrogen receptors

Ligand binding characteristics of hER K-362E and hER K-362E/A-382V were compared with HEGO by Scatchard analysis of [3H] 17β–oestradiol binding to receptors expressed in Cos-1 cells. 5υg Cos-1 extract was incubated at room temperature for 2 hours and specific binding determined for duplicate samples. The average of duplicate determinations is shown. Kd was calculated from the slope of the Scatchard plots.

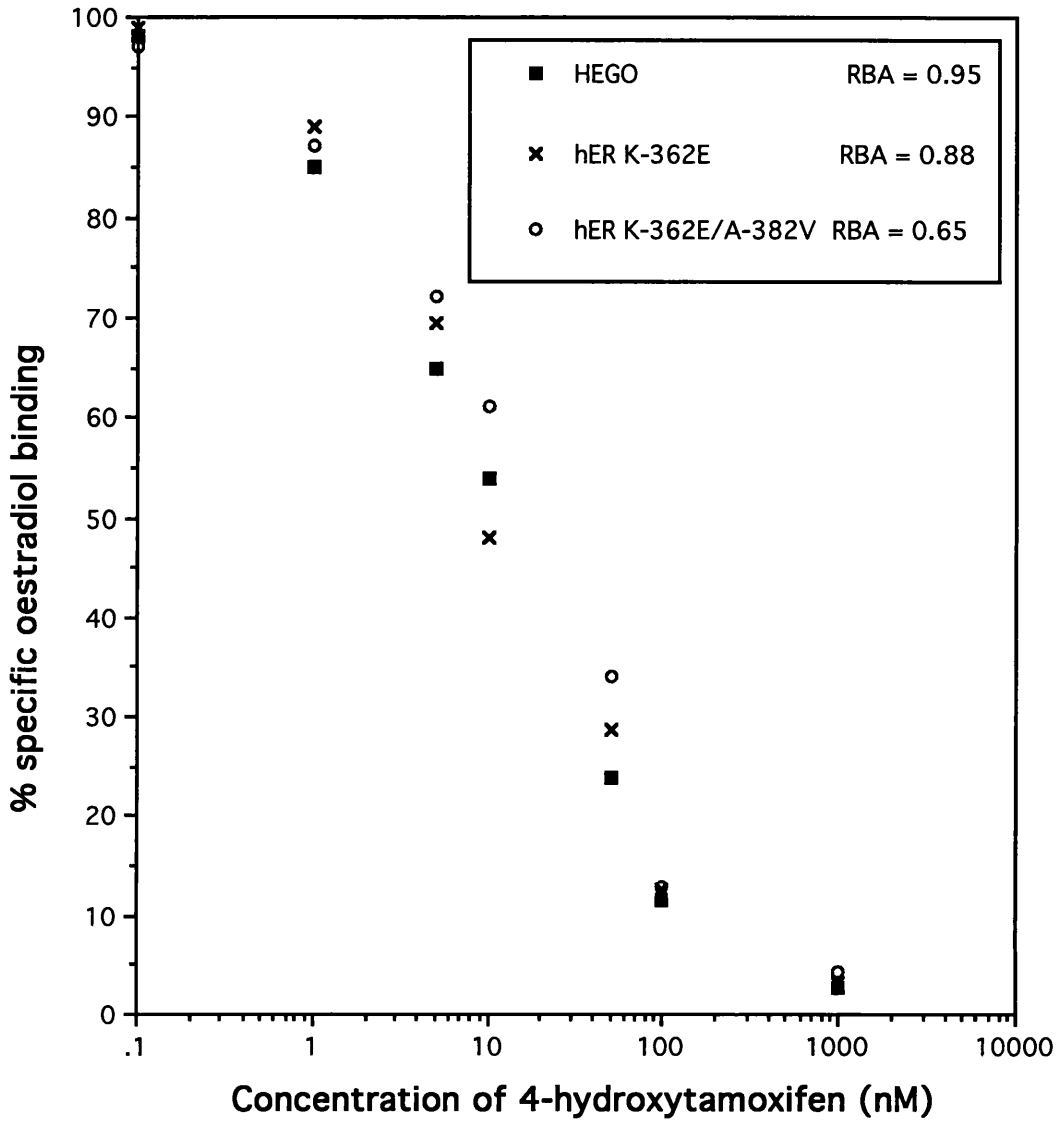


Fig 5.6

Competition assay of wild type and mutant oestrogen receptors

The ability of 4-hydroxytamoxifen to compete with 17β-oestradiol for oestrogen receptor was assessed by incubating receptors with 10 nM [^{3}H] oestradiol and increasing concentrations of unlabeled competitor ligand (4-hydroxytamoxifen) at room temperature for 2 hours. The specific binding of oestradiol was determined and the % of specific binding determined at each concentration of competitor ligand. Mutant receptors hERK-362E(×) and hERK-362E/A-382V (○) were compared to HEGO (■). Receptors were expressed in Cos-1 cells and 5 μg protein extract used. Data shown represents the average of duplicate determinations. Calculated relative binding affinity is shown for each receptor in the inset panel.

chosen because we wished to monitor the agonist and antagonist properties of 4-hydroxytamoxifen.

Dose response to ligands

The dose dependent response to both E2 and OHT was studied when either HEGO or either of the two mutant receptors were transiently expressed in CEF cells with an oestrogen responsive reporter ERE pBL CAT. A representative example of these experiments is summarised in figure 5.7. It can be seen that in the absence of added hormone HEGO produces a higher basal activity compared to either of the mutant receptors. In response to E2, HEGO produces a dose dependent response reaching a plateau at 10^{-9} M. The mutant receptors are also responsive to E2 but in contrast to the activity in yeast have similar maximal responses to HEGO. The dose dependency of this response is however dissimilar, in hER K-362E the dose response curve is left shifted by approximately 10 fold (compared to HEGO), but in hER K-362E/A-382V the dose response curve is shifted to the right approximately 1000 fold (compared to HEGO). When OHT is used as ligand we see no difference in response between HEGO and hER K-362E, but the response of hER K-362E/A-382V is again shifted to the right by at least 1000 fold. The magnitude of the maximal response to OHT is similar for all three receptors. In the experiment shown although the basal activity of HEGO is higher than for the mutant receptors there is an increase in activity seen with OHT. This is not always observed because there is variability in the basal activity seen which is thought to be due to differences in the efficiency with which oestrogens are removed from the serum used to prepare the medium.

4-hydroxytamoxifen as an antagonist

The ability of OHT to antagonise the stimulation by oestradiol was assessed in CEF cells, the results of a transient transfection experiment are shown in Fig 5.8. In this experiment 100 fold excess OHT was used to antagonise E2 stimulated activity. At the doses tested there was no apparent difference between HEGO or the two mutant receptors in the ability of 4-OHT to antagonise the stimulation of a reporter transiently expressed in CEF cells, OHT was unable to fully suppress reporter activity consistent with the known moderate partial agonist activity of OHT in this system.

Transcriptional activity of AF-2 defective mutant MOR in yeast.

From the above data is clear that there are differences between the transcriptional activity of mutant oestrogen receptors in yeast and animal systems, we therefore decided to analyse the activity in yeast of an ER mutant with defective transcription in mammalian systems. The mutant MOR M-547A/L-548A discussed extensively in previous chapters and known to have defective AF2 activity was studied as the N-terminal truncated 121-599 form to remove AF-1 activity. This receptor and its wild type counterpart were transferred into a galactose inducible yeast expression vector by V. Cavallies (see appendix). The ligand induced activity of this receptor was analysed and results shown in fig 5.9. It was observed that the mutant receptor MOR 121-599 M-547A/L-548A could induce reporter activity in a dose dependent manner from an ERE U3 reporter. Stimulation was to a similar degree as truncated wild type receptor MOR 121-599. However, the dose response curve for the AF2 mutant was slightly different with wild type receptor showing a small response to low concentrations of E2 ($10^{-10}/10^{-11}$ M). There was no response at these concentrations from the mutant receptor, there was a small difference in basal activity seen with wild type showing higher basal activity. The plateau of activation was however seen at the same concentration (10^{-8}M). When OHT is used as an agonist MOR 121-599 and MOR 121-599 M-547A/L-548A show similar responses. Stimulation is modest in comparison to E2 and is only seen at doses of 10^{-7}M and above with a plateau at 10^{-6}M.

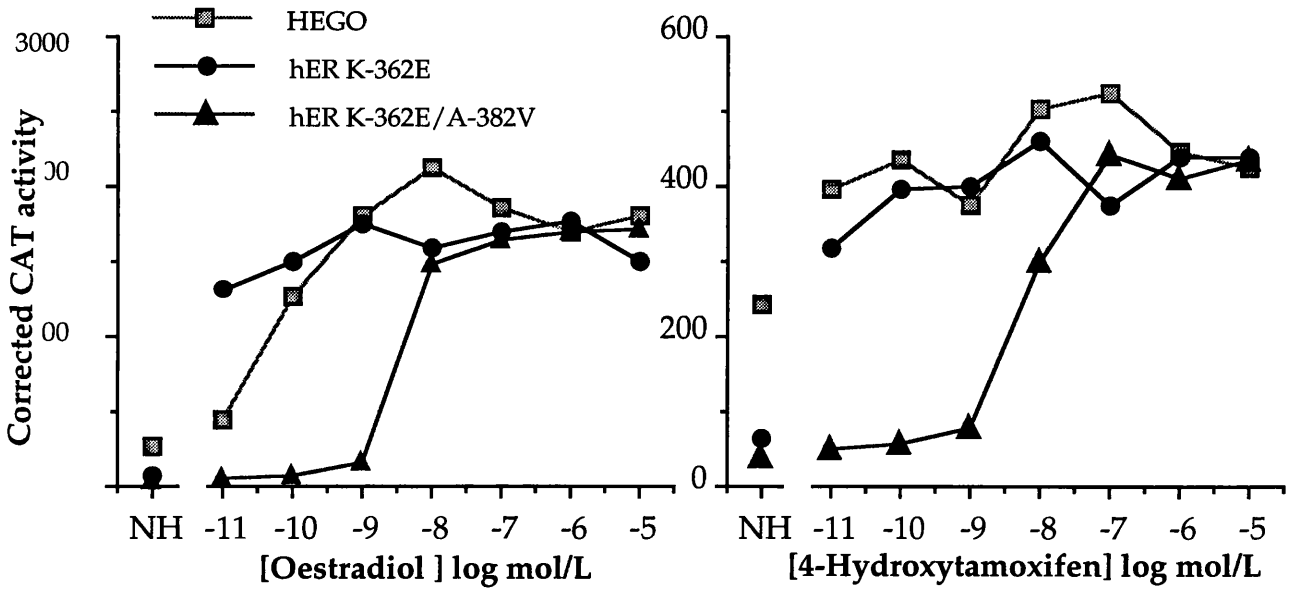


Fig 5.7

Transient transfection of chicken embryo fibroblasts with mutant and wild type oestrogen receptors

The activity of mutant oestrogen receptors was assesed by transient transfection in chicken embryo fibroblasts. Cells were transfected overnight with 5 µg ERE pBL CAT,1 µg pJ7 luciferase internal control and 0.5 µg pSG5hER. Containing HEGO (■), hERK-362E (●) or hER hERK-362E/A-382V (▲). Total DNA was made up to 10 µg per plate with pSG5. After transfection cells were incubated for 24 hours in the pressence of ethanol carrier or increasing concentrations of oestradiol (left hand plot) or 4-hydroxytamoxifen (right hand plot). Cells were harvested and luciferase and chloramphenical acetyl transferase activity recorded. Results shown are a representitive plot of CAT activity divided by luciferase activity to correct for transfection efficiency. Data points represent the average of duplicate transfections.

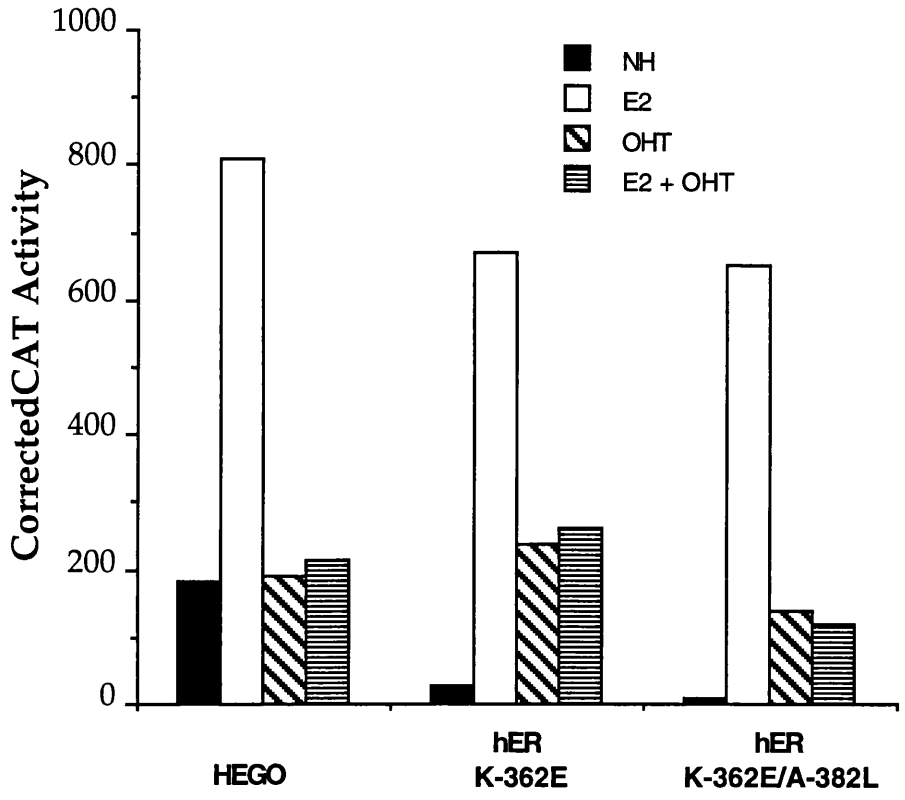


Fig 5.8

Antagonist activity of 4-hydroxytamoxifen in mutant oestrogen receptors.

Chicken embryo fibroblasts were transfected and harvested as described in fig legend 5.7 This figure shows data from an experiment in which cells are incubated with both oestradiol and an excess of 4-hydroxytamoxifen. Cells were treated with ethanol carrier alone (■) 10^{-8} M oestradiol (□), 10^{-6} M 4-hydroxytamoxifen (▨) or 10^{-8} M oestradiol and 10^{-6} M 4-hydroxytamoxifen together (▤). Data shown is the average of duplicate determinations and is a representitive example from several experiments.

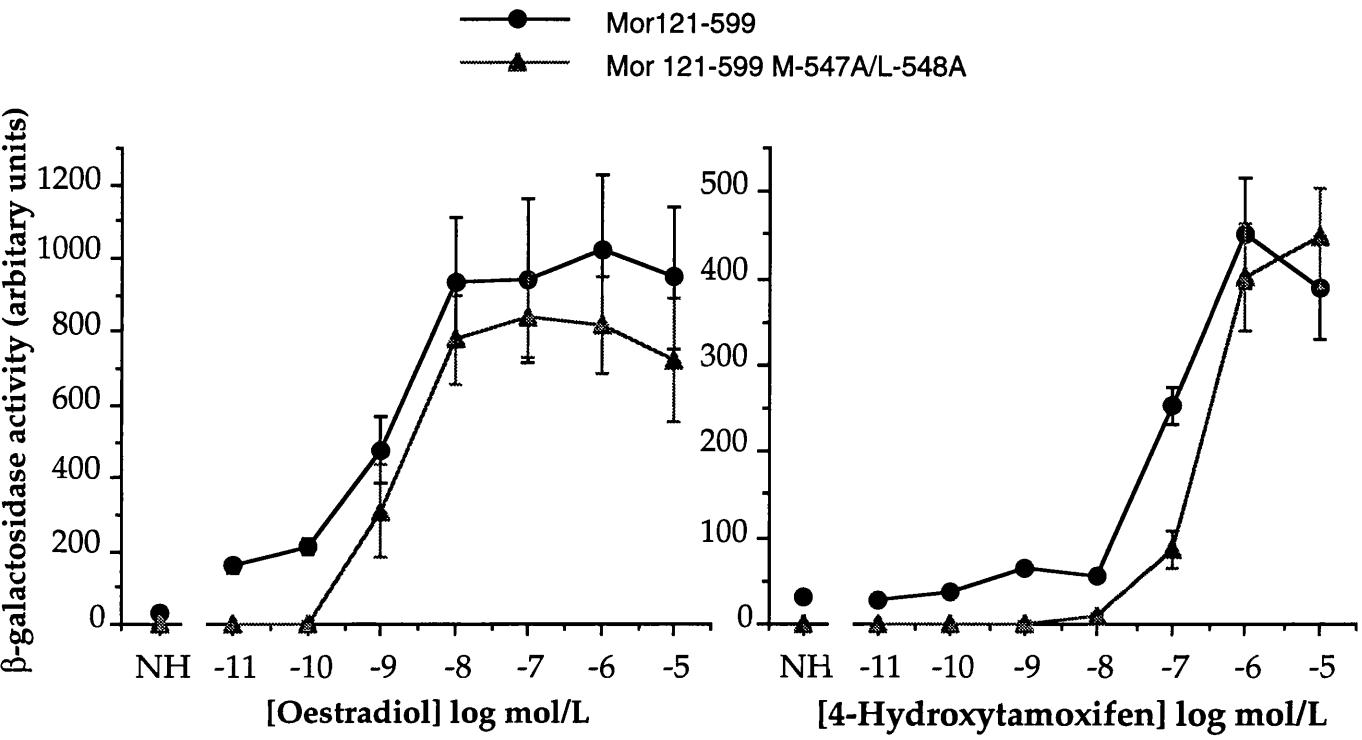


Fig 5.9

Transcriptional activity of mouse oestrogen receptor with mutations abolishing AF2 activity in mamalian cells

The activity of mutant mouse oestrogen receptors was tested in *saccharomyces cerrevisiae* The N-terminal truncated receptor121-599 was expressed on a galactose inducible expression vector as either wild type MOR121-599 (●) or containing mutations of 2 hydrophobic residues in the hormone binding domain MOR121-599 M-547A/L-548A (▲) and the ERE U3 lacZ reporter construct was used to monitor ligand induced activity. Transformed cells were grown in galactose under double selection in the presence of increasing concentrations of oestradiol or 4-Hydroxytamoxifen for 15 hours. Cells were analysed for β-galactosidase activity. The data shown represents the average of three independent experiments performed in duplicate with error bars indicating standard error.

Summary and conclusions

A system for phenotypic screening of mutant ER was developed and used to identify a point mutant ER that could induce transcription of an E2 responsive reporter but in contrast to both wild type and a super active mutant receptor could not be activated by OHT in yeast. However in an animal cell system a response to E2 and OHT could be elicited from the mutant receptor although only at higher concentrations. There was no difference in receptor stability in the presence of different ligands. The ligand binding characteristics of this mutant can not explain the findings since there was no significant difference in affinity of the mutant for E2, and OHT was able to compete for receptor binding sites with a similar RBA as wild type receptor.

The transcriptional activity of the AF2 defective receptor MOR M-547A/L-548A was studied in yeast as a truncated receptor lacking AF-1. It was found to be transcriptionaly active in the presence of E2. OHT was also capable of stimulating this truncated mutant receptor and it's wild type counterpart but the maximal response was weak in comparison to E2.

It appears therefore that there is a difference in the mechanism by which ER induces transcription in yeast and animal cells since a receptor that can not be stimulated by OHT when expressed in yeast is stimulated by OHT in animal cells. A truncated mutant ER that is transcriptionaly inactive in mammalian cells in the presence of E2 can induce transcription in yeast in the presence of E2.

Chapter 6

Discussion

The role of the oestrogen receptor in antioestrogen resistance

The oestrogen receptor has proved a valuable tool to determine prognosis and is routinely used in clinical practice to make therapeutic decisions for breast cancer patients. In this respect it is almost unique as a molecular marker in oncology. In trying to understand the nature of acquired antioestrogen resistance the oestrogen receptor has been an obvious area for investigation. Acquired tamoxifen resistance is frequently associated with continued sensitivity to alternate forms of hormonal manipulation in both clinical and experimental settings, and has been described in the introduction. This retained sensitivity implies that in this situation the hormonal axis remains intact and there is therefore a potential role for the ER in antioestrogen resistance. The ER may be directly responsible through aberrant activity arising from variant or mutant forms of the receptor, or indirectly as a result of alterations in factors that can influence ER function. These include alterations in signalling pathways that affect ER function, alterations in pathways that are affected by the ER and alterations in cofactors important in mediating the ER response. These possibilities are considered with particular reference to the findings presented in the last three chapters.

The potential role of splice variant ER in antioestrogen resistance

While the exon 5 variant has been the centre of most attention it is also appropriate to review the commonly detected splice variant receptors since these may also have important properties. The most frequently reported splice variants lacking exons 2,3,4 or7 are examined individually below The exon 5 variant is considered in most detail since it has been a major focus of this thesis and is the final variant to be discussed.

Exon 2 splice variant ER

The exon 2 deletion encodes a 152 amino acid protein, consisting of the first 151 amino acids of the A/B region of the hER and one unique amino acid, threonine. It has no DNA binding domain or hormone binding domain and it is therefore unable to act as a DNA dependent transcription factor. It is theoretically possible that it could interact with proteins involved

in mediating AF1 activity. Therefore if present at high enough levels this variant could have a negative influence on wild type AF1 activity through a squelching mechanism. There is however no evidence for this. In transient transfection of HeLa cells the exon 2 variant appears to have no intrinsic activity, nor does it interfere with wild type responses (Wang *et al.* 1991). This may not be the case in situations where AF-1 is more important in mediating transcriptional activity but at present there is no evidence to support a role of this variant in antioestrogen resistance.

Exon 3 splice variant ER

The exon 3 deletion results in an in frame splice from nucleotide 642 to 759. The splice variant therefore codes for a protein lacking amino acids 215-253 which form the central region of the DBD, removing the entire second zinc finger, destroying the ability of the receptor to bind DNA (Wang and Miksicek 1991). The variant receptor is transcriptionally inactive in transient transfection assays but represses the ligand induced activity of the wild type receptor (Wang and Miksicek 1991). The mechanism proposed is that the variant forms heterodimers with the wild type receptor, which are unable to bind efficiently to response elements, thus reducing transcriptional activation. This has not as yet been distinguished from the alternative possibility of the exon 3 variant forming inactive complexes, which squelch the wild type response by competing for cofactors. This variant therefore has the potential to interfere with normal ER responses. Although the activity of this variant on antioestrogen action has not been evaluated it is difficult to envisage how this variant may be involved in antioestrogen resistance.

Exon 4 splice variant ER

The exon 4 deletion produces an in frame variant that lacks amino acids 254-365. This protein contains both zinc fingers of the DBD, but lacks the last 10 amino acids of the DBD. The entire D region and the first 63 amino acids of the ligand-binding domain are absent. This variant is unable to bind DNA *in vitro* and is transcriptionally inert in transient transfection assays (Koehorst *et al.* 1994). It remains to be determined what function may

be served by this variant. It does not contain the major nuclear localisation signals and is unlikely to be able to bind ligand yet it forms the major ER transcript in rat brain and is present in ER negative PR positive meningioma (Koehorst *et al.* 1993; Skipper *et al.* 1993).

Exon 7 splice variant ER

Two groups identified the exon 7 splice variant. In one case from an ER positive PR negative breast tumour sample (Fuqua *et al.* 1992) and also from the T47D breast cancer cell line (Wang and Miksicek 1991). The variant mRNA encodes a receptor containing the first 457 amino acids of the ER and a further 10 non-ER residues. The variant is predicted to be unable to bind ligand as it lacks residues essential for ligand binding, it also lacks residues essential for AF-2 activity. In a yeast functional assay the exon 7 variant is found to be transcriptionally inactive and in addition appears to exert a dominant negative effect on the ligand induced transcriptional activity of the full length receptor (Fuqua *et al.* 1992). However in transient transfection experiments using HeLa cells the exon 7 variant was found to be functionally inert (Wang and Miksicek 1991) It is not clear why this variant behaves as a dominant negative in yeast but not in HeLa cells. The protein does not contain the major dimerisation interface and it is not clear why this variant acts as a dominant negative in yeast when in the same system the exon 5 variant is constitutively active.

Exon 5 splice variant ER

The structure and expression of this variant has been described in the introduction and the rationale for detailed functional analysis has also been explored.

Since virtually all the information concerning variant expression has relied on PCR analysis of RNA expression, the question of whether the variants are expressed at the protein level is unclear. Evidence in support of variant expression comes from analysis of a subline of the BT 20 breast cancer cell line which expresses predominantly exon 5 splice variant mRNA. Immunoprecipitation with an N-terminal antibody to the ER reveals a 43 kD band consistent with the size of the exon 5 variant protein. (Castles *et al.*

1993). However in a parallel experiment using MCF-7 cells expressing more variant ER mRNA in absolute terms it was not possible to demonstrate a variant band. The preliminary experiments described in chapter three using the specific exon 5 ER antibody MP40 in human tumour samples have not been able to demonstrate expression of the exon 5 variant in tumours.

The experiments in chapter three demonstrate that the exon 5 splice variant ER has been successfully expressed in several different systems. The expression of variant sequence protein has been confirmed by the raising and purification of a polyclonal antibody that specifically recognises the unique C terminus of the exon 5 variant amino acid sequence.

Exon 5 variant activity on oestrogen responsive reporter genes.

The ability of the exon 5 variant to stimulate oestrogen responsive reporter genes has been tested in transient transfection experiments. Constitutive activity has been clearly demonstrated in chicken embryo fibroblasts, where AF1 is recognised as an important contributor to overall activity (Lees *et al.* 1989; Tora *et al.* 1989; Berry *et al.* 1990). In MCF-7 breast cancer cells the variant does not result in significant constitutive activation of the ERE-tk-luciferase reporter but there is weak activation of the ERE-TATA-CAT reporter. This finding is consistent with the observations that ERE-TATA-CAT is more sensitive to AF-1 stimulation than ERE-tk-CAT (Tora *et al.* 1989).

Since the exon 5 variant expressing breast cancer cells described in chapter 3 also contain wild type ER there is a possibility of interaction between the two receptors. The var-1 cell line allows us to observe the effects of coexpression of wild type and variant receptor. Since there is no effect on oestradiol induced transcriptional activity under the influence of stimulatory ligand, it is unlikely that the variant is able to significantly compete with full length receptor homodimers. However the variant does contain the weak DBD dimerisation domain and interaction is therefore a possibility. Band shift experiments were performed using extracts from var-1 cells, but did not show any evidence of heterodimeric complex formation (data not shown).

One interesting observation that may indicate a degree of interaction *in vivo* is the observation that uninduced var-1 cells show more activity in the presence of pure antioestrogen than in the absence of added ligand (Fig 3.13). Since the uninduced var-1 cells do contain some variant receptor one possible explanation is that pure antioestrogen down regulates wild type ER and relieves a repressive influence of the unliganded wild type receptor revealing a minor constitutive activity from variant receptor. On inducing excess expression of variant, there is sufficient variant receptor to overcome the wild type repressive effect and constitutive activity is then unaffected by wild type ER.

Some of the major findings from these studies are contrasted by the findings of Fuqua and colleagues. Her group have transiently transfected MDA-MB-231 ER negative breast cancer cells and report a strong constitutive stimulation of ERE-tk-luciferase by the exon 5 variant when transiently expressed (Fuqua *et al.* 1995). This is difficult to reconcile with our own results. Constitutive agonist activity in the presence of the exon 5 variant in these cells suggests that there could be significant differences between MCF-7 cells and MDA-MB 231 cells in the way they respond to truncated ER. We attempted to explore the possibility that there were differences in AF-1 sensitivity between MCF-7 cells and MDA-231 cells, but we found this cell line technically difficult to transfect and were unable to generate any data to explore differences between these two breast cancer cell lines. Others have used MDA-MB-231 cells in transient transfections using wild type ER. In these studies the stimulation by hydroxytamoxifen in the presence of ER is minimal (Jiang and Jordan 1992; Catherino and Jordan 1995). Even in an MDA-MB 231 derived stable transfectant containing wild type receptor there is minimal stimulation by hydroxytamoxifen (Catherino and Jordan 1995). It appears therefore that the transcriptional activity of full length ER in response to oestrogen and antioestrogen is similar in MDA MB231 cells and MCF-7 cells. It is therefore difficult to resolve the different results obtained using these two different breast cancer cell lines. There is some indirect and incomplete evidence of possible differences between these two cell lines. The MDA 231 cell line has been used to examine the behaviour of point mutants in the HBD, both the valine 400 (HEO) and tyrosine 351 (HETO) ER mutants show modest tamoxifen stimulated activity (Jiang and Jordan 1992; Catherino and Jordan 1995). The mechanism by which these mutants

become more responsive to hydroxytamoxifen is not understood. It does indicate that the MDA 231 cell line is able to mount an antioestrogen induced agonist response in some circumstances and could form the basis for the differences reported in the exon 5 variant activity betweenMCF-7 and MDA 231 cells. Parallel experiments have not been performed in MCF-7 cells and would of course be complicated by the presence of wild type receptor

Effects of exon 5 variant on breast cancer cell proliferation

Our findings are that expression of the exon 5 variant in MCF-7 cells does not result in any detectable alteration in proliferation in the absence of ligand. There is no alteration in response to antioestrogens or of the ability of antioestrogens to antagonise the growth stimulation of oestradiol. These observations are challenged by the findings of Suzanne Fuqua's group who have conducted similar but less detailed studies of the proliferative effects of expressing the exon 5 variant in MCF-7 cells. They have established stable cell lines expressing exon 5 variant under the control of a constitutive promoter (CMV promoter) and the inducible metalothionien promoter. In their experiments using the inducible promoter they show that the growth of MCF-7 cells expressing the variant is not suppressed by hydroxytamoxifen but is suppressed by ICI 164,384 (Fuqua *et al.* 1995). In these experiments, the control cells show a minimal proliferative response to oestradiol, this contrasts with the marked effect seen in our experiments. Hydroxytamoxifen and ICI 164,384 inhibit growth of the control cells. While the most likely explanation for the differences in observations between laboratories is incomplete removal of oestrogens from growth medium, it could also reflect growth stimulation by cadmium (Garcia-Morales *et al.* 1994), or be a reflection of 'clonal drift' between the parental MCF-7 cells used in our respective laboratories. Comparing growth response of control and exon 5 expressing cells, in the Fuqua experiments, there does not appear to be a difference in proliferation of unstimulated cells. Given the poor growth response to oestradiol this is not suprising but makes assessment of a possible constitutive effect on growth as a result of exon 5 variant expression impossible to assess. The cells were all induced with cadmium. They do not show any data comparing growth of induced and uninduced cells therefore they have not demonstrated that the hydroxytamoxifen

resistance seen is mediated through the exon 5 variant receptor. It is possible that the hydroxytamoxifen resistance is a reflection of some other process. They have only demonstrated variant mRNA production and not examined protein expression and have not examined the expression time course to assess the duration of increased variant expression.
An important observation which is in agreement with our data is that the cells remain sensitive to pure antioestrogen. This implies that if the exon 5 variant is influencing growth then this is likely to be dependent on an interaction with wild type receptor to produce growth stimulation, and that this interaction while unaffected by the presence of hydroxytamoxifen, is inhibited by pure antioestrogen.

In their experiments using the constitutive CMV promoter similar results are reported, with tamoxifen resistance demonstrated in the exon 5 expressing cells (Fuqua and Wolf 1995). The fact that two exon 5 expressing stable cell lines show antioestrogen resistance is in favour of this being a variant receptor mediated effect, but this is not conclusive and not supported by our data. We have examined the effect of antioestrogens on the growth of three separate clones in induced and uninduced conditions covering a wide range of wild type to variant receptor ratios and have confirmed maintained expression of variant receptor over a prolonged period.

Despite criticism of the less rigorous examination of the proliferative effects of the variant receptor by Fuqua compared with our studies there do appear to be differences in our results and it is difficult to explain these adequately. It is conceivable that there are additional differences either in the exon 5 variant stable transfectants that show hydroxytamoxifen resistance that are responsible for the resistance seen, or that facilitate resistance through the variant receptor. These changes may be present (but not apparent) in the stocks of MCF-7 cells used to generate the variant expressing lines in Fuqua's laboratory

Effect of exon 5 variant on endogenous gene expression

The effect of exon 5 variant expression on ER and PR has been examined by Suzanne Fuqua's group. They have demonstrated increased PR expression in exon 5 expressing MCF-7 cells (Fuqua and Wolf 1995). As with the cell proliferation data, they do not present any data comparing

induced and uninduced cells to confirm that this difference is driven by variant expression. It would also be of considerable interest to see if the increased PR expression reported was influenced by antioestrogens. Our data using induced and uninduced cells does not support the role of exon 5 in inducing PR and is further underscored by the absence of any variant effect on pS2 expression.

The reasons for the different results obtained from two groups is not clear but may be similar to the reasons for differences seen in proliferative response, arising as a result of differences between the parental MCF-7 cell lines used by each laboratory, such that the variant is able to promote gene expression and growth in one subline but not another. One possibility being for instance, a difference in expression of cofactors involved in ER responses.

The basic model of tamoxifen action described in the introduction in which AF-1 is stimulated by tamoxifen suggests that AF-1 does not contribute strongly to growth stimulation in normal MCF-7 cells since hydroxytamoxifen does not stimulate cell growth and is a potent antagonist of E2 stimulated growth. Therefore it can be argued that growth stimulation would not be expected in the presence of constitutive AF-1 stimulation. The failure of the exon 5 variant to reverse the growth inhibition seen in the presence of the pure antioestrogen is seen by both groups and suggests that independent AF-1 mediated activity of the variant is not sufficient to affect proliferation in MCF-7 cells.

Variant ER and growth factor responses

The N-terminus of the ER is important in mediating the stimulation of ER by growth factors. The variant may therefore have a role in mediating growth factor responses. Attempts to examine this with transient transfections were not successful since we found that EGF or PKA activation produced very large changes in all tested internal control plasmids and on reporter constructs, even those lacking an ERE. It was therefore not possible to interpret any small changes associated with variant expression acting through an ERE reporter. We did not see any striking effects but this area of research should be regarded as incomplete.

Expression of variant ER in other tissues

The first description of the exon 5 splice variant from breast cancers also describes isolation from normal uterine tissue. It is possible that if a physiological role exists for this variant it may be in non breast tissue but there is no convincing evidence for this. There do not appear to be any major differences in the occurrence of splice variant mRNA species in normal and malignant uterine tissue (Hu *et al.* 1996). There is however a commonly detected variant involving a partial deletion of exon 8 indicating a possible cryptic splicing sequence within exon 8. There is no additional data to suggest a role for splice variant RNA in uterine malignancy.

Exon 5 variant mRNA has been isolated from hepatocellular carcinomas and surrounding non neoplastic cirrhotic liver tissue (Villa *et al.* 1995). There was a sex difference with variant receptor more often expressed in male specimens and the variant is not found in the female non neoplastic liver tissue. The significance of this observation is unclear but of interest since tamoxifen can be used to treat hepatocellular carcinoma. The sex differences suggest that there is an element of control over splice variant production in normal tissue. None of the published data on exon 5 variant expression in breast cancers appears to include male breast cancer tissue.

Splice variant ER is commonly found in meningioma. These tumours frequently express PR but are usually ER negative Initially exon 4 and exon 7 deleted splice variants were reported (Koehorst *et al.* 1993) but exon 5 deleted variant has now also been described (Blankenstein *et al.* 1995). The significance of variant receptor expression in meningioma remains uncertain. Exon 4 deletions have been isolated from other brain sources (Skipper *et al.* 1993). Rat pituitary tissue contains unique RNA isoforms lacking exons 1-4 and is induced by oestrogen, but is only expressed in female rats, again demonstrating that regulation of variant expression occurs in non malignant tissue. The function of this isoform has not been explored (Friend *et al.* 1995).

Evidence for exon 5 variant expression in antioestrogen resistance.

Several other groups have investigated the possible role of variant ER in antioestrogen resistance. Exon 5 variant receptor mRNA has been detected in all ER positive cell lines examined and at least two ER negative lines (Castles *et al.* 1995). The amount of exon 5 variant varies greatly and

even varies amongst different stocks of the same cell line (Klotz *et al.* 1995). Using semiquantitative PCR, the exon 5 variant to wild type ratio has been shown to be higher in malignant breast tissue compared to normal breast (Leygue *et al.* 1996). However when primary tumours are compared to tamoxifen resistant tumour material no significant differences in wild type to exon 5 variant mRNA is seen unless subgroups are analysed (Daffada *et al.* 1995). In this study higher exon 5 to wild type ratio is seen in ER negative tumours expressing either PR or pS2. In resistant tumours that were ER, pS2 and PR positive a higher ratio of variant to wild type was seen compared to the primary tumours. Since ratios were analysed the absolute exon 5 levels are not known. It is probable therefore that in the ER negative tumours the absolute amount of exon 5 variant mRNA remains small the high ratio being a product of low levels of wild type ER mRNA. The finding of a difference between resistant and primary tumours in the ER, pS2, PR positive subgroup is potentially important. This difference appears to be caused by a small number of resistant tumours with marked increases in wild type to variant ratios, prompting speculation that there may be a causal relationship in a minority of tamoxifen resistant cancers. However, there is no support for this from analysis of *in vitro* generated tamoxifen resistant cell lines. In antioestrogen resistant cell lines the major splice variant species (including exon 5 deletion) were analysed by RNase protection and have been compared to parental lines. No significant increasses in any of the splice variants has been detected from analysis of 10 separate resistant cell lines (Madsen *et al.* 1997). In one case the amount of exon 5 variant message was paradoxically reduced in the tamoxifen resistant clone (Madsen *et al.* 1995).

The detection of mRNA does not imply expression of protein and all work on variant expression involves PCR amplification and there remains some doubt over the reliability of this technique for quantitative analysis. Experiments by two groups using the MP40 antibody on paraffin embedded tumour samples have not demonstrated the expression of variant receptor in breast cancer sections, which raises serious doubts over the importance of this variant.

Splice variants of the glucocorticoid receptor

A splice variant of the glucocorticoid receptor in which there is failure to remove intron sequences between exon 7 and 8 has been described in multiple myeloma and is associated with glucocorticoid insensitivity (Moalli *et al.* 1993; Krett *et al.* 1995). In breast cancers there does not appear to be any evidence for splice variant GR demonstrating that the generation of splice variant nuclear receptors in tumours is a specific process rather than a non specific relaxation in the general control of RNA splicing (Pfeffer *et al.* 1995). This sugests that splice variant generation may have a physiological role but it remains unclear what that role might be.

Summary of splice variant ER in breast cancer

Splice variant ER mRNA is found readily in both normal and malignant breast tissues. The evidence for expression of the splice variants at the protein level is weak. There is considerable quantitative variation in the amount of variant mRNA produced in different tumours. The functional significance of splice variant ER in breast cancers is difficult to assess. The exon 5 deleted variant has been the subject of extensive evaluation since it has the potential to act as a constitutive activator. There is conflicting data from experimental models of the effect of the exon 5 variant receptor. The degree of transcriptional activation of reporter genes in breast cancer cells appears to depend on the cell line used and even within the same cell line there is dispute over the effect of the variant on endogenous gene expression and on proliferative responses, particularly on the response to hydroxytamoxifen. It is clear however that in cell culture models overexpression of the exon 5 variant is unable to overcome the growth inhibitory effects of pure oestrogen antagonists. While the exon 5 variant has been shown to have a variable degree of constitutive activity in breast cancer cells there is very little evidence to support the hypothesis that splice variant ER expression is a cause of antioestrogen resistance in human breast cancers.

Mutant oestrogen receptors as a mechanism of antioestrogen resistance in breast cancer

In the introduction I have discussed the properties of a number of mutant ERs generated through site directed mutagenesis. These mutations

can have profound effects on the properties of these receptors and have been highly influential in shaping our understanding of ER function. The experiments described in chapter four have explored a possible role for functionally significant mutations of the hormone binding domain in mediating antioestrogen resistance in breast cancer. We have analysed the transcriptional activity of mutants with known alterations in their ability to respond to oestrogen receptor ligands and examined human breast cancer samples for similar mutations within a limited region of the ligand binding domain. We have demonstrated that introducing mutant ER into breast cancer cells can result in a change in response to both triphenylethylene antioestrogens and pure antioestrogens. Analysis of this region did not reveal any naturally occurring mutations in breast cancers.

The transient transfection of the AF2 defective mutant oestrogen receptors into MCF-7 cells has demonstrated weak agonist responses to antioestrogens. This response is dependent on the amount of expression vector DNA used giving a dose response relationship. We did not see any suppression of activity with oestradiol, which would be expected, on transfection of excess AF2 defective receptor. A dominant negative effect has been reported for similar but not fully analogous hER mutations in this region in both CHO and MCF-7 cells (Ince *et al.* 1993; Ince *et al.* 1995). However different mutations show a variable degree of dominant negative activity and interestingly the dominant negative effect of the hER AF2 mutants is dependent on the presence of AF1 since dominant negative activity is lost when the A/B region is deleted. The absence of a dominant negative effect in our experiments is suprising and while there is no entirely satisfactory explanation. It may be because the wild type and mutant receptors do not heterodimerise with wild type hER efficiently *in vivo* (the mutants used were MOR mutants). If they do form heterodimers, the perhaps they do not disrupt the E2 stimulated transcriptional activity of the wild type component of the heterodimer in an analogous situation to the hER AF2 mutants when AF1 is deleted as described above.

Yeast as a model of ER function in eukaryotic cells

Yeast has been used extensively to study the function of mammalian proteins and in many instances yeast homologs of mammalian proteins exist. Steroid hormone receptors have been found to retain many functions when

expressed in yeast cells. The ER binds DNA and hormone with similar affinity when expressed in yeast or mammalian cells and is able to activate transcription in a ligand dependent manner when expressed in yeast (Metzger *et al.* 1988). The yeast model is therefore potentially a useful system to use to study the effects of mutations of the ER. Using a colour change phenotypic screen it is possible to use yeast as a screening system to identify mutant ER with specific phenotypic characteristics.

The experiments described in chapter 5 were aimed at using yeast to identify mutant oestrogen receptors with abnormal response to ligand, specifically to identify mutants that were unable to bind hydroxytamoxifen. Such mutations would be potential candidates for antioestrogen resistance and could potentially be identified in breast cancers. Although technical failure resulted in the recovery of only a limited number of mutations the technique has been demonstrated to have the capacity to identify functionally abnormal ER mutations. We may possibly have had more success by selecting a much broader range of phenotypic abnormalities such as constitutive activity which while seen and collected during our screening assays, was not our prime focus and these clones did not survive long term storage.

The specific mutation identified with abnormal properties K-362E/A-382V shows oestradiol sensitivity but no stimulation by hydroxytamoxifen in yeast. In chicken embryo fibroblasts there is a response to oestradiol and hydroxytamoxifen but only at higher concentrations. However the ligand binding at 25^0C is similar to wild type. This data is difficult to interpret but may be partly explained but postulating a temperature sensitive effect on ligand binding such that oestradiol is bound less well at higher temperature analogous to the mutation at G400 (Tora *et al.* 1989). This however does not explain the insensitivity to hydroxytamoxifen in yeast. The ability of tamoxifen to penetrate the cell wall may be limited as is the case for pure antioestrogens (Zysk *et al.* 1995). This probably explains the difference in right shift of the dose response curves in yeast between oestradiol and hydroxytamoxifen, but does not explain the total absence of activity of the K-362E/A-382V mutant in response to up to 10 micromolar hydroxytamoxifen. The competition assay does not show any evidence of differential binding between hydroxytamoxifen and oestradiol and therefore we must conclude that there are differences in the ability of yeast to respond to this mutant

compared with CEF cells and that these differences are not seen in the wild type receptor. For this reason we concluded that phenotypic preservation between yeast and animal cells was unreliable when screening for comparatively subtle effects and did not pursue this screening system further. Yeast has been used by other groups to characterise mutant ER and in most cases the phenotype has been conserved when reanalysed in mammalian cells (Pierrat *et al.* 1992; Wrenn *et al.* 1993). However there are differences reported. Notably the τ2 region between 302 and 339 which when exposed by deletion mutants results in powerful ligand independent activity in yeast but not mammalian cells (Pierrat *et al.* 1994). A further point of interest is that a mutation at 383 (W-383R) adjacent to the mutation identified by ourselves was identified by mutational screening in yeast. This mutation is completely unresponsive to oestradiol and hydroxytamoxifen in both yeast and Chinese hamster ovary cells. This mutant binds oestradiol but with around 10 fold lower affinity than wild type. Clearly mutations in this viscinity do have important consequences for ER function. This region forms part of helix 6 important in forming the ligand-binding pocket (Brzozwski *et al.* 1997) although neither W-383 nor K-382 make contacts with ligand directly.

AF2 defective mutations are active in yeast

The experiments shown in fig 5.9 clearly show that the AF2 mutant M-547A/L-548A is stimulated in yeast by both oestradiol and hydroxytamoxifen. The stimulation by oestradiol is of interest since it implies that the oestradiol induced activation is not dependent on the same interactions in yeast as in mammalian cells. However recent data from our own laboratory using different reporters suggests that this finding may not be generally applicable (Heery personal communication).

Are ER mutations present in breast cancers?

I have described much of the work searching for mutations of the ER in breast cancers and breast cancer cell lines. With the exeption of a few notable examples the overwhelming majority of breast cancers appear to contain wild type receptor. This impression has been confirmed with the recent publication of a further detailed screening of 96 tumours from a mixture of sources (both primary and metastatic tumours). This study

describes a new polymorphism in the N terminus of the ER, but no somatic mutations of the ER were detected (Anderson *et al* 1997)*. Given the laboratory-based evidence of the functional consequences of ER mutation this is a surprising result. The studies including the recent study described above have all relied on indirect techniques to detect mutations, using either, single strand chain polymorphism (SSCP,) or constant denaturant gel electrophoresis (CDGE). Using these techniques, only aberrantly migrating bands are selected for more detailed analysis by direct sequencing. Neither of these techniques is fully reliable and there is therefore a possibility that commonly occurring specific mutations are not detected with these techniques. The alternative to using these techniques is to either laboriously sequence genomic DNA or RT PCR products from breast tumours, across the entire coding region. Or limit the examination to specific areas where there is a high probability of functionally significant mutations. The majority of studies have concentrated on primary tumour material since this is more generally available than metastatic tumour tissue. There is therefore a danger that we are not analysing the appropriate material. However since the primary tumours can become resistant and give rise to resistant metastasis they could harbour a subpopulation of cells with mutant ER. These subpopulations would also be missed by SSCP and CDGE. It was for these reasons that we decided to use an alternative technique to concentrate on an area of the ER where it was known that mutations could give rise to functionally different receptors and could respond to antioestrogens. The technique described in chapter 4 has the potential to detect this subpopulation which could only otherwise be identified by a direct sequencing programme on a very large scale. Despite our best endeavours screening both treatment naïve and established tamoxifen resistant tumour samples we found no evidence of mutations in critical residues in H12 of the ER.

Why are ER mutations uncommon in breast cancer?

The search for mutations within the ER in breast tumours performed by several groups has produced very few mutations and those that have been identified have not been characterised for their functional significance. The possible explanations for this are of necessity speculative. Since the ER is a positive regulator of cell growth and that in the untreated state there is

*Reference is not listed in alphabetical order see page 195

always an oestrogenic signal present albeit a weak signal in postmenopausal women. There may not be sufficient growth advantage to a dominant positive mutation of the ER such as mutation of tyrosine 537 to encourage clonal expansion. Other mutations producing more significant growth advantage would be more likely to predominate and the acquisition of mutations leading to oestrogen independence would result in progressively less advantage to ER mutations. When exposed to antioestrogens the situation is different in that there is then no ER mediated mitogenic signal and this environment may favour the production of dominant positive or antioestrogen stimulated ER mutations. However this is not seen to any significant degree. In prostate cancer a number of androgen receptor mutations have been isolated from hormone insensitive tumours and mutation of the AR appears to be a more frequent event. The prostate cancer cell line LNCaP contains a mutant AR containing a point mutation resulting in threonine substituting Alanine at position 868 (Veldscholte *et al.* 1990). This mutation results in altered steroid binding specificity such that it can respond to progestins and is also stimulated by some but not all antiandrogens (Veldscholte *et al.* 1992). Precisely the same mutation has been identified in other metastatic prostate tumours. 6 out of 24 tumours carried this mutation in one study (Gaddipati *et al.* 1994), and it has been identified by several other groups (Suzuki *et al.* 1993; Taplin *et al.* 1995). Furthermore other mutations involving residues 868 and 865, have been identified (Taplin *et al.* 1995). Both of these mutations also demonstrate increased sensitivity to other steroid hormones. In addition to this 'hot spot' numerous mutations have been identified within the HBD of the AR such as a valine to methionine mutation at residue 706, which is stimulated by adrenal androgens (Culig *et al.* 1993). Most of the AR mutations identified have not been subjected to functional analysis, but it is clear that AR mutations are a relatively frequent event in prostate cancer occuring in perhaps 40% of metastasis (Tilley *et al.* 1996). Although they have been found in all stages of prostate cancer they are more frequent in advanced disease (Taplin *et al.* 1995). The prognosis in tumours containing AR mutations is poor and is associated with a poor response to hormone therapy (Tilley *et al.* 1996). In addition amplification of the AR gene locus is a relatively common event occurring after relapse from androgen deprivation therapy (Visakorpi *et al.* 1995).

A clear difference exists between the apparent frequency of AR mutations and gene amplification in prostate cancer and ER mutations / amplification in breast cancer. Most mutational analysis of breast cancers has been from primary tumour material. Much of the prostate work has been performed on metastatic material isolated from bone marrow and this may account for part of the difference. We may need to examine breast cancer specimens from more inaccessible sites to establish the contribution of mutant ER to antioestrogen resistance in life threatening metastatic sites.

There may be physicochemical reasons why the ER is less susceptible to mutation compared to the AR related to its position within the chromosome, it may be folded or relatively protected by the local organisation of chromatin.

While different tumour types are known to display different patterns of oncogene expression for instance K-ras is frequently mutated in colorectal carcinoma but H-ras is rarely involved whereas H-ras is commonly mutated in bladder cancer (Bos 1988). These patterns are often a reflection of the pathways important in the development or maintenance of the tissue of origin. It is therefore difficult to consider categorising the ER as a pathway that is not mutated in breast cancer because it's functional significance is unimportant. Further investigation is clearly needed before we can understand why ER mutations are uncommon in breast cancer.

Other signaling pathways and tamoxifen resistance

There is the possibility that antioestrogen resistance could be mediated by growth factor mediated influence on ER function. I have described the effect of PKA and EGF stimulation on the enhancement of agonist effects of hydroxytamoxifen and ligand independent activation. Clearly there is a potential route towards tamoxifen stimulated cell growth through growth factor influence on ER function. While alterations in growth factor receptors or alterations in growth factor signalling pathways are associated with poor prognosis there is as yet no clear evidence that these alterations are involved in mediating tamoxifen resistance through an ER mediated effect. This is area requires further study

The influence of ER on AP1 activity is a further area in which manipulation of the signalling environment could result in ER producing a different mitogenic signal in response to antioestrogen. The importance of

ER involvement in modulating AP1 activity remains uncertain but this is an additional area where further research is required to more clearly define the physiological role of ER in modulation of AP1 effects and to study possible alterations in disease states.

Cofactor expression

The recent proliferation in the number of identified coactivators and repressors of nuclear hormone receptors is a highly significant step forward in our understanding of how these receptors function and is a further avenue that requires exploration in respect of antioestrogen resistance. The finding of increased expression of A1B1 in breast and ovarian tumour tissue (Anzik *et al* 1997), may be an indication of the role these factors may play in the overall pathogenesis of cancers in hormone responsive tissue. It is clearly an area that is in need of examination with respect to hormone antagonist sensitivity and resistance.

Concluding remarks

Despite major research efforts, the molecular mechanisms underpinning the acquisition of antioestrogen resistance in breast cancer remain unexplained. It seems unlikely that there is a significant contribution through either oestrogen receptor mutations or through the generation of splice variant ER. We should therefore concentrate on the areas outlined above to define the importance of other molecules that mediate the complex network of pathways that are implicated in oestrogen and antioestrogen action. An understanding of these mechanisms will it is hoped provoke the development of new therapeutic strategies to control breast cancer.

Although our understanding of antioestrogen resistance has been slow, the recent therapeutic advances in the hormonal treatment of breast cancer have been encouraging. The widespread availability of specific aromatase inhibitors providing an important option in second line hormone therapy and the encouraging phase II results of the pure antioestrogen compound ICI 182,780 (faslodex), leading to anticipation of further options in the near future. These two examples demonstrate how scientific understanding of biological processes result in the development of advances that are of direct benefit to patients and provide justification for further endeavour.

Appendix

Abbreviations

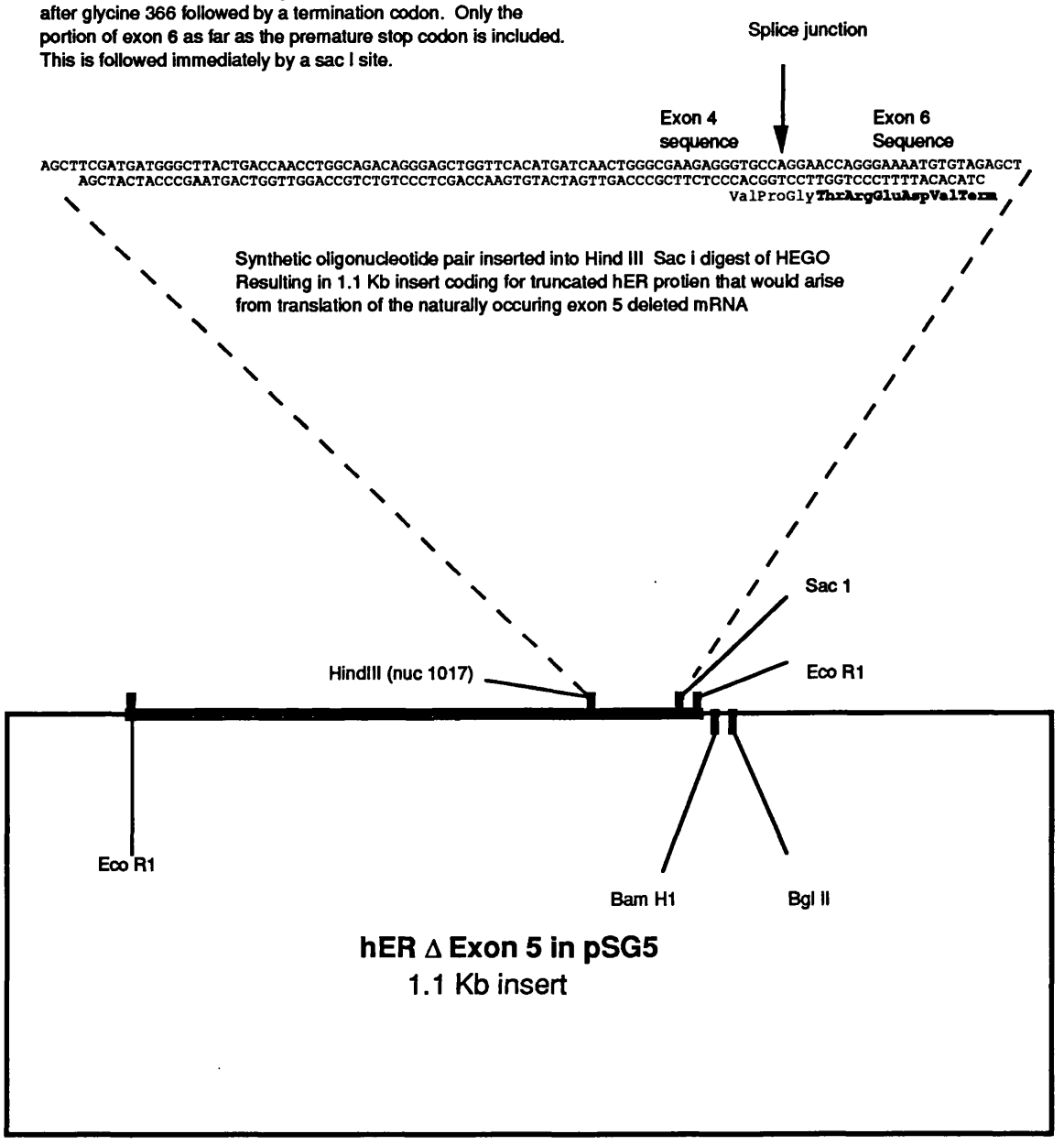
AF1/AF2	activation function 1/activation function 2
ATP	adenosine 5' triphosphate
AR	androgen receptor
BES	N, N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CDGE	constant denaturant gel electrophoresis
C-terminal	carboxyl terminal
CTD	carboxyl terminal domain
CTP	cytidine 5'-triphosphate
CMV	cytomegalovirus
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DCC	dextran coated charcoal
DEAE	diethylaminoethylamine
DMBA	dimethylbenzanthrycine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DR+X	direct repeat with X nucleotide spacing
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
ER	oestrogen receptor
ER+X	everted repeat with X nucleotide spacing

ERE	oestrogen response element
FCS	foetal calf serum
FRE	SF-1 response element
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GST	glutathione-S-transferase
GTP	guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine N'-2-ethansulphonic acid
hsp	heat shock protein
HRE	hormone response element
ICI 164,384	N-n-butyl-11-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)-N-methylundecanamide
ICI 182,780	7α-[9-(4,4,5,5,5-pentaflouropentylsulphinyl) nonyl]oestra1,3,5,(10)-triene-3,17β-diol
IR+X	inverted repeat with X nucleotide spacing
Kb	kilobase
kD	kilodalton
Kd	dissociation constant
lacZ	β-galactosidase gene
MMTV	mouse mammary tumor virus
MR	mineralocorticoid receptor
mRNA	messenger RNA
NBRE	NGFI-B response element
NGFI-B	nerve growth factor inducible protein-B
NMR	nuclear magnetic resonance
NP40	nonidet p40
N-terminal	amino terminal
ODx	optical density at wavelength of x nm
PAGE	polyacrylamide gel electrophoresis
PBSA	phosphate buffered saline A
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
RAR	retinoic acid receptor

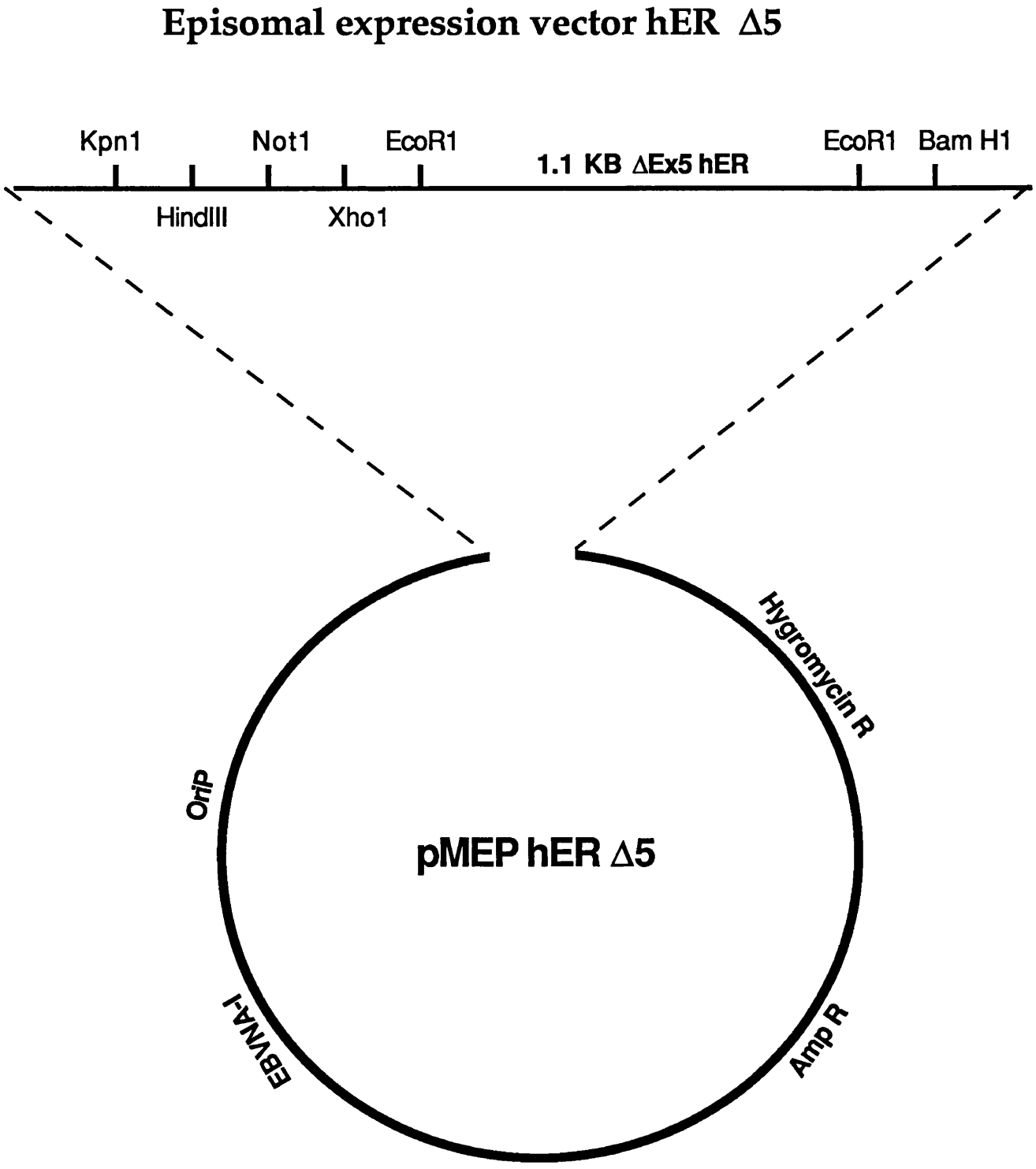
RARE	retinoic acid response element
RNA	ribonucleic acid
RNase	ribonuclease
RXR	retinoid X receptor
S	Svedberg units
SDS	sodium dodecyl sulphate
SSCP	single strand chain polymorphism
SV40	simian virus 40
T3	3, 5, 3'-triiodo-L-thyronine
TAF	TBP associated factor
TBP	TATA binding protein
TEMED	*N'N'N'N'*-tetramethylethylenediamine
TK	thymidine kinase
TR	thyroid hormone receptor
TRE	thyroid hormone response element
TREpal	palindromic thyroid hormone response element
Tris	tris(hydroxymethyl)aminoethane
Triton X-100	octyl phenoxy polyethoxyethanol
Tween-20	polyoxyethylenesorbitan monoluarate
UAS	upstream activating sequence
UTP	uridine 5'-triphosphate
UV	ultra violet
VDR	vitamin D receptor

CONSTRUCTION OF EXON 5 DELETION EXPRESSION VECTOR

This oligo pair consists of exon 4 and exon 6 sequence corresponding to a Precise deletion of Exon 5 sequence resulting in reading frame shift and coding for 5 unique amino acid residues after glycine 366 followed by a termination codon. Only the portion of exon 6 as far as the premature stop codon is included. This is followed immediately by a sac I site.



EXON 5 VARIANT SEQUENCE TRANSFERED TO pMEP4



References

Ali, S., D. Metzger, J.-M. Bornert, *et al.* (1993). EMBO J **12**(3): 1153-1160.

Allan, G. F., X. Leng, S. Y. Tsai, *et al.* (1992). J Biol Chem **267**(27): 19513-19520.

Anderson, D. E. (1972). J Natl Cancer Inst **48**: 1029-1034.

Anderson, D. E. (1976). Cancer **34**: 1090-1097.

Anolik, J., C. Klinge, R. Bambara, *et al.* (1993). J Steroid Biochem Mol Biol **46**(6): 713-30.

Anzik, S., J. Kononen, R. Walker, *et al.* (1997). Science **277**: 965-968.

Arbuckle, N. D., S. Dauvois and M. G. Parker (1992). Nucleic Acids Res **20**(15): 3839-3844.

Armstrong, B. and R. Doll (1975). Int J Cancer **15**: 617-631.

Aronica, S. M. and B. Katzenellenbogen (1993). Molecular Endocrinology **7**: 743-752.

Aronica, S. M., W. L. Kraus and B. S. Katzenellenbogen (1994). Proc Natl Acad Sci USA **91**: 8517-8521.

Astruc, M. E., C. Chabret, P. Bali, *et al.* (1995). Endocrinology **136**(3): 824-832.

Bates, S. E., N. E. Davidson, E. M. Valverius, *et al.* (1988). Molecular Endocrinology **2**: 543-545.

Beato, M. (1989). Cell **56**: 335-344.

Beatson, G. T. (1896). Lancet **ii**: 104-107.

Bentley, A., I. S. Fentiman, R. D. Rubens, *et al.* (1992). Lancet **339**: 71-85.

Beral, V., C. Hermon, G. Reeves, *et al.* (1995). Lancet **345**: 1642-3.

Berrino, F., M. Sant, A. Verdecchia, *et al.* (1995). Survival of cancer patients in Europe, IARC.

Berry, M., D. Metzger and P. Chambon (1990). EMBO J **9**(9): 2811-2818.

Blankenstein, M., S. Koehorst, C. van der Kallen, et al. (1995). J Steroid Biochem Mol Biol 53(1-6): 361-5.

Bos, J. L. (1988). Mutation Research **58**: 196-255.

Bourguet, W., M. Ruff, P. Chambon, *et al.* (1995). Nature **375**(6530): 377-382.

Bowler, J., T. J. Lilley, J. D. Pittam, *et al.* (1989). Steroids **54**(1): 71-99.

Brou, C., S. Chaudhary, I. Davidson, *et al.* (1993). EMBO J **12**: 489-499.

Brunner, N., D. Bronzert, L. L. Vindelov, *et al.* (1989). Cancer Res **49**: 1515-1520.

Brunner, N., G. Clarke, M. Lippman, *et al.* (1990). Models forstudying the progression of hormone dependentto independentgrowth. Growth regulation of cancerII. G. Clarke and M. Lippman. New York, Alan Liss**:** 115-125.

Brunner, N., T. L. Frandsen, C. Holst-Hansen, *et al.* (1993). Cancer Res **53**: 3229-3223.

Brunner, N., M. Johnson, Holst-Hansen, *et al.* (1995). Endocrine related cancer **2**(1): 27-36.

Brzozwski, A., A. Pike, Z. Dauter, *et al.* (1997). Nature **389**: 753-758.

Bucalossi, P., U. Veronesi and A. Pandolfi (1954). Tumori **40**: 365-402.

Bunone, G., P. Briand, R. Miksicek, *et al.* (1996). EMBO J **15**(9): 2174-83.

Butta, A., K. MacLennan, K. C. Flanders, *et al.* (1992). Cancer Res **52**: 4261-4264.

Caroll, K. K. and G. J. Hopkins (1979). Lipids **14**: 155.

Castles, C. G., S. A. W. Fuqua, D. M. Klotz, *et al.* (1993). Cancer Res **53**(24): 5934-5939.

Castles, C. G., D. M. Klotz, S. A. W. Fuqua, *et al.* (1995). Br J Cancer **71**(5): 974-980.

Catherino, W. H. and V. C. Jordan (1995). Cancer Lett **90**(1): 35-42.

Catherino, W. H., D. M. Wolf and V. C. Jordan (1995). Mol Endocrinol **9**(8): 1053-63.

Cavaillès, V., S. Dauvois, P. S. Danielian, *et al.* (1994). Proc Natl Acad Sci USA **91**: 10009-10013.

Cavailles, V., S. Dauvois, F. L'Horset, *et al.* (1995). EMBO J **14**: In press.

Chen, C. and H. Okayama (1987). Mol Cell Biol **7**: 2745-2752.

Cho, H. and B. S. Katzenellenbogen (1993). Molecular Endocrinology **7**: 441-452.

Clarke, R., N. Brunner, Katz, *et al.* (1989). Mol Endo: 3372-3380.

Clarke, R., N. Brunner, B. Katzenellenbogen, *et al.* (1989). Proc Natl Acad Sci U S A **86**(10): 3649-53.

Clarke, R., N. Brunner, E. Thompson, *et al.* (1989). J Endocrinol **122**(1): 331-40.

Clarke, R., R. B. Dickson and M. E. Lippman (1991). The role of steroid hormones and growth factors in the control of normal and malignant breast.

Nuclear Hormone Receptors. M. Parker. London, Academic Press Limited: 297-319.
Cole, M., C. Jones and I. Todd (1971). Br J Cancer **25**(270-275).
Collaborative group on hormonal factors in breast cancer (1996). Lancet **347**(9017): 1713-27.
Constantino, J. P., L. H. Kuller, D. G. Ives, *et al.* (1997). Journal of the National Cancer Institute **89**(11): 776-782.
Cowley, S., S. Hoare, S. Mosselman, *et al.* (1997). J Biol Chem **272**(32): 19858-62.
CRC (1996). Cancerhelp UK **website**(http://medweb.bham.ac.uk/cancerhelp).
Culig, Z., A. Hobisch, M. Cronauer, *et al.* (1993). Molecular Endocrinology **7**: 1541-1550.
Cuzick, J., D. Y. Wang and R. D. Bulbrook (1986). Lancet **1**(8472): 83-6.
Dabre, P. and R. Daly (1989). Proceedings of the Royal Society of Edinburgh. **95B**: 119-132.
Daffada, A. A. I., S. R. D. Johnston, I. E. Smith, *et al.* (1995). Cancer Res **55**: 288-293.
Daly, R. and P. Darbre (1990). Cancer Res **50**(18): 5868-75.
Danielian, P. S., R. White, S. A. Hoare, *et al.* (1993). Mol Endocrinol **7**: 232-240.
Danielian, P. S., R. White, J. A. Lees, *et al.* (1992). EMBO J **11**: 1025-1033.
Dauvois, S., P. S. Danielian, R. White, *et al.* (1992). Proc Natl Acad Sci USA **89**: 4037-4041.
Dauvois, S., R. White and M. G. Parker (1993). Journal of Cell Science **106**: 1377-1388.
De Wet, J. R., Wood K.V., M. Deluca, *et al.* (1987). Mol Cell Biol **7**(2): 725-737.
Dearnaley, D., J. Sloane, M. Ormerod, *et al.* (1981). Br J Cancer **44**(1): 85-90.
Defranco, D., A. Madan, Y. Tang, *et al.* (1995). Vitam Horm **51**: 315-38.
DeFriend, D., E. Anderson, J. Bell, *et al.* (1994). Br-J-Cancer. **70**: 204-211.
Dhingra, K. and G. N. Hortobagyi (1996). Semin Oncol **23**(4): 436-45.
Dickson, R. and M. Lippman (1991). Growth factors and oncogenes. Medical management of breast cancer. T. S. Powles, I. London, Martin Dunnitz.
Dickson, R. B., M. E. McManaway and M. E. Lippman (1986). Science **232**(4757): 1540-3.

Diel, I., M. Kaufmann, S. Costa, *et al.* (1996). J Natl Cancer Inst **88**(22): 1652-8.
Dorssers, L. C. J., T. van Agthoven, A. Dekker, *et al.* (1993). Molecular Endocrinology **7**: 870-878.
Dotzlaw, H., M. Alkhalaf and L. C. Murphy (1992). Mol Endo **6**: 773-785.
Dotzlaw, H., E. Leygue, P. Watson, *et al.* (1997). J Clin Endocrinol Metab **82**(7): 2371-4.
El-Tanani, M. and C. Green (1997). Mol Endocrinol **11**(7): 928-37.
Emmas, C. E., S. E. Fawell, S. A. Hoare, *et al.* (1992). J Steroid Biochem Mol Biol **41**: 291-299.
Encarnacion, C., D. Ciocca, W. McGuire, *et al.* (1993). Breast Cancer Res Treat **26**(3): 237-46.
Evans, R. M. (1988). Science **240**(4854): 889-895.
Ewertz, M. (1996). Maturitas **23**(2): 241-6.
Fawell, S. E., J. A. Lees, R. White, *et al.* (1990). Cell **60**: 953-962.
Fawell, S. E., R. White, S. Hoare, *et al.* (1990). Proc Natl Acad Sci USA **87**: 6883-6887.
Fentiman, I. S., M. Caleffi, A. Rodin, *et al.* (1989). Br J Cancer **60**: 262-264.
Freedman, L., B. Luisi, Z. Korsum, *et al.* (1988). Nature **334**: 543-546.
Friend, K. E., L. E. Ang and M. A. Shupnik (1995). Proc Natl Acad Sci USA 92: 4367-4371.
Fuqua, S. A., S. D. Fitzgerald, G. C. Chamness, *et al.* (1991). Cancer Res **51**: 105-109.
Fuqua, S. A. W., S. D. Fitzgerald, D. C. Allred, *et al.* (1992). Cancer Res **52**: 483-486.
Fuqua, S. A. W., C. Wiltschke, C. Castles, *et al.* (1995). Endocr Relat Cancer **2**(1): 19-25.
Fuqua, S. A. W. and D. M. Wolf (1995). Breast Cancer Research & Treatment **35**: 233-241.
Furr, B. J. A. and V. C. Jordan (1984). Pharmacol Ther **25**: 127-205.
Gaddipati, J., D. McLeod, H. Heidenberg, *et al.* (1994). Cancer Res **54**(11): 2861-4.
Garcia, T., S. Lehrer, W. D. Bloomer, *et al.* (1988). Mol Endo **2**: 785-791.
Garcia-Morales, P., M. Saceda, N. Kenney, *et al.* (1994). J Biol Chem **269**(24): 16896-16901.
Gaub, M.-P., M. Bellard, I. Scheuer, *et al.* (1990). Cell **63**: 1267-1276.

Gibson, M. K., L. A. Nemmers, W. C. Beckman Jr, *et al.* (1991). Endocrinology **129**(4): 2000-2010.
Gorski, J., D. Toft, G. M. Shyamala, *et al.* (1968). Recent Prog Horm Res **24**: 45-80.
Gottardis, M. M. and V. C. Jordan (1988). Cancer Res **48**: 5183-5187.
Gotteland, M., G. Desauty, J. Delarue, *et al.* (1995). Molecular and Cellular Endocrinology **112**: 1-13.
Graham, M. L., N. L. Krett, L. A. Miller, *et al.* (1990). Cancer Res **50**: 6208-6217.
Green, S. and P. Chambon (1987). Nature **325**(6099): 75-8.
Green, S. and P. Chambon (1988). Trends Genet **4**(11): 309-314.
Green, S. and P. Chambon (1991). The oestrogen receptor: From perception to mechanism. Nuclear Hormone Receptors. Molecular Mechanisms, Cellular Functions, Clinical Abnormalities. M. G. Parker. London, Academic Press**:** 15-38.
Green, S., P. Walter, V. Kumar, *et al.* (1986). Nature **320**(6058): 134-9.
Greene, G. L., N. B. Sobel, W. J. King, *et al.* (1984). J Steroid Biochem **20**(1): 51-6.
Gronemeyer, H. (1991). Annu Rev Genet **25**: 89-123.
Ham, J. and M. G. Parker (1989). Current Opinion in Cell Biology **1**: 503-511.
Hancock, D. C. and G. I. Evan (1992). Production and characterization of antibodies against synthetic peptides. Methods in Molecular Biology. M. Manson. Totowa, NJ, The Humana Press, Inc. **10:** 33-41.
Hard, T., K. Dahlman, J. Carlstedt-Duke, *et al.* (1990). Biochemistry **29**: 5358-5364.
Harlow, K. W., D. N. Smith, J. A. Katzenellenbogen, *et al.* (1989). J Biol Chem **164**: 17476-17485.
Heery, D. and M. Parker (1997). Retinoids **13**(1): 26-30.
Henderson, I. (1990). Endocrine therapy for metastatic breast cancer. Breast diseases. J. Harris, s. Hellman, I. Henderson and D. Kinne. Philladelphia, JB Lippincott.
Hernandez, N. (1993). Genes Dev **7**(7): 1291-308.
Horwitz, K., T. Jackson, D. Bain, *et al.* (1996). Mol Endocrinol **10**(10): 1167-77.
Horwitz, K. B., W. L. McGuire, Pearson, *et al.* (1975). Science **189**: 726-727.

Howell, A., D. Defriend and E. Anderson (1995). Endocrine related cancer **2**(2): 131-139.

Howell, A., D. DeFriend, J. Robertson, *et al.* (1996). Br J Cancer **74**(2): 300-8.

Howell, A., D. DeFriend, J. Robetson, *et al.* (1995). The Lancet **345**: 29-30.

Howell, A., D. Dodwell, H. Anderson, *et al.* (1992). Ann Oncol **3**(8): 611-7.

Hu, C., S. Hyder, D. Needleman, et al. (1996). Mol Cell Endocrinol 118(1-2): 173-9.

Huggins, C., L. Grand and F. Brillantes (1961). Nature **189**: 204-207.

Ignar-Trowbridge, D. M., K. G. Nelson, M. C. Bidwell, *et al.* (1992). Proc Natl Acad Sci USA **89**: 4658-4662.

Ignar-Trowbridge, D. M., C. T. Teng, K. A. Ross, *et al.* (1993). Mol Endo **7**: 992-998.

Ince, B. A., M. M. Montano and B. S. Katzenellenbogen (1994). Mol Endocrinol **8**(10): 1397-1406.

Ince, B. A., D. J. Schodin, D. J. Shapiro, *et al.* (1995). Endocrinology **136**(8): 3194-9.

Ince, B. A., Y. Zhuang, C. K. Wrenn, *et al.* (1993). J Biol Chem **268**(19): 14026-14032.

Jensen, E. V., T. Suzuki, T. Kawashima, *et al.* (1968). Proc Natl Acad Sci USA **59**: 632-638.

Jiang, S. Y. and V. C. Jordan (1992). J Natl Cancer Inst **84**: 580-591.

Joel, P., A. Traish and D. Lannigan (1995). Mol Endocrinol **9**(8): 1041-52.

Johnston, S., B. Haynes, I. Smith, *et al.* (1993). Lancet **342**(8886-8887): 1521-2.

Johnston, S. R. D., G. Saccanijotti, I. E. Smith, *et al.* (1995). Endocr Relat Cancer **2**(1): 105-110.

Jordan, V. (1997). J Natl Cancer Inst **89**(11): 747-9.

Jordan, V. C. (1984). Pharmacol Rev **36**(4): 245-76.

Jordan, V. C., M. M. Collins, L. Rowsby, *et al.* (1977). J Endocrinol **75**: 305-316.

Kamei, Y., L. Xu, T. Heinzel, *et al.* (1996). Cell **85**(3): 403-14.

Kampert, J. B., A. S. Whittemore and R. J. Paffenbarger (1988). Am J Epidemiol **128**(5): 962-79.

Karnik, P. S., S. Kulkarni, X. P. Liu, *et al.* (1994). Cancer Res **54**(2): 349-353.

Kato, S., H. Endoh, Y. masuhiro, *et al.* (1995). Science **270**: 1491-1494.
Katzenellenbogen, B. S., J. F. Elliston, F. J. Monsma, *et al.* (1987). Biochemistry **26**(8): 2364-73.
Keen, J., E. Miller, C. Bellamy, *et al.* (1994). Lancet **343**(8904): 1047-8.
Kern, F., A. Cheville and Y. Liu (1990). Semin Cancer Biol **1**(5): 317-28.
King, W. J. and G. L. Greene (1984). Nature **307**(5953): 745-7.
Klein-Hitpass, L., M. Schorpp, U. Wagner, *et al.* (1986). Cell **46**: 1053-1061.
Klotz, D., C. Castles, S. Fuqua, *et al.* (1995). Biochem Biophys Res Commun **210**(2): 609-15.
Knabbe, C., M. E. Lippman, L. M. Wakefield, *et al.* (1987). Cell **48**: 417-428.
Knight, W., R. Livingston, E. Gregory, *et al.* (1977). Cancer Res **37**(12): 4669-71.
Koehorst, S. G. A., H. M. Jacobs, J. H. H. Thijssen, *et al.* (1993). J Steroid: 227-233.
Koehorst, S. G. A., J. J. Cox, G. H. Donker, *et al.* (1994). Mol & Cell Endocrinol **101**: 237-245.
Koehorst, S. G. A., H. M. Jacobs, J. H. H. Thijssen, *et al.* (1993). J Steroid Biochem Molec Biol **45**: 227-233.
Koga, M. and R. Sutherland (1987). Biochem Biophys Res Commun **146**(2): 739-45.
Krett, N., S. Pillay, P. Moalli, *et al.* (1995). Cancer Res **55**(13): 2727-9.
Krust, A., S. Green, P. Argos, *et al.* (1986). EMBO J **5**(5): 891-7.
Kuiper, G., E. Enmark, M. Pelto-Huikko, *et al.* (1996). Proc Natl Acad Sci U S A **93**(12): 5925-30.
Kumar, V. and P. Chambon (1988). Cell **55**: 145-156.
Kumar, V., S. Green, G. Stack, *et al.* (1987). Cell **51**(6): 941-951.
Laron, Z., Kauli and Pertzelan (1989). Proceedings of the Royal Society of Edinburgh. **95B**: 13-22.
Lees, J. A., S. E. Fawell and M. G. Parker (1989). J Steroid Biochem **34**: 33-39.
Lees, J. A., S. E. Fawell and M. G. Parker (1989). Nucleic Acids Res **17**: 5477-5488.
Legault-poisson, S., J. Jolivet, R. Poisson, *et al.* (1979). Cancer treatment reports **63**: 1839-1841.
Leygue, E., P. Watson and L. Murphy (1996). J Natl Cancer Inst **88**(5): 284-90.

Lieberman, M. E., R. A. Maurer and J. Gorski (1978). Proc Natl Acad Sci USA **75**: 5946.
Lillenfield, A. (1956). Cancer **9**: 927-934.
Lippman, M. and R. Dickson (1989). Proc Royal Soc Edinburgh **95B**: 89-106.
Love, R., H. Barden, R. Mazess, *et al.* (1994). Arch Intern Med **154**(22): 2585-8.
Love, R., R. Mazess and H. Barden (1992). N Engl J Med **326**: 852-856.
Love, R. R., R. B. Mazess, D. C. Tormey, *et al.* (1988). Breast Cancer Res Treat **12**: 297-302.
Lykkesfeldt, A. (1996). Acta Oncol **35 Suppl 5**: 9-14.
Lykkesfeldt, A., S. Larsen and P. Briand (1995). Int J Cancer **61**(4): 529-34.
Lyons (1958). Proceedings of the Royal Society **149**: 303-325.
MacMahon (1970). Buletin of the World Health Organisation **43**: 209.
Madsen, M., B. Reiter, S. Larsen, *et al.* (1997). Cancer Res **57**(4): 585-9.
Madsen, M., B. Reiter and A. Lykkesfeldt (1995). Mol Cell Endocrinol **109**(2): 197-207.
Mahfoudi, A., E. Roulet, S. Dauvois, *et al.* (1995). Proc Natl Acad Sci USA **92**: 4206-4210.
Martinez, E. and W. Wahli (1991). Characterization of hormone response elements. Nuclear Hormone Receptors. Molecular mechanisms, cellular functions, clinical abnormalities. M. G. Parker. London, Academic Press: 125-154.
McDonald, C. C. and H. J. Stewart (1991). Br Med J **303**: 435-437.
McGuire, W. (1975). Cancer **362**(638-44).
Meir-Ewart, S. (1994). pHD Thesis. London, Imperial College and ICRF Lincoln's Inn Fields.
Metzger, D., M. Berry, S. Ali, *et al.* (1995). Mol Endocrinol **9**(5): 579-591.
Metzger, D., J. H. White and P. Chambon (1988). Nature **334**: 31-36.
Moalli, P., S. Pillay, N. Krett, *et al.* (1993). Cancer Res **53**(17): 3877-9.
Montano, M., K. Ekena, K. Krueger, *et al.* (1996). Mol Endocrinol **10**(3): 230-42.
Morabia, A., M. Bernstein, S. Heritier, *et al.* (1996). Am J Epidemiol **143**(9): 918-28.
Mosselman, S., J. Polman and R. Dijkema (1996). FEBS Lett **392**(1): 49-53.
Murphy, L. (1994). Breast cancer research and treatment **31**: 61-71.
Murphy, L. C. and H. Dotzlaw (1989). Mol Endo **3**: 687-693.

Musgrove, E. A., C. S. Lee, M. F. Buckley, *et al.* (1994). Proc Natl Acad Sci U S A **91**(17): 8022-6.
Musgrove, E. A. and R. L. Sutherland (1993). Eur J Cancer.
Mustacchi, Ramazini, Rigoni, *et al.* (1961). Archives of internal medicine **108**: 195-.
Nembrot, M., B. Quintana and J. Mordoh (1990). Biochem Biophys Res Commun **166**(2): 601-7.
Onate, S., S. Tsai, M. Tsai, *et al.* (1995). Science **270**: 1354-1357.
Osborne, C. K., E. Coronado, D. C. Allred, *et al.* (1991). J Natl Cancer Inst **83**(20): 1477-1482.
Osborne, C. K. and S. A. W. Fuqua (1994). Breast Cancer Research & Treatment **32**: 49-55.
Osborne, C. K., V. J. Wiebe, W. L. McGuire, *et al.* (1992). J Clin Oncol **10**(2): 304-310.
Pakdel, F. and B. Katzenellenbogen (1992). J Biol Chem **267**(5): 3429-37.
Parker, M. G. (1993). Current Opinion in Cell Biology **5**: 499-504.
Parklin, D. M., J. Stjernesward and C. C. Muir (1984). Bulletin of the World Health Organisation **62**: 163.
Pavlik, E., K. Nelson, S. Srinivasen, *et al.* (1992). Cancer Research **52**: 4106-4112.
Peto, J., D. F. Easton, F. E. Matthews, *et al.* (1996). Int J Cancer **65**(3): 275-83.
Pfeffer, U., E. Fecarotta and G. Vidali (1995). Cancer Res **55**: 2158-2165.
Pham, T. A., J. F. Elliston, Z. Nawaz, *et al.* (1991). Proc Natl Acad Sci USA **88**(8): 3125-9.
Philips, A., D. Chalbos and H. Rochefort (1993). J Biol Chem **268**(19): 14103-14108.
Phillips (1980). J Natnl Cancer Inst **65**: 1097-1007.
Pierrat, B., D. Heery, Y. Lemoine, *et al.* (1992). Gene **119**: 237-245.
Pierrat, P., D. M. Heery, P. Chambon, *et al.* (1994). Gene **143**: 193-200.
Power, R. F., S. K. Mani, J. Codina, *et al.* (1991). Science **254**: 1636-1639.
Pratt, W. B. (1993). Role of heat-shock proteins in steroid receptor function. Steroid Hormone Action. M. G. Parker. Oxford, IRL Press**:** 64-93.
Renaud, J., R. Natcha, M. Ruff, *et al.* (1995). Nature **378**: 681-689.
Roodi, N., L. R. Bailey, W.-Y. Kao, *et al.* (1995). Journal of the National Cancer Institute **87**(6): 446-450.

Russo, J., L. K. Tay, D. R. Ciocca, *et al.* (1983). Environ Health Perspect **49**(185): 185-99.
Sabbah, M., F. Gouilleux, B. Sola, *et al.* (1991). Proc Natl Acad Sci USA **88**: 390-394.
Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press.
Schwabe, J. W., D. Neuhaus and D. Rhodes (1990). Nature **348**(6300): 458-61.
Schwabe, J. W. R., L. Chapman, J. T. Finch, *et al.* (1993). Structure **1**(3): 187-204.
Scott, G., P. Kushner, j. Vigne, *et al.* (1991). J Clin Investigation **88**: 700-706.
Sheldon, M. and D. Reinberg (1995). Curr Biol **5**(1): 43-6.
Sillero-Arenas, M., M. Delgado-Rodriguez, R. Rodigues-Canteras, *et al.* (1992). Obstet Gynecol **79**: 286-94.
Skipper, J., L. Young, J. Bergeron, *et al.* (1993). Proc Natl Acad Sci U S A **90**(15): 7172-5.
Sleigh, M. J. (1986). Anal Biochem **156**(1): 251-256.
Smith, C. L., O. M. Conneely and B. W. O'Malley (1993). Proc Natl Acad Sci USA **90**: 6120-6124.
Smith, D. F. and D. O. Toft (1993). Mol Endocrinol **7**(1): 4-11.
Soule, H. D. and McGrath (1980). Cancer Letters **10**: 177-189.
Steinberg, K. K., S. B. Thacker, S. J. Smith, *et al.* (1991). Jama **265**(15): 1985-90.
Sutherland, R. L., J. A. Hamilton, K. J. Sweeney, *et al.* (1995). Ciba Found Symp **191**(218): 218-28.
Sutherland, R. L., C. S. Lee, R. S. Feldman, *et al.* (1992). J Steroid Biochem Mol Biol **41**(3-8): 315-21.
Suzuki, H., N. Sato, Y. Watabe, *et al.* (1993). J Steroid Biochem Mol Biol **46**(6): 759-65.
Szabo, C. I. and M. C. King (1995). Hum Mol Genet(1811).
Taplin, M., G. Bubley, T. Shuster, *et al.* (1995). N Engl J Med **332**(21): 1393-8.
Thompson, E., N. Brunner, J. Torri, *et al.* (1993). Clin Exp Metastasis **11**(1): 15-26.
Thompson, E. W., D. Katz, T. B. Shima, *et al.* (1989). Cancer Res.

Thompson, M. E., R. A. Jensen, P. S. Obermiller, *et al.* (1995). Nat Genet **9**(4): 444-50.
Tilley, W., G. Buchanan, T. Hickey, *et al.* (1996). Clinical Cancer Research **2**: 277-285.
Toft, D. and J. Gorski (1966). PNAS **5**: 1574-1581.
Toh, Y., I. S. Penci and Niccolson (1994). Gene.
Toi, M., A. L. Harris and R. Bicknell (1993). Br J Cancer **68**: 1088-1096.
Tonetti, D. A. and V. C. Jordan (1995). Anticancer Drugs **6**(4): 498-507.
Tora, L., A. Mullick, D. Metzger, *et al.* (1989). EMBO J **8**(7): 1981-1986.
Tora, L., J. White, C. Brou, *et al.* (1989). Cell **59**(3): 477-487.
Torchia, J., D. Rose, J. Inostroza, *et al.* (1997). Nature **387**(6634): 677-84.
Trichopoulos, D., B. MacMahon and P. Cole (1972). JNCI **48**: 605-613.
Turken, S., E. Siris, E. Seldin, *et al.* (1989). J Natl Cancer Inst **81**: 1086-1088.
Tzukerman, M. T., A. Esty, D. Santisomere, *et al.* (1994). Mol Endocrinol **8**(1): 21-30.
Veldscholte, J., C. A. Berrevoets, C. Ris-Stalpers, *et al.* (1992). J Steroid Biochem Molec Biol **41**(3-8): 665-669.
Veldscholte, J., C. Ris-Stalpers, G. Kuiper, *et al.* (1990). Biochem Biophys Res Commun **173**(2): 534-40.
Veronesi, U., A. Goldhirsch and J. Yarnold (1995). Breast Cancer. Oxford Textbook of Oncology. Peckham. Oxford, Oxford University Press.
Villa, E., L. Camellini, A. Dugani, et al. (1995). Cancer Research 55: 498-500.
Visakorpi, T., E. Hyytinen, P. Koivisto, *et al.* (1995). Nat Genet **9**(4): 401-6.
Voegel, J., M. Heine, C. Zechel, *et al.* (1996). EMBO **15**: 3667-3675.
vom Baur, E., C. Zechel, D. Heery, *et al.* (1996). EMBO J **15**(1): 110-24.
Vonderhaar, B. K. (1988). Regulation and developmentof the normal mammary gland by hormones and growth factors. Breast Cancer Cellular and Molecular Biology. Lippman and Dickson. Boston, Kluwer Academic Press: 251-266.
Wagner, R., J. Apriletti, M. McGrath, *et al.* (1995). Nature **378**: 690-697.
Wakeling, A. E. and J. Bowler (1987). J Endocrinol **112**: R7-R10.
Wakeling, A. E. and J. Bowler (1988). J Steroid Biochem **30**: 141-148.
Wakeling, A. E., E. Newboult and S. W. Peters (1989). J Mol Endocrinol **2**: 225-234.

Walker, P., J. E. Germond, Brown, *et al.* (1984). Nucleic Acids Res **12**(22): 8611-26.
Wang, Y. and R. J. Miksicek (1991). Molecular Endocrinology **5**: 1707-1715.
Wassink, W. F. (1935). Genetika(17): 103-144.
Waterhouse, J. (1976). Cancer incidence in five continents. Lyon, IARC.
Watts, C. K., K. J. Sweeney, A. Warlters, *et al.* (1994). Breast Cancer Res Treat **31**(1): 95-105.
Webb, P., G. N. Lopez, R. M. Uht, *et al.* (1995). Mol Endo **9**: 443-456.
Weis, K., K. Ekena, J. Thomas, *et al.* (1996). Mol Endocrinol **10**(11): 1388-98.
Weisz, A. and R. Rosales (1990). Nucl Acids Res **18**: 5097-5106.
Welsch (1982). Hormones and tumourigenesis, an historical view. Steroid hormones. H. r. o. m. tumours. Montreal, Eden Press. **1**.
Westley, B. R. and F. E. B. May (1995). Endocrine-Related Cancer **2**(1): 37-44.
White, R., J. A. Lees, M. Needham, *et al.* (1987). Mol Endocrinol **1**(10): 735-744.
White, R., M. Sjoberg, E. Kalkhoven, *et al.* (1997). EMBO J **16**(6): 1427-35.
Wilding, G., M. E. Lippman and E. P. Gelmann (1988). Cancer Research **48**: 802-805.
Wolf, D., S. Langan-Fahey, C. Parker, *et al.* (1993). J Natl Cancer Inst **85**(10): 806-12.
Wolf, D. M. and V. C. Jordan (1994). Breast Cancer Res Treat **31**(1): 129-138.
Wrenn, C. K. and B. S. Katzenellenbogen (1993). J Biol Chem **268**(32): 24089-24098.
Ylikomi, T., M. T. Bocquel, M. Berry, *et al.* (1992). EMBO J **11**(10): 1-14.
Zawel, L. and D. Reinberg (1993). Prog Nucleic Acid Res Mol Biol **44**: 67-108.
Zhuang, Y., B. S. Katzenellenbogen and D. J. Shapiro (1995). Mol Endocrinol **9**(4): 457-466.
Zilliacus, J., A. Wright, J. Carlstedt-Duke, *et al.* (1995). Mol Endo **9**: 389-400.
Zysk, J. R., B. Johnson, B. A. Ozenberger, *et al.* (1995). Endorcinology **136**(3): 1323-1326.

Anderson, T., R. Wooster, K. Laake, *et al.* (1997). Hum Mutat **9**(6): 531-6.

Clark, G., J. Harvey, C. Osborne, *et al.* (1997). Proc Am Soc Clin Onc **16**: abstract 454 p129a.

Elledge, R. and C. Osborne (1997). British Medical Journal **314**: 1843-4.