The Regulation of wild-type Estrogen Receptor mRNA and Clone 4 Estrogen Receptor-like mRNA by 6α-methyl-17α-hydroxyprogesterone acetate (MPA) and Phorbol 12-myristate 13-acetate (TPA) in T47D5 Human Breast Cancer Cells

By

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A Thesis submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirement for
the Degree of Master of Science

Department of Biochemistry and Molecular Biology
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Abstract

Patients with breast tumours which are estrogen receptor (ER) positive are more likely to respond to endrocrine therapy; however, approximately 30% of ER-positive tumours are resistant to endocrine therapy and many tumours which are initially responsive will eventually develop resistance. Since many resistant tumours continue to express the estrogen receptor, abnormal/variant estrogen receptors may exist which result in altered responsiveness to hormonal therapy and may be one mechanism by which breast tumours progress from hormone dependence to hormone independence.

Although unequivocal evidence for the presence of ER-like proteins has not been reported, the possibility of their expression has been suggested due to the detection of variant ER-like mRNAs in a wide range of normal and neoplastic estrogen target tissues and cell lines. The generation of altered transcripts may result from rearrangement of the ER gene, from alternative splicing of the wild-type (wt) ER primary transcript or *trans*-splicing of the transcripts derived from the wt ER gene and the transcript of another gene.

Clone 4 was originally isolated and cloned from RNA isolated from human breast tumours and was later found in human breast cancer cell lines, normal breast tissue, Ishikawa (human endometrial carcinoma) cells and normal human uterine tissue. Clone 4 contains exons 1 and 2 from the normal ER mRNA then diverges to sequences that are similar to LINE-1 sequences. The aim of this research was to address the hypothesis that alternatively spliced ER mRNA expression depends on the transcription of the wt ER gene. To examine this, I have studied the regulation of clone 4 ER-like mRNA steady-state levels by agents known to alter the steady state levels of wt ER mRNA. The regulation of steady-state levels of clone 4 ER-like mRNA relative to wt ER mRNA by the progestin, 6α-methyl-17α-

hydroxyprogesterone acetate (MPA), and the phorbol ester, phorbol 12-myristate 13-acetate (TPA), was examined using reverse transcription-polymerase chain reaction (RT-PCR) assays and RNase protection analysis. The treatment of T47D5 cells with 10 nM MPA over a 72 hour time course resulted in a decrease of wt ER mRNA to approximately 51% of control steady-state levels by 24 hours and a parallel decrease of clone 4 ER-like mRNA steady-state levels to approximately 38% by 24 hours when examined by RT-PCR. Similar results were obtained when the time course was analyzed by RNase protection assays where steady-state levels of the wt ER and clone 4 ER-like transcripts fell to approximately 32% and 44% of control levels, respectively. When T47D5 cells were treated with 1 μM TPA over a 72 hour time course, wt ER mRNA and clone 4 ER-like mRNA steady-state levels decreased in parallel to approximately 54% and 55% of controls, respectively, by RT-PCR analysis and to approximately 37.5% and 46% of control steady-state levels, respectively between 24-48 hours. Therefore, agents known to decrease steady-state levels of wt ER mRNA were also shown to decrease the steady-state levels of clone 4 ER-like mRNA in an apparently parallel fashion. In conclusion, this thesis provides evidence that the generation of clone 4 ER-like transcripts is regulated in a similar fashion to wt ER transcripts and is therefore likely to depend on the transcription of the wt ER gene.

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List of Abbreviations

Chemicals:

$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium chloride
TEMED	N, N, N', N'-tetramethylethylenediamine
KOH	Potassium Hydroxide
Tris	Tris (hydroxymethyl) aminomethane
HCl	Hydrochloric acid
SDS	Sodium dodecyl sulphate
Na_2HPO_4	Sodium monophosphate
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
DTT	Dithiothreitol
EDTA, Na_2EDTA	Sodium ethylenediaminetetraacetic acid
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
GITC	Guanidinium isothiocyanate
CsCl	Cesium chloride
NaAc	Sodium acetate
ddH2O	double distilled water
EtBr	Ethidium Bromide
Mg^{2+}	Magnesium ions
SSC	Standard saline citrate
SSPE	Standard saline phosphate EDTA
MOPS	Morpholinopropane sulphonic acid
TBE	Tris borate EDTA
PAGE	Polyacrylamide gel electrophoresis
DMSO	Dimethyl sulphoxide
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
GRB	Gel running Buffer

Units:

cm^2	square centimeters
M, mM, µM, nM	Molar, millimolar, micromolar, nanomolar
L, mL, µL	Litre, millilitre, microlitre
g, mg, µg, ng, pg	grams, milligrams, micrograms, picograms
mm, nm	millimeter, nanometer
Ci, µCi	Curie, microcurie
IU	International units
rpm	Revolutions per minute
cpm	Counts per minute

s	Seconds
°C	Degrees Celsius
bp, kb	Base pairs, kilobase pairs
mmole	millimole
kDa	kilodaltons
%	percent

Nucleotide Bases:

G	Guanine
A	Adenine
T	Thymine
C	Cytosine

Amino Acids:

Ala	Alanine
Asp	Aspartate
Gly	Glycine
Ser	Serine
Tyr	Tyrosine
Val	Valine

Acronyms:

AR, ARE	Androgen receptor, androgen response element
ER, ERE	Estrogen receptor, estrogen response element
GR, GRE	Glucocorticoid receptor, glucocorticoid response element
MR, MRE	Mineralocorticoid receptor, mineralocorticoid response element
PR, PRE	Progesterone receptor, progesterone response element
RAR, RARE	Retinoic acid receptor, retinoic acid response element
T_3R, TRE	Thyroid hormone receptor, thyroid hormone response element
$VitD_3$	Vitamin D_3
HRE	Hormone Response Element
ER+, ER-	ER-positive, ER-negative
PR+, PR-	PR-positive, PR-negative
DNA, cDNA	Deoxyribonucleic acid, complementary DNA
RNA, mRNA	Ribonucleic acid, messenger RNA
NTP, dNTP	nucleotide triphosphate, deoxy NTP
cAMP	cyclic adenosine monophosphate
DNase I	Deoxyribonuclease I

RNase, RNase P	Ribonuclease, ribonuclease protection assay
GF	Growth factors
FGF	Fibroblast growth factor
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
EGF, EGFr	Epidermal growth factor, EGF receptor
IGF	Insulin-like growth factor
ACTH	Adrenocorticotropin hormone
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
LHRH	Luteinizing hormone releasing hormone
GnRH	Gonadotropin releasing hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
AE	Antiestrogen
RT	Reverse Transcription
PCR	Polymerase Chain Reaction
M-MLV	Muloney Murine Leukemia Virus
UDG	Uracil DNA Glycosylase
UTR	Untranslated region
wt	wild-type
E2	estrogen
TPA	Phorbol 12-myristate 13-acetate
MPA	6α-methyl-17α-hydroxyprogesterone acetate
CAT	Chloramphenicol acetyl transferase
TAF, TAF-I, TAF-2	*Trans*-activation function
TATA	TATA box
CAT	CAT box
HBD	Hormone binding domain
NLS	Nuclear localisation signal
DBD	DNA binding domain
DMEM	Dulbecco's modified essential medium
FBS	Fetal bovine serum
MW	Molecular Weight
v/v	Volume per volume
w/v	weight per volume
α	alpha
β	beta
α-^{32}-P	radiolabelled phosphorus
VLDL	very low density lipoprotein

Introduction

Breast Cancer

One in nine women will develop breast cancer during the course of her lifetime (Volkers, 1992) making breast cancer the most common cancer amongst women in western society. Based on the worldwide yearly increase of breast cancer incidence, over one million breast cancer cases per year will be diagnosed by the year 2000 (Miller et al, 1986). Despite increased awareness concerning breast cancer which has led to early detection and diagnosis of fewer advanced tumours (Miller et al, 1991), the incidence is still increasing. Extensive research is being carried out to identify risk factors, improve treatments, and determine the etiology of breast cancer in order to prevent the disease.

Risk of Developing Breast Cancer

A number of factors have been linked to an increased risk of developing breast cancer (reviewed in Marshall, 1993) (see Figure 1). The number of years a woman is exposed to hormones significantly influences her risk (Marshall, 1993). From the onset of menarche to menopause, the risk steadily increases with both an early age of menarche initiation and late onset of menopause increasing the risk 1-2 fold. North American and northern European women have a 2-4 fold higher risk of developing breast cancer than women in Asia or Africa. This is also true for women in a higher socioeconomic class or those who are obese post-menopausally. The dietary differences between these geographical areas and economic classes implicated high fat diets as a potential cause of breast cancer although research has demonstrated no significant link between high fat diets and breast cancer (Willett, 1989). First pregnancy occurring before the age of 20 appears to have a protective effect,

Risk Factors for Breast Cancer in Females			
Factor	**High Risk**	**Low Risk**	**Magnitude of Differential**
Age	Old	Young	ooo
Country of Birth	North America Northern Europe	Asia, Africa	oo
Socioeconomic class	Upper	Lower	oo
Marital Status	Never Married	Ever Married	o
Place of residence	Urban	Rural	o
Place of residence	Northern U.S.	Southern U.S	o
Race	White	Black	o
Age at first full-term pregnancy	Older than 30	Younger than 20	oo
Oophorectomy	No	Yes	oo
Body build, postmenopausal	Obese	Thin	oo
Age at menarche	Early	Late	o
Age at menopause	Late	Early	o
Family history of premenopausal bilateral breast cancer	Yes	No	ooo
History of cancer in one breast	Yes	No	ooo
History of fibro-cystic disease	Yes	No	oo
Any first-degree relative with breast cancer	Yes	No	oo
History of primary cancer in ovary or endometrium	Yes	No	oo
Radiation to chest	Yes	No	oo
Key to Magnitude of risk differential ooo= relative risk of greater than 4.0; oo=relative risk of greater than 2.0-4.0; o=relative risk of between 1.1 and 1.9.			

Figure 1. Breast Cancer Risk Factors. Various risk factors and the relative difference between high risk and low risk are assessed. The key to the magnitude of differential expressed in fold-increase is shown at the bottom of the figure. This figure was reproduced from Marshall, 1993.

although the pregnancy must go full-term suggesting an association to an early age of mammary cell differentiation by hormonal changes associated with pregnancy (Cairns, 1975 and Preston-Marin et al, 1990; Layde et al, 1989; Pathak et al, 1986). A 2-4 fold increased risk is associated with first pregnancy after 30.

Family history also appears to play a role in the likelihood of developing breast cancer. Having any first degree relative with breast cancer, a history of ovarian or endometrial primary cancers or fibrocystic disease increases a woman's risk 2-4 fold. The highest risk is associated with a woman who has had cancer in one breast or has a familial history of breast cancer in both breasts occurring pre-menopausally, where the risk is increased 4 fold. Family histories indicate some women may be genetically predisposed to getting breast cancer (Anderson, 1974). For some women, however, common environmental factors may be responsible for this apparent link (rev. in Marshall, 1993).

Breast cancer may be hereditary or sporadic. *BRCA1* is believed to be responsible for familial breast cancer where the chance of a woman inheriting a defective *BRCA1* gene is 1 in 200 and accounts for approximately 5% of all breast cancer cases (Narod et al, 1991; Hall et al, 1990; Roberts, 1993). Loss of function of *BRCA1* conveys a lifetime chance of approximately 87% of developing breast cancer and a 47% chance of developing ovarian cancer for women who belong to families with high incidence of breast and ovarian cancers (Easton et al, 1993; Ford et al, 1994). *BRCA1* is believed to be a tumour suppressor gene and a putative zinc finger domain in the *BRCA1* sequence suggests it functions as a transcription factor (Miki et al, 1994). Thomson et al (1995) hypothesize that *BRCA1* is a negative regulator with respect to growth of mammary epithelial cells since the growth of both normal and malignant mammary cells is enhanced when *BRCA1* antisense oligonucleotides are

used to inhibit *BRCA1* expression. The autosomal dominant *BRCA1* gene was localized to chromosome 17q21 (Miki et al, 1994). In a recent study, the molecular weight of the *BRCA1* protein was found to be 190 kDa in breast epithelium (Jensen et al, 1996). In these experiments, *BRCA1* appeared to be localized to the Golgi although a small amount of nuclear immunoreactivity was observed (Jensen et al, 1996). The identification of sequences in *BRCA1* similar to the 10 amino acid granin consensus sequence suggested *BRCA1* is a secreted protein (Jensen et al, 1996). Further studies showed that *BRCA1* is a post-translationally modified protein and is secreted when cAMP is activated. Furthermore, this protein is also induced by estradiol (Jensen et al, 1996). Studies also showed that overexpression of *BRCA1* inhibited the growth of breast and ovarian cancer cell lines *in vitro* while mutant and truncated *BRCA1*, proteins which were almost full length, had no effect on cell growth (Holt et al, 1996). The finding that Brca1 mRNA is induced in mice during pregnancy (Marquis et al, 1995; Lane et al, 1995) in conjunction with the proposed tumour suppressor activity of *BRCA1* suggests the protective effect during lactation and pregnancy against breast cancer may be mediated by *BRCA1* (Jensen et al, 1996). The discovery of the *BRCA1* gene was a major breakthrough. However, the link between *BRCA1* and sporadic breast and ovarian cancers remains controversial and requires further research (Boyd, 1995).

The risk for breast cancer associated with oral contraceptives and estrogen replacement therapy (ERT) is still unresolved. It is believed that the use of estrogen replacement therapy slightly increases the risk of breast cancer development while women are taking ERT pills, however, the risk decreases upon cessation of use (rev. in Marshall, 1993). Armstrong (1988), however, found no increased risk of breast cancer with ERT. The slightly increased risk

associated with oral contraceptives also decreased upon withdrawal from use (rev. in Marshall, 1993).

Research suggests the risk of developing breast cancer may also be affected perinatally. Maternal obesity, severe nausea, late age of pregnancy and large size of fetus have been linked to high estrogen levels during pregnancy which may increase the daughter's risk of developing breast cancer (Ekbom et al, 1992; McFayden et al, 1982; Petridon et al, 1990).

Risk factors appear to account for approximately 40% of all breast cancer cases diagnosed, leaving 60% with unidentified causes (Marshall, 1993; Seidman et al, 1982).

Development of Normal Mammary Tissue

A number of hormones and peptide growth factors have been shown to be important in mammary development. Mammary gland development begins during the fetal period arising from dense mammary bands of epithelial tissue which develop into mammary buds several weeks following conception (rev. in Topper and Freeman, 1980). Interestingly, Raynaud (1950) found mammary development is not altered by destruction of the ovaries, implying ovarian hormones are not necessary for the initial phases of mammary development. Up to this point, male and female development are identical. Androgen production by the fetal testes inhibits the development of the lactiferous ducts. In females, fetal testes are destroyed (Raynaud, 1950) and the lactiferous ducts push towards the apex of the nipple (Drews and Drews, 1977).

At this stage, response to a combination of glucocorticoid, prolactin and insulin results in milk protein production in mammary cells *in vitro* (Ceriani, 1970). Partial differentiation of mammary cells and secretion by the secretory cells occurs during the period shortly prior to birth until

approximately four weeks post-natally as a result of stimulation by placental steroids and fetal prolactin (Friesen, 1973). Following this period, however, the mammary glands revert to a dedifferentiated state until puberty.

Slightly more differentiated cells are produced during adolescence. Adipose tissue is deposited and stromal connective tissue develops. The development of intricate ducts also occurs which is dependent on the combination of estrogen and growth hormone or prolactin but independent of progesterone and insulin (Topper and Freeman, 1980). The ductal end buds of ovariectomized mice were stimulated to grow by exogenous estrogens (Bresciani, 1968), however, the combination of ovariectomy, hypophysectomy and adrenalectomy abolished the stimulative effect of estrogen (Lieberman et al, 1978; Lyons, 1958).

Pituitary hormones are not necessary for maintaining the lactiferous ducts if adrenal and ovarian secretion is present and, interestingly, pituitary hormones may be substituted for by thyroid hormones in dwarf mice (Piscott and Nandi, 1961). Glucocorticoids appear to be required for maximal ductal growth, although growth occurs in its absence (Richardson, 1955).

Not until pregnancy does the mammary gland become fully differentiated although cyclical changes occur during the menstrual cycle (Bresciani, 1965). Differentiation results in the development of lobuloalveolar structures which are epithelial cells organized into spheres (Kaplan and Schenken, 1990). Lobuloalveolar development requires progesterone, is stimulated by thyroid hormone (Vonderhaar and Greco, 1979), insulin-like growth factor-I and II, transforming growth factor-alpha and epidermal growth factor and enhanced by glucocorticoids (Nandi, 1958) while general growth of the mammary gland is stimulated by estrogens (Topper and Freeman, 1980). Maturation and maintenance of the mammary gland structure requires growth hormone, prolactin or placental lactogen (Nandi, 1958; Talwalker and Meites, 1961;

Topper, 1970; Franks et al, 1977; Neilson et al, 1979) which is supported by evidence showing the complete loss of alveoli that occurs with hypophysectomy or ovariectomy (Nandi, 1958).

Cyclic responsiveness by mammary cells to insulin occurs during development in that insulin sensitivity is obtained during pregnancy and remains until cessation of lactation then gains sensitivity with the next pregnancy (Oka et al, 1974).

Although progesterone plays an important role in mammary development, this hormone is also responsible for blocking secretion by the gland (Topper and Freeman, 1980). Initiation of postpartum lactation is believed to be promoted by estrogen (Topper and Freeman, 1980) while maintenance of lactation is achieved by decreased progesterone and increased prolactin and glucocorticoid levels (Lyons, 1958; Topper and Freeman, 1980). Estrogen is not required in postpartum lactation maintenance and ER levels decrease despite the stimulation of prolactin and its receptor by estrogen. Oxytocin, however, also promotes the release of prolactin (Lumpkin et al, 1983) and is necessary for milk ejection by promoting myoepithelial contraction.

The regulation of hormones and growth factor expression during mammary development is a very intricate process. The role of estrogen in mammary gland development and maturation begins at the time of adolescence and continues until full gland maturation at the initiation of postpartum lactation. The role of estrogens in development suggests that estrogen has a potential role in the development of breast cancer.

Steroid Hormone Receptors

The ER belongs to the superfamily of nuclear hormone receptors. Based on DNA binding domain amino acid homologies and similarities between

hormone response element sequences, the superfamily can be divided into 2 subfamilies. The first subfamily consists of PR, GR, AR, mineralocorticoid receptor and the second of ER, $VitD_3$, T_3R and RAR.

The human ER gene has been cloned (Walter et al, 1985), sequenced (Green et al, 1986) and the single gene copy localized to chromosomal position 6q24-27 (Walter et al, 1985). S1 nuclease mapping and primer extension localized the transcription start site which is defined as nucleotide +1 (Green et al, 1986). Analysis of the ER gene has yielded information regarding the 5' and 3' ends of the gene. Green et al (1986) described a TATA-like box and a CAAT-like box upstream from the transcription start site at position -27 and -103, respectively. Further sequences upstream were analyzed by Piva et al (1992) who found another TATA box at -2391, a CACC motif at -2372 and a capsite at -2360. In addition, a putative 459 bp ORF is located approximately 2 kb upstream of the presently designated exon 1 (Piva et al, 1992; Keaveney et al, 1991). This exon was not found in the chicken estrogen receptor upstream sequences (Nestor et al, 1994). Interestingly, the -2776 to -38 region of the human ER gene containing the TATA box, CACC motif and capsite located upstream from the putative start site of the long ORF shows a 77.5% homology to the 512 bp of the 5' flanking sequence and first exon sequence of the mouse ER gene (Piva et al, 1992).

Depending on the species, the mRNA ranges in length from 6.2 to 7.5 kb and the 589-600 amino acid protein has a molecular mass of approximately 66.5 kDa. The human estrogen receptor has 8 exons and 7 introns. The introns of the human estrogen receptor are very large ranging in size from >16 kb to >32 kb (Ponglikitmongol et al, 1988). The 5'-untranslated region of the ER mRNA is 232 nucleotides long and one hypothesis suggests that this region provides a mechanism for post-transcriptional regulation of the ER (rev. in Gronemeyer et al, 1988). The long 3'-untranslated region of the ER mRNA

may also have physiological significance in the post-transcriptional regulation of the receptor (Keaveney et al, 1993). Studies of the rat GR gene demonstrated DNA binding sites within the 3'-untranslated region which were recognized by the GR (Okret et al, 1986). The third polyadenylation signal of the 5 found in the ER mRNA is used for adding the poly A tail (Walter et al, 1985).

The nuclear steroid hormone receptors are modular in nature allowing the assignment of distinct functional domains. The functional domains of the human ER are shown in figure 2 (rev. in Green et al, 1986; Beato, 1989; Evans, 1988). The 5'-untranslated region is encoded by part of exon 1. Domain A/B is encoded by exon 1 and part of exon 2 while the rest of exon 2, all of exon 3 and part of exon 4 encode domain C which is the DNA binding domain. The two zinc fingers of the DBD are encoded by different exons. Domain D, or the hinge domain, is encoded by exon 4. Exons 5-7 and part of exon 4 and 8 encode domain E while the balance of exon 8 encodes domain F and the 3'-untranslated region.

Region A/B contains a non-acidic *trans*-activation function (TAF-I) which exhibits cell- and promoter-specific activity in transient transfection studies using wild-type and deletion mutant receptor constructs (Tzukerman et al, 1994). This region may be required to maximally stimulate estrogen-responsive genes (Kumar et al, 1987). A ligand-inducible, non-acidic *trans*-activation function (TAF-2) has also been found in region E, the hormone binding domain, using chimeric receptor studies with the GAL4-DNA binding domain and the hormone binding domain of ER (Webster et al, 1988; Kumar et al, 1987). TAF-1 and TAF-2 have been determined to be independent and functionally distinct from each other (Tora et al, 1989; Tasset et al, 1990 ; Tzukerman et al, 1994), however, further investigation revealed synergistic activity when the TAFs were placed in separate vectors and

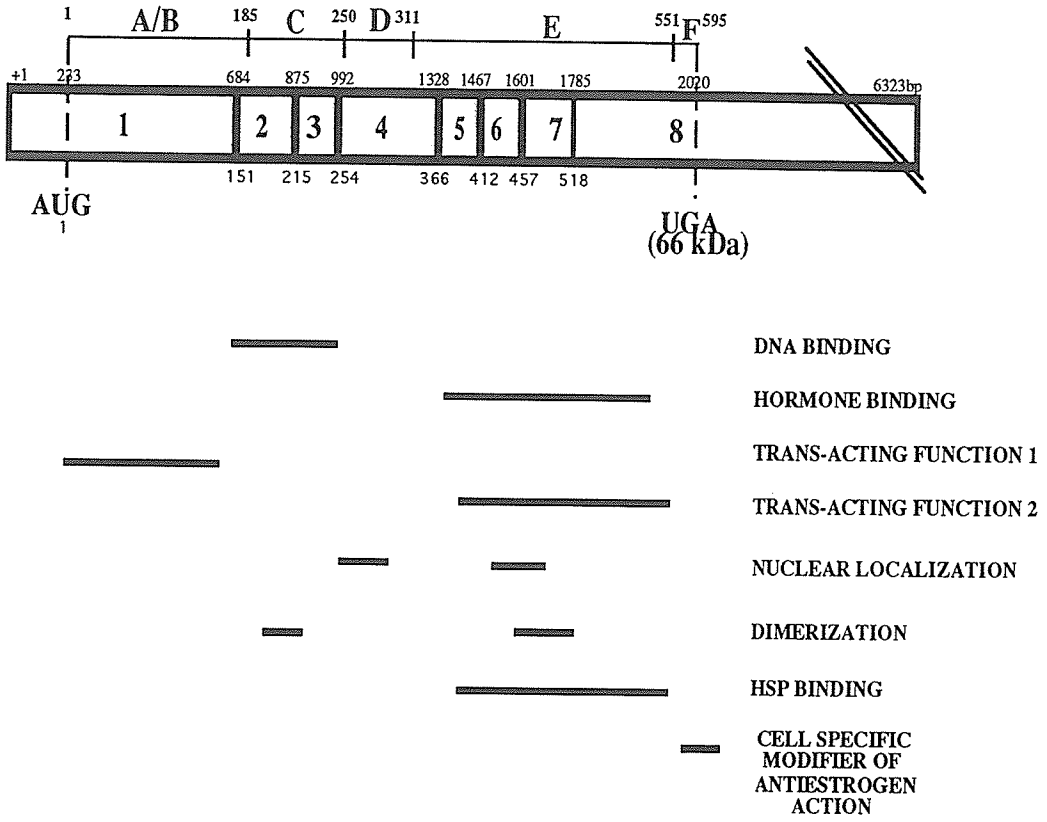


Figure 2. Domains of the estrogen receptor. The human estrogen receptor may be divided into functional domains. This figure shows the 8 exons of the mRNA, the nucleotide and amino acid positions at the exon boundaries and the amino acid positions at the boundaries of the functional domains of the estrogen receptor. The sequences involved in the specified functions (eg. DNA binding) are indicated by bars below the mRNA structure. This figure was adapted from information derived from Parker, 1993 and Ponglikitmongkol et al, 1988 and Montano et al, 1995.

transiently transfected into cells (Tora et al, 1989). TAF-2 appears to be formed by the 3 dimensional configuration of the receptor rather than encoded by contiguous ER segments (Webster et al, 1989). The presence of two *trans*-activation functions may provide a means to finely regulate the interaction of cellular factors with estrogen-responsive genes (Tora et al, 1989).

Domain E contains sequences responsible for ligand binding and receptor dimerization. This region is mainly hydrophobic and hormone binding likely requires multiple receptor (domain E)-ligand interactions (Kumar et al, 1986).

Domain C contains two putative DNA-binding fingers, each of which is formed by the coordinate interactions of 4 cysteine residues with a zinc molecule (Evans, 1988; Umesono and Evans, 1989; Evans and Hollenberg, 1988). The fingers are thought to interact with specific sequences located in the hormone response elements of estrogen-responsive genes (rev. in Green and Chambon, 1986; Gronemeyer, 1991). Experiments suggest the existence of constitutive DBD and ligand-activated HBD dimerization functions (Kumar and Chambon, 1988).

Domain D is proposed to act as a hinge between the DBD and HBD. Deletions or insertions have not been shown to inactivate the ER and, therefore, a precise conformation of this region does not appear necessary for ER activity (Kumar et al, 1987). The glucocorticoid receptor contains two distinct nuclear localization signals. One signal is found adjacent to the second zinc finger (region C-D), while the other signal is hormone-dependent and localized to the hormone binding domain (domain E) possibly overlapping the steroid binding domain (Picard and Yamamoto, 1987). The NLS of region D may be conserved between the family of steroid hormone receptors (Fawell et al, 1990) and, therefore, present in the ER. These signals

may be important for intranuclear localization or nuclear retention of the receptor.

Regulation of the magnitude of transcription in response to ligands has been assigned to domain F. Studies by Montano et al (1995) used ER constructs either deleted or point-mutated in the F domain in transient transfection assays. Their results suggest the F domain is important in determining the conformation of the ER when ligand binds, thus determining the magnitude of response to the ligand. Response to estrogen and antiestrogens by deleted constructs was cell specific suggesting a role for domain F in interacting with cell specific transcription factors or other transcription-associated proteins (Montano et al, 1995).

Other members of the steroid hormone receptor family are modular in nature and organized in a similar manner to the estrogen receptor, however, the degree of conservation between similar domains of different receptors varies (Green et al, 1986; Evans, 1988; Beato, 1989). The most highly conserved region is domain C, the DBD, where, for example, the conservation of this domain between chicken and human ER is 100%, (Kumar et al, 1986; 1987; Green and Chambon, 1987). Less conservation is seen in domain E where the domain is conserved 94% between human and chicken ER and very little conservation is seen in the rest of the domains with the A/B domain being the least conserved.

Steroid Action

The classical theory of steroid hormone receptor activation holds that estrogen diffuses into the cell and binds to the ER in the nucleus or cytoplasm inducing a conformational change in the receptor and tight nuclear association. The conformational change results in the dissociation of receptor associated proteins, the formation of homodimers and binding of the

homodimer to the hormone responsive element (rev. in Green and Chambon, 1986).

A model proposed by Gorski et al (1993) suggests that ER interacts with the ERE in the absence of ligand and may also bind to the ERE as a monomer. In this model, monomeric ER interaction with the ERE occurs in the absence of ligand and ligand binding induces a conformational change in the receptor stimulating transcription of estrogen-responsive genes through the *trans*-activation functions of the ER (Gorski et al, 1993). This conformational change allows the interaction of the receptor with additional *trans*-activation factors, transcription factors or other proteins associated with the chromatin (Gorski et al, 1993) resulting in a heteromeric complex that stabilizes the transcription complex. The conformational change induced by estrogens and antiestrogens is different, resulting in different responses to the ligands (Gorski et al, 1993; Montano et al, 1995). The ER has been localized to the nucleus in the absence of ligands (Welshons et al, 1984) suggesting ligands may interact with the receptor in the nucleus and may not be responsible for nuclear translocation of the ER/ligand complex to the nucleus and also supports the unliganded model of Gorski et al (1993).

Previous research contributing to the classical model of steroid hormone action used the ER expression construct HEO which was later determined to have a single point mutation in the hormone binding domain. This point mutation resulted in a more labile receptor with altered E2 affinity (Tora et al, 1989b; Gronemeyer, 1991). It was previously believed that liganded ER homodimers resulted in a stronger association of the complex with the ERE (Kumar and Chambon, 1988), however, studies using the wt ER construct (HEGO) have not found ligand-bound receptor to have a higher affinity than unliganded receptors for the ERE when examined *in vitro* using mobility shift or avidin-biotin-DNA complex assays (Gorski et al, 1993). An interesting

finding suggests E2 is necessary for ER/ERE interaction in the presence of Mg^{2+} whereas association occurs with or without E2 in the absence of Mg^{2+} (Brown and Sharp, 1990).

A ligand-inducible, non-acidic *trans*-activation function (TAF-2) was localized to the hormone binding domain (Webster et al, 1988; Kumar et al, 1987) and a cell and promoter-specific *trans*-activation function (TAF-I) localized to domain A/B (Tzukerman et al, 1994). TAF-I and TAF-2 have been determined to be independent and functionally distinct from each other (Tora et al, 1989; Tasset et al, 1990; Tzukerman et al, 1994), however, further studies revealed a synergistic effect between TAF-I and TAF-2 (Tora et al, 1989). The presence of two *trans*-activation functions may provide a means to finely regulate the interaction of cellular factors with estrogen-responsive genes (Tora et al, 1989). Although previous research suggested that the dimerization function of the HBD is necessary for dimer formation, studies of an ER construct deleted in the HBD was demonstrated to form dimers (Kumar and Chambon, 1988) and was shown to be constitutively active (Didier et al, 1990). In contrast to HBD deleted receptors, TAF-2 mutated receptors are unable to form dimers, bind the ERE very weakly if at all, and are also unable to transactivate the receptor (Didier et al, 1990). These results suggested the DBD dimerization domain could function alone and, furthermore, that this domain could be disrupted by the unliganded HBD dimerization function (Kumar and Chambon, 1988).

The hormone binding domain consists of two zinc fingers capable of interacting with the DNA. The fingers interact with a palindromic estrogen-responsive element allowing specific activation of estrogen-dependent genes (Kumar et al, 1987). The first finger contains three residues which are responsible for discriminating between a GRE (TGTTC) and an ERE (TGACC) (Mader et al, 1989; Fawell et al, 1990) while the residues in the second zinc

finger appear necessary to generate the appropriate spacing between the half-sites necessary for ER binding. This most likely occurs through protein-protein interactions rather than sequence recognition (Umesono and Evans, 1989). Interaction of the receptor first with the downstream half-site may facilitate receptor binding to the upstream HRE half-site (Tsai et al, 1988; Ponglikitmongkil et al, 1990). Dimeric interaction of the ER with the ERE *in vitro* has been suggested (Kumar and Chambon, 1988; Klein-Hitpass et al, 1989). The consensus ERE (5'-AGGTCA-n_3-TGACCT-3' where n is any nucleotide) is a perfect palindrome. Other EREs are shown in figure 3. Interestingly, ER interactions with half-sites separated by over 100 nucleotides have also been demonstrated (Kato et al, 1992). Evidence for dimer/ERE interaction was demonstrated by *in vitro* methylation interference assays showing the guanine residues in both ERE half-sites on both faces of the DNA directly interacted with the ER (Klein-Hitpass et al, 1989). However, Kumar and Chambon (1988) showed ER homodimer/ERE interaction of intact and HBD-deleted ER constructs. Gorski et al's model is further reinforced, by the description of monomeric ER/ERE interaction with a perfect palindromic ERE but also with an ERE which had a one nucleotide substitution destroying the dyad symmetry of the response element (Medici et al, 1991). Further studies demonstrated that the oligomeric form of the receptor was dictated by the spacing between the half-sites (Naar et al, 1991; Umesono et al, 1991; Forman et al, 1992). ER, T_3R, RAR and $VitD_3$ receptor response elements are identical except for the orientation and spacing with respect to half-sites (Naar et al, 1991). For example, half-sites may be positioned head to head (eg. AGGTCA-n_3-TGACCT) and interact with ER, tail to tail (eg. TGACCT-n_3-AGGTCA) and interact with T_3R or be directly repeated (eg. AGGTCA-n_3-AGGTCA) and interact with RAR (Naar et al, 1991). Changes in spacing between direct half-site repeats such as n=3, 4 or 5 resulted in the activation of

A	**Response Element**		**Consensus Sequence**	
	GRE/PRE/ARE/MRE		GGTACA-n_3-TGTTCT	(1)
	TRE/RARE/DRE (half-site)		TCAGGTCA-	(1)
	ERE		AGGTCA-n_3-TGACCT	(1)
B	Vitellogenin A1	*Xenopus*	GGTCA-n_3-TGACC	(2,3)
	Vitellogenin A2	*Xenopus*	GGTCA-n_3-TGACC	(2,3)
	Vitellogenin B1a	*Xenopus*	AGTTA-n_3-TGACC	(2,4)
	Vitellogenin B1b	*Xenopus*	AGTCA-n_3-TGACC	(2,4)
	Vitellogenin II	Chicken	GGTCA-n_3-TGACC	(2,3)
	ApoVLDL II	Chicken	GGTCA-n_3-TGACT	(2)
	Ovalbumin (half sites)	Chicken	$(TGACC)_4$ GGTCA	(5)
	Prolactin	Rat	TGTCA-n_3-TGTCC	(6)
	β-Luteinizing Hormone	Rat	GGACA-n_5-TGTCC	(7)
	Uteroglobin	Rabbit	GGTCA-n_3-TGCCC	(8)
	pS2	Human	GGTCA-n_3-TGGCC	(9)
	Oxytocin	Human	GGTGA-n_3-TGACC	(10)
	Lactoferrin	Human	GGTCA-n_3-CGATC	(11)
	c-fos	Human	CGGCA-n_3-TGACC	(12)
	Hageman factor XII	Human	GGGCA-n_3-TGACC	(13)
C	c-jun (Human)		GCAGA-n_3-TGACC	(14)
	c-fos (Murine)		GGTCA-n_3-CAGCC	(15)
	cathepsin D (Human)		GGGCCGGGCTGACCCCGCGGG	(16)
	creatinine kinase (Rat)		nGGTCA-n2-CACCCn	(17)
	c-myc (Human)		GGGCA-n_5-TCTCA	(18)
	prothrombin (Human)		CTGACC CTC TGACCT	(19)
	ribosomal protein L7 and primase (Mouse)		GGTTGCCTTGACA	(19)
	calmodulin (Human,Rat)		AGGTCACaGGGTCAG	(19)

Figure 3. Steroid Hormone Receptor Hormone Response Elements. Part A shows the consensus sequences for the glucocorticoid, progesterone, androgen and mineralocorticoid receptors (GRE/PRE/ARE/MRE), as well as the thyroid hormone, retinoic acid and vitamin D3 receptor half-sites (TRE/RARE/DRE) compared to the estrogen receptor (ERE). **Part B** shows genes in which ERE sequences have been identified. **Part C** shows non-classical ERE sequences that have been identified. References in brackets from above are: (1) Beato, 1989 (2) Walker et al, 1984 (3) Klein-Hitpass et al, 1988 (4) Martinez and Wahli, 1989 (5) Kato et al, 1992 (6) Waterman et al, 1988 (7) Shupnik et al, 1989 (8) Slater et al, 1990 (9) Berry et al, 1989 (10) Richard and Zingg, 1990 (11) Teng et al, 1992 (12) Weisz and Rosales, 1990 (13) Farsetti et al, 1995 (14) Hyder et al, 1995 (15) Hyder et al, 1992 (16) Augereau et al, 1994 (17) Wu-Peng et al, 1992 (18) Dubik and Shiu, 1992 (19) Dana et al, 1994. This figure was reproduced from Miller, 1994 and updated.

$VitD_3$, T_3R and RAR, respectively, in an assay examining the ability of steroid hormone receptors to *trans*-activate reporter genes (Umesono et al, 1991). The oligomeric form of receptor binding was also examined with RAR. Monomeric binding of RAR to HREs with direct repeats containing no spacing between half-sites or palindromic repeats positioned 5 or 10 nucleotides apart was observed, whereas dimers predominated when palindromes had 0 or 3 spacing nucleotides separating the half-sites (Forman et al, 1992). This provides evidence that receptor interaction is a finely regulated process.

Studies yielding results which bring the classical model of steroid hormone receptor activity into question have been discussed. Research demonstrating *in vivo* (Gorski et al, 1993) and *in vitro* (Medici et al, 1991) interaction of monomeric ER with EREs has been shown. Furthermore, this monomeric binding may occur in the absence of ligand (Gorski et al, 1993; Brown and Sharp, 1990), although, ligand may be required to activate the *trans*-activation functions of the receptor and induce transcription of E2-responsive genes. The receptor monomer may form a stable heteromeric complex by interacting with other proteins (Gorski et al, 1993) or the oligomeric form of receptors may be dictated by half-site spacing and orientation. This is in contrast to the interaction of liganded homodimers with the ERE proposed by the classical model. Table 1 lists some estrogen-responsive genes.

Regulation of Gene Expression

Transcriptional, post-transcriptional, translational and post-translational events are all steps were the regulation of gene expression may occur.

Transcription may be increased or decreased by various agents. Post-transcriptionally, pre-mRNA processing, mRNA transport to the cytoplasm and, mRNA stability may all be controlled. Studies examining steady state

Gene	Reference
ER	Saceda et al, 1988
PR	Horwitz et al, 1985
pS2	Rio et al, 1987
cathepsin D	Cavailles et al, 1991
TGF-alpha	Bates et al, 1988
TGF-beta	Knabbe et al, 1987
IGF-I and II	Huff et al, 1988
hepatocyte growth factor	Liu et al, 1994
apoVLDL	Dashti et al, 1983
uteroglobin	Cato and Beato, 1985
human Hageman factor XII	Gordon et al, 1991
preproenkephalin	Spampinato et al, 1989
complement factor c3	Sundstrom et al, 1989
ovalbumin	Lai et al, 1983
lactotransferrin	Teng et al, 1989
oxytocin	Adan and Burbach, 1992
BRCA1	Jensen et al, 1996
c-erb-B2	Grunt et al, 1995
Zif268	Suva et al, 1991
c-myc	Dubik et al, 1987
c-fos	Loose-Mitchell et al, 1988
GABA receptor subunit A	Herbison and Fenelon, 1995
Nerve growth factor receptor	Sohrabji et al, 1994
galanin	Vrontakis et al, 1993
cyclin B1	Thomas and Thomas, 1994
retinoic acid receptors	Roman et al, 1993
epidermal growth factor receptor	Lingham et al, 1988
gas-1	Ferero and Cairo, 1993
1,25-dihroxyvitamin D_3 receptor	Duncan et al, 1991
gonadotropin-releasing hormone	Radovick et al, 1991
c-jun	Weisz et al, 1990
vitellogenin	Skipper et al, 1977
actin	Cicatiello et al, 1992
creatinine kinase-B	Pentecost et al, 1990
luteinizing hormone	Shupnik et al, 1989
prolactin	Shull and Gorski, 1989

Table 1. Estrogen-responsive genes. This table lists some estrogen-responsive genes and groups that examined them.

mRNA levels show transcriptional and post-transcriptional events play an important role in regulating gene expression. The stability of individual transcripts is highly variable and steady-state levels are a result of the net transcripts resulting from transcript production versus transcript degradation. For example, the half-life of the nuclear transcription factors c-fos and c-myc is approximately 30 minutes (Shaw and Kamen, 1986; Caput et al, 1986) whereas beta-globin (important for carrying oxygen in blood) mRNA is very stable and has a half-life of about 16.5 hours (Ross and Pizaro, 1983). Studies have identified sequences within the 3' untranslated region of some genes which may dictate the susceptibility of the mRNA to degradation. Proto-oncogenes and lymphokines contain the sequence AUUUA which render them unstable (Shaw and Kamen, 1986; Caput et al, 1986). Cellular nucleases have also been hypothesized to affect mRNA half-life (Wreschner et al, 1981). The AU-rich region in the 3'- untranslated region may act as a substrate for the cellular nucleases leading to degradation of the transcripts. Physiological responses may result in differential regulation of mRNA stability. For example, a 30-50 fold variation in histone transcripts has been demonstrated to occur during the cell cycle. Although transcription plays a small part in this variation, the main mechanism appears to be changes in mRNA stability (Atwater et al, 1990; Ross, 1989). The 3' terminal sequence of the transcript contains the destabililizing sequence which forms a stem-loop structure. Further studies are required to determine the exact mechanisms by which transcriptional and post-transcriptional mechanisms regulate gene expression.

Regulation of translation and amino acid chain elongation, as well as protein stability, are also important in regulating gene expression. Several possible mechanisms for regulating protein expression have been proposed by other researchers (Meyuhas et al, 1987). One mechanism involves the

sequestration of mRNA by packaging of the transcripts into ribonucleoproteins, making the transcripts unavailable to the ribosome for translation. Another mechanism proposed involves the disruption of mRNA secondary structure by binding of a protein to the transcript which affects the rate of initiation of polypeptide synthesis or, thirdly, the repression of translation by a newly synthesized protein present in the 5' ORF of the gene.

The steady-state protein levels of a cell are determined by the difference between protein synthesis and protein degradation. For example, proteolysis of 3-hydroxy-3-methylglutaryl-coenzyme A reductase is regulated by sterols such as cholesterol. A short-lived protein and the endoplasmic reticulum play roles in the regulated degradation (Chun et al, 1990). Post-translational control may also be manifested in post-translational modifications, such as phosphorylation or methylation, which may be necessary for protein function.

Transcriptional and Post-transcriptional ER Regulation

The main control mechanisms involved in regulating expression the of the estrogen receptor occur transcriptionally and post-transcriptionally. As previously discussed, the ER contains sequences in the 5' and 3' UTR which are essential in the regulation of expression of the ER (see Steroid Hormone Receptors). The 5' region of the ER gene contains an ORF of 459 bp, designated exon 1* by Piva et al (1992), approximately 2 kb upstream from the start site designated by Green et al (1986) (Piva et al, 1992). The ORF is preceded by promoter sequences (see Figure 4) consisting of a CAAT-like motif, a TATA-like motif and a putative CAP site indicating this ORF may be transcribed. A splice site was identified at -1884 and a potential acceptor splice site at +164 (Keaveney et al, 1991). Use of the upstream promoter would result in an

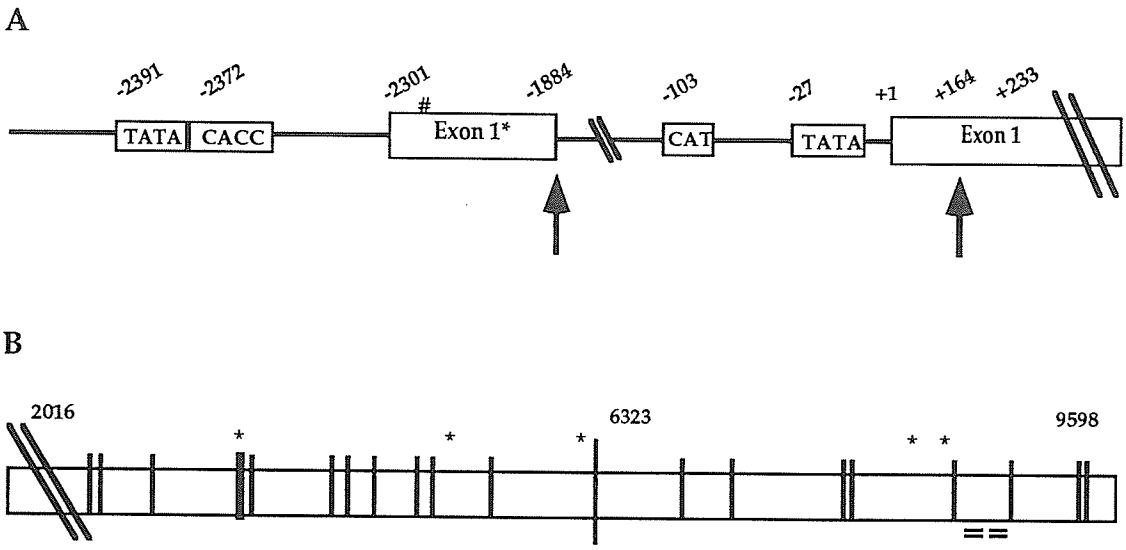


Figure 4. Structure of the 5' and 3' region of the ER gene. The 5' and 3' regions contribute to the transcriptional and post-transcriptional regulation of the ER. **A**. The 5' UTR of the ER contains a TATA-like box motif at -2391 (TTAAATTATATT), a CACC-like motif at -2372 (CCACCCA), a putative cap site at -2360 (TAG), and an ORF of 459 bp from -2301 to -1884 (Exon 1*). The donor splice site (-1884) and the acceptor splice site (+164) are indicated by the arrows. **B**. The 3'-UTR of the ER gene shows vertical bars representing ATTTA repeats which are believed to be a destabilizing sequence, * representing the five putative polyadenylation sites at 4418, 5493, 6306, 8205, 8440 and parallel horizontal bars representing Alu repetitive elements (nucleotides 8480-8774 and 8835-9146) in a head-to-tail inverse orientation. This figure was designed from information from Piva et al (1992), Keaveney et al (1991), Keaveney et al (1993), Green et al (1986) and Weigel et al (1995).

ER transcript which contains sequences from exon 1* spliced to exon 1 in the 5'-UTR. No alteration of the ER protein would result since the normal translation start site at nucleotide +233 is maintained. Weigel et al (1995) identified transcripts which were derived from this upstream promoter in MCF-7, BT-474 and MDA-MB-361 human breast cancer cell lines. These transcripts accounted for up to 30% of the ER transcripts detected in these cell lines. However, ZR-75-1 and BT-20, also ER-positive breast cancer cell lines, expressed very low levels of the exon 1*-exon 1 containing ER transcript (Weigel et al, 1995). Further studies are required to elucidate the conditions which determine the site of transcription initiation.

Regulation of the ER may also occur through the 3' region of the ER. Keaveney et al (1993) subcloned fragments of the 3'-UTR into CAT expression vectors and found all fragments analyzed reduced CAT expression suggesting an important role for the 3'-UTR in regulating ER expression. The 3'-UTR of the ER gene contains 13 copies of the sequence ATTTA (Keaveney et al, 1993) which is believed to promote instability of transcripts. Also found in the 3' region are half-site EREs which may be involved in the regulation of ER expression. This region also contains a number of sequences which could potentially form stem and loop structures which may affect stability of the ER transcript as in the post-transcriptional regulation of the transferrin receptor through stem and loop structures (Mullner and Kuhn, 1988).

The regulation of the estrogen receptor at the transcriptional level was demonstrated when MCF-7 cells were treated with estrogen (Saceda et al, 1989). The initial level of ER transcription fell by 90% and was followed by a decrease in ER mRNA expression and ER protein expression. The continued suppression of ER transcript levels, however, did not appear to be the result of reduced gene transcription since three hours after treatment, transcription was increased to a level greater than control levels (Hadcock and Malbon,

1991). Interestingly, this increase in transcription did not lead to an increase in the steady state levels of ER transcripts suggesting a concommitant increase in the degradation of ER mRNA was occurring. The post-transcriptional regulation of ER in the presence of E2 occurred independent of protein synthesis (Saceda et al, 1989). However, induction of an RNA intermediate capable of RNA digestion may mediate the decrease in steady state levels of ER transcripts as opposed to the decrease being a result of a direct regulation of ER mRNA by the ER (Saceda et al, 1989). These experiments suggest a predominately post-transcriptional mechanism for regulating ER transcripts. Autoregulation of other steroid hormone receptors by their ligands such as progestins (Wei et al, 1988), thyroid hormones (Lazar and Chin, 1988), and glucocorticoids (Kalinyak et al, 1987) has also been demonstrated.

The down-regulation of ER mRNA was also observed when cells were treated with phorbol esters (Ree et al, 1991; Ree et al, 1992; Saceda et al, 1991; Lee et al, 1989). Further studies have provided evidence for the generation of catalytic RNA molecules which may degrade ER mRNA since down-regulation of ER mRNA by TPA requires on-going RNA synthesis but not protein synthesis (Ree et al, 1992).

Other compounds have been shown to alter ER expression. Melatonin is believed to interfere with transcription of the ER gene. Using nuclear run-on experiments, Molis et al (1994) demonstrated a decrease in ER gene transcription of greater than 50% within 4 hours of treatment with physiological (10^{-9}M) concentrations of melatonin. These researchers hypothesized that this inhibition may occur through the activation of signal transduction pathways as a result of the interaction of melatonin/melatonin receptor which may inhibit the ER gene transcriptional machinery. Alternatively, melatonin may diffuse to the nucleus and inhibit ER gene

transcription by direct interaction of this agent with promoter sequences on the ER gene (Molis et al, 1994).

Progestins have also been shown to decrease steady-state levels of ER mRNA (Alexander et al, 1990; Berkenstram et al, 1989; Read et al, 1989). A recent study demonstrated liganded PR repressed ER-mediated transcription of E2-responsive genes (Kraus et al, 1995). This interference appears to occur through a quenching mechanism. Quenching interferes with the ability of the ER to interact with the necessary transcriptional machinery. This group suggests the PR must bind to the PRE of the E2-responsive gene and either directly or indirectly interfere with productive ER interaction with the preinitiation complex (Kraus et al, 1995). In addition, progestins have been shown to block estrogen-induced ER protein synthesis in uterine decidual cells (Bhakoo and Katzenellenbogen, 1977; Takeda and Leavitt, 1986).

A recent study identified a DNA-binding factor present in ER-positive cell lines (DeConnick et al, 1995). Low levels of this protein were detected in normal human mammary epithelial cells while abundant expression was found in breast cancer cell lines and endometrial carcinomas that were ER-positive. Transcriptional regulation of the ER may occur by binding of this DNA-binding protein, estrogen receptor factor-I (ERF-I), to two binding sites found in the 5'-untranslated region of the ER. Augmented expression of the ER was found when studies were conducted using the 75 bp region of the 5'-untranslated region of the ER promoter (DeConnick et al, 1995). ERF-I does not appear to directly interact with the ER. This was concluded following studies examining ER and ERF-I. Supershifting of ERF-I complex with an ER-antibody did not occur, the ERF-I 30 kDa protein identified in South-western blotting is distinct from the ER protein, the ERF-I site found in the 5'-untranslated region of the estrogen receptor gene is not similar to an ERE, and BT-20 cells produce an ERF-I complex even though this cell line expresses

a truncated 43 kDa ER-like protein. ERF-I was not found in ER-negative cell lines. This protein may play an important role in the regulation of the ER, and possibly other genes, in breast cancer as well as in normal tissue. In addition, ERF-I expression may become a useful prognostic marker for tumors which will respond favorably to hormonal therapy.

Studies have demonstated the apparent correlation between ER mRNA expression and protein expression (Barrett-Lee et al, 1987), hence, a corresponding decrease in ER protein was also seen in experiments discussed above.

Translational and Post-translational Regulation of ER

Research has shown that ER protein steady state levels may be regulated translationally and posttranslationally. ICI 164,384 is believed to block translation of the ER transcript and decrease the half-life of the ER protein although the mechanism is unknown at this time (Dauvois et al, 1992). In light of the mechanisms proposed for general translational control, the ER has been further examined. The 5'-untranslated region of the ER transcript potentially contains a signal sequence which would target the transcript to the endoplasmic reticulum. Signal peptide sequences are bound by a signal recognition particle (Walter and Blobel, 1983; Walter and Lingappa, 1986) following the translation of the signal sequence by the ribosome. The signal recognition particle/ribosome complex then binds to a signal recognition peptide receptor present in the endoplasmic reticulum membrane and the translated peptide is targeted to the cytoplasm of the organelle. Interestingly, the ER contains a 5' ORF of approximately 20 amino acids in the 5' untranslated region which may represent the signal sequence. Alternatively, this ORF may produce a protein which may inhibit translation as described for carbamoyl-phosphate synthetase A mRNA. The 5' ORF translation

product forms a complex with a regulatory protein which blocks the ribosome from scanning further than the first ORF (Werner et al, 1987). This remains to be studied.

ER protein degradation has been observed in mouse uterus (Horigome et al, 1988). This degradation was stimulated by estrogen. Cleavage of the ER from the 66 kDa form to a 54 kDa form appeared to be mediated by a cysteine proteinase and further cleavage to a 37 kDa form by an unidentified proteinase, different from the first cleavage enzyme, was also observed over a longer incubation period. Another study reported a Ca^{2+}-activated proteinase which was stimulated by E2 (Puca et al, 1977). This proteinase was found to be calpain which is a protein expressed in a large variety of tissues (Murayama et al, 1984). Although protein degradation does not appear to be a major mechanism in the regulation of ER expression, it may contribute to the regulation.

The ER is a phosphoprotein. The role of phosphorylation in the transcriptional activity of the estrogen receptor has not been clearly defined. Since the ER is an autoregulatory protein, phosphorylation may play an important role in regulating the expression of the ER. Phosphorylation of steroid hormone receptors may play a role in transcriptional activation (Ali et al, 1993; Denner et al, 1990b), DNA binding (Denton et al, 1992), and receptor processing and shuttling (Orti et al, 1992; DeFrance et al, 1991). The ER contains a number of sites with the potential to act as phosphorylation substrates for protein kinases (Green et al, 1986). Studies have suggested that ligands alter the phosphorylation state of receptors such as GR, PR and Vit D_3 (Hoeck et al, 1989; Denner et al, 1990; Brown and DeLuca, 1990). Altered transcriptional activation may result from increased receptor phosphorylation (Aronica and Katzenellenbogen, 1993), however, overall receptor phosphorylation is not directly related to increased receptor

transcriptional activity. Binding of the receptor to specific DNA sequences has also been suggested to occur due to receptor phosphorylation (Denner et al, 1989). Denton et al (1992) proposed that phosphorylation of serine residues of the ER following hormone binding increases the affinity of the ER for the ERE and may increase the association time of the DNA/receptor complex, increasing the effectiveness of transcriptional activation.

Examination of mouse ER phosphorylation was performed recently by Lahooti et al (1994). This group suggested that phosphorylation of specific, critical amino acids occurs in stages as a result of DNA or ligand binding. The increased phosphorylation in the DNA-binding-dependent stage may be an increase in the total number of receptors that are phosphorylated rather than a qualitative change (ie. new sites) of phosphorylation. This group further suggests that phosphorylation may play a role in regulation of ER activity, however, not in transcriptional activity, but possibly another step, such as nucleocytoplasmic shuttling. This was suggested since a transcriptionally defective mutant was equally phosphorylated when compared to the transcriptionally active wt receptor. The role of phosphorylation remains to be elucidated although it may play a role in the activation of the ER and, therefore, expression of the estrogen receptor.

Phosphorylation sites are shown in Figure 5. Serines 104, 106 (LeGoff et al, 1994), and 167 (Arnold et al, 1995a; Arnold et al, 1994) have been shown to be phosphorylated upon estradiol treatment (LeGoff et al, 1994). Tyrosine 537 is phosphorylated independent of ligand and is believed to be important for binding of the ER to the ERE (Arnold et al, 1995b; Arnold et al, 1995c). Serine 118 was found to be phosphorylated in the presence of hormone by LeGoff et al (1994) and Joel et al (1995) and independent of ligand by Kato et al (1995).

Although the main mechanism for regulating ER expression appears to be post-transcriptional, the other steps discussed may contribute to the overall

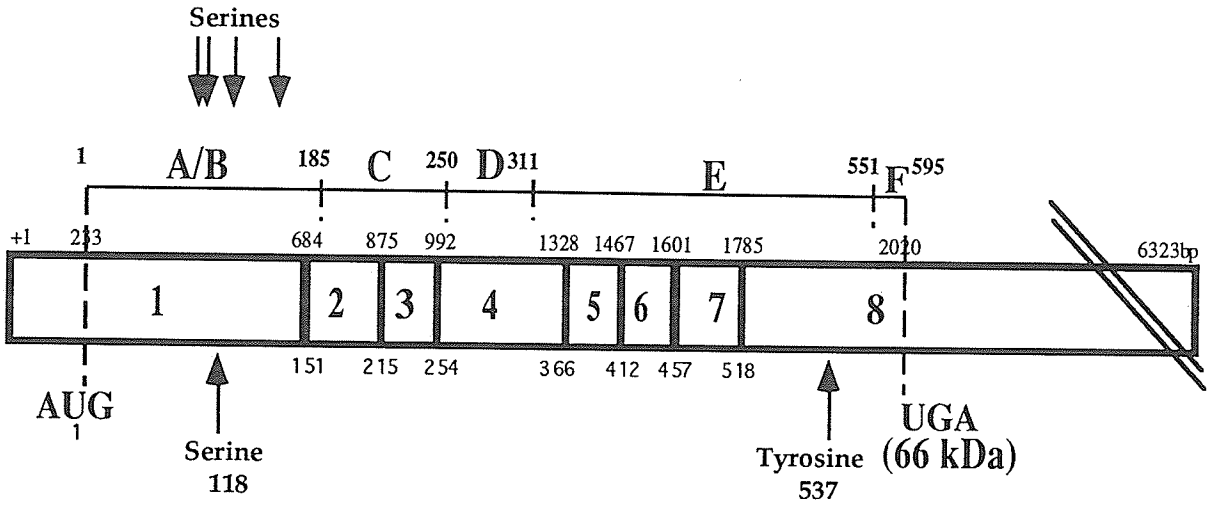


Figure 5. Phosphorylation sites of the ER protein. This figure shows the nucleotide and amino acid positions at the exon boundaries and the functional domains of the ER protein. Arrows on top indicate the positions of phosphorylation sites which are ligand-dependent (Serines 104 (a), 106 (a), 118 (a,b,c), 167 (d,e)). The bottom arrows indicate the amino acids believed to be phosphorylated independent of ligand and are believed to be important in binding of the ER to EREs (Serine 118 (c) and Tyrosine 537 (f,g)). Serine was found to be a hormone-dependent phosphorylation site by LeGoff et al (1994) and Joel et al (1995) but a ligand-independent site by Kato et al (1995). (a) LeGoff et al, 1994 (b) Joel et al, 1995 (c) Kato et al, 1995 (d) Arnold et al, 1995a (e) Arnold et al, 1994 (f) Arnold et al, 1995b (g) Arnold et al, 1995c.

expression of the ER. Many more studies are needed to fully understand how regulation of gene expression occurs.

Estrogen in Breast Cancer

A case for the role of estrogen in the development of breast cancer is demonstrated by the observations that males and ovariectomized or nonestrogenized females rarely develop breast cancer (Vorherr, 1980). High levels of endogenous estrogens in males are linked to an increased risk of breast cancer (Thomas, 1993). The importance of estrogen in breast cancer was demonstrated as early as 1896 when breast tumour regression was achieved by ovariectomy of pre-menopausal women, thereby removing the main source of estrogens (Beatson, 1896). Furthermore, estrogen has been shown to increase the proliferation of ER-positive breast cancer cells in culture (Dickson and Lippman, 1988) as well as the growth of these cells as tumours in nude mice (Soule and McGrath, 1980)

Breast cancer appears to originate as an estrogen-dependent disease which progresses to hormone-independence resulting in endocrine resistant tumours (Brenner et al, 1988). Approximately 70% of breast cancer patients are ER-positive upon presentation and of these, approximately 60% will respond to endocrine therapy, (Horwitz et al, 1975) leaving the remainder of primary tumours resistant to endocrine therapy. Furthermore, originally responsive tumours will eventually develop resistance although many continue to express the ER. Approximately 10% of ER-negative tumours will also respond to endocrine therapy (Horwitz et al, 1975). This has made understanding the mechanisms of endocrine therapy and the development of hormone-resistant tumours crucial for the development of new and better treatments.

Biopsy Sample Analysis

The decision to use endocrine therapy is often dependent upon the ER content in the biopsy sample. Estrogen and progesterone receptors are prognostic markers and often predict if a patient will respond to endocrine therapy (Horwitz et al, 1975; Blanco et al, 1984, Barbi et al, 1987; Bonadonna and Valagussa, 1988). Reduced aggressiveness of the tumour and response to endocrine therapy appear to be directly correlated to ER quantity in the tumour; tumours with high levels of ER respond very well, while tumours with low levels respond poorly (McGuire et al, 1978). The presence of ER and PR denotes a tumour which will be more responsive to hormonal therapies (Horwitz et al, 1975; McGuire et al, 1978).

Biopsy samples are subjected to ligand-binding analysis and immunoassays (Holmes et al, 1990) to determine ER and PR content. Ligand-binding analysis uses radioactive estrogen to bind to ER, while immunoassays use antibodies typically specific for the carboxy-terminus of the estrogen receptor to determine ER content. One drawback to using assays aimed at the carboxy-terminus of the ER is that only apparently intact ERs will be detected. Tumours containing receptors with defects that render them less active or inactive relative to the wild-type receptor will be classified as ER-positive tumours, yet not be equally affected by E2 or antiestrogens (Sluyser and Witliff, 1992). Studies have shown that ER charged with ligand sometimes cannot bind the nucleus and that unliganded ER may often bind the nucleus (Raam et al, 1988).

In addition to ER and PR measurements, tumour size, tumour grade, regional lymph node status and the presence of distant metastasis are examined to be used for prognostic evaluation of tumours. Taking these factors into consideration, the mode of treatment is decided upon.

Endocrine Therapies

In 1896, Beatson published a report that oophorectomy resulted in breast tumour regression. Following this discovery, many attempts to manipulate the hormonal milieu were made (rev. in Howell et al, 1993). Surgical removal of key glands in hormone production and control such as irradiation of the ovaries, adrenalectomy, and hypophysectomy were developed. Administration of synthetic hormones, inhibitors of hormones or inhibitors of key enzymes in hormone production were also tried such as androgens, estrogens, progestins, antiestrogens, aromatase inhibitors, LHRH analogues, antiprogestins, pure antiestrogens, somatostatin analogues and prolactin inhibitors (see Table 2).

Given the dependence of tumours on estrogens for growth, endocrine therapies are targeted to preventing the production or activity of estrogens. Figure 6 shows the effect of estrogen on the cell and sites where hormonal therapies may be targeted. Treatments focus on depriving the tumour of estrogen in a manner which is the least toxic to the patient. Research indicates that combining endocrine therapies does not increase response or survival time (Santen et al, 1990). However, resistance to endocrine therapy may be overcome by employing an alternative endocrine therapy. Unfortunately, some endocrine therapies targeted at the level of the receptor, for example the antiestrogen tamoxifen, in some cases may eventually promote tumour growth since this growth ceases upon withdrawal of the agent (Howell et al, 1992). The reason for treatment failure may be due to the emergence of cells resistant to the agent (Goldie and Coldman, 1984), although studies show that resistant tumours may continue to express the ER (Darbre and King, 1987; Taylor et al, 1982; Manni et al, 1980). Although tumours may respond following withdrawal and administration of the same agent or a different endocrine therapy, the development of resistance limits

Type	Mechanism	Used or in Clinical Trials
Oophorectomy	Decrease production of estrogens	Used
Ovarian irradiation	Decrease production of estrogens	Used
Androgens	gonadotrophin release suppressed followed by decreased estrogen levels direct effect on tumour cells	Rarely Used
Synthetic Estrogens High Dose	unknown mechanism	Rarely Used
Progestins eg. MPA, megestrol acetate	effect autocrine and paracrine growth factor loops, direct antiproliferative effect, increase estrogen metabolizing enzyme, suppresses ER levels	Second-line therapy
Adrenalectomy	prevents production of androstenedione, the precursor of estradiol removing source of estradiol precursor	Rarely Used
Hypophysectomy	prevents release of GnRH from hypothalamus decreasing release of LH and FSH from pituitary and stimulation of ovarian function	Rarely Used
Antiestrogens eg. Tamoxifen Toremifene Droloxofene	inhibit binding of estrogen to ER decrease stimulatory peptide GFs increase inhibitory peptide GFs	Used
Aromatase Inhibitors eg. Aminoglutethimide	blocks estradiol synthesis by inhibiting conversion of androstenedione to estrone	Third or Fourth-line therapy
LHRH analogues	inhibit pituitary gonadotropin release leading to inhibition of ovarian function	Used
Antiprogestins eg. RU 486	acts though PR or GR to inhibit cell proliferation	ClinicalTrials
Pure Antiestrogens eg. ICI 182780	inhibits estrogen binding to ER may increase ER degradation may increase inhibitory peptide GFs	ClinicalTrials
Somatostatin analogues eg. octreotide	inhibits cell proliferation through somatostatin receptors reduces IGF-I decreases EGF	Clinical Trials
Prolactin release Inhibitors eg. Bromocriptine	may inhibit proliferation of breast cancer cells (through prolactin receptor)	Clinical Trials

Table 2. Endocrine Therapies. This table shows endocrine treatments available and in clinical trials. Information was taken from Pollak (1996), Santen et al (1990) and references discussed in the text.

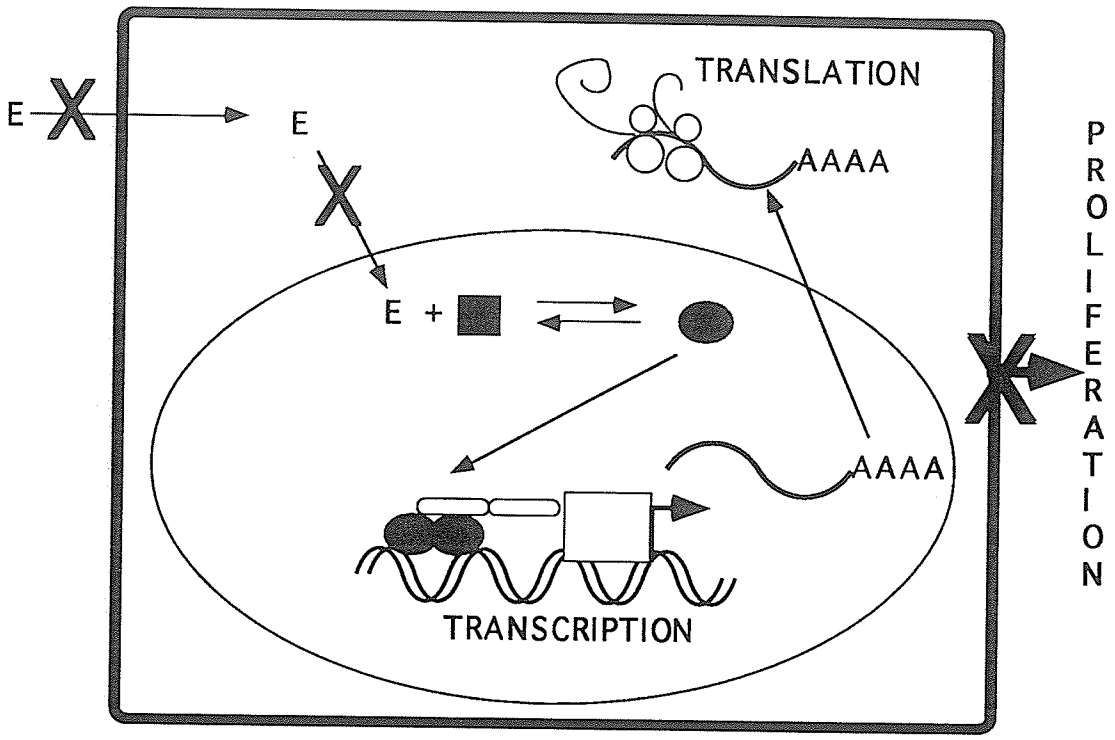


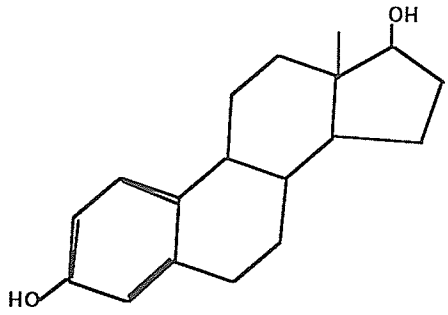
Figure 6. Estrogen activity and sites targeted for endocrine therapy. Estrogen diffuses into the cell where it interacts with the ER in the nucleus. Following homodimerization, the ER activates transcription of estrogen-responsive genes which are translated in the cytosol. Endocrine therapies may inhibit the diffusion of estrogen into the cell, into the nucleus or may inhibit cellular proliferation by other cellular processes as described in the text. The square represents unliganded ER, the oval, liganded ER and the two ovals, ER homodimers.

the usefulness of these agents (McGuire, 1980). The proposed mechanisms by which endocrine therapies work will be discussed, followed by proposed mechanisms for the development of tumours which are resistant to endocrine therapy.

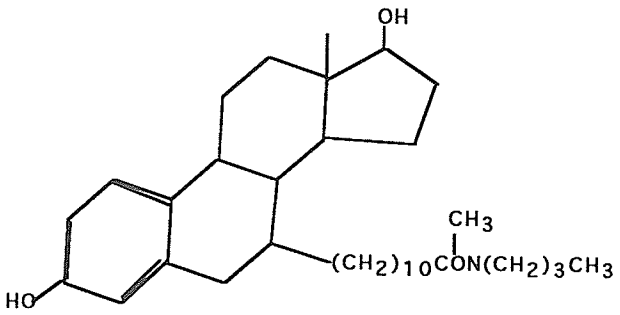
Non-steroidal Antiestrogens

Tamoxifen (Nolvadex) is a non-steroidal, triphenylethylene derivative antiestrogen widely used as adjuvant treatment following surgery for breast cancer and for advanced disease (Jordan et al, 1987). The tumouristatic effect of tamoxifen is primarily manifested through its interaction with the ER (Furr and Jordan, 1984; Epstein, 1988). The major metabolite of tamoxifen is 4-hydroxytamoxifen (4OH-Tam) which competes with estradiol for high affinity binding to the ER (Jordan et al, 1977). Figure 7 shows the structure of estradiol, tamoxifen, 4-hydroxytamoxifen, and the pure antiestrogen (discussed in the following section) ICI 164,384. Tamoxifen has been shown to be extremely effective in ER-positive breast cancer treatment (Sunderland and Osborne, 1991) and as effective as ovariectomy or hypophysectomy in blocking the growth-stimulative activity of estrogens in hormone-dependent breast cancer (Furr and Jordan, 1984). Treatment of breast cancer patients with tamoxifen is believed to increase the disease-free interval (Rutqvist et al, 1987; Baum et al, 1988), the overall survival time of patients (Early Breast Cancer Trialist's Collaborative Group, 1988), and decrease the risk of developing contralateral primary breast tumours in breast cancer patients (Cuzick and Baum, 1985). Research concerning tamoxifen administration to women at high risk for developing the disease is also being done (Powles et al, 1989; rev. in Jordan, 1995).

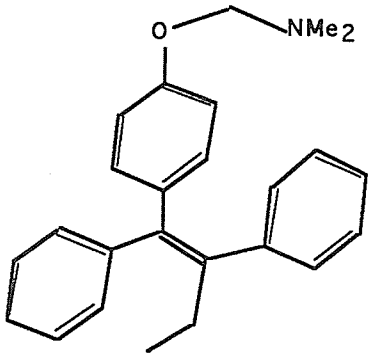
Tamoxifen is a very popular choice for therapy since nausea and hot flashes are the most common side effects while severe side effects are rare. In a study by Patterson and Butterbsy (1981), only 3% of patients had severe



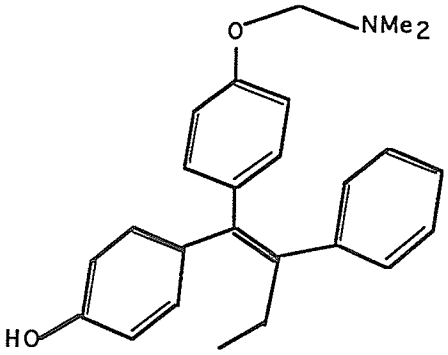
ESTRADIOL



ICI 164,384



TAMOXIFEN



4-HYDROXYTAMOXIFEN

Figure 7. The structures of Estradiol, ICI 164,384, Tamoxifen and 4-hydroxytamoxifen. Me_2 = Two methyl groups attached to the nitrogen atom.

enough side effects to withdraw from treatment. Tamoxifen has been shown to have partial agonist activity in the cardiovascular system as well as in bone, averting the risk of developing osteoporosis which would be expected by an antagonist of the ER (Jordan et al, 1987). However, in a large number of patients, tamoxifen also stimulates the growth of endometrial cells (Kedar et al, 1994). Another short-coming of tamoxifen is the almost inevitable failure of treatment within 5 years (Osborne et al, 1992).

Using *in vitro* studies of chimeric receptors in heterologous mammalian cells, McDonnell et al (1995) suggested that the 4-OH-Tam/ER complex is delivered to the DNA and that the complexes formed by 4-OH-Tam/ER, the pure antiestrogen, ICI 164,384 and the ER and estradiol/ER are distinct, allowing the cell to discriminate between the ligands. Furthermore, activity of these compounds may be cell and promoter-specific (McDonnell et al, 1995) (see Table 3). Research has determined that the *trans*-activation functions of the ER are cell and promoter-specific where TAF-1 and/or TAF-2 may be required to mediate the activity of the ER (Tzukerman et al, 1994; Kastner et al, 1990; Tasset et al, 1990). Tamoxifen was shown to block TAF-2 activity, hence acting as an antiestrogen in a TAF-2 cell- and promoter-requiring system (Tzukerman et al, 1994). In contrast, a system that requires only TAF-1 would have TAF-2 inhibited but TAF-1 functioning or allowed to interact with transcription factors, resulting in the partial agonist activity of antiestrogens (Reese and Katzenellenbogen, 1992; Danielian et al, 1993; Tzukerman et al, 1994). Research by Montano et al (1995) also supports the hypothesis that distinct conformational changes are induced by antiestrogens from that of estradiol and further suggests different cells have different coactivators or repressors which may contribute to the variability of antiestrogen activity. Also, studies have identified antiestrogen binding sites in ER-positive and ER-negative breast cancer cells (Miller et al, 1986). These

Tamoxifen Activity	Cell Type	Promoter	Reference
Agonist	MDA-MB-231	ERE-pS2-CAT	Montano et al, 1995
Agonist	CHO	ERE-pS2-CAT	Montano et al, 1995
Antagonist	3T3	ERE-pS2-CAT	Montano et al, 1995
Antagonist	HeLa	ERE-pS2-CAT	Montano et al, 1995
Agonist	HepG2	C3 promoter	Tzukerman et al, 1994
Antagonist	HeLa	Vit-tk-CAT	Webster et al, 1988
Antagonist	MCF-7	(ERE)-TATA-CAT (ERE)2-TATA-CAT pS2-CAT	Fujimoto and Katzenellenbogen, 1994
Agonist	HeLa	collagenase-CAT	Webb et al, 1995
Agonist	HeLa	ERE-collagenase-CAT	Webb et al, 1995
Antagonist	HeLa	ERE-tk-CAT	Webb et al, 1995
Agonist	CEF CV-1 MDA-MB-453	classical ERE-CAT	Webb et al, 1995
Agonist	Ishikawa 3T3 HepG2	collagenase-CAT	Webb et al, 1995
Antagonist	ZR-75 MCF-7	collagenase-CAT	Webb et al, 1995

Table 3. Cellular and promoter-dependent activity of Tamoxifen. This table shows the cell type transiently transfected with the specified promoter and wt ER (except MCF-7 cells which express the ER). The cells were treated with mono-hydroxytamoxifen and the effect on the stated promoter/reporter gene determined. MDA-MB-231 = ER-negative human breast cancer cells, MCF-7 = ER-positive human breast cancer cells, HeLa = human cervical cancer cells, HepG2 = human hepatocellular carcinoma cells, CHO = chinese hamster ovary cells, 3T3 = mouse fibroblast cells, CV-1 = monkey kidney cells, ZR-75 = ER+ human breast cancer cells, Ishikawa = human endometrial carcinoma cells, MDA-MB-453 = ER+ human breast cancer cells, CEF = chicken embryo fibroblast cells.

sites are binding sites distinct from the estrogen receptor (Sutherland and Foo, 1979; Kon, 1983; Sudo et al, 1983; Fishman, 1983; Clark et al, 1987) and do not bind estrogens (Sutherland et al, 1980). Evidence suggests that these binding proteins are not direct mediators of antiestrogen action (Miller and Katzenellenbogen, 1983; Katzenellenbogen et al, 1985; Teske et al, 1987; Sheen et al, 1985; Miller et al, 1984). Pavlik et al (1992) examined the ratio of binding sites (AEBS) to ER in AE resistant but estrogen sensitive LY-2 cells versus MCF-7 cells which are AE and E2 sensitive. The ratio found in the resistant cells was approximately 3 times that of the MCF-7 cells. The effectiveness of tamoxifen as an antiestrogen in tumours may therefore be determined in part by the expression of AEBS in the tumour cells of the patient receiving treatment.

Tamoxifen is an antagonist of the estrogen receptor blocking the growth-promoting effect of estrogen on breast tumours, however, numerous studies have identified alternative pathways of tamoxifen action (see figure 8). Both ER-positive and ER-negative tumours may be eradicated by the stimulation of natural killer cells in the vicinity of the tumour by tamoxifen (Berry et al, 1987). Also, within the cell, tamoxifen may affect chemicals in the intracellular signalling pathway that are necessary for cell growth and proliferation. The level or activity of calmodulin, which regulates cellular pools of calcium, is decreased by tamoxifen (Lam, 1984; Gulino et al, 1986). Tamoxifen, as well as its major metabolites 4-hydroxytamoxifen and N-desmethyltamoxifen, (O'Brian et al, 1986) also decrease the activity/level of protein kinase C.

Growth factors are also affected by tamoxifen. *In vitro*, IGF-I has been demonstrated to have a mitogenic effect on breast cancer cells (rev. in Sara and Hall, 1990). In a clinical setting, Pollack et al (1990) found the concentration of IGF-I in the serum of patients administered tamoxifen was

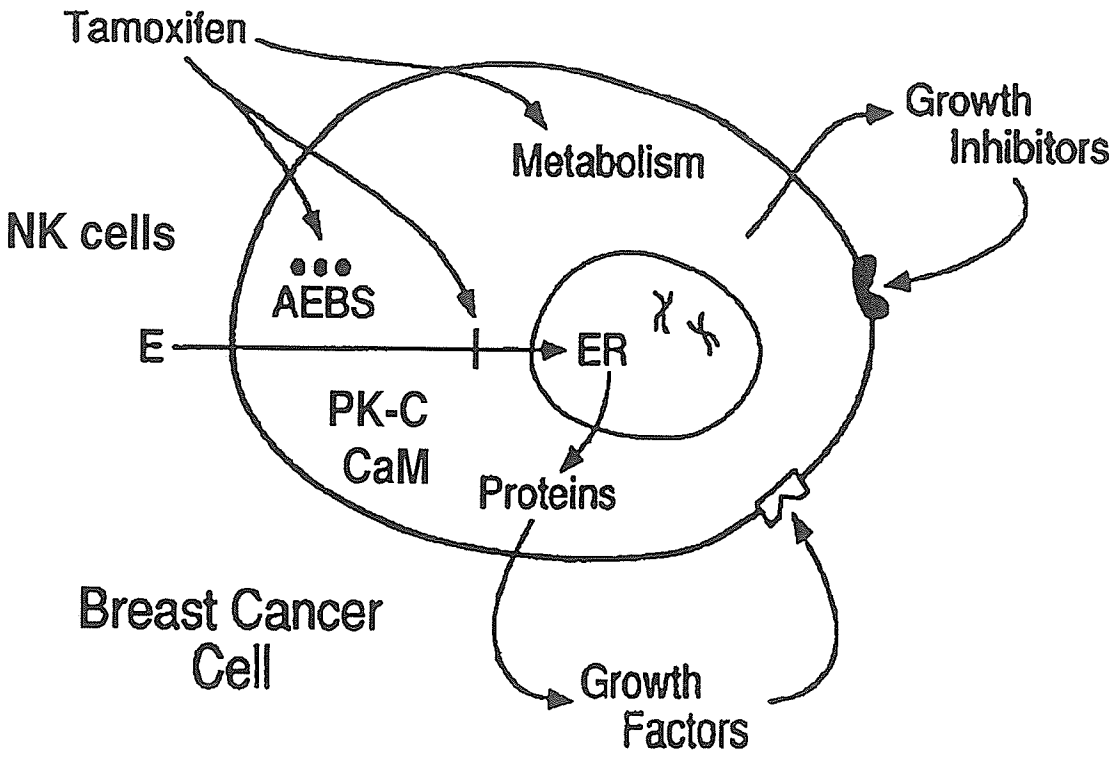


Figure 8. Factors Affected by Tamoxifen. This diagram illustrates the multiple interactions of tamoxifen affecting intracellular and extracellular processes. AEBS= antiestrogen binding sites, CaM= calmodulin, E= estrogen, PK-C= protein kinase C, NK cells= natural killer cells. This figure was reproduced from Osborne and Fuqua, 1994 and modified by additional information referenced in the text.

decreased. Another study demonstrated significant decreases in serum IGF-I levels in post-menopausal women following tamoxifen treatment (Friedl et al, 1993). The mRNA of another growth factor, TGF-β is increased by tamoxifen (rev. in Osborne and Fuqua, 1994). This growth factor inhibits growth of breast cancer cells that have been stimulated with estrogens in culture (Knabbe et al, 1987). Autocrine or paracrine loop activity of TGF-β may also provide a mechanism by which tamoxifen may exert its antitumoural properties. The attraction of natural killer cells, macrophages, as well as the promotion of growth of stromal cells which have been found to increase stromal TGF-β in tamoxifen-treated patients' biopsy samples (Butta et al, 1992), may contribute to tamoxifen's activity (Sporn et al, 1987).

TGF-α secretion is stimulated by estradiol (Bates et al, 1988). The ability of TGF-α to interact with EGFr provides an autocrine loop through which estradiol can stimulate TGF-α which, in turn, interacts with EGFr to stimulate cell proliferation. When estradiol activity is blocked, for example, by addition of tamoxifen, one pathway for growth stimulation is interrupted. The production of TGF-α was decreased in patients with ER-positive tumours following tamoxifen treatment (Noguchi et al, 1993). Paracrine stimulation of ER-positive cells may also be blocked by tamoxifen further contributing to the effectiveness of tamoxifen as an antiestrogen (Freiss et al, 1990).

Disparate evidence has been observed for tamoxifen's effect on the growth factor receptor, c-erbB-2. In nude mice and ZR-75-1 cells, tamoxifen stimulated c-erbB-2 mRNA expression (Warri et al, 1991). In patients treated with tamoxifen for 3 weeks prior to surgery, c-erbB-2 mRNA expression was decreased compared to controls, however, this study was done with patients who were ER-negative. No change was observed in ER-positive patients (LeRoy et al, 1991).

At the present time, tamoxifen is a popular choice for the treatment of advanced breast cancer or as an adjuvant treatment, however, resistance to this compound has prompted re-evaluation of older compounds and development of new ones.

Pure Antiestrogens

ICI 182,780 and 164,384 are steroidal compounds that have been examined for their efficacy as antiestrogens. These agents have a 7α-alkylamide sidechain on the steroid B ring (Bowler et al, 1989; Wakeling et al, 1991) (see Figure 7). The difference between the two compounds is the fluorine atoms present on the side chain of ICI 182,780 which increases its solubility and affinity for the ER relative to ICI 164,384 (Wakeling et al, 1991). Research has shown that estrogen activity in all target tissues examined was totally blocked following treatment with ICI 182,780 (Hu et al, 1993). These compounds were developed to circumvent the partial agonist activity seen with tamoxifen. Tumours resistant to tamoxifen are not cross-resistant to the pure antiestrogens (Hu et al, 1993) prospectively making these compounds useful following failure of tamoxifen treatment. Other research suggests that these compounds may be more effective than tamoxifen due to their different mechanism of action (rev. in Howell et al, 1993).

The mechanism by which the pure antiestrogens exert their effect remains controversial. Some studies conclude that ICI binds to sites similar to estradiol and the 7α-alkylamide side chain interferes with receptor dimerization since the receptor dimerization domain is localized to the HBD of the ER (see figure 2) and, therefore, interferes with ICI/ER DNA binding (Dauvois et al, 1992; Parker, 1993). It was speculated that a reduction in affinity of the complex for the ERE occurred (rev. in Howell et al, 1993). These studies were performed in insect and mammalian cells over-expressing the

ER. However, DNA binding by ICI was not found to be inhibited in systems not over-expressing the ER (eg. yeast and transiently transfected cells) (Webster et al, 1988; Tsai et al, 1991) suggesting the compounds exert their action prior to DNA binding. Studies have also found an increase in receptor turnover via a decreased half-life of the ER protein following treatment with ICI 182,780 and ICI 164,384 which may account for the effectiveness of these compounds as antiestrogens (Dauvois et al, 1992; Gibson et al, 1991; McDonnell et al, 1992; Parker, 1993; Arbuckle et al, 1992). Other studies, however, have determined that the ICI/ER complex is delivered to the DNA (McDonnell et al, 1991; Pham et al, 1991; Berry et al, 1990) and the activity of the compound is cell and promoter-dependent. The cell and promoter context of antiestrogens was also seen by Montano et al (1995) when using chimeric receptors. McDonnell et al (1995) suggest that steps prior to the binding of the ICI/ER complex to DNA are important for conferring the antiestrogenic properties of the pure antiestrogens although interference with DNA binding and increased receptor turnover are also necessary mechanisms. Additionally, the mRNA levels of the inhibitory growth factor TGF-β_2 are increased 10-fold in MCF-7 human breast cancer cells treated with ICI 164,384 (Paik et al, 1994) which may account for the antiestrogenic activity of the compound.

Regardless of the mechanism, research appears to indicate that pure antiestrogens are effective growth inhibitors. Studies in athymic nude mice implanted with MCF-7 cells and treated with E2, as well as female nude mice implanted with Br10 human breast cancer cells, resulted in growth inhibited tumours when treated with ICI 182,780 (Wakeling et al, 1991). Furthermore, Gottardis et al (1989) showed tamoxifen-stimulated growth in MCF-7/nude mice to be inhibited by ICI 164,384. Results indicate the potential for these compounds to act as effective agents in the treatment of breast cancer.

In phase I clinical trials, ICI 182,780 had little toxicity and reduced cell proliferation when used in patients prior to surgery (DeFriend et al, 1994). In light of the antiuterotrophic activity in rats, Wakeling (1993) suggested that peripheral organs, such as the uterus, were more sensitive to the antiestrogenic effects of ICI 182,780 than central estrogen target tissues, such as the pituitary. In contrast to what would be predicted for pure antiestrogens, no significant bone density change was observed (rev. in Howell et al, 1993).

Pure antiestrogens may find a place as adjuvant therapy to tamoxifen or in tumours failing to respond to tamoxifen. However, resistance to pure antiestrogens has been shown to occur using cells in culture which suggests that resistance may occur *in vivo* and limit the efficacy of pure antiestrogens (Lykkesfeldt et al, 1995).

Progestins

Progesterone is important in the development of female reproductive organs as well as in regulating biological functions. The belief that progesterone is an antagonist of estrogen may explain why hormone-responsive breast tumours may be effectively treated with the synthetic progestin, MPA, with a response rate reaching almost 70% in PR-positive tumours (Sutherland et al, 1988). The progestins, MPA and megestrol acetate, are agents used as second- or third-line therapy in the clinical treatment of breast cancer.

Numerous studies have been conducted to determine the effect of progestins in breast cancer, although the mechanism of action remains unclear. Progestins may act indirectly to alter the hormonal environment. Suppression of ACTH release from the pituitary which decreases the availability of E2 precursors (Mathews et al, 1970), decreased circulating levels of estrogen, gonadotropins, and sex hormone binding globulin following

progestin treatment may deprive the tumour of estrogen (Alexieva-Figusch et al, 1984). Estrogen is know to inhibit tumour cell proliferation at pharmacological doses. Increased levels of urinary estrogens were detected in some progestin-responsive patients following MPA treatment (Sadoff and Lusk, 1974), therefore, progestins may also exert their antitumoural properties through *in vivo* conversion of MPA to estrogens (Sadoff and Lusk, 1974). Progestins may also increase 17β-hydroxysteroid dehydrogenase, the enzyme responsible for converting estrogen to the less active estrogen, estrone (Liu and Tseng, 1979). This effect may be mediated through progestin interaction with the PR (Fournier et al, 1985).

Progestins have been demonstrated to directly inhibit the proliferation of human breast cancer cell lines (Horwitz and Freidenberg, 1985; Allegra and Keifer, 1985) and inhibit estrogen-stimulated growth (Allegra and Keifer, 1985). Down-regulation of estrogen receptor levels has also been observed (Clark and Peck, 1979).

Interestingly, comparison of megestrol acetate and tamoxifen used in post-menopausal breast cancer patients yielded similar findings with respect to antitumoural results, while at conventional doses (<500mg/day), MPA is less effective than both agents. Increasing the dose of MPA (>500mg/day) improves the antitumoural properties of the agent (rev. in Santen et al, 1990), however is likely to increase the side effects experienced by the patients.

Although the effects of progestins are believed to be mediated through the PR, most studies have not found a significant correlation between PR status and response to therapy (Tseulings et al, 1980; Ettinger et al, 1986; Haller and Glick, 1986). An alternative pathway may involve progestin interaction with the AR and GR (Tseulings et al, 1980). At high doses, the side effects seen in treatment are glucocorticoid-related (rev. in Santen et al, 1990) and commonly include weight gain, edema and increased appetite (rev. in Santen et al, 1990).

Aromatase Inhibitors

Another method of reducing the amount of estrogen available to the tumour is to inhibit the synthesis of estrogen by inhibiting the activity of aromatase (rev. in Howell et al, 1993). Aminoglutethimide and 4-hydroxyandrostenedione are used clinically, however, the low specificity of the first and the poor bioavailability of the latter render them less likely to be used as a first-line therapy. The main target for aminoglutethimide is extra-adrenal aromatase, however, this drug also affects several steroid hydroxylation enzymes of the adrenal and enzymes that cleave side chains of cholesterol. Aminoglutethimide reduces levels of circulating estradiol to approximately 40%. However, in premenopausal women, this drug is ineffective since ovarian aromatase is only partially inhibited.

4-hydroxyandrostenedione is 60 times more active than aminoglutethimide, acting as an androstenedione analogue. Androstenedione is converted to estrogen by aromatase. 4-hydroxyandrostenedione reduces serum estradiol to approximately 66% with few side effects. Side effects of aromatase inhibitors occur in approximately 25% of patients and include rash, fatigue, lethargy and nausea (rev. in Howell et al, 1993).

Antiprogestins

Clinical trials are being conducted with antiprogestins. These agents are growth inhibitory or stimulatory depending on the dose, culture condition and cell line being examined (Michna et al, 1992). Compounds being tested include RU38486 and the newer agents ORG 31710 and 20058 as well as RU4886. These agents inhibit the estrogen-induced growth of cultured cell lines by up to 74% (Bardon et al, 1985). In rats with tumours, comparison of progestins, tamoxifen and antiprogestins shows the latter to be slightly more

active (rev. in Howell et al, 1993). Clinical studies demonstrated a partial response or stabilization of disease (12% and 46% of patients, respectively) in response to RU486 as a second or third-line treatment (Romieu et al, 1987; Klijn et al, 1989).

As progestins may act as agonists for the GR, antiprogestins may accordingly antagonize the GR resulting in antiglucocorticoid effects. Circulating estrogen levels increase as a result of increased ACTH, cortisol and the precursor in estrogen synthesis, androstenedione (rev. in Howell et al, 1993). Further research and development of antiprogestins may yield more promising agents which may provide another group of drugs that will be effective in the treatment of human breast cancer.

Others

LHRH analogues are given by injection on a monthly basis. These compounds inhibit the pituitary release of gonadotropin which in turn inhibits the stimulation of estrogen production by the ovary. LHRH agonists or antagonists are used in the treatment of premenopausal, hormone-responsive patients with metastatic breast cancer. In addition to their systemic effect, evidence suggests LHRH receptors may be expressed in neoplastic cells and directly interact with LHRH analogues (rev. in Pollak, 1996).

Prolactin receptors have been detected in 20-50% of human breast cancer samples. Stimulation of tumour growth by prolactin *in vitro* has been demonstrated, therefore, prolactin inhibitors are presently in clinical trials (rev. in Santen et al, 1990). Also in clinical trials are somatostatin analogues. These compounds may directly inhibit cell proliferation through somatostatin receptors. Indirectly, these agents have been shown to reduce

circulating EGF and IGF-I levels which normally stimulate cell proliferation (rev. in Santen et al, 1990).

Endocrine resistance

Unfortunately, with the treatment of breast cancer, "cure" is defined as the number of years of disease free survival. Even after 15 years, treatment failure is still a possibility despite the decreasing chance of recurrence over time (Bonadonna and Valgussa, 1988).

Breast tumours are believed to originate as hormone-dependent tumours then progress to hormone-independence. Some mouse mammary and rat prostatic tumours have been shown to progress from hormone-dependence to hormone-responsive but independent growth then further progress to a hormone-independent and unresponsive phenotype (Matsuzawa et al, 1983; Humphries and Isaacs, 1982; Brunner et al, 1989). A simple explanation of the mechanism by which tumours develop resistance to endocrine therapy would be the outgrowth of an ER-negative population of cells or loss of expression of the ER. This has been found in ovarian cell lines and tumours which are hormone-independent and unresponsive (Sainsbury et al, 1985; Davidson et al, 1987). However, other studies have shown that hormone-independent and unresponsive tumours do not show an altered expression of estrogen receptor upon acquisition of hormone-independence (Darbre and King, 1987; Taylor et al, 1982; Manni et al, 1980). Over 60% of resistant human breast tumours continue to express ER and PR (rev. in Osborne and Fuqua, 1994).

The question of endocrine resistance is complicated and probably involves multiple factors. Tumours that respond to second-line endocrine therapy after failure to the first are considered partially resistant, however, tumours

may also be completely resistant to hormonal therapy (rev. in Johnston et al, 1992). Resistance to endocrine therapies will be discussed further.

Research has been ongoing to determine how initially responsive tumours become resistant to endocrine therapy. As mentioned previously, tamoxifen may behave as a partial agonist in bone, the cardiovascular system (Jordan et al, 1987) and uterine tissue (Kedar et al, 1994). Additional research has shown tamoxifen-stimulated growth of cells originally responsive to the tumouristatic effect of tamoxifen. Various models and research suggest interactions between a variety of factors leading to resistance (see Table 4).

Gottardis and Jordan (1988) and Gottardis et al (1989) used an MCF-7/nude mouse model to demonstrate the acquisition of resistance following several months of tamoxifen treatment. Furthermore, tamoxifen-stimulated growth continued when the tumours were transplanted into other athymic nude mice, but growth was arrested by cessation of tamoxifen therapy. Also, tumours resistant to tamoxifen were sensitive to the growth-inhibitory effects of ICI 182,780 (Gottardis et al, 1989). One possible explanation is that tamoxifen is metabolized to some estrogenic compounds which promote tumour growth through the ER (Gottardis et al, 1989). Anecdotal evidence suggests that some patients have shown similar responses to tamoxifen, being initially responsive then stimulated by the agent. In 10-30% of patients, cessation of treatment resulted in inhibition of tumour growth (Howell et al, 1990; Caney et al, 1987). Tamoxifen is converted to a number of metabolites in the cell where 4-OH-Tam is the main active antiestrogen (see Figure 7). A metabolite of potential importance is monophenol tamoxifen or metabolite E. Although this estrogenic metabolite is found at low levels, isomerization of this compound may occur, generating a more potent compound which has been shown to stimulate cell growth (Wiebe et al, 1992). The estrogenic activity of metabolite E was also demonstrated by Jordan et al (1983) in the rat

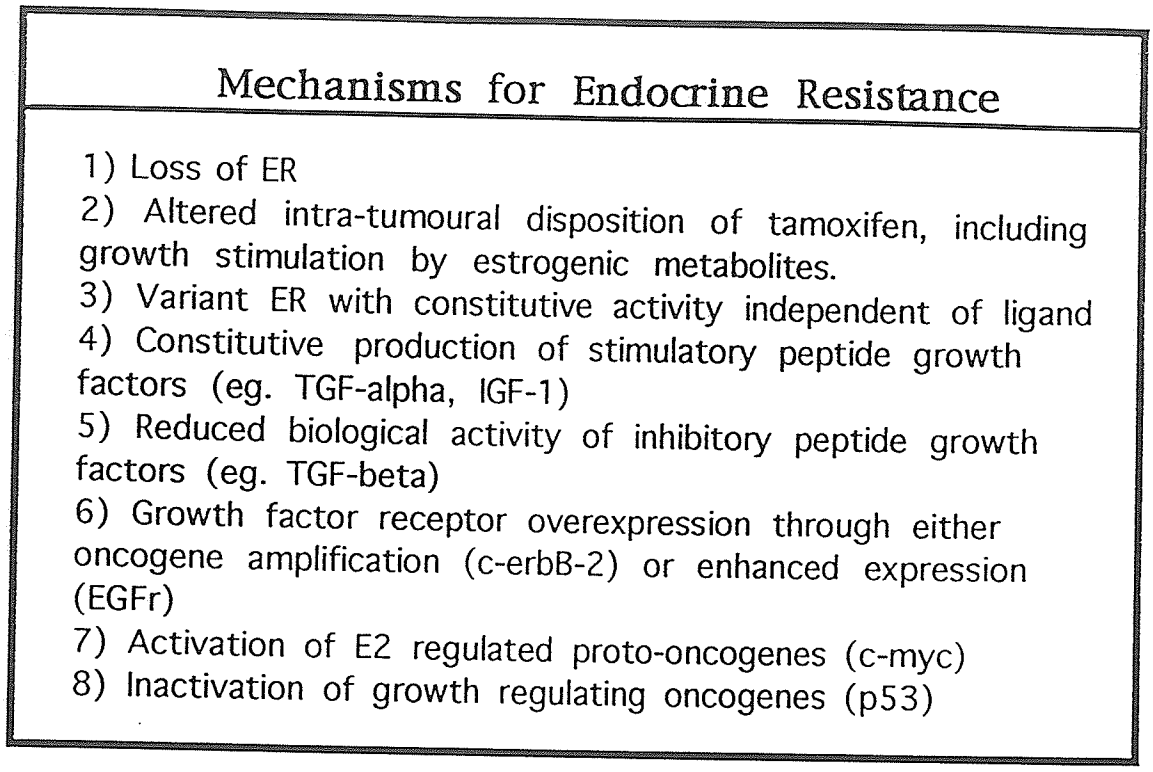


Table 4. Mechanisms for Endocrine Resistance. This figure lists mechanisms which have been proposed to result in endocrine resistance. This figure was reproduced from Johnston et al, 1992.

uterine model. Interestingly, tamoxifen stimulated growth occurred in the presence of the compound deoxytamoxifen which cannot be metabolized to metabolite E (Osborne and Fuqua, 1994). Furthermore, other studies have shown that serum metabolite E levels are not elevated in patients even when relapsing (Murphy et al, 1987). These results agree with studies showing no alteration in the metabolic profile in patients where tamoxifen and its major metabolites in the serum remained at constant levels even following several years of therapy (Fahey et al, 1989) as well as in mice (Osborne and Fuqua, 1994). These findings bring into question the validity that metabolite E is a major mechanism in the development of tamoxifen resistance.

Rather than the result of resistance, two studies suggest tamoxifen-stimulated growth is the cause of resistance (Pritchard et al, 1980; Hoogenstraten et al, 1984). Ovarian ablation in premenopausal women who developed resistance to tamoxifen resulted in tumours that regressed (Pritchard et al, 1980). However, if tamoxifen treatment was continued following ovarian ablation, regression was not seen (Hoogenstraten et al, 1984) suggesting agonistic activity of tamoxifen supported further tumour growth.

Although serum levels appear to remain constant, altered intratumoural accumulation of tamoxifen provides another possible mechanism by which tumours may develop tamoxifen-stimulated growth or resistance to tamoxifen. Multidrug resistance involves P-glycoprotein which pumps cytotoxic drugs from the cell. This protein has not been found to be overexpressed in resistant tumours although it does bind tamoxifen (rev. in Osborne and Fuqua, 1994). MCF-7 cells transplanted into nude mice which acquired tamoxifen resistance were found to have lower intratumoural levels of tamoxifen than in tamoxifen-responsive tumours (Osborne et al, 1991). Similar results were obtained from human tumours (rev. in Osborne and

Fuqua, 1994; Johnston et al, 1992). In contrast, another study found increased levels of tamoxifen in tamoxifen-resistant-stimulated tumours in MCF-7 cell xenografted athymic nude mice (Maenpaa et al, 1994). The significance of the tumour tamoxifen content experiments is questionable since the studies in mice showing tumour regression of a tamoxifen-stimulated tumour following cessation of treatment indicated even low levels of tamoxifen are capable of stimulating growth (Gottardis et al, 1989; Osborne et al, 1991), therefore, simply decreasing the intratumoural concentration of drug and metabolites may not be a plausible mechanism for resistance.

High affinity binding sites for antiestrogen have been found in ER+ and ER- breast cancer cells (Miller et al, 1986). These sites are binding sites distinct from the estrogen receptor (Sutherland and Foo, 1979; Kon, 1983; Sudo et al, 1983; Fishman, 1983; Clark et al, 1987) and do not bind estrogens (Sutherland et al, 1980). Evidence suggests that these binding proteins are not direct mediators of antiestrogen action (Miller and Katzenellenbogen, 1983; Katzenellenbogen et al, 1985; Teske et al, 1987; Sheen et al, 1985; Miller et al, 1984). Pavlik et al (1992) examined the ratio of antiestrogen binding sites (AEBS) to ER in AE resistant but estrogen sensitive LY-2 cells versus MCF-7 cells which are AE and E2 sensitive. The ratio found in the resistant cells was approximately 3 times that of the MCF-7 cells. In breast carcinoma samples, AEBS may bind AEs preventing them from acting as antiestrogens.

Tumours are heterogeneous in their cellular composition, especially with respect to ER/PR expression and hormonal sensitivity (Osborne, 1985). Selective pressure or selection of clones of cells that react to tamoxifen as an agonist may account for tamoxifen-stimulated tumour growth. A tumour containing more cells sensitive to growth-inhibition by tamoxifen will regress (Howell et al, 1990; Graham et al, 1992) whereas cells not inhibited will grow. Clonal selection and expansion has been demonstrated in ZR-75 cells

(Graham et al, 1992) and in patient tumours (Baildan et al, 1987; Horwitz, 1993). MCF-7 hormone-dependent breast cancer cells deprived of estrogens were shown to select for a population of cells which were estrogen-independent and insensitive to antiestrogens (Katzenellenbogen et al, 1987). Similarly, T47D cells maintained in estrogen-free conditions failed to express the ER, however continued to grow in the absence of hormones (Murphy et al, 1990). ER-positive ZR-75-1 cells were rendered antiestrogen resistant by random insertional mutatgenesis. An integration site for the retroviral breast cancer antiestrogen locus-1 (bcar-1) is present. These cells no longer express ER, however, cell growth may be controlled by this site resulting in antiestrogen resistance. Growth may occur in the absence of receptor via this site (Dorssers et al, 1993).

The reduction of the expression of ER in patients exposed to tamoxifen treatment has been demonstrated (Taylor et al, 1982) which, theoretically, should lead to a predominantly ER-negative tumour, however, this study found 10/14 patients who relapsed remained significantly ER-positive although these receptors may not have remained functional. In light of this and studies previously mentioned, mutations in the estrogen receptor may, therefore, provide a significant mechanism for development of tamoxifen-resistant ER-positive tumours.

Studies have shown androgen and progesterone receptors that are mutated may alter the response of cells to antagonists; responding to them as agonists (Veldsholte et al, 1990; Vegeto et al, 1992). Several ER mutants have been identified which confer the ability of cells to respond to antiestrogens as estrogen agonists. Transfection of an ER mutated in the hinge domain by insertion of 2 short synthetic alpha-helices into HeLa cells demonstrated that this mutant is constitutively active. The mutant ER binds the ERE more tightly than the wt receptor and is able to respond to the antiestrogens ICI

164,384 and trans-hydroxytamoxifen as agonists on the ERE-TATA-CAT promoter (Xing et al, 1994). The ER cloned from MCF-7 cells was demonstrated to contain a single point mutation, Gly_{400}->Val_{400}, in the hormone binding domain. This receptor has a lower affinity for ligands than the wild-type receptor (Tora et al, 1989). When stably transfected into the ER-negative breast cancer cell line MDA-MB-231 and treated with E2, 4-OH-Tam or RU39,411 (which is an antiestrogen), the cells responded to each as an agonist (Jiang et al, 1992;1993). This is similar to results observed in the MCF-7/nude mouse model of Gottardis and Jordan (1988) discussed above where tamoxifen stimulated tumour growth. Using the MCF-7/nude mouse model stimulated by tamoxifen, Wolf and Jordan (1994) identified the tamoxifen-stimulated tumour line MCF-7/MT2, which contains mutant ER. The single point mutation of Asp_{351}->Tyr_{351} is in the hormone binding domain (see Figure 9). In transfection studies of this mutant into MDA-MB-231 ER-negative cells, E2 and nonsteroidal antiestrogens act as agonists (rev. in Jordan, 1995). Other tamoxifen-stimulated tumour lines studied by Wolf and Jordan (1994), however, possessed wild-type ER suggesting mutant ERs are not the only mechanism conveying hormone resistance to tumours.

The expression of variant ER mRNA has been analyzed. Compared to the MCF-7 parent cell line, the tamoxifen resistant cell line MCF-7/TAMR-1 line was shown to express high levels of ER mRNA deleted in exon 2 and lower levels of exon 5-deleted ER mRNA (Madsen et al, 1995). If a protein was produced by the exon 2-deleted ER mRNA, only TAF-1 of the wt ER would remain since deletion of exon 2 results in a translation frame shift which introduces a translation stop signal following exon 2 (see Figure 9). Another recent study using tumour samples, however, failed to show a difference between the expression of exon 5-deleted mRNA in tamoxifen resistant tumours versus primary breast carcinomas (Daffada et al, 1995). Exon 5-

deleted mRNA has been shown to be constitutively active in a yeast expression system (Fuqua et al, 1992). Interestingly, immunoprecipitation of ER proteins from BT-20 cells, a breast cancer cell line determined to be ER-negative by ligand binding assay, and yeast using the ER-specific monoclonal antibody H226 (which recognizes the amino-terminus of ER) yielded two proteins, one corresponding to the 65 kDa wt ER protein and the other to the predicted 42 kDa truncated protein derived from the deleted-5 ER transcript. Although variant protein was not detected in MCF-7 cells which express the exon 5-deleted transcript, exon 5-deleted stably transfected MCF-7 cells expressed the variant protein at a level equivalent to the wt protein (Fuqua et al, 1992 (abstract)). These transfected cells remained estrogen-responsive, but developed resistance to tamoxifen suggesting that variant expression or overexpression may contribute to resistance (Fuqua et al, 1992 (abstract)).

Variants lacking exon 4, exon 7 as well as a double deletion of exons 4 and 7 were also identified (see Figure 9) in the tamoxifen resistant MCF-7/TAMR-1 cell line (Madsen et al, 1995). Analysis of the cDNA of ER for the presence of mutations was performed using 40 breast tumours, half resistant to tamoxifen, half sensitive to tamoxifen. Only 2/20 tumours resistant to tamoxifen had mutations. One tumour showed a 42 base pair replacement in exon 6 and the other showed no mutation in the primary tumour, however, the tamoxifen-resistant metastatic tumour contained a single bp deletion in exon 6 (Karnik et al, 1994).

The presence of ER mutations in antiestrogen resistant tumours suggests ER variants may play a role in the development of endocrine resistance, however, studies demonstrating other mechanisms suggest mutation of the ER is not the sole mechanism, but rather a contributor to the development of resistance to endocrine therapy.

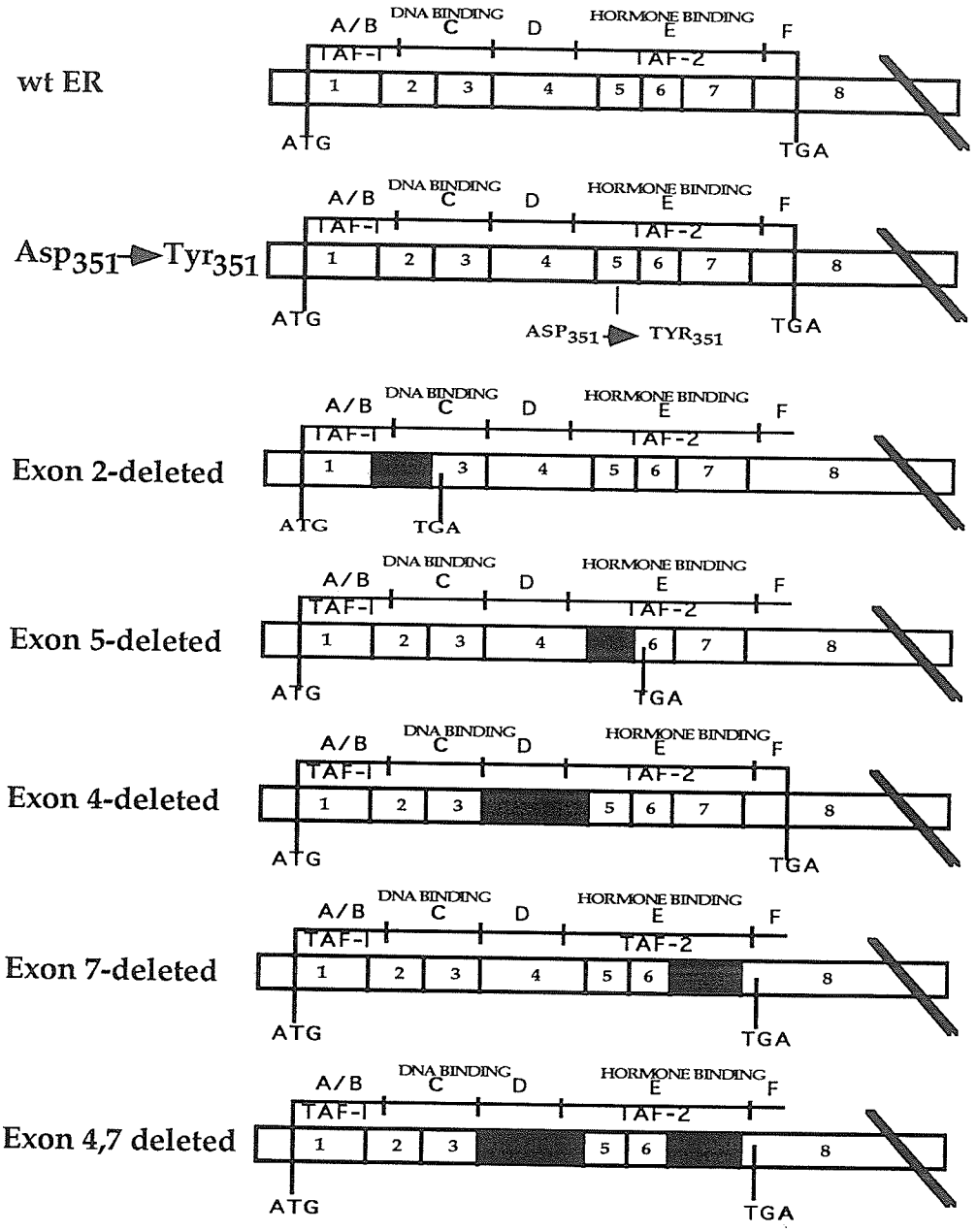


Figure 9. Variant ER mRNA detected in tamoxifen resistant tumours and cells lines. The aspartate to tyrosine substitution in the HBD was detected in MCF-7/MT2 cells (Wolf and Jordan, 1994). Exon 2-, 4-, 5- and 7-deleted as well as a double deletion of exons 4 and 7 were detected in MCF-7/TAMR-1 tamoxifen resistant cells by Madsen et al (1995). The exon 5-deleted transcript was also detected in human breast tumours, however, was not correlated to tamoxifen resistance (Daffada et al, 1995).

Tamoxifen resistance has also been studied in LY-2 cells. MCF-7 cells are estrogen-dependent, antiestrogen-sensitive human breast cancer cells. By growing these cells in media with the antiestrogen LY117018, these cells gradually developed resistance to the antiestrogenic effects of this compound, as well as 4-OH-Tam or ICI 164,384. The resistant phenotype of these cells was maintained even following 4 years of growth in the absence of antiestrogen. These cells are interesting since they continue to express the ER, albeit at a level approximately 30% of the MCF-7 parent cell line. No point mutations have been detected in the ER (Mullick and Chambon, 1990) and pS2, anestrogen-responsive gene, remains inducible in these cells (Davidson et al, 1986). The PR, however, is not expressed. Paik et al (1994) suggest that a defect in cellular pathways used by antiestrogens rather than the development of new pathways leads to endocrine resistance.

The identification of a nuclear cofactor necessary for steroid hormone ligand-dependent transcriptional activity suggests the loss of such factors may result in the presence of an ER which is unable to *trans*-activate (Yoshinaga et al, 1993). Alternatively, loss of these factors may result in the inability of cells to activate growth inhibitory pathways required for antiestrogen activity (rev. in Paik et al, 1994). Furthermore, given the cell and promoter-specific activities of antiestrogens, simply an alteration of the cofactor profile may provide LY-2 cells or breast tumours with the ability to respond to antiestrogens as estrogen agonists (Wolf and Jordan, 1994; Jordan, 1994).

Studies have revealed that although cAMP is normally decreased by tamoxifen administration, increased levels of cAMP result in a tamoxifen/ER complex that is weakly estrogenic. Fujimoto and Katzenellenbogen (1994) demonstrated the agonistic properties of tamoxifen in MCF-7 cells treated with 8-bromo-cAMP or agents which increased cAMP, isobutyl methylxanthine plus cholera toxin. The antiestrogen-ER complex was able to

activate transcription of a reporter gene with the (ERE)-TATA-CAT or $(ERE)_2$-TATA-CAT and pS2-CAT promoters (Fujimoto and Katzenellenbogen, 1994). This finding further demonstrates the cell and promoter specific activities of antiestrogens.

Given the wide range of effects tamoxifen has in the cell, many mechanisms may be interconnected and responsible for the development of tamoxifen resistance. Expression of peptide growth factors and/or receptors may be altered in tumours leading to tamoxifen resistance. Also, crosstalk between growth factor receptors or hormones and the ER signalling pathway occurs (Power et al, 1991) suggesting resistance may result from the disruption of the delicate balance between these signalling systems. The ability of TGF-α to interact with EGFr provides an autocrine loop through which estradiol can stimulate TGF-α which, in turn, interacts with EGFr to stimulate cell proliferation. When estradiol activity is blocked, for example, by addition of tamoxifen, one pathway for growth stimulation is interrupted. In hormone-independent cell lines, studies have shown TGF-α mRNA and EGFr mRNA are expressed constitutively at very high levels (Zajchowski et al, 1988). However, other studies have shown that transfection and overexpression of TGF-α in MCF-7 cells failed to confer estrogen-independent growth to the cells (Clarke et al, 1989) although tamoxifen-responsive (ie. growth-inhibited) T47D and MCF-7 cells became growth stimulated upon addition of EGF demonstrating a paracrine mechanism for peptide growth factors. Loss of regulation of these factors may provide a route for unregulated tumour growth bypassing the tumour requirement for E2 (Koga and Sutherland, 1987). Other evidence for autocrine/paracrine mechanisms of resistance has been obtained. MCF-7 cells cocultured with MDA-MB-231, a hormone-independent breast cancer cell line, developed resistance to tamoxifen and

were stimulated to grow by paracrine factors secreted by the MDA-MB-231 cells (Robinson and Jordan, 1989).

In vitro breast cancer cells are stimulated by IGF-1 which acts as a potent mitogen (Sara and Hall, 1990). However, tamoxifen causes circulating IGF-1 levels to decrease (Pollack et al, 1990). Resistance may occur by increased levels of IGF-1 in the serum or by paracrine activities of IGF-1 on adjacent cells. Xenografts of MDA-MB-231 cells in nude mice were growth inhibited when antibodies to IGF-1 receptor were used (Arteaga et al, 1989). With respect to IGF-II, overexpression conferred estrogen-independent growth *in vivo* and affected other estrogen and antiestrogen sensitive genes (Daly et al, 1991; Cullen et al, 1992).

Breast cancer cells grown *in vitro* are inhibited by the inhibitory growth factor, TGF-β. Breast cancer cells stimulated by estrogens have been shown to increase the production of TGF-β mRNA following tamoxifen treatment (Knabbe et al, 1987). In LY-2 cells, TGF-β was not found to be inducible, however the growth of LY-2 cells could be inhibited by exogenous addition of TGF-β (Knabbe et al, 1987). Unexpected results were obtained in patients where resistance to tamoxifen was accompanied by increased expression of TGF-β mRNA as opposed to the expected decrease (Thomson et al, 1991). Further research was conducted in hormone-independent cell lines where addition of exogenous TGF-β had less effect on the inhibition of cell growth than normally encountered (Sainsbury et al, 1987). This may be the result of TGF-β receptor downregulation or an alteration of the pathway through which TGF-β exerts its inhibitory effect (King and Darbre, 1989). Evidence for this was seen when IGF-1 and TGF-α, as well as elevated levels of TGF–β_1, were found in media of MCF-7 cells transfected with the v-Ha-ras oncogene (Dickson et al, 1987). These cells were estrogen-independent and antiestrogen resistant (Kasid et al, 1985).

Attention has recently been given to fibroblast growth factors (FGFs) which have been found to be amplified in approximately 20% of human breast tumours (Adnane et al, 1991). Interesting results were obtained when athymic/nude mice were implanted with MCF-7 cells which had been transfected with FGF-4. These cells were able to grow in the absence of estrogen supplementation and in the presence of tamoxifen. Futhermore, coinjection of these cells with normal MCF-7 cells was performed. Under normal conditions, the MCF-7 cells were unable to grow, however, the FGF-4 transfected cells appeared to support their growth and when FGF-4 was overexpressed, could also confer resistance to adjacent MCF-7 cells (rev. in Kern et al, 1994). Analysis of MCF-7 cells demonstrated expression of mRNA for all 4 members of the FGF receptor family and stimulation of cells by addition of exogenous FGF *in vitro* (McKleskey et al, 1994; Briozzo et al, 1991).

Although FGFs provide an additional pathway through which tumour cells may acquire resistance to endocrine therapy, further research needs to be done to determine the expression of FGFs, their receptors and the effects on breast tumours.

Increased levels of EGFr, which binds the mitogenic growth factor TGF-α, may result from transcriptional activation (Davidson et al, 1987) rather than gene amplification of the c-erbB-1 gene which encodes the EGF receptor. Increased EGFr has been observed in breast tumours (Downward et al, 1984). Many studies have demonstrated the inverse relationship between EGFr and ER which may provide a step in the progression of tumours from hormone-dependence to hormone-independence or may act as a marker of the stage of progression of the tumour.

Amplification of c-erbB-2, an oncogene coding for a transmembrane protein similar to EGFr, has been detected in human breast cancer cell lines and tumours (Van de Vijver et al, 1987) and may be at least partially

responsible for endocrine resistance when overexpressed (Wright et al, 1992). High expression of c-erbB-2 in patients following tamoxifen treatment suggests a poor prognosis and previous studies suggested tumours acquired reduced sensitivity to tamoxifen when cells were transfected with c-erbB-2 cDNA (rev. in Paik et al, 1994). ER-negative patients treated with tamoxifen prior to surgery showed decreased expression of c-erbB-2 mRNA compared to controls (LeRoy et al, 1991) while expression following tamoxifen treatment was stimulated in nude mice and ZR-75 breast cancer cells (Warri et al, 1991). LY-2 cells showed a decreased expression of erbB-2 protein rather than the predicted increase. Furthermore, examination of 900 ER+ breast cancer patients showed no correlation between erbB-2 expression and response to tamoxifen (Paik et al, 1994). However, overexpression of erbB-2 in MCF-7 cells resulted in estrogen independence and antiestrogen resistance (Pietra et al, 1995). This estrogen-independence was observed by transient transfection of ERE-CAT constructs into MCF-7 cells and treatment of the cells with heregulin (the ligand for c-erbB-2 or HER-2 receptors) in the absence of estrogen (Pietra et al, 1995). Heregulin was able to elicit an estrogen-independent activation of the ERE-CAT reporter gene which was abolished by pre-incubation of transfected cells with the pure antiestrogen, ICI 182,780. Furthermore, MCF-7 cells overexpressing HER-2 were able to activate the ERE-CAT reporter gene and the activation was enhanced by estradiol treatment (Pietra et al, 1995). The involvement of c-erbB-2 in the acquisition of breast tumour resistance to tamoxifen, therefore, remains controversial.

Additional studies have examined ligand-independent activation of the ER. Ignar-Trowbridge et al (1993) found EGF stimulated transcription of an estrogen-responsive gene from an ERE in the absence of estrogen. Using ER deletion mutant constructs, the sequences necessary for EGF activation of the ER were localised to amino acids 1-339. Interestingly, estradiol did not

activate this construct (Ignar-Trowbridge et al, 1993). Okadaic acid (a protein phosphatase 1 and 2A inhibitor) and dopamine were shown to activate an estrogen-responsive reporter gene in the absence of estrogen at a level equivalent to that induced by estradiol in monkey kidney CV1 transiently transfected cells (Powers et al, 1991). Aronica and Katzenellenbogen (1993) demonstrated estrogen-independent activation of an ERE_2-TATA-CAT reporter gene when transiently transfected immature rat uterine cells were treated with IGF, 8-bromo-cAMP or cholera toxin plus isobutylmethyxanthine. This activation was equivalent to the ligand-induced CAT activity seen upon estradiol stimulation. These studies provide further support for cross-talk between cell-signalling pathways. The tight link between various signalling systems suggests a role exists for peptide growth factor or growth factor receptor overexpression, constitutive activity or amplification of genes coding for receptors in the development of endocrine therapy resistance in breast cancer.

Loss of control of genes responsible for regulating the cell cycle may also play a role in the development of resistance to endocrine therapy. The oncogene, c-myc, a nuclear transcription factor which is regulated by estrogen in MCF-7 ER+ cells was found to be amplified in up to 32% of breast cancer cases (Escot et al, 1986). c-myc expression is decreased by tamoxifen (LeRoy et al, 1991; Santos et al, 1988), therefore, unregulated expression of c-myc may provide a pathway through which resistance may develop. Cyclin D is another factor involved in the progression of cells through the cell cycle. Cyclin D1 was placed under the control of a zinc-responsive metallothionein promoter in T47D breast cancer cells and the cells treated with zinc (Musgrove et al, 1994). In this experiment, cyclin D was shown to increase the rate of cells transitioning from G1 to S phase. Cyclin D1, therefore, is another factor which may be altered during resistance. p53 is thought to act as a tumour

suppressor gene and regulate DNA synthesis (rev. in Johnston et al, 1992). It has been suggested that the loss of an allele for p53 may result in breast cancer. Mutant p53 proteins have been detected in human bladder and lung cancers as well as melanomas and may be involved in Li-Fraumeni syndrome (rev. in Johnston et al, 1992).

The development of resistance to endocrine therapy may result from the disruption of a number of factors important in conferring hormone responsiveness to tumours. The loss of the estrogen receptor, altered intratumoural disposition of tamoxifen, the presence of variant ERs, constitutive production of stimulatory growth factors, decreased production of inhibitory growth factors, overexpression of growth factor receptors, activation of E2 regulated protooncogenes and the inactivation of growth regulating oncogenes may all provide necessary steps in the acquisition of hormone resistance. The identification of variant ERs and their possible role in hormone resistance will be further examined.

Variant ER

Variant-sized ER-like transcripts have been identified in a wide range of tissues and cell lines by Northern blotting, RNase protection assays and RT-PCR. The variants appear to occur in the presence of wild-type ER mRNA (Carruba et al, 1994; Dotzlaw et al, 1992; Koehorst et al, 1993; Pfeffer et al, 1993; Zhang et al, 1993; Castles et al, 1993) and in the absence of deletions or gross alterations (Murphy and Dotzlaw, 1989; Vacca et al, 1989) or amplification of the ER gene (Koh et al, 1989).

Identification of genetic ER mutations was previously discussed (see Endocrine Resistance section). Other mutations and genetic polymorphisms will be discussed here followed by ER-like mRNA. Transfection studies are described with each transcript and the protein structure is also discussed,

although it should be noted that these are putative protein structures. See Table 5 for an overview of identified variant ER mRNA. ER-like proteins that have been identified in breast tumours and cell lines will be discussed later.

Alterations of the ER Gene

Using RNase protection and RNase A, cleavage of single or multiple base mismatches between RNA/RNA hybrids was performed in 71 human breast tumours (Garcia et al, 1988). A silent mutation in the B domain of the estrogen receptor was detected at base 261 where there was a G->C substitution. This mismatch was discovered in a subpopulation of tumours and correlated with a low level of estrogen binding although no alterations were found in the hormone binding domain. No correlations were found between expression of the B variant (B') and progesterone binding, family history of breast cancer, or degree of tumour heterogeneity. The variant was also identified in normal human myometrium. Association of this variant gene with spontaneous abortion and hypertension has been demonstrated (Lehrer et al, 1990; 1993). In a comparison of obstetric records for breast cancer patients, 50% of women with the B variant allele had spontaneous abortions compared to 10% for women homozygous for the wt ER gene (Lehrer et al, 1990). However, this relationship was only true in women with ER-positive breast cancer and not in women with ER-negative breast cancer or women without cancer (Lehrer et al, 1993b). Also, women heterozygous for the ER B variant allele (BB') showed a higher incidence of hypertension (48% incidence) at a significantly younger age than the women in the study homozygous for the wt ER gene (13-31%) (Lehrer et al, 1993). Heterozygous women were also significantly taller (Lehrer et al, 1994). The BB' genotype

may be a marker for breast cancer susceptibility (Lehrer et al, 1993b). This allele may affect mRNA stability or translation of the generated ER mRNA.

A number of studies have also examined a *Pvu*II restriction fragment length polymorphism in the human estrogen receptor gene which results in one fragment of 0.6 kb and one of 1.6 kb on Southern blotting (Castagnoli et al, 1987; Hill et al, 1989). Hill et al (1989) localized this site to the DNA binding domain or the hormone binding domain of the ER. They also showed that absence of the 0.6 kb fragment was usually associated with no or very low ER levels although the reason for this has not been determined (Hill et al, 1989).

In studies done in human meningiomas where variant estrogen receptor transcripts have been detected, *Eco*R 1 restriction enzyme analysis of the DNA of 23 tumours showed 5 tumours to have 2 additional fragments that the others did not. These fragments were localized to the DNA-binding domain, however, these variant fragments were attributed to point mutations in the gene since no gross alterations of the ER gene was found (Vacca et al, 1989).

Variant ER mRNAs

Exon 2-Deleted Transcripts

Exon 2-deleted transcripts were detected using RNase protection and cDNA synthesis-PCR of total RNA from T47D and MCF-7 cells (Wang and Miksicek, 1991; Miksicek et al, 1993). This was done using RT-PCR to amplify ER and ER-like transcripts followed by hybridization with exon-specific radiolabeled oligonucleotides by Pfeffer et al (1995) in MCF-7 cells, neoplastic breast tissue and normal breast tissue (also found by Leygue et al, 1996). Only mild inhibition of wt ER activation of an ERE-TK-CAT reporter plasmid was seen when the wt ER construct was cotransfected into HeLa cells with the exon 2-deleted ER construct (Wang and Miksicek, 1991). This is not surprising since a frame shift mutation is introduced by the deletion of exon 2, resulting in a

Variant	Domain Deleted	Frame	Identifiers	Predicted Protein (kDa)	Tissues
delE2	a/b,c,D,E	out	2,7,11,12,13	16.3	m,T,t,N,m(r)
delE3	c	in	4,7,12	61.8	t,m,T
delE4	D,e	in	6,7,8,9,10,11,13	53.7	m,T,N,t,z,U,P,B
delE5	e	out	1,4,5,7,11,12, 13,14,15	41	m,T,N,t,z,b,H,u U,m(r)
delE6	e	out	7	61	m
delE7	e	out	3,4,7,11,12,13,15	51.8	m,T,N,t,U,u,m(r)
delE4+7	D,e	out	11,13	39.3	m,T,N
delE3-5	c,D,e	out	11	23.9	m
delE5-7	e	out	11	41.4	m
delE5+	?	?	11	?	m
delEx	?	?	11	?	m
delE2-3	b,c	?	12	?	N,B
del4/7	E	out	19	?	U,T
dupE6	E	out	17	51.4	B
dupE3,4	e,F	out	17	83.3	B
dupE6,7		in	18	80	m(2A)
clone4	c,D,E		16	24	N,B,T5,I
clone24	D,E		16	37	B

Table 5. Variant ER transcripts detected in cell lines and tissues. This figure shows some variant ER transcripts which have been identified. Other variants are discussed in the text. delE2 = exon 2-deleted transcripts, del4+7 = deletion of both exons 4 and 7, del5-7 = deletion of exons 5 to 7, del5+ = deletion of exon 5 as well as sequences from another exon, delEx=deletion of a short sequence between exons 3 and 6, delE4/7 = deletion of part of exon 4 and part of exon 7 and joining of sequences remaining from the two exons together. Completely deleted domains are in capital letters while partially deleted domains are in lower case. The cell lines and tissues examined are as follows: m = MCF-7 cells T = human breast tumours, N = normal human breast tissue, b = BT-20 cells, t = T47D cells, z = ZR75-1 cells, U = normal uterine tissue, B = rat brain tissue, P = prostate cancer cells, H = hepatocellular carcinoma, u = uterine carcinoma cells m(r) = MCF-7/TAMR-1 tamoxifen resistant breast cancer cells, m(2A) = MCF-7:2A estrogen-independent MCF-7 subline. The numbers correspond to : (1) Fuqua et al, 1991 (2) Wang and Miksicek, 1991 (3) Fuqua et at, 1992 (4) McGuire et al, 1992 (5) Castles et al, 1993 (6) Koehorst et al, 1993 (7) Miksicek et al, 1993 (8) Pfeffer et al, 1993 (9) Skipper et al, 1993 (10) Carruba et al, 1994 (11) Pfeffer et al, 1995 (12) Leygue et al, 1996 (13) Madsen et al, 1995 (14) Villa et al, 1995 (15) Hirata et al, 1995 (16) Murphy and Dotzlaw, 1989 (17) Murphy et al, 1996 (18) Pink et al, 1996 (19) Daffada et al, 1995b. This figure was adapted from Pfeffer et al (1995) and modified according to above references.

truncated 152 amino acid putative protein. Exon 2-deleted transcripts were also detected in the tamoxifen resistant cell line, MCF-7/TAMR-1, however the significance of the transcript in the development of resistance is unknown at this time (Madsen et al, 1995).

Exon 3-Deleted Transcripts

Conflicting results in transfection experiments have been reported with respect to the inhibitory properties of the protein encoded by the precise mRNA deletion of exon 3. This transcript was originally identified in T47D cells using cDNA synthesis-PCR (Wang and Miksicek, 1991) and later in MCF-7 cells (Miksicek et al, 1993), breast cancer biopsy samples and normal uterine tissue (Fuqua et al, 1993) and normal mammary tissue (Leygue et al, 1996). Exon 3 encodes the second zinc finger of the estrogen receptor, therefore, deletion of these sequences would yield a protein incapable of binding DNA (Miksicek et al 1993; Wang and Miksicek, 1991) in ER-ERE gel mobility shift assays or gel retardation assays (Fuqua et al, 1993). Both groups also agree that the exon 3-deleted protein is unable to activate transcription of an estrogen-regulated reporter gene. Using transient cotransfection into HeLa cells of the cloned deleted-3 variant and wild-type plasmids, results showed the variant interfered with wt ER transcriptional activation of an ERE-tk-CAT reporter gene. The variant reduced transcriptional activation of the wt ER by almost 70% when the variant was in a 3-fold excess of wt ER plasmid (Wang and Miksicek, 1991) and by nearly 80% when in a 10-fold excess (Miksicek et al, 1993). By mixing varying amounts of the *in vitro* translation products from wt ER and the exon 3-deleted constructs and subjecting the mixture to gel mobility shift analysis, a progressive inhibition of ER binding was observed (Wang and Miksicek, 1991) while cotranslation of the two

transcripts abolished binding in this assay (Wang and Miksicek, 1991; Miksicek et al, 1993).

Fuqua et al (1993), however, found exon 3-deleted translated products to be inactive (ie. did not inhibit ER *trans*-activation) in a yeast expression assay. This assay requires cloning of cDNA into a yeast expression vector. The variant and wt ER expression vectors were cotransformed into yeast with an estrogen-responsive reporter gene. Using wt ER cloned into a yeast vector, this system has been demonstrated to be strictly dependent upon estradiol for activity (Fuqua et al, 1992). Deleted-3 ER coexpression with wt ER did not affect wt *trans*-activation of the reporter gene even when the variant plasmid was in excess of wt ER plasmid in this assay (Fuqua et al, 1993). A GAL4 transcriptional interference assay was performed in a yeast expression system to further test the variant. By placing GAL4 and the ERE promoter sites upstream from a reporter gene such that the promoter sites overlap, only one promoter may be bound by a transcription factor at a time. Therefore, this assay measures the ability of variant ER constructs to bind to an ERE. Mild but not statistically significant inhibition of GAL4 activity by cells transformed with the deleted-3 construct was observed (Fuqua et al, 1993).

In T47D cells, Miksicek et al (1993) estimated the level of the exon 3-deleted transcript at 25% of wt ER levels implying a possible role for the transcript in altered estrogen responsiveness. However, Fuqua et al (1993) found very low levels of the deleted-3 transcript which was equivalently expressed in all ER-positive tumours suggesting it is unlikely to be related to altered estrogen responsiveness *in vivo*. Interestingly, Wang and Miksicek (1991) was the only group to find the exon 3-deleted transcript in MCF-7 cells. Pfeffer et al (1995) did not detect this deletion in cell lines and human normal and neoplastic breast tissue using RT-PCR, or Southern blot analysis. However, this group did detect a variant deleted in exons 3-5. The explanation they

suggest for this discrepancy is that Miksicek et al (1993) and Wang and Miksicek (1991) used RNase protection to analyze their samples using a probe containing parts of exons 2 and 3 which may have picked up the variant exon 3-5-deleted transcript detected by Pfeffer et al (1995). However, cDNA containing an exon 3 deletion was obtained, cloned and sequenced by Wang and Miksicek (1991).

Exon 4-Deleted Transcripts

Meningiomas are benign intracranial tumours that express high levels of PR while ER is not detected or is detected at very low levels. Koehorst et al (1992, 1993) used RT-PCR on RNA isolated from ER-/PgR+ human meningiomas to look for an ER variant capable of binding DNA which is potentially constitutively active. Overexpression of a transcript precisely deleted in exon 4 was detected in these tumours and was also expressed in MCF-7 cells (Koehorst et al, 1992, 1993). This precise deletion was also detected by Pfeffer et al (1993) in MCF-7 and ZR-75-1 ER-positive but not the ER-negative MDA-MB-231 breast cancer cell lines, by Skipper et al (1993) in normal lizard and rat tissues with high levels in the hypothalamus and trace amounts in the uterus; by Miksicek et al (1993) in T47D cells and MCF-7 cells, and by Carruba et al (1994) in PC3 human prostate cancer cells. Recent studies by Pfeffer et al (1995) confirmed the presence of this transcript in MCF-7 cells, as well as in breast tumour tissue and normal breast tissue. This study showed the exon 4-deleted mRNA to be expressed at the highest level of all expressed variants which is in contrast to Miksicek et al (1993) who found exon 3 and exon 7-deleted transcripts to be expressed at the highest level with exon 4-deleted transcripts being detected at a very low level. Pfeffer et al (1995) attribute this difference to the methods used. Miksicek et al (1993) used RNase protection whereas Pfeffer et al (1995) detected the transcript using 3

different primer pairs (located in exons 3 and 5, exons 1 and 6, and exons 3 and 8) with similar intensities obtained from each pair.

Deletion of exon 4 would result in an in-frame deletion. Therefore, a protein missing the nuclear localization signal, the hinge domain and part of the HBD (see figure 9) would be predicted. Residues encoded by exon 4 are believed to be important for binding a heat shock protein, hsp90, to form the non-DNA binding form of the ER (Chambraud et al, 1990). Deletion of these residues may result in a ligand-independently activated receptor which could account for low ER levels by classical detection, but high PR levels. Also present in exon 4 is a putative nuclear localization signal (Fawell et al, 1990), however the size of the deleted 4 protein would theoretically allow its passage through the nuclear pores by passive diffusion (Koehorst et al, 1993).

Exon 5-Deleted Transcripts

Like the unusual meningioma phenotype of ER-/PgR+, this apparently paradoxical phenotype has been identified in human breast tumours (Fuqua et al, 1991). These tumours are ER-negative by ligand binding analysis, yet express the ER-dependent gene product, PR. RT-PCR and RNase protection analysis revealed an ER transcript precisely deleted in exon 5. Although the exon 5-deletion was detected in all ER-positive tumours and 3 ER-positive cell lines (T47D, ZR-75-1 and MCF-7 cells) as well as normal uterine endometrium and endometrial cancer (Hirata et al, 1995), the overexpression of this transcript in some apparently ER-/PgR+ tumours relative to the wt transcript was found and may result in ligand-independent ER protein. A stop codon would be introduced at the 5' end of exon 6 due to the deletion of exon 5 (Fuqua et al, 1991), thereby generating a truncated protein which would not bind hormone yet be able to bind DNA. Exon 5-deleted transcripts were also expressed at a significantly higher level relative to the wild-type transcript in human breast tumours compared to normal breast tissue

(Leygue et al, 1996). Recently, this transcript was also identified by a number of other groups in the MCF-7/TAMR-1 tamoxifen resistant cell line (Madsen et al, 1995), in cancerous breast tissue (Pfeffer et al, 1995), in normal breast tissue (Pfeffer et al, 1995; Leygue et al, 1996), and in tamoxifen resistant tumours (Daffada et al, 1995) although the significance is unknown at this time.

In the yeast expression assay, the deleted-5 variant demonstrated 10-15% transcriptional activity in the absence of ligand compared to the liganded wt ER activation of an E2-responsive reporter gene. Western blotting of yeast extracts using an ER-specific antibody identified the expected 40 kDa truncated protein (Fuqua et al, 1991), however, this variant was not detected in the normal breast cell line HBL-100 (Fuqua et al, 1991) and neither the variant nor wt transcripts were detected in ER-/PgR- tumours (Fuqua et al, 1991; Zhang et al, 1993), the MDA-MB-231 ER-negative cell line (Zhang et al, 1993) or human meningiomas (Koehorst et al, 1993).

The human breast cancer cell line BT-20 is classified as ER-negative by ligand binding but expresses low levels of ER mRNA although the transcript appears to be slightly smaller than ER transcripts from ER-positive cell lines (6.3 kb vs. 6.5 kb) (Castles et al, 1993). The BT-20 cell line was found to express the deleted-5 transcript at a level 5-fold higher than the wt transcript. In a yeast expression experiment, in the absence of estradiol, the variant was 40-45% as active as the E2-activated wild type receptor. This is a higher level than determined in previous experiments, however, the discrepancy may be attributed to a greater yeast transformation efficiency of deleted-5 plasmids in this experiment, or simply due to previously non-optimized yeast expression system conditions resulting in variation between experiments. Interestingly, immunoprecipitation of ER proteins from BT-20 cells and yeast cells using the ER-specific monoclonal antibody H226 (which recognizes the amino-

terminus of ER) yielded two proteins, one corresponding to the 65 kDa wt ER protein and the other to the predicted 42 kDa truncated protein derived from the deleted-5 ER transcript. Although variant protein was not detected in MCF-7 cells which express low levels of exon 5-deleted transcript, MCF-7 cells stably transfected with an exon 5-deleted expression construct expressed the variant protein at a level equivalent to the wt protein (Fuqua et al, 1992 (abstract)). These transfected cells remained estrogen-responsive, but developed resistance to tamoxifen suggesting that variant expression or overexpression may contribute to resistance (Fuqua et al, 1992 (abstract)). This is in contrast to results observed by Rea and Parker (1996). This study also examined MCF-7 cells transfected with the exon 5-deleted construct. Although the variant had weak transcriptional activity in transient transfection assays, no effect on the expression of pS2 or PR, which are E2-responsive genes, was seen. Furthermore, this study failed to demonstrate an alteration of cell responsiveness to estrogens or antiestrogens as was seen by Fuqua et al, (1992 (abstract)). Therefore, the role of the variant in the development of resistance remains unclear.

From yeast expression assay analysis, the exon 5-deleted variant protein appears to function in a dominant positive manner. In the absence of ligand, it is capable of forming dimers, binding to DNA and constitutively activating transcription from TAF-1. When coexpressed with the wt ER, the variant may form heterodimers (Castles et al, 1993).

The exon 5-deleted transcript has also been found in hepatocellular carcinoma cells. Carcinoma cells from human males showed an increased expression of ER compared to the normal level, however, the tumours showed poor response to antiestrogen therapy. By RT-PCR, Southern blotting and hybridization analysis, carcinomas from females were found to express mostly wt ER, however, males expressed exon 5-deleted variant and wt ER

transcripts in cirrotic tissue and almost exclusively the deleted-5 variant in tumours (Villa et al, 1995).

Exon 6-deleted mRNA

Low levels of a putative exon 6-deleted ER transcript were found in MCF-7 cells. Since this transcript was not sequenced, the detection of a precisely deleted exon 6 transcript remains tentative (Miksicek et al, 1993). Pfeffer et al (1995) and Leygue et al (1997) failed to detect an exon 6-deleted transcript in the tissues they analyzed.

Exon 7-deleted mRNA

The identification of ER-like proteins in some human breast tumours which could be supershifted by antibodies to the N-terminus (H226) and C-terminus (D75) but not antibodies directed more 5' in the C-terminus (H222) in gel shift assays using an ERE led to further investigation and identification of exon-7 deleted transcripts (Fuqua et al 1992). The classification of tumours as ER+/PgR- prompted studies to identify ER-transcripts that when translated would yield proteins capable of binding estrogen but unable to activate transcription of E2-responsive genes. Using PCR, an ER transcript precisely deleted in exon 7 was detected in 2 of 23 ER+/PgR- tumours (Fuqua et al, 1992). Exon 7 deleted ER transcripts were also found in T47D, MCF-7 cells (Miksicek et al, 1993), normal uterine tissue (Fuqua et al, 1992; Hirata et al, 1995), human endometrial cancer (Hirata et al, 1995), in the tamoxifen resistant MCF-7/TAMR-1 cell line (Madsen et al, 1995), in MCF-7 cells, breast tumours (Pfeffer et al, 1995) and normal breast tissue (Pfeffer et al, 1995; Leygue et al, 1996). Comparison of 12 ER+/PR+ and 15 ER+/PR- tumours using RNase protection assays showed a significantly more abundant ratio of variant deleted-7 to wt transcripts in some ER+/PR- tumours (Fuqua et al, 1992). The deletion of exon 7 would yield a truncated protein due to a frame

shift mutation. This would result in a protein deleted in the carboxyl-terminus dimerization domain (Fawell et al, 1990) and part of the hormone binding domain.

In a yeast expression system, the deleted-7 ER plasmid was unable to activate an E2-responsive reporter gene in the presence or absence of E2. However, cotransformation of the wt ER construct and increasing amounts of the deleted-7 plasmid resulted in a progressive inhibition to almost 60% of wt E2-reporter gene activation (Fuqua et al, 1992). In a yeast expression system, a GAL4 transcriptional interference assay was performed to measure the ability of variant ER contructs to bind to an ERE. GAL4 and ERE promoters overlap such that only one promoter may be bound by a transcription factor at a time. Yeast cells transformed with the deleted-7 variant construct showed a 64% reduction of GAL4 activity in the presence or absence of hormone versus an 85% GAL4 inhibition by yeast cells transformed with the wt ER plasmid in the presence of hormone. This indicates the deleted-7 variant may exert a dominant negative effect on wt ER by competing for ERE binding sites. In contrast, the same study included gel retardation assays which showed that variant-7 deleted ER protein displayed reduced or deficient DNA binding while coexpression of wt ER and deleted-7 variant reduced wt ER binding capacity suggesting the formation of inactive heterodimers. Both wt and variant proteins were equivalently expressed when yeast extracts were analyzed by Western analysis (Fuqua et al, 1992).

Expression of exon 7-deleted ER transcripts may account for a small percentage of ER-positive tumours which do not express PR. Furthermore, elevated variant levels may interfere with normal ER function resulting in an estrogen or antiestrogen resistant tumour.

Clone 4

In our laboratory, several ER-like cDNAs have been isolated including clone 4. This ER-like transcript was first identified in human breast cancer biopsy samples using Northern blotting and its abundance led to further investigation of the transcript through cDNA cloning (Dotzlaw et al, 1992). Clone 4 has also been identified in T47D5 human breast cancer cells, Ishikawa (human endometrial carcinoma) cells (Leygue et al, 1996), normal human mammary cells (Leygue et al, 1996; 1996b) and normal human uterine tissue (Murphy et al, 1993).

Sequencing of clone 4 cDNA revealed that the 2333 bp transcript begins 6 bps 5' of the normal ER transcription start site (Green et al, 1986) and contains exons 1 and 2 from the normal ER mRNA then diverges to sequences that are similar to LINE-1 sequences (Dotzlaw et al, 1992) (see Figure 10). LINE-1 sequences are interspersed repeats of DNA sequences which contain active transposable elements. Since 10^4-10^5 copies of LINE-1 sequences are present in the human genome, the origin of the clone 4 LINE-1-like sequences has been difficult to determine. Any attempt at hybridizations with parts of the LINE-1 elements has resulted in smearing of the hybridization bands (Dotzlaw et al, 1992; Murphy et al, 1993). LINE-1 sequences may be present in intron 2 and 3 of the normal ER gene and be incorporated into the transcript through alternative splicing or may be a result of trans-splicing LINE-1 sequences from another gene to exons 1 and 2 of the normal ER transcript.

The putative clone 4 protein contains several open reading frames. The major ORF encodes 220 amino acids and would be identical to the normal ER up to residue 214 followed by an additional six unique residues. The predicted 24 kDa protein has been observed using *in vitro* transcription/translation and Western blotting of extracts from Cos-1 transiently transfected cells. However, transient transfection of clone 4 under

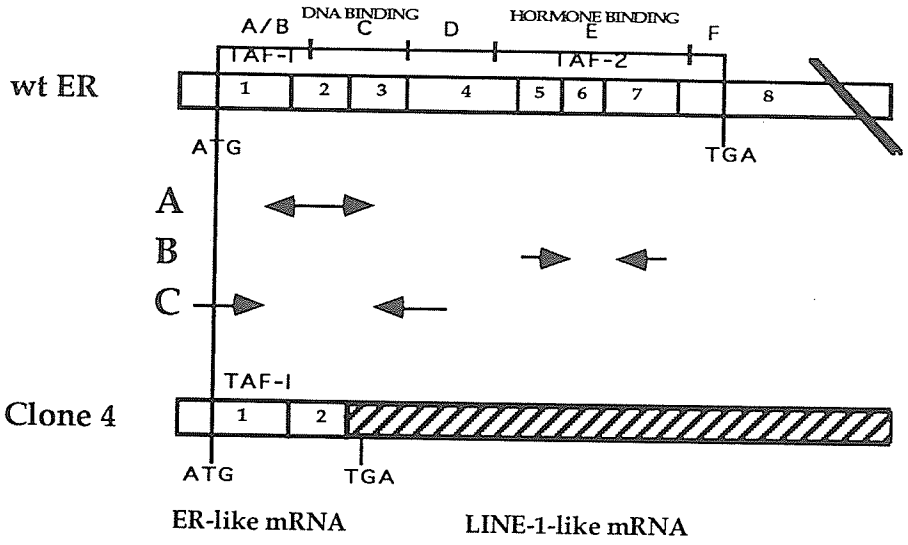


Figure 10. Clone 4 ER-like mRNA. This figure shows the exons and domains of the wt ER mRNA (top) in comparison to clone 4 ER-like mRNA (bottom). The first 2 exons of the clone 4 ER-like mRNA are identical to the wt ER sequence. However, following exon 2, the sequence of clone 4 diverges to LINE-1-like sequences (illustrated by the hatched rectangle). **A.** The riboprobe used to detect both wt (257bp) and clone 4 ER-like transcripts (302bp). **B.** The PCR primers used to detect the wt ER mRNA. **C.** The PCR primers used to detect clone 4 ER-like mRNA.

the SV-40 promoter into Cos-1 cells and MCF-7 cells failed to activate an ERE-reporter gene and was also unable to interfere with the activity of normal ER (Dotzlaw et al, 1992). The putative clone 4 protein would contain TAF-1 and the first zinc finger of the normal ER (see figure10). Previous research indicates both zinc fingers are necessary for DNA binding. Therefore, the inability of clone 4 to activate transcription of an E2-responsive reporter gene is not surprising. Clone 4, however, may interfere with normal ER function by competing for transcription factors or accessory proteins.

In a study by Murphy et al (1995), a group of 106 human breast cancer biopsy samples was subdivided according to ER and PR status (ER+/PR+, ER+/PR-, ER-/PR-). The RNA from these tumours was analyzed for the presence of wt ER and clone 4 variant transcripts by RNase protection. Significantly elevated levels of clone 4 mRNA relative to the wt ER mRNA were detected in PR-negative tumours relative to PR-positive tumours regardless of other prognostic factors. Elevated clone 4 mRNA relative to the wt ER mRNA was also detected in tumours with "poor" prognosis (tumour size>2cm, aneuploid, %S phase fraction>11%, or %S phase fraction>6.7% and diploid, 4 or more positive lymph nodes) versus "good" prognosis tumours (tumour size<2cm, diploid, %S phase fraction<6.7%, node negative) (Murphy et al, 1995). These results are consistent with those of Leygue et al (1996b) where clone 4 expression relative to wt ER mRNA expression in normal breast tissue was significantly lower than in breast tumours which were ER+, PR+, and node-negative.

Other Deletions

Other deletions within transcripts identified include a double deletion of exons 4 and 7 (Madsen et al, 1995; Pfeffer et al, 1995), an exon 2-3 double deletion (Leygue et al, 1996), exons 3-5 deletion, exons 5-7 deletion, exon 5

plus sequence from another part of the transcript, and a deletion of a small amount of sequence between exons 3 and 6 (Pfeffer et al, 1995). Daffada et al (1995b) recently identified an ER-like transcript deleted in part of exons 4 and 7 and all of exons 5 and 6. This transcript appeared to use a donor and acceptor splice site present in exon 4 and 7, respectively. The predicted protein would be homologous to the wt ER up to the novel splice site of exon 4. The remaining transcript would be translated out of frame resulting in 32 novel (non-ER) amino acids and termination at the exon 7/8 boundary due to the generation of a stop codon. This protein would lack the HBD and TAF-2, however, the DBD, NLS and TAF-1 would be unaffected. The exon 4/7-deleted transcript is believed to be tissue specific since it was detected in normal endometrium and breast cancer tissues, but not liver samples where the deleted-5 transcript was previously identified (Villa et al, 1995). Even with all of these findings, not every research group agrees with the existence of variant mRNAs (Rio et al, 1987) or their potential involvement in the progression of breast cancer from hormone-dependent to hormone-independent (Rio et al, 1987).

Exon Duplications

Exon-duplicated ER transcripts have also been identified (Murphy et al, 1996). Duplication of exon 6 was found. This transcript would encode a truncated protein with a molecular weight of 51.4 kDa since an in-frame stop codon is present in the second exon 6. The protein would be truncated in the middle of the E domain (the hormone binding domain) and, therefore, unlikely to bind hormone. The protein would also be missing the TAF-2 and the HBD dimerization domains, however, TAF-1 and the DBD dimerization domain as well as the NLS would remain intact (see figure 2). Another example of exon duplication was found in which both exons 3 and 4 were

duplicated and predicted to encode a putative protein of 83.3 kDa. This protein would not be truncated, but instead would contain a third zinc finger and part of a second hinge region. This protein and that derived from the exon 6 duplicated transcript may have some function, however, at this time, that is pure speculation since the conformation of the receptors may alter the ability of the receptor to function. Both of these exon duplicated transcripts were isolated from human breast cancer biopsy samples, however, a third exon duplicated ER-like transcript was identified in an estrogen-independent MCF-7 subline (Pink et al, 1996). The in-frame duplication of exons 6 and 7 was found following identification of an approximately 80 kDa ER-like protein in MCF-7:2A cells. This variant wil be further discussed under ER-like Proteins which follows.

Other Insertions

One insertion identified involved a 69 bp insertion of sequence between exons 5 and 6 of the wt ER mRNA. The inserted sequence is not part of the wt ER mRNA, however, the sequence may be part of intron 5 (Murphy et al, 1996). Although this insertion is in-frame between amino acids 412 and 413 of the wild type estrogen receptor, the sequence encodes 23 novel amino acids. The structure of the 68.8 kDa predicted protein may be altered which may result in an ER with altered function.

Studies examining ER transcripts in T47Dco human breast cancer cells have shown frame shift/termination mutations in ER mRNA. These transcripts would encode proteins that would be truncated in the hormone binding domain or the DNA binding domain (Graham et al, 1990). A mutant with an in-frame deletion in the hinge region and part of the hormone binding domain was also detected. This was an interesting discovery since these cells express very low levels of the ER, are hormone-indendent yet

continue to express a very high level of the estrogen-dependent gene product, PR. Graham et al (1990) also identified a number of variant transcripts. One variant was approximately 2 kb long. When analyzed, this transcript was found to contain approximately 1 kb of intron 5 upstream of exon 6. Another transcript with an insertion in exon 5 revealed the presence of sequences 70-80% homologous to the Alu repetitive elements found in humans. A variant containing exons 1, 2 and 3 of the wt ER mRNA, followed by sequences found in the 5'-UTR of the ER then 4 codons of exon 4 upstream of the wt ER exons 5-8 was also identified. The predicted protein lacks amino acids of the DBD, domain D and part of domain E (Graham et al, 1990). Karnik et al (1994) identified a 42 bp replacement in exon 6. The 42 bases are present in exon 5. This replacement generates a 5 bp deletion resulting in a frame shift which generates an ER transcript with a translation termination codon in exon 6 at amino acid 454. A truncated protein would result which is defective in the HBD and, therefore, unable to bind ligand. If translated, this protein may constitutively activate estrogen-responsive genes and induce estrogen-independent growth. This hypothesis is consistent with the observation that although the patient expressing this variant had high ER levels, this patient progressed on tamoxifen and developed resistance to the drug (Karnik et al, 1994).

ER-like Proteins

Grunder Strain mice are used as a model for progression of breast cancer from hormone-dependence to hormone-independence. Several proteins similar to the estrogen receptor have been found. Variant sized proteins (50 kDa and 35 kDa) were found in Grunder Strain mice which could bind monoclonal antibodies for the C-terminus of the estrogen receptor and could also bind hormone (Moncharmant et al, 1991). The progression to hormone

independence is accompanied by an increase in the 35 kDa ER protein and a decrease in the 50 kDa ER protein (Moncharmont et al, 1991).

Other studies in ER-positive primary breast tumours with ER expression ranging from ER-poor to ER-rich demonstrated expression of the 67 kDa ER protein, however, this protein did not bind DNA when ER-ERE binding was examined (Montgomery et al, 1993). This group suggested a post-translational modification of the estrogen receptor protein such as the formation of sulphydryl bonds between cysteine residues of the DNA binding domain zinc fingers which destroys the conformation required by the ER to bind DNA (Montgomery et al, 1993).

In a similar study, Scott et al (1991) identified 50 kDa homodimers and 67 kDa-50 kDa heterodimers which reacted to ER antibodies in Western analysis, however, the 50 kDa protein did not bind DNA. Analysis of the ER mRNA from one tumour showed an unaltered transcript suggesting that a post-translational modification was responsible for the lack of DNA binding. This is in contrast to Montgomery et al (1993) who found 40-50 kDa ER proteins which exhibited tight DNA binding.

Pink et al (1995) have identified a protein which cross-reacts with 3 ER antibodies. MCF-7 human breast cancer cells were grown in the absence of estrogens to yield the cell line MCF-7:2A. This cell line remains growth inhibited by antiestrogens, but is able to grow in the absence of estrogen. In this cell line, an 80 kDa protein has been identified which does not appear to be down-regulated to the same extent as the 66 kDa ER suggesting this protein may be involved in conferring growth responsiveness to the cells in the absence of estrogen and may also be responsible for constitutively activating ER reporter genes (Pink et al, 1995). Analysis of the MCF-7:2A cell line revealed an ER-like mRNA which contains a precise duplication of exons 6 and 7 (Pink et al, 1996). Further study showed MCF-7:2A cells contain 4-5

copies of the ER gene where 3 copies contain the wt ER gene and one contains an ER gene which has been rearranged. This rearrangement results in the inframe duplication of exons 6 and 7 producing an 80 kDa ER-like protein when transcribed and translated (Pink et al, 1996).

Generation of Variant Transcripts

Numerous studies have identified wt ER mRNA with variant ER mRNA (Koehorst et al, 1993; Pfeffer et al, 1993; Castles et al, 1993; Zhang et al, 1993; Dotzlaw et al, 1992; Carruba et al, 1994). Although it is not known at this time whether the same cells are expressing each variant transcript or if different cell populations are present, each of which expresses a different transcript, it appears that the variant transcripts are derived from the normal ER gene (Murphy et al, 1993; Murphy and Dotzlaw, 1989, Vacca et al, 1989) which is alternatively spliced.

In the case of exon deletions, the precise deletion of exons may result from the use of a splice signal located at an exon-intron boundary further upstream than the intended splice site. For example, precise deletion of exon 5 of the ER would result from the use of the 3' splice donor site of exon 4 and the 5' splice acceptor site of exon 6. In the case of non-ER sequences being present in the transcript, a splice site within an intron may be used, thereby adding intronic sequences to the normal ER transcript. The analysis of a transcript deleted within exons was performed (Daffada et al, 1995b). This transcript was found to contain exons 1-3 of the wt ER mRNA, then part of exon 4 spliced to part of exon 7 followed by exon 8. The localization of the splice sites identified novel acceptor and donor sites within exons 4 and 7, respectively, suggesting splicing of transcripts may occur at sites other than consensus wt ER splice sites (Daffada et al, 1995b). Variant transcripts may result in a truncated putative protein or, with non-ER-exonic sequences, in a transcript

containing several exons of the ER followed by novel sequences which may have been part of an intron in the ER gene. Since the introns of the ER have not been completely sequenced, the origin of incorporated non-ER-exonic sequences into transcripts remains unknown at this time.

Alternatively, *trans*-splicing may play a role in the generation of variant ER transcripts. *Trans*-splicing has been demonstrated in a vertebrate system (Sullivan et al, 1991). Exons 1-5 of the fetal rat liver androgen-binding protein (ABP) and sequences from histamine decarboxylase (HDC) mRNA were found to be spliced together using an acceptor splice site from one gene and a donor splice site from the other gene to form a hybrid transcript. The transcript encodes the first 240 amino acids of ABP and 643 residues of the HBC enzyme which would encode a novel 98 kDA protein (Sullivan et al, 1991). One mechanism suggested for generating these hybrid transcripts is the complementary base pairing of repetitive elements (eg. LINE-1 sequences) found in the introns of different RNAs allowing intermolecular splicing to occur. This would generate a hybrid transcript. This process has been examined *in vitro* (Konarska et al, 1985; Solonick, 1985). Evidence supporting this hypothesis is the identification of repetitive elements in the introns of both HDC and ABP genes (McKinnon et al, 1986; Milner et al, 1984).

In the case of exon duplications, the mechanism is unknown, however, it is believed to be different than the alternative or *trans*-splicing mechanisms proposed for exon deleted and truncated ER transcripts (Murphy et al, 1996). In the case of exon duplications detected by Murphy et al (1996), an alteration probably exists in the ER gene which is transcribed to produce the detected variant mRNA. Simultaneous expression of wt ER and exon-duplicated or inserted ER-like mRNAs in the tumours studied suggests only one allele of the ER gene is affected (Murphy et al, 1996). Alternatively, two cell

populations may be represented where the wt ER gene is expressed in one population and the altered ER gene in the other.

Analysis of the ER-like mRNA encoding the 80 kDA ER-like protein detected in a subline of MCF-7 cells yielded an ER-like transcript duplicated in exons 6 and 7 (Pink et al, 1996). The ER gene was localized to the normal position for the ER gene, which is chromosome 6, however, MCF-7:2A cells contain 4-5 copies of the ER gene. One of these copies contains the gene for the exon 6-7 duplication which was further examined. To rule out a trans-splicing mechanism in the generation of the exon 6-7 duplication, genomic PCR was performed from exon 7 to exon 8. This revealed an intron of approximately 5.9 kb between exon 7 and the duplicated exon 6 (exon 6') which may be derived from the intron preceding exon 6 (which is approximately 17 kb) or possibly from sequences added to the intron following the duplicated exon 7 (exon 7') since that intron is only about 3.5 kb. Interestingly, Pink et al (1996) suggest this intron may be the site of the gene rearrangement which resulted in the inframe duplication of exons 6 and 7. These findings suggest altered/mutated ER genes may also be involved in the generation of ER-like transcripts/proteins.

Importance of Variant Transcripts

Although there is an abundance of evidence suggesting the existence of variant ER mRNA, the translation of many of these into stable proteins remains speculative although evidence for their expression appears to be mounting. Many of these variant ER mRNAs have been cloned, subcloned, sequenced, transfected into various cell lines, *in vitro* transcribed and translated and even expressed in yeast expression assay systems. The results from these experiments show that *in vitro*, these variants can be translated and perhaps may possess activity *in vivo*.

The presence of variant-sized ER mRNAs in a wide range of tissues has led to speculation by many researchers of variant ER mRNA significance in the progression of human breast cancer from hormone dependent to hormone independence. With variant ER-like transcripts also being detected in normal tissues, the role of these transcripts is being further questioned. If abnormal/variant ER proteins are produced from the variant transcripts, these proteins may contribute to the development of hormone resistance or independence by forming non-functional homodimers or by forming non-functional heterodimers between normal and variant proteins. Although this is still hypothetical at the moment, the presence of variant transcripts in itself may play a role in the development of hormone resistance by squelching transcription factors and, therefore, interfering with normal ER transcription. The balance between the various transcripts in each tissue may be important in determining the sensitivity of each cell-type to estrogen and altering the balance between the transcripts may result in altered estrogen responsiveness.

Research Aim

The aim of this research was to address the hypothesis that alternatively spliced variant ER mRNA expression depends on the transcription of the normal ER gene. This predicts that altered transcription of the ER gene should lead to altered levels of the alternatively spliced variant ER mRNA. To do this, I have investigated the effect of various agents known to alter steady state ER mRNA levels in the estrogen receptor positive breast cancer cell line, T47D5. I compared the effect these agents had on the steady state levels of a truncated ER-like variant mRNA, clone 4, compared to wt ER mRNA levels.

Materials

Dulbecco's Modified Eagle's Medium (DMEM) and supplements used in cell culture medium were obtained from GIBCO/BRL Life Technologies Inc. (Burlington, Ontario, Canada). Phorbol 12-myristate 13-acetate (TPA) and 6α-methyl-17α-hydroxyprogesterone acetate (MPA) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Tissue culture flasks were from Corning, New York, USA and dishes from Nunclon (Canadian Life Technologies, Burlington, Ontario, Canada). Reagents used in reverse transcription were obtained from GIBCO/BRL except deoxynucleotides (also used in the PCR reactions) which were purchased from Pharmacia Biotech Inc. (Baie d'Urfe, Quebec, Canada). Uracil DNA Glycosylase from GIBCO/BRL and 2'-deoxy-uridine-5'-triphosphate from Boehringer Mannheim Canada (Laval, Quebec, Canada) were used in combination with Promega 10 x thermophilic buffer, magnesium chloride and Taq DNA polymerase (Promega Corporation, Madison, Wisconsin, USA) for PCR reactions. Radiolabelled α-^{32}P-CTP and α-^{32}P-dCTP were purchased from ICN (Costa Mesa, California, USA). Rat GAPDH cDNA insert, a gift from Dr. R. Shiu (University of Manitoba), was used for nick translation using an Amersham nick translation kit (Amersham Life Sciences, Oakville, Ontario, Canada) containing nick translation enzyme and deoxynucleotides (dATP, dGTP, dTTP). Clone 4 cDNA was subcloned into pSP72 and linearized using *Xho*I for RNase protection assay (clone 4 template) by Helmut Dotzlaw. Ambion RPA II Ribonuclease Protection Kit was purchased from Ambion, Inc. (Austin, Texas, USA). T7 RNA polymerase, nucleotides and RNase-free DNase I for RNase protection assays were obtained from Pharmacia, RNase inhibitor from Promega, spermidine for T7 buffer and salmon sperm DNA for Northern blot hybridization buffer from Sigma. BioRad Laboratories Ltd.

(Mississauga, Ontario, Canada) nucleotide sequencing gel apparatus were used for running denaturing PAGE gels.

Methods

Cell line and culture conditions

The human breast cancer cell line, T47D5 (Reddel et al, 1988) was chosen to study the regulation of clone 4 variant ER mRNA relative to wild-type ER mRNA. These cells express both transcripts at a reasonably high level as determined by RT-PCR analysis. This cell line has been shown to express very low levels of TGF-α and undetectable levels of EGF. The ER level is comparable to the level in T47D cells at approximately 85,000 ER binding sites/cell (Murphy and Dotzlaw, 1989).

Maintaining and Plating cells

T47D5 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and referred to as complete medium (CM) when supplemented with 5% (v/v) FCS, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 0.3% glucose. 150 cm^2 flasks containing cells were incubated at 37°C and 5% CO_2 and in a humidified atmosphere.

To remove cells from the monolayer in stock flasks, medium was aspirated off and 5-6 mL of Earle's/EDTA (5.3 mM KCl, 117 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO4, 5.6 mM D-glucose, 1 mM EDTA) solution was added. Gentle tapping and pipetting of solution along the sides of the flask aided in cell removal and also separation of clumped cells. The cell line was maintained by transferring 1-2 mL (approximately 3 x 10^6 cells) of cell suspension into a fresh flask containing CM.

To set up a time course experiment, cells from several flasks were pooled

in a 50 mL centrifuge tube (Corning, New York, USA), made up to 20 mL with CM and an aliquot counted with a Coulter Counter (Coulter Electronics Ltd., Ontario, Canada). The total number of cells in the centrifuge tube was calculated from the Coulter Counter corrected count multiplied by the appropriate dilution factors. Cell number was adjusted to 2-3 x 10^6 cells/25 mL of CM and 25 mL of this cell suspension was aliquoted into each 150 mm x 40 mm tissue culture dish.

Cell Treatment

Medroxyprogesterone acetate (MPA) was dissolved in absolute ethanol to a concentration of 10^{-2}M. Phorbol 12-myristate 13-acetate (TPA) was prepared in DMSO to obtain a 5 mg/mL stock. Working MPA and TPA concentrations were prepared from these stocks at 1000x the required final concentration (MPA at 10^{-5} M, TPA at 10^{-3} M).

Immediately before treating the cells (3 days after plating), media was aspirated from the 150 x 40 mm dishes and replaced with 25 mL of fresh media. Pipetting of 25 µL of the appropriate treatment or vehicle directly into the media of each dish resulted in a 1000-fold dilution of the working stock concentration giving the final MPA concentration of 10 nM and TPA of 1 µM).

Cells were harvested at the appropriate time points over 72 hours by pouring off the media, scraping the dish with a rubber policeman and pipetting cells into a 50 mL conical tube. The plate was rinsed with 1-2 mL of CM to maximize cell recovery from each dish. Cells were pelleted by centrifugation at 2500 rpm and 4°C for 4 minutes allowing excess media to be poured off and the cell pellet stored at -70°C until RNA isolation.

RNA Isolation

Total RNA was isolated from T47D5 cells using the method of Chirgwin et

al (1979). All solutions, containers and pipette tips used when dealing with RNA were autoclaved prior to use in order to denature and inactivate RNases which would degrade RNA. Frozen cell pellets were thawed in 8 mL of GITC buffer pH 7.0 (4 M GITC, 2.8 mL per litre β-mercaptoethanol (Sigma), 0.5% (w/v) N-lauroylsarcosine and 25 mM sodium citrate pH 7.0). Cells in GITC were passaged 7-10 times through a 21-gauge needle attached to a 10 mL syringe to aid cell lysis and to shear DNA. The resulting lysate was layered onto a 5.7 M CsCl/0.1 M EDTA pH 7.5 cushion in a Beckman Polyallomer Quick Seal™ centrifuge tube. The tubes were topped up with 4 M GITC buffer, heat sealed and centrifuged at 25,000 rpm for 21 hours at 22°C in a Beckman Ti 70.1 rotor to pellet the RNA. Following centrifugation, a 16-guage needle was used to make a hole in the top of the tube and a needle and syringe was inserted into the tube approximately 1/3 from the bottom to draw the supernatant containing cellular debris and proteins from the upper part of the tube. The top two-thirds of the tubes were cut off using a red hot scalpel and liquid was removed using a sterile pasteur pipette to remove excess salt solution and DNA fragments. The Quick Seal tube was cut again close to the pellet using a red hot scalpel to remove any remaining DNA or salt that may be adhered to the portion of the tube above the pellet. The pellet was transferred to a 13 mL sterile polypropylene test tube by rinsing the remaining piece of Quick Seal tube twice with 250 µL of 10 mM Tris-1 mM EDTA pH 8.0 which had been warmed to 65°C. The test tube volume was made up to 1 mL with 10 mM Tris-1 mM EDTA and the samples placed at 65°C for 15 minutes to dissolve RNA.

Precipitation of RNA was performed by addition of 70 µL 3 M NaAc pH 5.5 and 2.5 mL absolute ethanol. The samples were incubated at -20°C for a minimum of 20 minutes, then centrifuged at 11,000 rpm and 4°C for 40 minutes using a Beckman JA-17 rotor. The polypropylene tubes were drained

then dessicated in a speed-vacuum and the resulting pellet resuspended in a predetermined volume of sterile ddH_2O to give an RNA concentration of approximately 1 µg/µL and incubated at 65°C until RNA dissolved.

Total RNA was quantitated spectrophotometrically by taking a 1 in 250 dilution of each sample and reading the OD at a wavelength of 260 nm. The concentration was determined using 1 OD unit = 40 µg/mL of RNA (Sambrooke et al, 1989) and multiplying by the dilution factor of 250.

Northern Analysis

From each sample, 15 µg of total RNA was transferred into a sterile 1.5 mL microfuge tube and the volume made up to 10 µL with ddH_2O. Samples were denatured by addition of 20 µL deionized formamide, 7 µL of 37% (v/v) formaldehyde and 4 µL of 5 x GRB (1 x GRB is 40 mM MOPS pH 7.0, 10 mM NaAc pH 5.5, 1 mM EDTA pH 7.5) and incubation at 65°C for 20 minutes. The samples were placed on ice to minimize annealing and refolding into a secondary structure which may alter the migration of the RNA through the gel. Prior to loading, 4 µL of 10 x loading buffer (0.1% cyanogen bromide, 0.1% xylene blue, 50% glycerol) was added to each sample.

The samples were loaded onto a 1.5% (w/v) agarose gel containing 30 mL of 5 x GRB, 25 mL formaldehyde (37% v/v) and 7 µL ethidium bromide (25 mg/ml) and run at 120 V for 10 minutes. This was done to run the samples into the gel before submerging the gel in 1 x GRB. The gel was then covered with 1 x GRB and run at 30 V for approximately 16 hours. RNA integrity was assessed by observing the presence of the 28S and 18S ribosomal RNA bands after placing the gel on a Trans-illuminator UV box.

This procedure was also used to verify the quality of RNA isolated from T47D5 cells prior to RT-PCR and RNase Protection analysis.

Transfer of RNA

The samples were transferred from the gel to nitrocellulose (Fisher Scientific, Nepean, Ontario, Canada) in order to probe for GAPDH mRNA (Thomas, 1980). The gel was placed face down on a glass plate covered with #1 Whatman filter paper with the filter paper ends submerged in a glass dish containing 20 x SSC (1 x SSC is 0.15 M NaCl and 15 mM sodium citrate pH 7.0). The gel was overlaid with the nitrocellulose membrane, 3 layers of #1 Whatman filter paper, a stack of paper towels and a heavy bottle to promote even transfer of RNA from gel to nitrocellulose membrane by capillary action. This was left to transfer for 24 hours.

The following day, the nitrocellulose was rinsed in 3 x SSC and the location of the 28S and 18S ribosomal bands marked upon UV trans-illumination. The nitrocellulose was air dried then baked under vacuum at 80°C for 2 hours.

Hybridization with GAPDH cDNA

Nick translation of a rat GAPDH cDNA insert was performed using approximately 200 ng of DNA, 2 μL Amersham nick translation enzyme (1U DNA polymerase I and 20 pg DNaseI in a buffer with Tris-HCl pH 7.5, magnesium chloride, glycerol and bovine serum albumin), 4 μL dNTPs from nick translation kit nucleotides (100 μM of each dGTP, dATP, dTTP in a Tris-HCl pH 7.8, magnesium chloride and 2-mercaptoethanol-containing nick translation buffer) and 7 μL of α-^{32}P-dCTP (specific activity of 800 Ci/mmole) and made up to a final volume of 20 μL with ddH_2O. This reaction was incubated at 16°C for 90 minutes and terminated by addition of 25 μL of stopping buffer (0.02 M EDTA and 0.2% SDS). A Sephadex G-50 (Pharmacia) column packed in a plastic 5 mL pipette was used to separate unincorporated nucleotides from the labeled probe. Labeled cDNA probe was eluted from the

column by addition of 10 mM Tris-1 mM EDTA pH 8.

Nitrocellulose filters that had been previously baked were placed in hybridization tubes and 10 mL/blot of hybridization buffer added (50% deionized formamide, 5 x SSPE pH 7.4 (1 x SSPE is 1.15 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA), 250 µg/mL denatured salmon sperm DNA, 5 x Denhardt's reagent (1 x Denhardt's reagent is 0.2% of each Ficoll, polyvinylpyrrolidone, bovine serum albumin) and 0.1% SDS. The blots were pre-hybridized at 42°C for 2 hours following which the probe was boiled to denature the cDNA, added to the hybridization tube and left to hybridize for 24 hours at 42°C. The blot was washed twice with 2 x SSC, 0.1% SDS for 20 minutes at room temperature followed by washing at 65°C with 0.1 x SSC, 0.1% SDS until hybridized bands were easily distinguishable from the background by Geiger counter monitoring. The blots were wrapped in Saran wrap and exposed to Kodak XAR film overnight to visualize GAPDH hybridized products.

Reverse Transcription

The quantitated total RNA isolated from treated T47D5 cells was diluted with ddH_2O to generate 25-50 µL stocks of RNA at a concentration of 100 ng/µL and stored with concentrated stocks at -70°C. Samples were thawed and denatured for 3 minutes at 65°C. 1 µL (100 ng) of each RNA sample was aliquoted into a 1.5 mL microfuge tube. As controls, one tube was set up with ddH_2O instead of RNA to ensure the reaction mixture was not contaminated with RNA or DNA, and another tube set up with RNA to which reaction mixture without reverse transcriptase was added to ensure samples or solutions were not contaminated with DNA. Total RNA was reverse transcribed in a final volume of 10 µL containing 1 x First Strand Buffer diluted from 5 x buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)

provided with Gibco/BRL M-MLV Reverse Transcriptase, 10 mM DTT, also provided with the enzyme, 0.5 mM dNTPs, and 2.5 μM random hexamers (from Gibco/BRL 0.09 OD_{260} units/μL random hexamer stock in 3 mM Tris-HCl pH 7.0, 0.2 mM EDTA), 100 U/reaction of M-MLV Reverse Transcriptase (stored in 20 mM Tris-HCl pH 7.5, 1 mM DTT, 0.01% (v/v) Nonidet-P40, 0.1 mM Na_2EDTA, 0.1 M NaCl, 50% (v/v) glycerol). The reverse transcriptase was added to the mixture immediately prior to aliquoting 9 μL of the mixture into each sample and incubating the samples at 37°C for 1 hour. The reaction was stopped by boiling the samples for 3 minutes which denatured the reverse transcriptase. RT products (cDNA of total RNA) were stored at -20°C.

Polymerase Chain Reaction

Reverse transcribed samples frozen at -20°C were placed at 65°C for 3 minutes to thaw and denature RT products then placed on ice. The final volume of the RT samples was made up to 40 μL with ddH_2O. Two μL of each RT sample were amplified by PCR. The reaction mixture for each sample consisted of 1 x magnesium free thermophilic buffer from 10 x buffer supplied with Promega Taq DNA polymerase (500 mM KCl, 100 mM Tris-HCl pH 9.0, 1% Triton X-100); 200 μM each of dATP, dCTP, and dGTP; 4 mM dUTP; 2 mM $MgCl_2$ (25 mM stock); 1.1 U/reaction Taq DNA polymerase (stored in 50% glycerol, 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 1.0% Triton X-100); 0.22 U/reaction Uracil DNA Glycosylase (stored in 30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% (w/v) TWEEN 20, 50% (v/v) glycerol) and 0.5 mM of each primer. The final reaction volume was 50 μL. Two drops of mineral oil were added to each sample to prevent evaporation of reaction components.

Optimal cDNA input was determined by using 0.5 μL to 4 μL of RT products from vehicle treated control T47D5 RNA for each PCR primer pair.

The same sample was also used to determine the optimum number of cycles. For a valid comparison of PCR products within a primer set and also between primer sets, exponential amplification is necessary. During exponential amplification, proportional amplification of the template input concentration will occur. This proportional increase ceases once primers and dNTPs are no longer in excess of templates resulting in linear amplification (Bloch, 1991). Using the predetermined input, a range of cycle numbers were run with each primer set and the optimum number of cycles for each primer pair selected to analyse the rest of the experiment.

PCR Parameters

The Perkin-Elmer Thermal Cycler was used to amplify reverse transcribed products. One drop of mineral oil was added to each well of the cycler to seal the space between the heating block and reaction tube thus ensuring efficient heat transfer between the block and the tube and maintaining the reaction tubes at the programmed temperature.

Previously amplified PCR product carryover is the main source of PCR contamination (Gibbs and Chamberlain, 1989, Kwok and Higuchi, 1989). To control carryover contamination from acting as a template for PCR primers, dUTP was substituted for dTTP in every PCR reaction and the enzyme, uracil DNA glycosylase was added to the reaction mixture (Longo et al, 1990). UDG eliminates the presence of uracil residues in the DNA resulting from the deamination of cytosine which would lead to a C->T mutation upon replication. UDG removes uracil residues from DNA leaving apyrimidinic sites on an intact DNA sugar-phosphodiester backbone (Duncan, 1981). DNA polymerases replication of these abasic strands is blocked and DNA repair enzymes recruited (Friedberg et al, 1981) *in vivo*. An important note is that UDG acts only on DNA longer than 4 nucleotides and not on RNA, dUMP,

uridine or free dUTP (Duncan, 1981).

The reaction tubes were placed at 20°C for 10 minutes to allow UDG to act on any contaminating DNA. The first denaturation step was performed at 94°C for 7.5 minutes to inactivate UDG and fragment contaminating DNA and was followed by the cycling steps. These steps included 45s at 94°C for denaturation, 90s at 56°C for primer annealing and 90s at 72°C for primer elongation. This cycling continued for the predetermined number of cycles as described above. At the end of the cycling, samples were left at 75°C since UDG has been shown to retain some of its enzymatic activity at lower temperatures even after heat denaturation (GIBCO /BRL UDG product leaflet).

Polymerase Chain Reaction Primers

Primer pairs for PCR were selected using Oligo version 3.4. by analyzing computer sequences derived from a semi-automated search for oligonucleotide primers that would be stable and not hybridize to each other. The output sequences were then compared for base composition and annealing temperatures and location of priming on the cDNA. Primer data output included the number of bases of each primer, the priming location on the cDNA, the theoretical ideal annealing temperature for each primer, the primer melting temperature, the difference between melting temperatures for each primer of the pair, the relative molecular weight of each primer, and the sequences within the primer which may anneal to the other primer to form primer dimers. Primer pairs that can anneal to each other will compete for priming with the cDNA resulting in hindered annealing of primers to the template, interfering with efficient PCR amplification.

Oligonucleotide primers were synthesized at the University of Manitoba in the Department of Physiology. The dry products were dissolved in 100 μL of

ddH_2O and quantitated spectrophotometrically. A dilution of 1 in 200 was performed and the absorbance of the dissolved primer read at 260 nm. The extinction coefficients for each base is as follows: A 1.54; T 0.88; G 1.17; C 0.73. The occurence of each nucleotide in the primer sequence was determined and the total extinction coefficient calculated by multiplying the number of times the base occurred in the primer by the extinction coefficient then adding together the total extinction coefficients for each nucleotide. The absorbance of the primer at 260 nm was multiplied by the dilution factor and divided by the total extinction coefficient for the primer x 10^4 (absorbance x dilution factor / total extinction coefficient x 10^4) Each primer was diluted with sterile ddH_2O to 50 μM. To examine clone 4 ER-like transcripts, a PCR primer was designed to recognize a 17 bp sequence in exon 1 of wild-type ER cDNA and the other to bind to its complementary 18 bp sequence in the ER-unrelated sequences of clone 4 cDNA (sense/upper primer 5'-TGCCCTACTACCTGGAGAA-3' and antisense/lower primer 5'-GGCTCTGTTCTGTTCCATT-3', respectively). The correct product generated was 337 base pairs. The wild-type ER transcripts were detected by primers positioned in exon 5 and exon 7 (sense/upper primer 5'-TGCTCCTAACTTGCTCTTGG-3' and antisense/lower primer 5'-TCCAGAGACTTCAGGGTGC-3', respectively) yielding a product of 198 base pairs. Sequences in these exons were chosen to exclude two commonly occuring ER variant mRNAs, exon 5-deleted and exon 7-deleted transcripts, from being detected as wt ER mRNA. GAPDH is a housekeeping gene which was also amplified and used as a loading control. The product generated was 169 base pairs using the sense/upper primer 5'-ACCCACTCCTCCACCTTTG-3' and antisense/lower primer 5'-CTCTTGTGCTCTTGCTGGG-3'.

PCR Products

40 µL of the PCR products were transferred to a clean 1.5 mL microfuge tube. Each sample amplified for GAPDH had 4 µL of 10 x loading buffer added, while samples amplified for clone 4 or wild-type estrogen receptor transcripts had 4 µL of 50% glycerol added to them. Products were stored at -20°C until gel electrophoresis was performed.

PCR products were electrophoresed on a 2% (w/v) agarose gel in TBE (1 x TBE is 0.09 M Tris-HCl, 0.09 M Boric Acid and 2 mM EDTA). Triple loading of the wells allowed the analysis of all three transcipts at each time point. GAPDH, the smallest PCR product, was loaded first and allowed to run for 10 minutes followed by the wild-type estrogen receptor samples which were run for 20 minutes then clone 4 samples which were run for 15 minutes. 18 µL of each sample were loaded and run at 120 V with 1 x TBE as the buffer.

Visualization of results required 30 minutes post-staining of the gel in a 5 µg/mL ethidium bromide solution and ultraviolet trans-illumination.

PCR Product Analysis

A photograph of the ethidium bromide stained gel was taken. This photograph was scanned using an Apple Colour OneScanner and the scanning program Ofoto 2.0 on a MacIntosh LC III Computer. Using the program Adobe Photoshop 2.5, the scanned photograph was inverted, making the bands black and the background white. Quantitation of the bands was performed using the program Image 1.49 following subtraction of the background. For each band, the program calculated the intensity, yielding results in "scan units". These results were transferred to a Microsoft Excel 3.0 worksheet for further calculations.

In Microsoft Excel, the wild-type ER and clone 4 cDNA products were normalized for loading by dividing each value by the GAPDH value for the

corresponding sample then multiplying by 100 to obtain whole numbers. Once values had been normalized for loading, results were further normalized to their respective time 0 control, the control being 100%. This was done to determine the percent change in the clone 4 or wild-type ER transcripts over time due to treatment.

Each time course experiment was analyzed by RT-PCR 3 to 7 times to yield a mean and standard deviation for that individual time course experiment. The mean for each transcript at each time point was averaged with the means obtained from the two other time course experiments to arrive at the overall mean and standard deviation.

Ribonuclease Protection Assay

The cDNA for clone 4 was subcloned into the pBluescript (Stratagene, PDI Bioscience Inc., Aurora, Ontario, Canada) vector by Helmut Dotzlaw. This vector was cut with *Pst*I and *Kpn*I and the resulting fragment inserted into pSP72 (Promega) between SP6 and T7 promoters which can be used for sense and antisense in vitro transcription, respectively. The vector was linearized using *Xho*I and used as the template to generate an antisense labelled riboprobe by in vitro transcription. This probe could detect both wild-type and clone 4 ER-like transcripts (see Figure 7).

In vitro trancription required 1 x T7 transcription buffer (40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 10 mM DTT, 0.4 mM of each ATP, GTP, UTP, 10 µCi α-^{32}P-CTP, 10 units RNase inhibitor (stored in HEPES-KOH pH 7.6, 50 mM KCl, 8 mM DTT and 50% glycerol), 1 µg linearized DNA template and 4 units T7 RNA polymerase (stored in 20 mM potassium phosphate pH 7.7, 100 mM NaCl, 1.0 mM EDTA, 10 mM DTT, 0.1% (v/v) Triton X-100, and 50% glycerol).

The *in vitro* transcription reaction mixture was incubated at 37°C for 30-45

minutes. 10 units of DNase I (stored in 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 50% glycerol) was added to the reaction and incubated for another 15 minutes at 37°C to digest the DNA template. Addition of 20 µg of yeast transfer RNA and water brought the volume to approximately 50 µL. The labeled template was extracted with phenol / chloroform/ isopentyl alcohol (25:24:1) and transferred to a clean 1.5 mL microfuge tube in which three precipitations and resuspensions of the sample in 50 µL of ddH_2O, 200 µL of 2.5 M ammonium acetate and 750 µL of absolute ethanol were performed to remove unincorporated nucleotides. The resulting pellet was rinsed with 75% absolute ethanol/25% 0.1 M sodium acetate pH 5.5. and dessicated in a speed-vacuum. Probe elution buffer (50 µL) was added to the dry pellet and 1 µL transferred to 5 mL Ecolite + scintillation fluid (ICN Biomedicals Inc, Irvine, California, USA) to determine the activity of the probe by liquid scintillation counting. The probe was then diluted to 0.5×10^6 cpm/µL. Ambion probe elution buffer is composed of 0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS.

RNA was prepared by precipitating 15 µg total RNA isolated from T47D5 cell experiments with 7 µL 3M sodium acetate pH 5.5 and 240 µL absolute ethanol. The resulting pellet was dessicated and resuspended in 19 µL of hybridization buffer (80% deionized formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4 and 1 mM EDTA). To each sample, 1 µL of diluted probe was added, boiled for 3 minutes, mixed, quickly centrifuged then placed at 45-50°C overnight to hybridize.

The following day, a 1 in 200 dilution of RNases from the Ambion kit was added (the Ambion solution is approximately 0.5 mg/mL RNase A and 10,000 units/mL RNase T1) to each sample and incubated at 37°C for 30 minutes to digest single stranded RNA. Precipitation of samples using 300 µL of the kit RNase inactivation and precipitation solution to separate hybridized

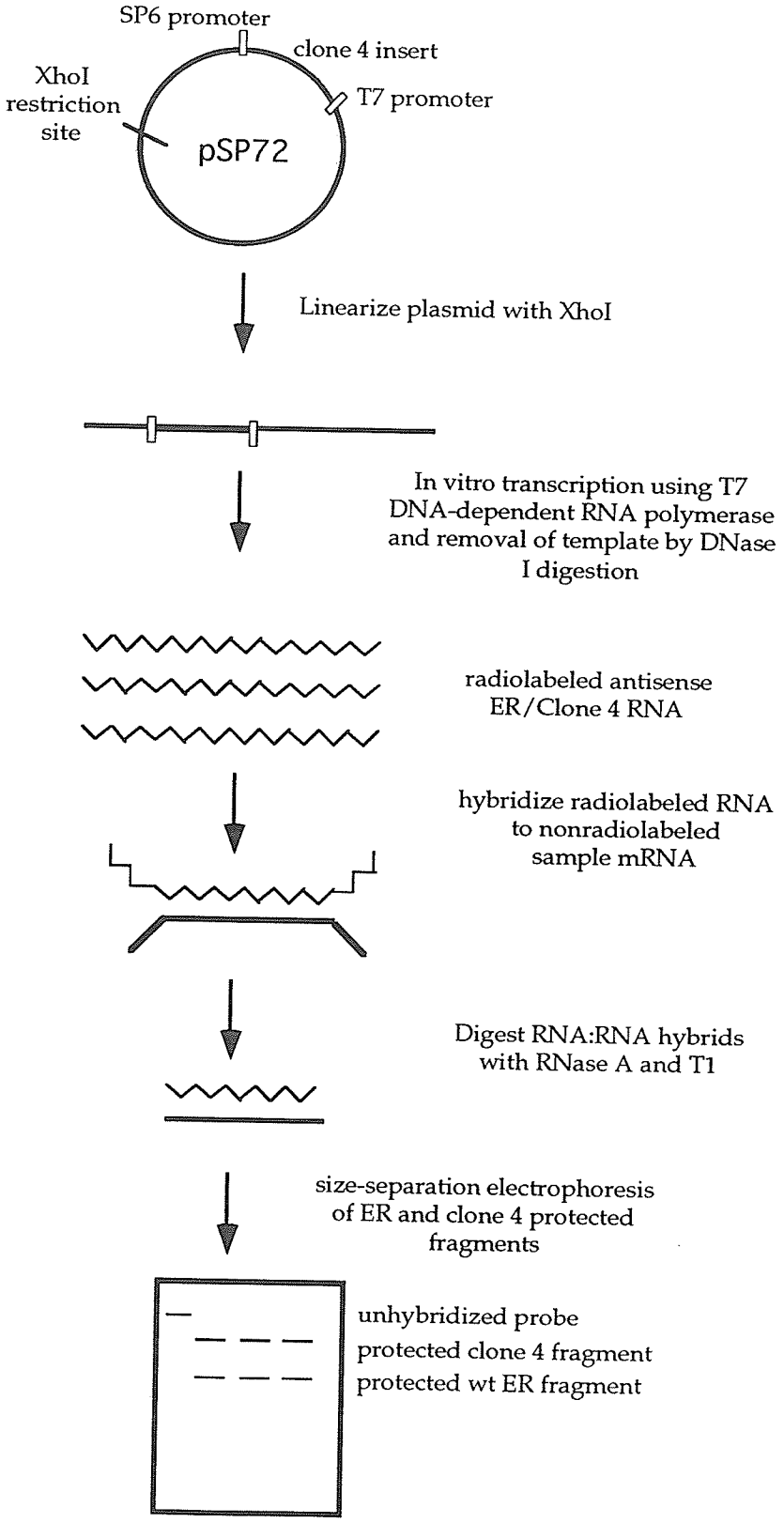


Figure 11. Schematic diagram of RNase protection assay

fragments from digested products. Following precipitation at -80°C, the tubes were centrifuged at 4°C for 15 minutes in an IEC MicroMB centrifuge. Supernatants were decanted, drained and the inside of the tube was carefully dried above the pellet using a Kimwipe to remove as much residual fluid as possible. The protected fragments were resuspended in 8 µL of loading buffer from the Ambion RNase Protection kit (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA), boiled for 3 minutes to dissolve pellet and separate copies of the probe, mixed, quickly centrifuged and loaded onto a 5% polyacrylamide-7 M urea gel.

Polyacrylamide Gels

Protected fragments were size separated by electrophoresis using a Bio-Rad sequencing gel apparatus. A 5% polyacrylamide gel was prepared with 29:1 acrylamide/bis-acrylamide, 1 x TBE and 7 M urea. Fresh gel solution was degassed using a vacuum pump to decrease the formation of air bubbles during casting. After degassing, 70 µL 25% ammonium persulphate and 50 µL TEMED were added to 50 mL of gel solution and cast immediately. The spacers used between the plates were 0.4 mm and the comb used was 0.4 mm thick with 16-wells.

The gel was allowed to polymerize for 1 hour then pre-run in 65°C pre-heated 1 x TBE at 50 watts for approximately 30 minutes to heat the gel. Prior to sample loading, wells were rinsed with a syringe to remove urea and non-polymerized acrylamide. The gel was run at 50 watts for ninety minutes.

Following electrophoresis, the gel plates were separated, the gel transferred to #1 Whatman filter paper and dried in a Bio-Rad gel dryer (80°C, 2 hours). Protected fragments were visualized by exposure of the gel to Kodak XAR film either overnight at room temperature or at -70°C for 3 nights, both with an intensifying screen. The developed film was used to locate, mark and cut

bands representing wild-type and clone 4 ER-like protected fragments on the gel. Each band was placed in a scintillation vial containing 5mL Ecolite + scintillation fluid. Counts per minute were transferred to a Microsoft Excel 3.1 spreadsheet and results expressed relative to their respective time 0 control where time 0 was given the arbitrary value of 100% and used to determine the relative change in ER and clone 4 mRNA levels due to treatment.

Each time course experiment was analyzed by RNase P 3 to 7 times to yield a mean and standard deviation for that individual time course experiment. The mean for each transcript at each time point was averaged with the means obtained from the two other separate time course experiments to arrive at the overall mean and standard deviation for each transcript at each time.

Experiments

Effect of MPA on ER mRNA and Clone 4 ER-like mRNA Steady-State Levels

Rationale

Estrogen plays a role in the proliferation of breast cancer cells, therefore, agents which affect the regulation of ER have received attention in hopes of elucidating the pathways involved in ER regulation. It is likely that clone 4 ER-like transcripts are generated by alternative splicing of the ER primary transcript, thereby requiring transcription of the ER gene. Altered transcription of the ER gene might be expected to alter the steady-state levels of variant ER mRNAs as well. At present, relatively few studies have been carried out to determine the effect of progestin treatment on ER mRNA in human breast cancer cell lines (see Table 6 for summary of studies). Research has shown that progestins downregulate ER protein and ER mRNA levels. Read et al (1989) used the synthetic progestin, R5020, to demonstrate a decrease of ER mRNA in T47D cells to 20% of control levels within 2 days of progestin exposure. Alexander et al (1990) found similar results with T47D cells treated with the synthetic progestins ORG 2058, R5020 and MPA, however, the decrease occurred after approximately 6 hours and was less marked (35-40% of control) than the decrease observed by Read et al (1989). This study also showed partial recovery of ER mRNA levels between 12 to 24 hours. Berkenstram et al (1989) used MCF-7 cells and $T47D_{co}$ (cells that contain ER but are estrogen unresponsive) and found similar results to Alexander et al (1990) using 100 nM R5020 with a decrease of ER steady-state transcript levels to approximately 45% of the control by 6 hours followed by a partial recovery. Furthermore, prior to the present study, no research has examined the regulation of clone 4 transcripts.

Cell Line	Progestin	Concentration (nM)	Results % control	Time (hr)	Group
T47D	R5020	10 nM	20%	48	Read et al (1989)
T47D & MCF-7	ORG2058 R5020 MPA	10 nM 10 nM 10 nM	35-40%	6	Alexander et al (1990)
T47Dco	R5020	100 nM	45%	6	Berkenstram et al (1989)

Table 6. Summary of research studying the effect of progestins on ER mRNA in human breast cancer cell lines. This table shows the group which studied the change in steady-state ER mRNA levels evoked by the specified progestin in the listed human breast cancer cell line.

Results

Experiments were performed to determine the effect of the synthetic progestin MPA on ER mRNA and clone 4 ER-like transcript steady-state levels in T47D5 human breast cancer cells. Cells were treated with 10 nM MPA and harvested at various times over 72 hours.

For RT-PCR experiments, clone 4 and wt ER products were normalized to GAPDH expression. GAPDH is a housekeeping gene which was also amplified by RT-PCR and used in these experiments as a loading control (see Materials and Methods). To validate the use of GAPDH as a loading control, GAPDH was examined by Northern analysis to determine if the expression of this gene was affected by MPA treatment. Figure 12A shows a photograph of the EtBr stained gel. Briefly, from a 10 nM MPA treated T47D5 cell time course experiment which had been analyzed by RNase P and RT-PCR, 10 μg of RNA was electrophoresed on a 1.5% agarose EtBr-containing gel then hybridized to nick-translated GAPDH cDNA (see Materials and Methods) and the hybridized fragments visualized by exposure to x-ray film. The two major bands seen in Figure 12A are the 28S and 18S ribosomal bands. Figure 12B shows the autoradiograph of the hybridized GAPDH fragments derived from the gel in Figure 12A following transfer of the gel to nitrocellulose and hybridization to the α-^{32}P-labeled GAPDH cDNA nick-translated probe. Following the transfer of the gel to nitrocellulose, both the gel and the nitrocellulose were examined under UV light to ensure the bands were completely transfered to the nitrocellulose. The poor quality of the photographs in Figure 12 is due to the deterioration of the photograph over time. Fixative to preserve the photo was not applied following the experiment, however, examination of the photographs upon completion of the experiments suggested variations in GAPDH expression appeared to correlate with changes in ribosomal band intensity (ie. the loading of each

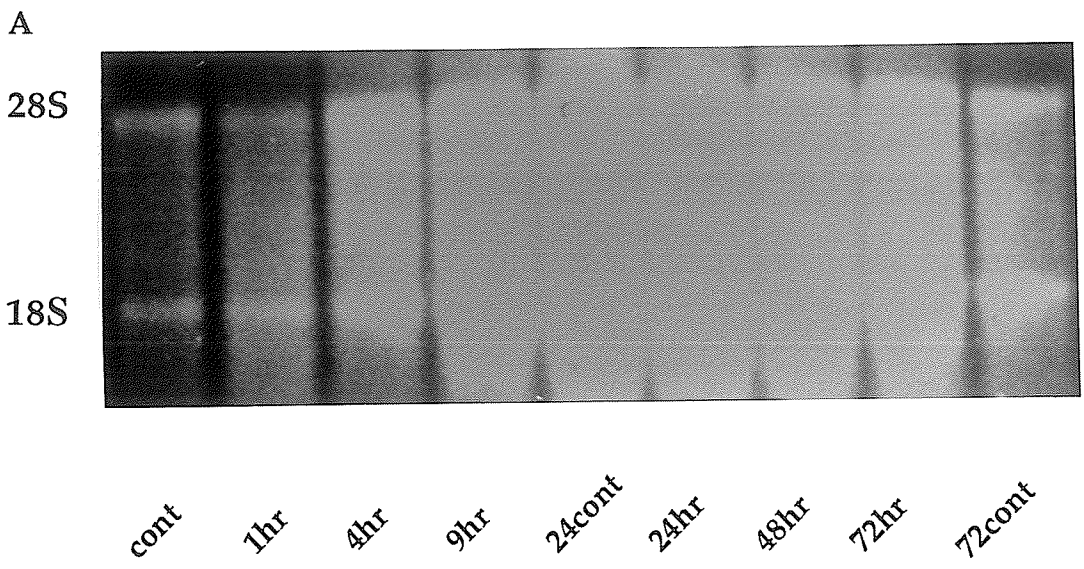
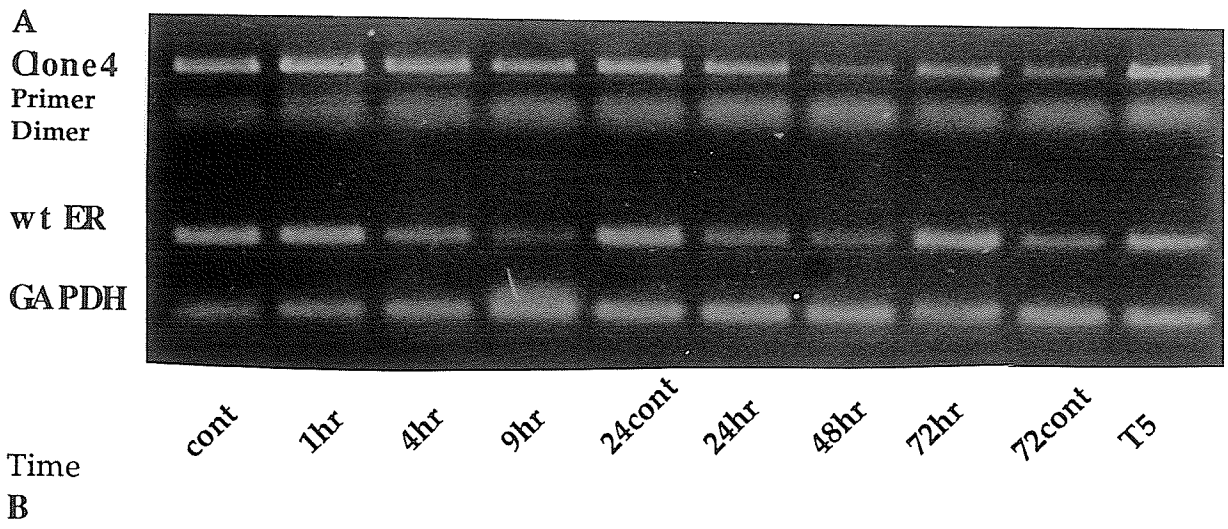


Figure 12. Effect of MPA treatment on GAPDH expression in T47D5 cells. T47D5 cells were treated with 10nM MPA and harvested at the indicated times. Total RNA was isolated and 15 μg subjected to Northern blot analysis.
A. EtBr-stained agarose gel representative of 3 separate experiments demonstrating the integrity of RNA.
B. Autoradiogram obtained following hybridization of the Northern blot from Part A to α-^{32}P-labeled GAPDH cDNA and exposure to x-ray film for 1 day at room temperature.

lane). This experiment was repeated yielding similar results. This suggests that MPA does not affect GAPDH expression.

Prior to RT-PCR analysis of the MPA-treated T47D5 time course experiment, optimal cDNA input and PCR cycle numbers were determined as described in Materials and Methods for ER, clone 4 and GAPDH primer pairs. RT-PCR analysis was performed using these parameters. Experiments were conducted as described in Materials and Methods. Following RT-PCR, 18 μL of PCR product were electrophoresed on a 2% agarose gel and post-stained with ethidium bromide. Figure 13A shows a representative experiment of ER, clone 4 and GAPDH RT-PCR products at various times after 10 nM MPA treatment. Clone 4 and ER products at each time point were normalized for loading to GAPDH (see Material and Methods). This normalized value was expressed relative to the time 0 vehicle treated control which was set to the arbitrary value of 100%. The bands labelled primer dimer on the RT-PCR gel result from the upper and lower primers of clone 4 annealing to each other. Figure 13B is a graphical representation of the mean of results for each time point calculated from 3 separate time course experiments. The ER and clone 4 transcripts remain relatively comparable to control expression until 6.5-9 hours. The lowest levels of both the wt ER and clone 4 transcripts occurred at approximately 24 hours (wt to 51% and clone 4 to 38% of controls) following treatment with 10 nM MPA. This decrease was statistically significant at the $p<0.05$ level at 24 hours for both wt ER and clone 4 transcripts when analyzed using a student's t-test. This decrease was followed by a partial recovery which occurred over the following 48 hours. These results demonstrate that the wt ER and clone 4 transcripts follow the same trend with respect to magnitude of response and response time following MPA treatment.

To validate the expression of the results relative to the time 0 vehicle treated control, additional vehicle treated controls at 12, 24 and 72 hours were



B

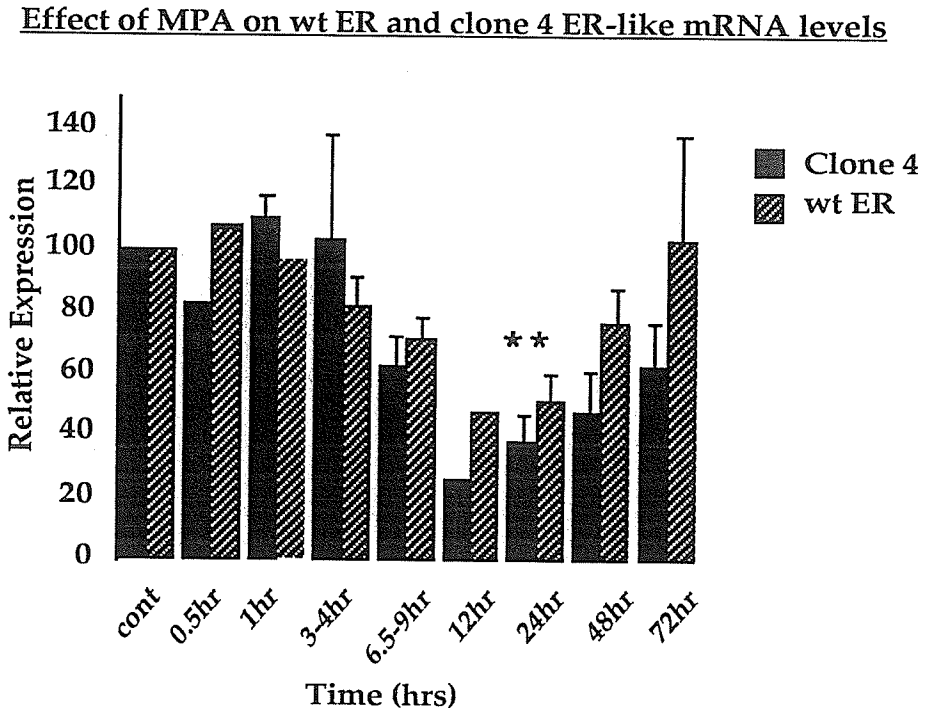


Figure 13. Time course of effect of MPA on wt ER mRNA and clone 4 ER-like mRNA steady-state levels. T47D5 cells were harvested at the indicated times following 10 nM MPA treatment. Total RNA was isolated and 100ng subjected to reverse transcription followed by PCR amplification of 1 μL of RT product .
A. An ethidium (EtBr)-stained agarose gel representative of 3 separate time course experiments where the cycle numbers for this experiment were as follows: clone 4=39, wt ER=29, GAPDH=21.
B. Histogram of RT-PCR results. Results are expressed as percentage of vehicle treated time 0 control. Mean ± standard deviation is shown where n=3 for all time points except 0.5 hours and 12 hours where n=1. * indicates a significant difference from vehicle treated control by student's t-test ($p<0.05$).

also analyzed. Figure 14 shows the results. Although slight variation is observed in control samples, expression of wt ER and clone 4 ER-like mRNAs at 24 and 72 hours were not significantly different when statistically analyzed by a student's t-test. Statistics were not performed with respect to the 12 hour control since this control was used in only one time course experiment.

To verify that a similar pattern of regulation was found using a methodology not relying on PCR amplification, the RNA obtained from T47D5 cell time course experiments was also subjected to RNase protection analysis. 15 μg of total RNA was hybridized to an α-^{32}P-labeled antisense probe which yielded protected fragments of clone 4 and wt ER mRNA (see Materials and Methods). The hybridized fragments were visualized by exposure of the 7 M polyacrylamide gel to x-ray film and the bands representing each fragment excised and quantitated by liquid scintillation counting. Figure 15A shows a representative autoradiogram of a time course experiment of T47D5 cells treated with 10 nM MPA. The mean results for each time point calculated from 3 separate experiments were plotted relative to the time 0 vehicle treated control which was set to the arbitrary value of 100%, and are shown in Figure 15B. As with RT-PCR analysis, steady-state transcript levels remained relatively comparable to control expression until 6.5-9 hours. The lowest levels of both wt ER and clone 4 transcripts occurred by approximately 24 hours (wt to 32% and clone 4 to 44% of controls). Transcripts were significantly different from the time 0 vehicle treated control at 24 hours for the wt ER and 48 hours for clone 4 at $p<0.05$ with each experiment. A slight recovery of the transcripts was observed over the remaining time course.

To validate the expression of the results relative to the time 0 vehicle treated control, expression of wt ER and clone 4 ER-like transcripts in the vehicle controls at 12, 24 and 72 hours were analyzed concurrently. The results

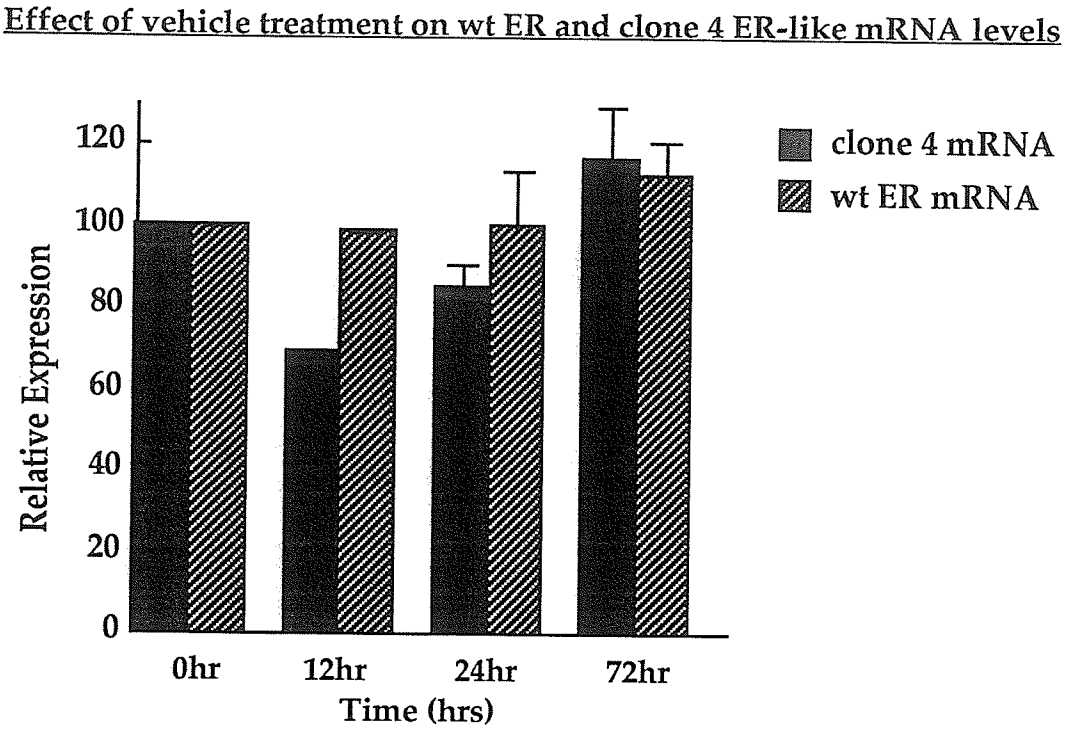


Figure 14. Effect of vehicle treatment (absolute ethanol) on steady-state levels of clone 4 ER-like mRNA and wt ER mRNA in control samples. T47D5 cells were treated with MPA vehicle treatment and harvested concurrently with MPA time course experiments (see Figure 13). Total RNA was isolated and subjected to RT-PCR analysis. Results are expressed as percent of time 0 vehicle treated control. Means $\pm$ standard deviations are shown where n=3 for all time points except 12 hours where n=1. No significant difference was found between vehicle treated control wt ER and clone 4 ER-like steady-state transcript levels when analyzed by student's t-test. PCR cycle numbers for this experiment were clone 4=37, wt ER=29, GAPDH=21.

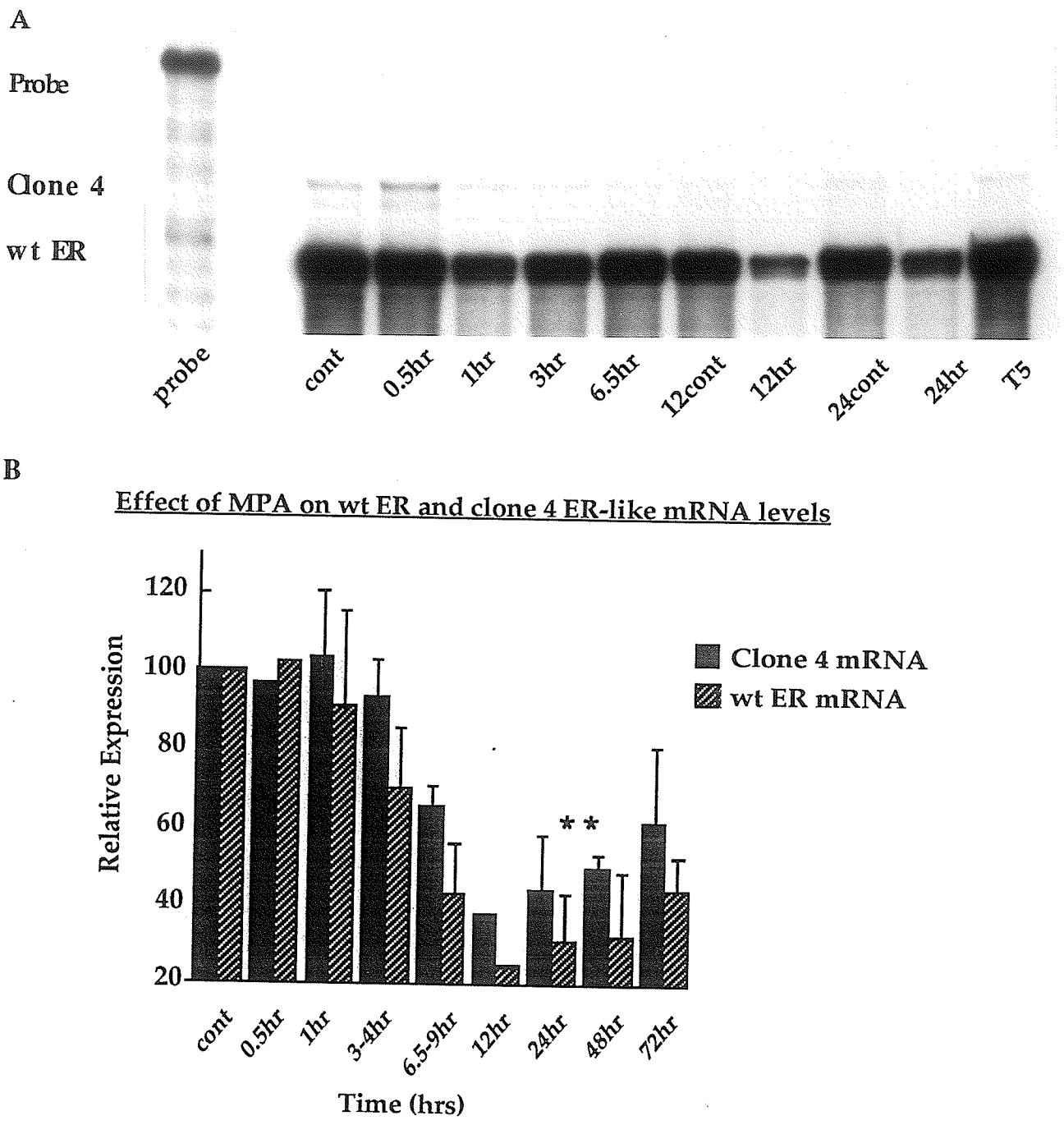


Figure 15. Time course of effect of MPA on wt ER mRNA and clone 4 ER-like mRNA steady-state levels. T47D5 cells were harvested at the indicated times following 10 nM MPA treatment. Total RNA was isolated and 15 μg subjected to RNase P analysis.
A. An autoradiogram representative of 3 separate time course experiments.
B. Histogram of RNase P results. Results are expressed as percentage of vehicle treated time 0 control. Mean ± standard deviation is shown, n=3 for all time points except 0.5 hours and 12 hours where n=1. * indicates a significant difference from vehicle treated control by student's t-test ($p<0.05$).

are shown in Figure 16. Variation was found in the controls where a statistically significant difference was found at 24 hours ($p<0.01$) for clone 4. Although MPA treated clone 4 ER-like mRNA levels were lower than the 24 hour vehicle treated control, further analysis was done. Scrutiny of data from individual experiments for this control showed high standard deviations in the results obtained from the individual experiments which were used to calculate the overall mean. The 3 individual means used to calculate the overall experimental mean were as follows: 66.85 ± 8.87, 63.26 ± 27.93, 59.40 ± 40.13. However, standard deviations calculated for individual experiments were not included in the overall calculation of the mean and standard deviation, therefore, this may have contributed to the apparent significant difference from the time 0 vehicle treated control of the final calculated mean for the 24 hour control for clone 4. Furthermore, since no significant difference was found using RT-PCR with the same samples, the significance of this difference is questionable. To further investigate the possible significance of the difference observed in the 24 hour control for clone 4, results obtained by RT-PCR and RNase P from the T47D5 time course experiments were compared. Figure 17 shows no significant difference between the two methods. The absence of a significant difference in RT-PCR controls and in the RT-PCR versus RNase P methods at 24 hours suggests the difference found in the clone 4 24 hour control by RNase P is due to experimental variation. Figure 17B shows the results for ER mRNA steady-state levels over 72 hours derived from RT-PCR versus RNase P. Statistical analysis (student's t-test) of these results show no significant difference between RT-PCR and RNase P at the $p<0.01$ level. (This was the statistical level used when examining the 24 hour control for clone 4). These results demonstrate that using the time 0 control to analyze the 72 hour MPA time course experiments is valid. Furthermore, the results obtained using RT-PCR

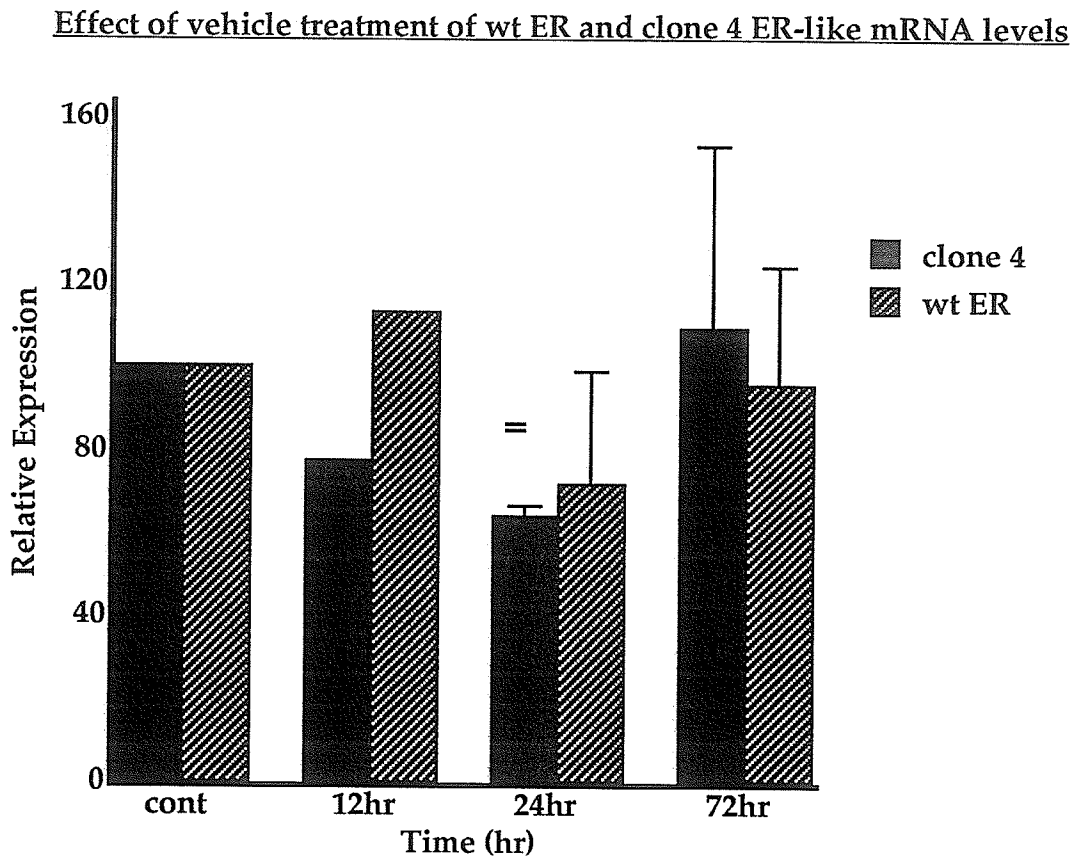
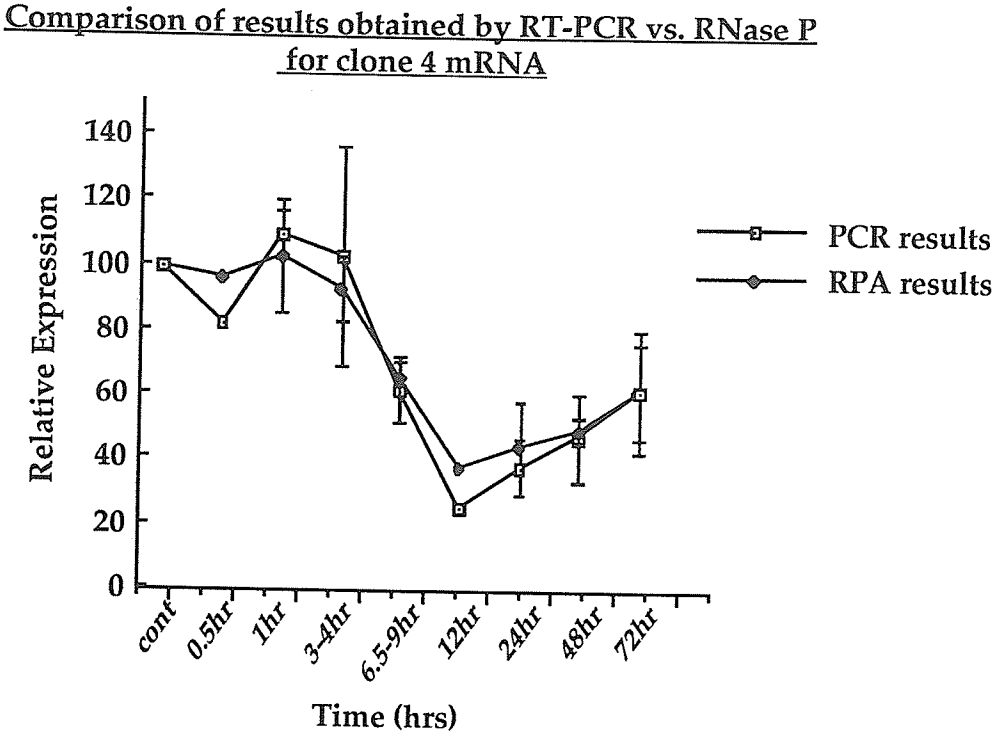


Figure 16. Effect of vehicle treatment (absolute ethanol) on steady-state levels of clone 4 ER-like mRNA and wt ER mRNA in control samples. T47D5 cells were treated with MPA vehicle treatment and harvested concurrently with MPA time course experiments (see Figure 15). Total RNA was isolated and subjected to RNase P analysis. Results are expressed percentage of vehicle treated time 0 control. Means $\pm$ standard deviations are shown where n=3 for all time points except 12 hours where n=1. A significant difference was found between time 0 controls and (=) the 24 hour clone 4 ER-like steady-state transcript levels when analyzed by student's t-test ($p<0.01$).

A



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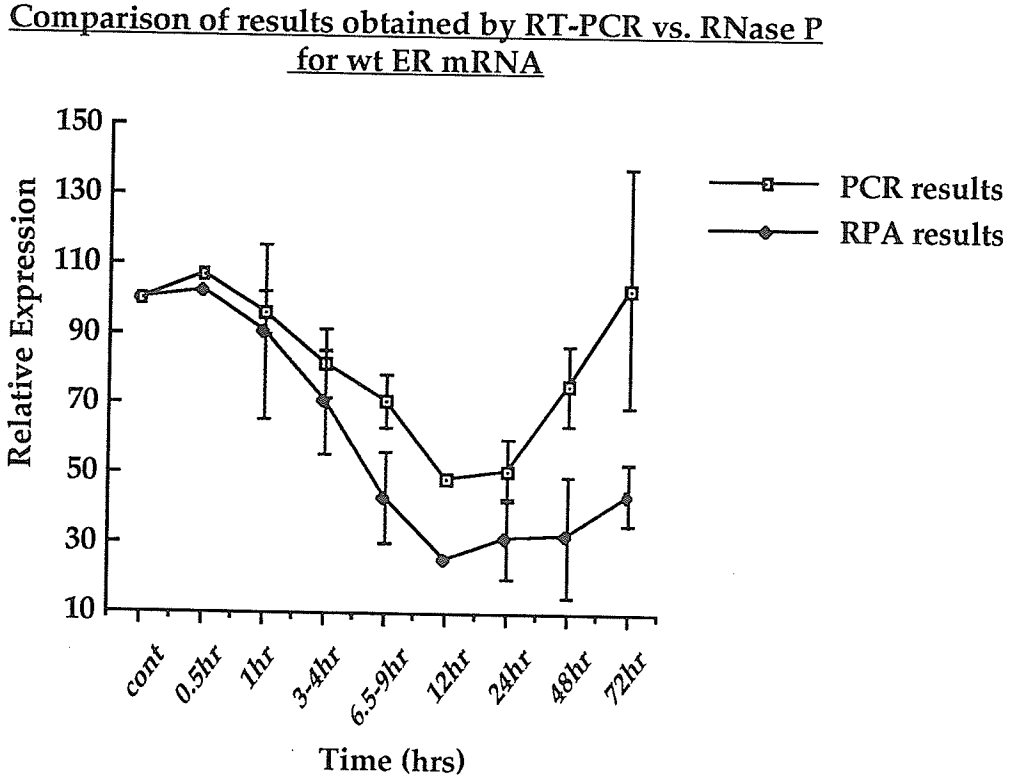


Figure 17. Comparison of results obtained by RT-PCR versus RNase P. Mean results from T47D5 cells which had been treated with 10 nM MPA and subjected to RT-PCR and RNase P analysis were compared. n=3 for all time points except 0.5 hours and 12 hours where n=1.
A. Mean (±standard deviation) RT-PCR vs. mean (± standard deviation) RNaseP results for clone 4 steady-state mRNA levels.
B. Mean (± standard deviation) RT-PCR vs. mean (± standard deviation) RNase P results for wt ER steady-state mRNA levels.

with those obtained using RNase P analysis.

To summarize, the treatment of T47D5 human breast cancer cells with 10 nM MPA resulted in a statistically significant decrease in the steady-state levels of wt ER and a parallel decrease in clone 4 ER-like mRNAs when analyzed by both RT-PCR and RNase P.

Effect of TPA on ER mRNA and Clone 4 ER-like mRNA Steady-State Levels

Rationale

Phorbol esters are tumour promoters which have been shown to affect mammalian tissues. The modulation of expression of ER mRNA by phorbol esters has been studied in human breast cancer cells. Lee et al (1989) treated MCF-7 human breast cancer cells with 10 nM TPA, a phorbol ester, and found a maximal decrease of ER mRNA steady-state levels to 15-20% of control levels between 12-24 hours following treatment. Levels remained depressed over the following 48 hours of the experiment. Saceda et al (1991) found similar results in MCF-7 cells although the maximum decrease was evident by 6 hours. Ree et al (1991; 1992) showed a decrease in steady-state levels of ER transcript levels in MCF-7 cells using 100 nM TPA. This decrease occurred by 9 hours where the transcript fell below detectable levels by Northern blot analysis. Furthermore, ER mRNA steady-state levels failed to recover over the 48 hour time course following removal of TPA at 10 hours (Ree et al, 1991). In the present study, the effect of 1 μM TPA on the steady-state levels of wt ER and clone 4 ER-like transcripts was examined in T47D5 human breast cancer cells. Cells were treated with a final concentration of 1 μM TPA or vehicle control (absolute ethanol) and harvested at appropriate time-points over 72 hours.

Results

For RT-PCR experiments, clone 4 and ER amplified products were normalized to GAPDH. GAPDH is a housekeeping gene which was also amplified by RT-PCR and used in these experiments as a loading control (see Materials and Methods). GADPH expression was examined using Northern analysis to determine if the expression of this gene was affected by TPA treatment. Figure 18A shows a photograph of the EtBr stained gel. Briefly, from a 1 μM TPA treated T47D5 cell time course experiment which had been analyzed by RNase P and RT-PCR, 10 μg of RNA was electrophoresed on a 1.5% agarose EtBr-containing gel then hybridized to nick-translated GAPDH cDNA (see Materials and Methods) and the hybridized fragments visualized by exposure to x-ray film. The two major bands seen in Figure 18A are the 28S and 18S ribosomal bands. Figure 18B shows the autoradiogram of the hybridized α-^{32}P-labeled GAPDH fragments derived from the gel in Figure 18A following transfer of the gel to nitrocellulose and hybridization to GAPDH cDNA nick-translated probe. Following the transfer of the bands to nitrocellulose, both the gel and the nitrocellulose were examined under UV light to ensure the bands were completely transfered to the nitrocellulose. The poor quality of the photographs in Figure 18 is due to the deterioration of the photograph over time. Fixative to preserve the photo was not applied following the experiment, however, examination of the photographs upon completion of the experiments suggested variations in GAPDH expression generally appeared to correlate with changes in ribosomal band intensity (ie. the loading of each). This experiment was repeated yielding similar results. Therefore, TPA does not appear to affect GAPDH expression. This is in contrast to one study which found a 10-fold increase in GAPDH expression over 24 hours following TPA treatment (Bohm et al, 1990), however, agrees with another study which detected no significant change in GAPDH

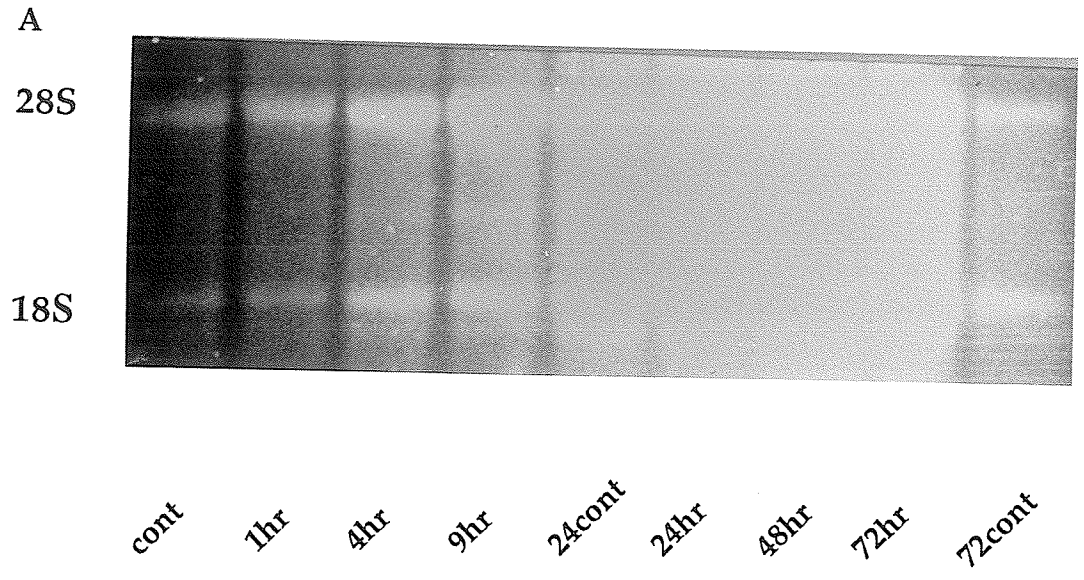
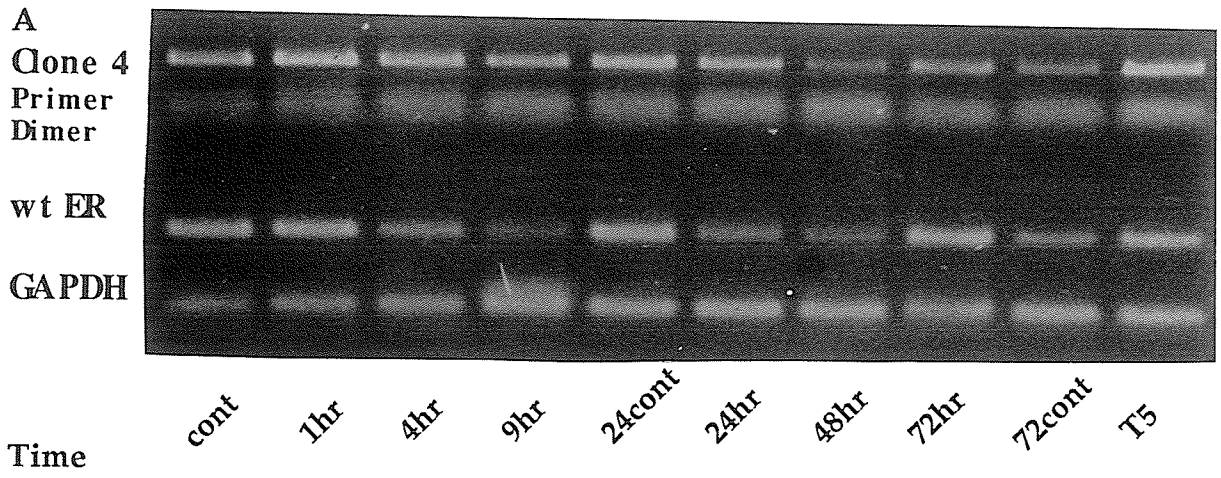


Figure 18. Effect of TPA treatment on GAPDH expression in T47D5 cells. T47D5 cells were treated with 1μM TPA and harvested at the indicated times. Total RNA was isolated and 15 μg subjected to Northern blot analysis.
A. EtBr-stained agarose gel representative of 3 separate experiments demonstrating the integrity of RNA.
B. Autoradiogram obtained following hybridization of the Northern blot from Part A to α-^{32}P-labeled GAPDH cDNA and exposed to x-ray film for 1 day at room temperature.

expression following TPA treatment (Zhu et al, 1991).

Prior to RT-PCR analysis of the TPA-treated T47D5 time course experiment, optimal cDNA input and PCR cycle numbers were determined as described in Materials and Methods for ER, clone 4 and GAPDH primer pairs. RT-PCR analysis was performed using these parameters. Experiments were conducted as described in Materials and Methods. Following RT-PCR, 18 μL of PCR product were electrophoresed on a 2% agarose gel and post-stained with ethidium bromide. Figure 19A shows a representative experiment of ER, clone 4 and GAPDH RT-PCR products at various times after 1 μM TPA treatment. As with MPA experiments, primer-dimers are labelled on the RT-PCR gel. Clone 4 and wt ER products were normalized for loading to GAPDH, a housekeeping gene which was also amplified by RT-PCR and used as a loading control in these experiments. Products at all points were expressed relative to the time 0 vehicle treated control which was set to the arbitrary value of 100%. Figure 19B is a graphical representation of the mean of results for each time point from 3 separate time course experiments. The steady-state levels of wt ER and clone 4 transcripts fell quickly relative to the vehicle treated control following treatment with TPA. The lowest levels of wt ER transcripts occurred at 24-48 hours following TPA treatment, while clone 4 transcripts reached the lowest level at 48-72 hours (wt to 55% and clone 4 to 53-56% of controls) although this change was not found to be statistically significant. This change in steady-state transcript levels remained relatively constant over the remaining time course. These results demonstrate that the wt ER and clone 4 transcripts follow the same trend with respect to magnitude of response and response time following TPA treatment.

To validate the expression of the results relative to the time 0 vehicle treated control, expression of wt ER and clone 4 ER-like transcripts in the vehicle controls at 24 and 72 hours were concommitantly analyzed. Figure 20



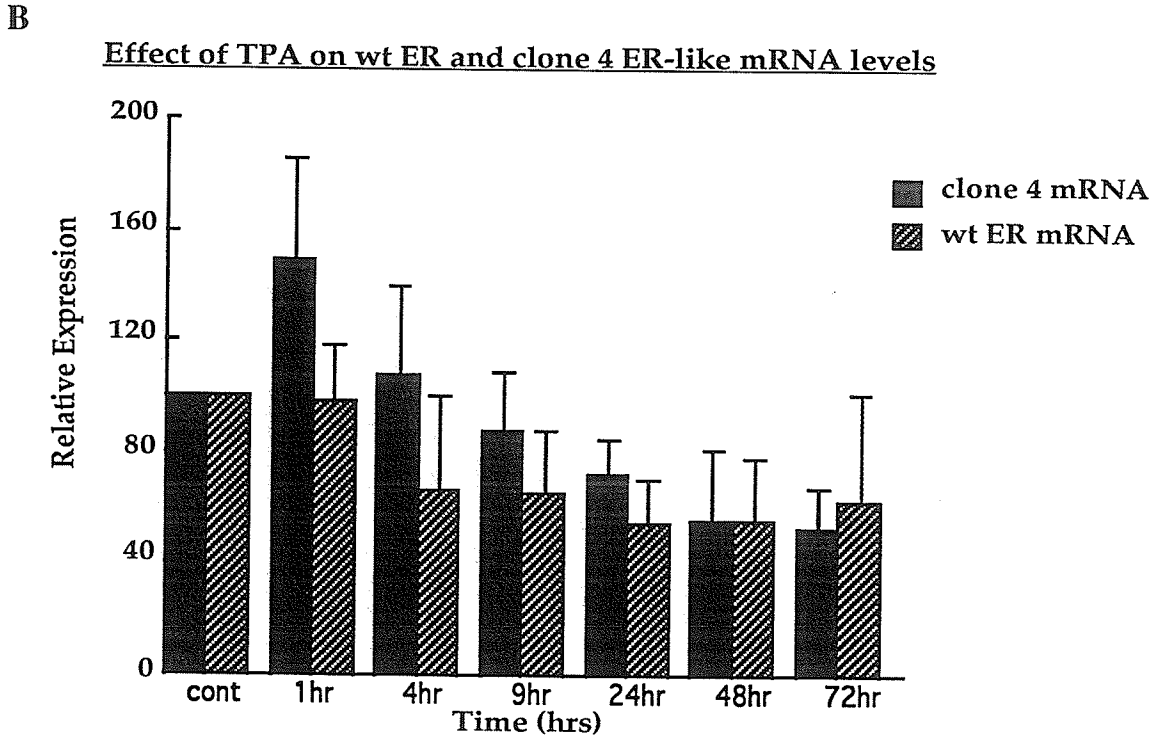


Figure 19. Time course of effect of TPA on wt ER mRNA and clone 4 ER-like mRNA steady-state levels. T47D5 cells were harvested at the indicated times following 1 μM TPA treatment. Total RNA was isolated and 100ng subjected to reverse transcription followed by PCR amplification of 1 μL RT products.
A. An ethidium (EtBr)-stained agarose gel representative of 3 separate time experiments where the cycle numbers were for this experiment were as follows: clone 4=37, wt ER=27, GAPDH=21.
B. Histogram of RT-PCR results. Results are expressed as percentage of vehicle treated time 0 control. Means ± standard deviations are shown where n=3 for all time points.

shows the results. Although slight variation is observed in control samples, wt ER and clone 4 ER-like mRNA levels at 24 and 72 hours were not significantly different when statistically analyzed by a student's t-test.

To verify that a similar pattern was found using a methodology not relying on PCR amplification, the RNA obtained from T47D5 cell time course experiments was also subjected to RNase P analysis. 15 μg of total RNA was hybridized to an α-^{32}P- labeled probe which yielded protected fragments of clone 4 and wt ER mRNAs (see Materials and Methods). The hybridized fragments were visualized by exposure of the 7 M polyacrylamide gel to x-ray film and the bands representing each fragment excised and quantitated by liquid scintillation counting. Figure 21A shows a autoradiograph of a time course experiment representative of the three T47D5 cell experiments treated with 1 μM TPA. The mean results for each time point calculated from 3 separate experiments were plotted relative to the time 0 vehicle treated control which was arbitrarily set to 100%. Results are shown in Figure 21B. As with RT-PCR analysis, transcript levels fell quickly following the treatment of cells with TPA. The lowest levels of both wt ER and clone 4 transcripts occurred by 24-48 hours (wt to 37-40% and clone 4 to 46% of controls). Transcripts were significantly different at 4 hours and 24 hours ($p<0.005$) for ER and 24 hours for clone 4 at $p<0.025$. No partial recovery was seen over the remaining time course.

A number of points from individual RNase P experiments were not included in the calculation of the mean for the steady-state levels of wt ER and clone 4 ER-like transcripts at each time point following TPA treatment. These are shown in Table 7. These points from individual experiments were apparent outliers when compared to results of repeat experiments within the same time course experiment when z-test analysis was done. These points were determined to fall at least 1.15 standard deviations from the mean.

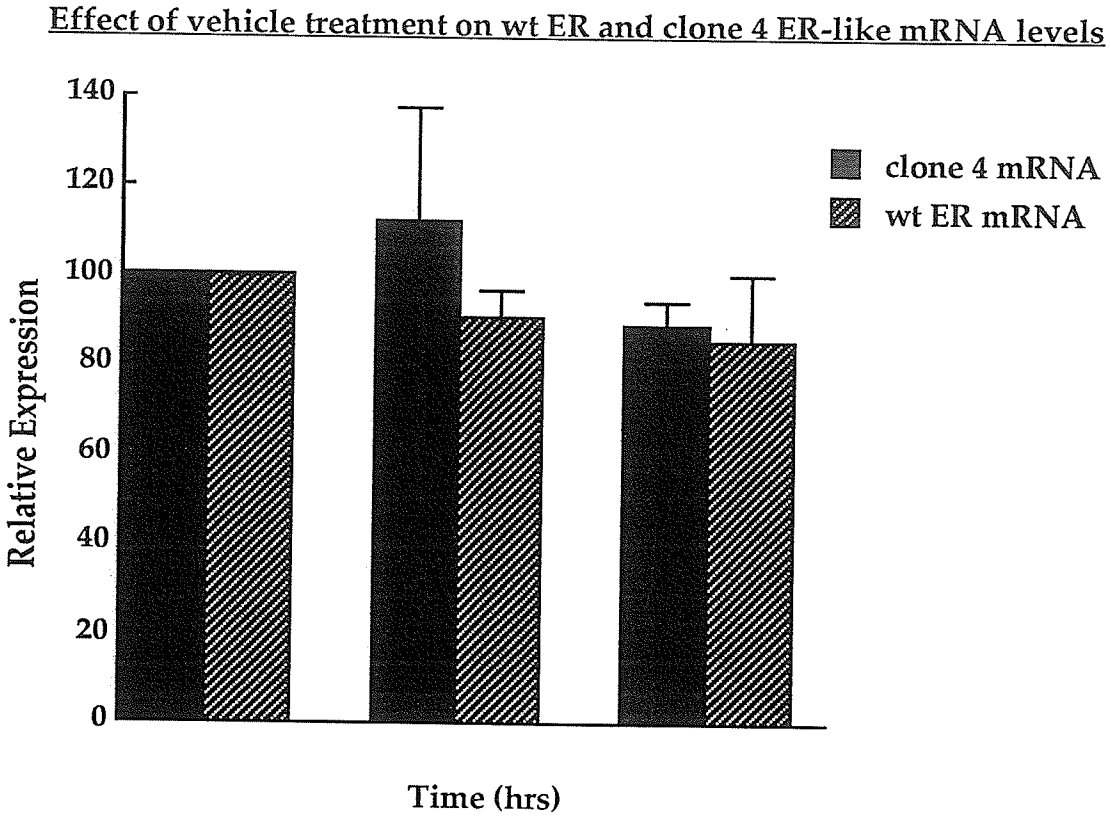


Figure 20. Effect of vehicle treatment (absolute ethanol) on steady-state levels of clone 4 ER-like mRNA and wt ER mRNA in control samples. T47D5 cells were treated with TPA vehicle treatment and harvested concurrently with TPA time course experiments (see Figure 19). Total RNA was isolated and subjected to RT-PCR analysis. Results are expressed as percentage of time 0 vehicle treated control. Means $\pm$ standard deviation are shown where n=3 for all time points. No significant difference was found between time 0 controls and 24 and 72 hour wt ER and clone 4 ER-like steady-state transcript levels when analyzed by student's t-test.

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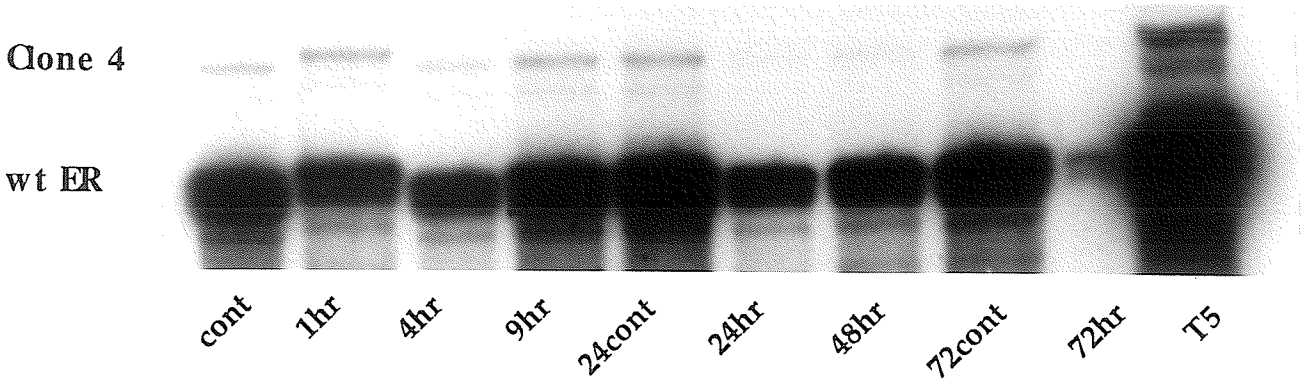

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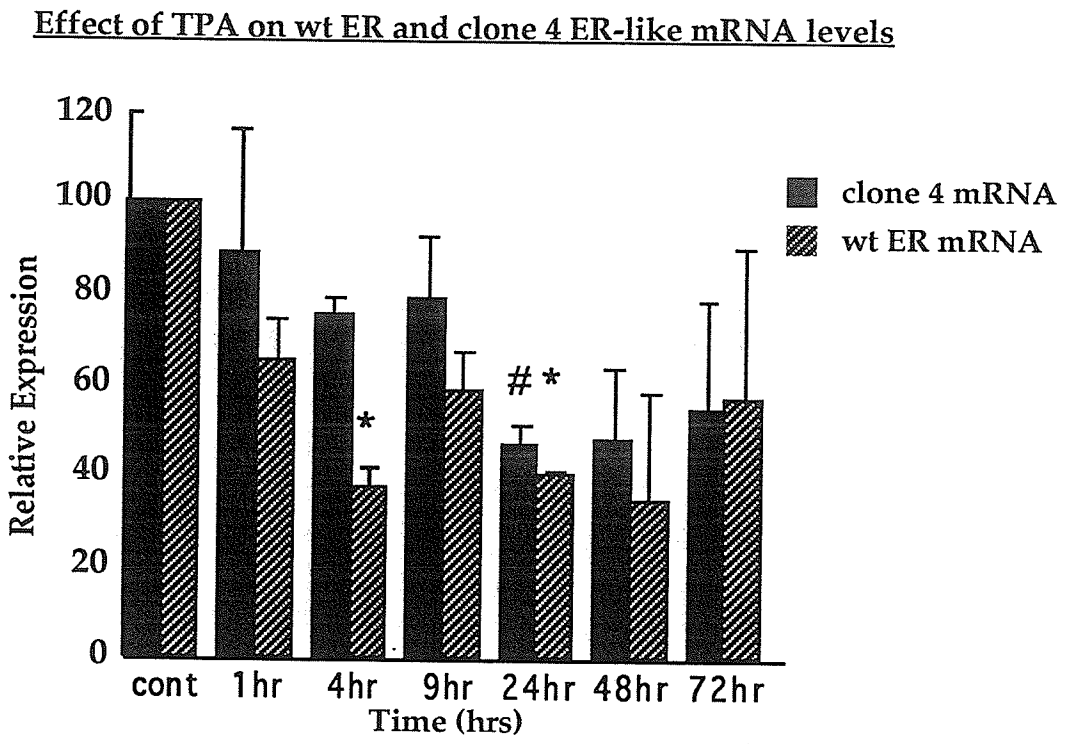

Figure 21. Time course of effect of TPA on wt ER mRNA and clone 4 ER-like mRNA steady-state levels. T47D5 cells were harvested at the indicated times following 1μM TPA treatment. Total RNA was isolated and 15μg subjected to RNase P analysis.
A. An autoradiogram representative of 3 separate time course experiments.
B. Histogram of RNase P results. Results are expressed as percentage of vehicle treated time 0 control. Mean ± standard deviation is shown where n=3 for all time points. * indicates a $p<0.005$ significant difference and # indicates a $p<0.025$ significant difference from vehicle treated control by a student's t-test .

Autoradiograms were examined revealing an apparent overloading of the lanes. In all but one case, both clone 4 and wt ER data obtained from the same lane were discarded. The exception is from the third time course experiment where the clone 4 results were not a gross outlier with respect to the repeated results, whereas the wt ER results were. Therefore, in this case, only the wt ER 72 hour control was discarded (see Table 7).

To validate the expression of the results relative to the time 0 vehicle treated control, expression of wt ER and clone 4 ER-like transcripts in the vehicle controls at 24 and 72 hours were analyzed concurrently. The results are shown in Figure 22. Variation was found in the vehicle treated controls where a statistically significant difference was found at 72 hours ($p<0.025$) for clone 4. One apparent explanation is the exclusion of the standard deviations obtained from the individual experiments in the calculation of the overall mean and standard deviation. The results from each time course experiment for the 72 hour clone 4 control time point were as follows: 77.94 ± 39.22, 81.36 ± 1.88, 83.21 ± 28.11. The standard deviation of the 72 hour control for clone 4 calculated from the individual experiments is small. However, exclusion of the standard deviation for the individual experiments may have contributed to the apparent significance of the 72 hour control for clone 4. Furthermore, since no significant difference was found using RT-PCR analysis with the same samples, the significance of this difference is questionable. To further investigate the significance of the results observed for the 72 hour clone 4 control, the results obtained by RT-PCR and RNase P for the T47D5 time course experiments were compared. Figure 23 shows there is a significant difference between RT-PCR and RNase P results. However, the significance was found at 4 hours ($p<0.01$) and 24 hours ($p<0.025$) for clone 4 (see Figure 23A) and 24 hours ($p<0.025$) for wt ER transcripts (see Figure 23B). The clone 4 expression at 72 hours in the 1 μM TPA treated time course experiments

wt ER	Expt. 1	Expt. 2	Expt. 3
time(hr)	4hr	72 control	72 control
discarded	431.55	520.67, 777.55	4.69
included	35.29 29.83 48.02 24.82 82.62	399.71 94.92 83.73	188.15 170.48
clone 4			
discarded	8313.93	418.73, 586.52	
included	70.65 50.75 87.06 52.74 127.93	80.20 80.35 83.53	

Table 7. Table of points discarded from RNase P TPA time course experiments. This table shows points excluded during the calculation of individual time course experimental means, as well as overall experimental means. These numbers are expressed as the percentage of the time 0 vehicle treated control. When compared to repeat experiments of the same time course, these points appeared to be outliers and, therefore, were discarded.

Effect of vehicle treatment on wt ER and clone 4 ER-like mRNA levels

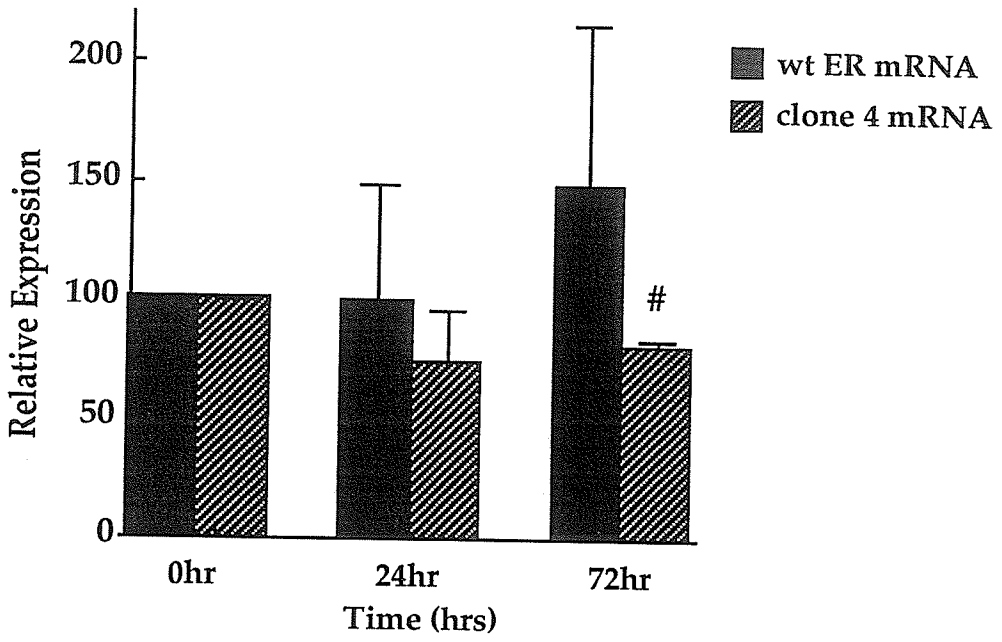

Figure 22 Effect of vehicle treatment (absolute ethanol) on steady-state levels of clone 4 ER-like mRNA and wt ER mRNA in control samples. T47D5 cells were treated with TPA vehicle treatment and harvested concurrently with TPA time course experiments (see Figure 21). Total RNA was isolated and subjected to RNase P analysis. Results are expressed as a percentage of the time 0 vehicle treated control. Means $\pm$ standard deviations are shown where n=3 for each time point. A significant difference was found between time 0 controls and the 72 hour clone 4 ER-like steady-state transcript levels when analyzed by student's t-test ($p<0.025$).

were not found to be statistically different when comparing results obtained by the two methods. Therefore, the significant difference of the 72 hour clone 4 control seen in RNase P experiments is most likely negligible when determining the validity of comparing all time course samples to the time 0 control. The statistically significant differences at the 24 hour time point for clone 4 and the 24 hour time point for both wt ER and clone 4 ER-like transcripts demonstrate some variability between results obtained by two different methods. This does not, however, discredit the use of the time 0 control for analyzing the steady-state transcript changes for the entire T47D5 TPA-treated time course experiments. Furthermore, the TPA treated steady state levels of wt ER and clone 4 ER-like transcripts were lower than the control treated levels, thereby maintaining the pattern induced by TPA treatment of T47D5 human breast cancer cells.

To summarize, treatment of T47D5 human breast cancer cells with 1 μM TPA resulted in a decrease in the steady-state levels of wt ER and a parallel decrease in clone 4 ER-like mRNA when analyzed by both RT-PCR and RNase P.

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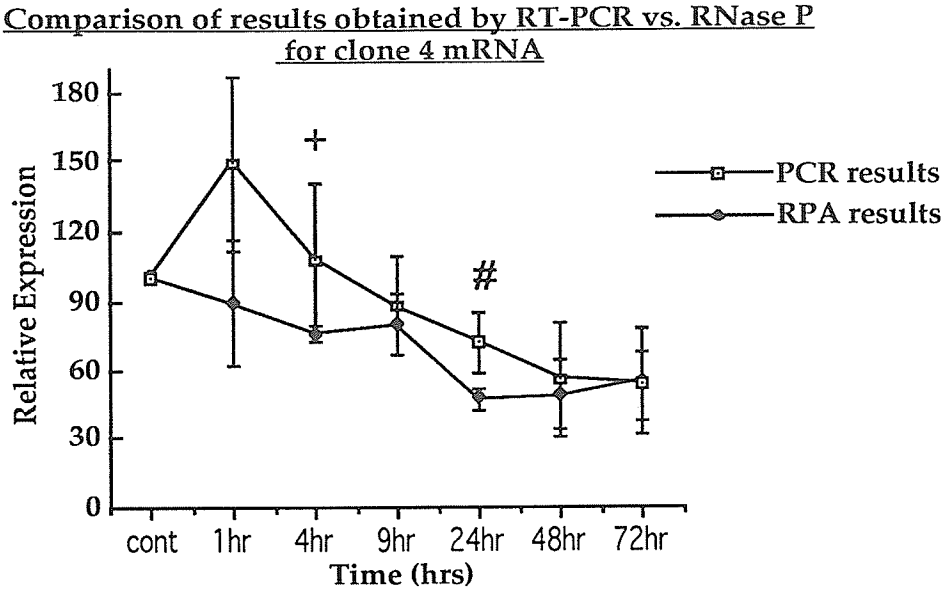

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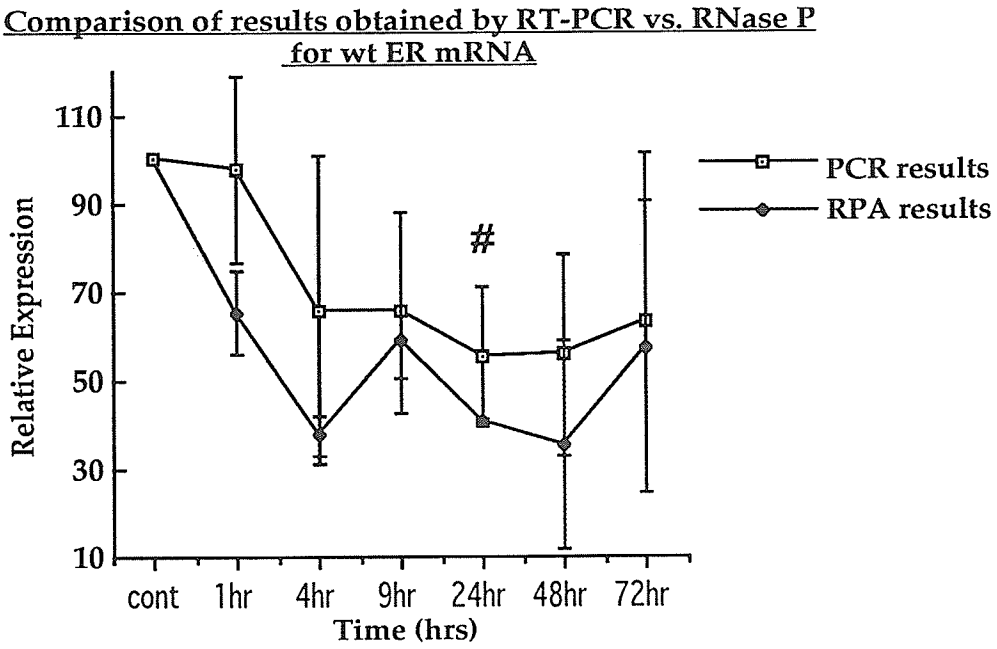

Figure 23. Comparison of results obtained by RT-PCR vs. RNase P. Results from T47D5 cells treated with 1μM TPA and subjected to RT-PCR and RNase P were compared.

A. Mean (± standard deviation) RT-PCR vs. mean (± standard deviation) RNase P results for clone 4 steady-state mRNA levels. A significant difference was found at (+) 4 hours ($p<0.01$) and (#) 24 hours ($p<0.025$).

B. Mean (± standard deviation) RT-PCR vs. mean (± standard deviation) RNase P results for wt ER steady-state mRNA levels. A significant difference was found at (#) 24 hours ($p<0.025$).

Discussion

During the course of her lifetime, one in nine women will develop breast cancer (Volkers, 1992) making breast cancer the most common cancer amongst women in western society. Despite increased awareness concerning breast cancer which has led to early detection and diagnosis of fewer advanced tumours (Miller et al, 1991), the incidence is still increasing.

Estrogen has been shown to play a role in the development of normal mammary tissue (Topper and Freeman, 1980; Bresciani, 1968), and also in the development and proliferation of breast tumours (Vorherr, 1980; Beatson, 1896; Dickson and Lippman, 1988; Soule and McGrath, 1980). Breast cancer appears to originate as an estrogen-dependent disease which progresses to hormone-independence, resulting in endocrine resistant tumours (Brenner et al, 1988). Approximately 70% of breast cancer patients are ER+ upon presentation and of these, approximately 60% will respond to endocrine therapy (Horwitz et al, 1975) leaving the remainder of primary tumours resistant to endocrine therapy. Furthermore, originally responsive tumours will eventually develop resistance although many continue to express the ER.

The ER interacts with EREs of estrogen responsive genes (Kumar and Chambon, 1988; Klein-Hitpass et al, 1989; Gorski et al, 1993). Therefore, endocrine therapies may be targeted at the ER, itself inhibiting the transcription of estrogen-responsive genes, or to reduce circulating levels of estrogens. In addition, endocrine therapies may also modulate peptide growth factors, growth factor receptors, and factors which regulate the cell cycle which would affect tumor cell growth. Unfortunately, with the treatment of breast cancer, "cure" is defined as the number of years of disease free survival. Even after 15 years, treatment failure is still a possibility despite the decreasing chance of recurrence over time (Bonadonna and Valgussa, 1988). Proposed mechanisms for endocrine resistance were discussed in the

introduction (see Endocrine Resistance). Loss of expression of the ER was suggested, however, studies have shown that hormone independent and unresponsive tumours do not show an altered expression of the estrogen receptor upon acquisition of hormone-independence (Darbre and King, 1987; Taylor et al, 1992; Manni et al, 1980). Over 60% of resistant human breast tumours continue to express ER and PR (rev. in Osborne and Fuqua, 1994). An alternative mechanism proposed to explain the development of resistance to endocrine therapy was the expression or altered expression of variant ER-like proteins. Although unequivocal evidence for the presence of ER-like proteins has not been reported, the possibility of their expression has been suggested due to the detection of variant ER-like mRNAs in a wide range of normal and neoplastic estrogen target tissues and cell lines.

Precise exon deletions from ER mRNA of exons 2, 3, 4, 5, 6, and 7, as well as double deletions of exons 4/7 and 2/3 have been detected (see Table 5 in Introduction). In addition, exon duplications of ER exons 3 + 4, exon 6 and exons 6 + 7 have been found, as have several point mutations, multi-base pair insertions and deletions (see Table 5 in Introduction). A number of ER-like proteins have been detected including an 80 kDA ER-like protein isolated from an MCF-7 estrogen-independent subline containing 4-5 copies of the ER gene (Pink et al, 1996). One of these copies contains an ER gene duplicated in exons 6 and 7 which is believed to generate the inframe duplication of exons 6 and 7 in the ER transcript and is thought to produce the 80 kDa ER-like protein upon translation (Pink et al, 1996). In addition to the discovery of the 80 kDa protein, BT-20 human breast cancer cells which are ER-negative by ligand binding assays, were shown to express high levels of the exon 5-deleted ER transcript. Furthermore, this cell line expressed a protein of approximately 43 kDa which is the predicted size of the truncated protein which would result from an exon 5-deleted transcript (Castles et al, 1993). A

study by Fuqua et al (1992 (abstract)) showed that MCF-7 cells stably transfected with the exon 5-deleted construct expressed the variant at a level equivalent to the wt protein. In addition, the transfected cells remained estrogen-responsive, but developed resistance to tamoxifen suggesting that variant expression or overexpression may contribute to resistance (Fuqua et al, 1992 (abstract)). However, a recent study found no alteration in the responsiveness of MCF-7 cells to estrogen or antiestrogens when transfected with the exon 5-deleted construct (Rea and Parker, 1996). Therefore, the role of variants in the development of resistance remains unclear.

The generation of variant transcripts may result from rearrangement of the ER gene, from alternative splicing of the wt ER primary transcript or *trans*-splicing of the transcripts derived from the wt ER gene and the transcript of another gene. Clone 4 was originally isolated and cloned from RNA isolated from human breast tumours and was later found in human breast cancer cell lines, normal tissue, Ishikawa (human endometrial carcinoma) cells and normal human uterine tissue (Dotzlaw et al, 1992). The 2333 bp transcript begins 6 bps 5' of the normal ER transcription start site (Green et al, 1986) and contains exons 1 and 2 from the normal ER mRNA then diverges to sequences that are similar to LINE-1 sequences (Dotzlaw et al, 1992). Due to the large number of copies of LINE-1 sequences present in the human genome, the origin of the clone 4 LINE-1-like sequences has been difficult to determine. Clone 4 may result from alternative splicing of exons 1 and 2 of the wt ER transcript to LINE-1 sequences present in the introns of the wt ER gene. Alternatively, clone 4 may be the result of *trans*-splicing of LINE-1 sequences from another transcript to wt ER exons 1 and 2.

Elevated levels of clone 4 ER-like mRNA relative to wt ER mRNA were found in PR-negative tumours versus PR-positive tumours (Murphy et al, 1995). Elevated levels of clone 4 mRNA were also found in "poor" prognosis

tumours versus "good" prognosis tumours (Murphy et al, 1995). Furthermore, Leygue et al (1996b) detected significantly lower levels of clone 4 transcripts in normal breast tissue than in ER+/PR+/node-negative breast tumours.

Given the significance of these results, this study further examined the origin of clone 4 ER-like mRNA. The aim was to address the hypothesis that alternatively spliced mRNA expression depends on transcription of the wt ER gene. This predicts that altered transcription of the wt ER gene should lead to altered levels of the alternatively spliced variant ER mRNA. To do this, I investigated the effect of various agents known to alter steady-state ER mRNA levels in the breast cancer cell line, T47D5. I compared the effect these agents had on the steady-state levels of the truncated ER-like variant mRNA, clone 4, relative to wt ER mRNA levels.

First, the effect of the synthetic progestin, MPA, on clone 4 and wt ER transcript steady-state levels was examined. Progesterone is important in the development of female reproductive organs as well as in regulating biological functions. The belief that progesterone is an antagonist of estrogen may explain why hormone-responsive breast tumours may be effectively treated with MPA with a response rate reaching almost 70% in PR-positive tumours (Sutherland at al, 1988). Progestins may act indirectly to alter the hormonal environment. It has been suggested that due to progestin administration, gonadotropins and ovarian function may be suppressed resulting in decreased circulating estrogen levels or intracellular estradiol levels may be reduced by an increase in 17β–hydroxysteroid dehydrogenase, the enzyme responsible for estradiol metabolism (rev. in Mauvais-Jarvis, 1986). Progestins may also act directly to affect tumor cell growth.

At present, relatively few studies have been carried out to determine the effect of progestin treatment on ER mRNA in human breast cancer cell lines

(see Table 6 for summary of studies). Research has shown that progestins down-regulate ER protein and ER mRNA levels. Read et al (1989) used the synthetic progestin, R5020, to demonstrate a decrease of ER mRNA in T47D cells to 20% of control levels within 2 days of progestin exposure. Alexander et al (1990) found similar results with T47D cells treated with the synthetic progestins ORG 2058, R5020 and MPA, however, the decrease occurred after approximately 6 hours and was less marked (35-40% of control) than the decrease observed by Read et al (1989). This study also showed partial recovery of ER mRNA levels between 12 to 24 hours. Berkenstram et al (1989) used MCF-7 cells and $T47D_{co}$ (cells that contain ER but are estrogen unresponsive) and found similar results to Alexander et al (1990) using 100 nM R5020 with a decrease of ER steady-state transcript levels to approximately 45% of the control by 6 hours and a partial recovery to 70% of control levels by 50 hours.

To examine the hypothesis that clone 4 ER-like transcripts are derived from the ER gene, regulation of ER mRNA and clone 4 ER-like mRNA by MPA was examined. Prior to the present study, no research had examined the regulation of clone 4 transcripts. Experiments were performed to determine the effect of the synthetic progestin MPA on ER mRNA and clone 4 ER-like transcript steady-state levels in T47D5 human breast cancer cells.

The down-regulation of wt ER mRNA observed in this study was similar in magnitude of response and time of lowest steady-state levels to studies by Alexander et al (1990) and Berkenstram et al (1989). When analyzed by RT-PCR, wt ER mRNA decreased to 51% of vehicle-treated control values by approximately 24 hours following treatment of T47D5 cells with 10 nM MPA. Furthermore, the steady-state levels of clone 4 ER-like mRNA decreased in parallel to the wt transcripts reaching the lowest level of 38% by approximately 24 hours. Samples were also analyzed using a method not

relying on PCR amplification to verify results obtained by RT-PCR. Using RNase P, similar results were obtained where wt ER and clone 4 ER-like transcripts fell to the lowest levels of 32% and 44% of control values, respectively, by approximately 24 hours. Consistent with the findings of Alexander et al (1990) and Berkenstram et al (1989), a partial recovery was seen over the remaining time course for steady state levels of the wt ER transcript. In addition, the clone 4 ER-like steady state levels recovered in parallel.

Alexander et al (1990) examined the steady state levels, as well as the half-life of wt ER mRNA, following progestin treatment. Since the half-life of the transcript was unaltered, the mechanism through which wt ER steady-state levels were decreased was proposed to be transcriptional although direct assays to determine this have not been reported. If PR directly alters ER gene transcription, the molecular mechanisms remain unclear although the apparent absence of a PRE in the 5'-UTR of the ER gene suggests the mechanism is not classical.

Examination of the 5'-UTR of the ER gene has revealed the presence of a CATT-like motif (-2372 bp), a TATA-like motif (-2391 bp) and a putative CAP site (-2360 bp) (Piva et al, 1992), as well as an ORF approximately 2 kb upstream from the trancription initiation site identified by Green et al (1996) (Piva et al, 1992; Keaveney et al, 1991). Green et al (1986) also described a TATA-like box and a CAAT-like box at bps -27 and -103, respectively. In addition, studies identified two half-site EREs at positions -895 and -871 as well as a retinoic acid response element at base pair +115 (Sullivan et al, 1995 (abstract)). Recent research studying the 5'-untranslated region of the ER gene described a fragment capable of activating its own ER promoter. Transient transfection assays of HeLa cells with a luciferase reporter gene driven by the -242 to -11 bp portion of the human ER gene promoter were performed

(Castles et al, 1995 (abstract)). Although HeLa cells do not express the ER, reporter gene activity was observed. Cotransfection of HeLa cells with a construct for the ER and the -242 to -11 fragment of the ER gene promoter upstream of the luciferase reporter gene showed increased luciferase activity. This activity was not enhanced when longer fragments of the ER gene promoter (up to bp -727) were used in the cotransfection experiments. Therefore, the ER is able to activate its own promoter through sequences present in the fragment -242 to -11 of the ER gene promoter. A half-site ERE and a binding site for TFIID have been localized to this fragment (Castles et al, 1995 (abstract)). However, as of yet, no PRE has been identified in the ER promoter. Therefore, the mechanism by which MPA down-regulated wt ER mRNA and clone 4 ER-like mRNA remains to be elucidated.

Kraus et al (1995) examined the effect of progestins on the transcription of estrogen responsive genes. A number of mechanisms have been proposed to result in transcriptional repression. Competitive repression occurs when a repressor protein binding site overlaps the transcription factor binding site. The repressor protein competes for DNA binding and inhibits binding by transcription factors like ER (Levine and Manley, 1989). Squelching occurs when the overexpression of a transcription factor, for example PR, sequesters transcriptional activators necessary for the activation of other transcription factors (Levine and Manley, 1989). Repressor proteins may also bind to a specific non-overlapping sequence in the promoter region of a gene interfering with the formation of the transcriptional complex (Levine and Manley, 1989). Lastly, quenching may also repress transcription. In quenching, activators required for transcription are prevented from interacting with the transcription complex by a repressor protein which binds to a site distinct from the activator binding site (rev. in Levine and Manley, 1989).

In the study by Kraus et al (1995), ER and PR constructs were transiently transfected into rat uterine cells and 3T3 mouse fibroblast cells. The activity of a CAT reporter gene attached to ERE/PRE-containing promoters was examined in the presence of E2, the progestin, R5020, or the antiprogestin, RU486 (Kraus et al, 1995). When cells were treated with R5020, or RU486, repression of ER-mediated transcription was observed. This repression was abolished when constructs lacking the upstream PRE were used suggesting PR interaction with PREs is necessary for transcriptional repression of ER-mediated transcription. Furthermore, the position of the PRE relative to the ERE-promoter was not a significant determinant of progestin-induced repression of ER-mediated transcription since the inhibition of CAT activity was seen when vectors containing PRE sequences were placed distal to the ERE-promoter sequences as well as when the PREs were placed adjacent to the ERE-promoter sequences in the vector (Kraus et al, 1995).

To determine if ligand-bound PR interfered with binding of EREs by ER, GAL4 binding sites were placed adjacent to two PREs and the distal PR promoter of the rat gene. This arrangement was similar to the ERE_2-PRE_2-promoter-CAT constructs used in the above experiments. The activity of GAL-VP16, a constitutive activator, was not inhibited by liganded PR. Therefore, binding of PR to PREs does not block adjacent binding sites. These results also suggest a specific subset of activators are required for liganded-PR repression of ER-mediated transcription since synergistic activation of GAL-VP16 activity was seen in a context similar to the previously observed CAT reporter gene repression driven by ERE_2-PRE_2-promoter vectors (Kraus et al, 1995). Therefore, direct transcriptional repression is not likely the mechanism by which progestins/antiprogestins exert their repressive effect on ER-mediated transcription. Further experiments demonstrated repression even at low concentrations of PR and the requirement for the PR to be

liganded to inhibit ER-mediated transcription suggesting squelching was also not likely a mechanism for repression in this case (Kraus et al, 1995).

From these results, Kraus et al (1995) deduced that the PR may directly or indirectly repress ER-mediated transcription when bound to PREs by interfering with its ability to interact with the preinitiation complex required for transcription. This is supported by the finding that ER mutated in the N-terminal region decreased the repression normally induced by agonist-bound PR (Kraus et al, 1995). Inhibitory factors may be recruited by PR or, alternatively, a component of the ER-signalling pathway may be disrupted resulting in quenching of ER-mediated transcription.

In contrast to that study, another group demonstrated PR binding to its response element was not necessary to repress transcription from the ER-responsive rabbit PR gene (Savouret et al, 1991). Progressive deletions of sequences from the 5' and 3' ends of the -2762 to +788 of the PR gene showed progestin-mediated down-regulation of progesterone receptor gene transcription did not require PRE binding by PR (Savouret et al, 1991) although it did require the presence of PR. This study suggests the PR may interact with the estrogen receptor or transcriptional complex proteins resulting in repression of ER-mediated PR gene transcription. Deletion experiments identified an ERE at position +698/+723, overlapping the translation initiation site, which is thought to be involved in the observed down-regulation. This ERE differs from the consensus ERE by one nucleotide at the 3' end (Savouret et al, 1991). Although a similar ERE was not identified in the human PR gene (Savouret et al, 1991), estrogen regulation of heterologous genes by the -711 to +31 fragment from the human PR gene has been demonstrated (Kastner et al, 1990).

The conflicting results obtained by the two research groups (Kraus et al, 1995; Savouret et al, 1991) suggests different mechanisms may be responsible

for repression of progestin-induced ER-mediated transcription in the two systems examined. Although studies have not identified a PRE in the ER gene promoter, PREs may be present but unidentifed as of yet. The study by Kraus et al (1995) demonstrated PREs placed distal to ERE-promoter sequences were able to inhibit ER-mediated transcription. Furthermore, cell and promoter specific activities were also seen by Kraus et al (1995) when comparing the repression seen using R5020, RU486 and the thymidine kinase, the distal progesterone receptor promoter of the rat PR gene and the pS2 promoter in rat uterine cells and mouse 3T3 fibroblast cells. Similar studies examining the activity of tamoxifen were discussed in the introduction (see Table 3). The agonist/antagonist activity of tamoxifen was dependent on the cell and promoter context used in the study.

Although the precise mechanism by which progestins decrease ER-mediated transcription is unclear, the parallel regulation of the steady-state clone 4 ER-like mRNA levels relative to the wt ER transcripts by progestins suggests that a similar promoter is regulating expression of clone 4 ER-like mRNA. This is consistent with the hypothesis that clone 4 is an alternatively spliced product of the wt ER transcript.

To further examine the regulation of clone 4 ER-like mRNA steady-state levels relative to wt ER mRNA steady-state levels, the effect of the phorbol ester, TPA, on these transcripts was examined. This agent was chosen since the proposed mechanism for phorbol ester down-regulation of ER mRNA is post-transcriptional. Phorbol esters have been shown to inhibit cell proliferation and stimulate cell differentiation (Smith et al, 1983; Rovera et al, 1979), but have also been found to stimulate cell proliferation and inhibit differentiation (Wang et al, 1975; Yuspa et al, 1978). Transcription of a number of genes such as c-myc, c-fos (Greenberg and Ziff, 1984), collagenase and stromolysin (Whitham et al, 1986) have been shown to be modulated by

phorbol esters. In addition, the modulation of expression of ER mRNA by phorbol esters has been studied in human breast cancer cells. Lee et al (1989) treated MCF-7 human breast cancer cells with 10 nM TPA and found the steady-state level of wt ER mRNA maximally decreased to 15-20% of control levels between 12-24 hours following treatment. Levels remained depressed over the remaining 48 hours of the experiment. Saceda et al (1991) found similar results in MCF-7 cells although the maximum decrease was evident by 6 hours. Ree et al (1991; 1992) showed a decrease in ER transcript steady-state levels in MCF-7 cells using 100 nM TPA. This decrease occurred by 9 hours where the transcript fell below levels detectable by Northern blot analysis. Furthermore, ER mRNA steady-state levels failed to recover over the 48 hour time course following removal of TPA at 10 hours (Ree et al, 1991). In the present study, the effect of 1 μM TPA on the steady-state levels of wt ER and clone 4 ER-like transcripts were examined in T47D5 human breast cancer cells. This study demonstrated a decrease of wt ER mRNA steady-state levels to approximately 55% of vehicle-treated control values by RT-PCR and 37-40% by RNase P. Although there appears to be a discrepancy in the magnitude of response obtained by the two different methods, the difference was not statistically significant when analyzed by a student's t-test. Furthermore, the decrease in wt ER mRNA transcripts was paralleled by a decrease in the steady-state levels for clone 4 mRNA. wt ER mRNA decreased to 53-56% of controls and clone 4 to 55% of controls by RT-PCR and wt ER transcripts to 37-40% of controls and clone 4 to 46% of controls by RNase P.

Experiments using nuclear run-on assays have shown that transcription of the ER gene was not affected by short-term TPA treatment of MCF-7 cells (Saceda et al, 1991). Since mRNA steady-state levels are determined by the equilibrium between transcript production and degradation, the mechanism

by which phorbol esters regulate ER mRNA expression appears to be post-transcriptional. Saceda et al (1991) demonstrated the half-life of the wt ER mRNA was shortened from approximately 4 hours in control-treated cells to approximately 40 minutes in cells treated with TPA. Ree et al (1992) found similar results where the half-life of the ER transcript was approximately 3 hours versus 12 hours in the presence of actinomycin D, an RNA synthesis inhibitor. Actinomycin D abolished the down-regulation of wt ER mRNA by TPA as did the RNA polymerase inhibitor 5, 6-dicholoro-1-β-D-ribofuranoylsylbenzimidazole (Ree et al, 1992). Puromycin and cycloheximide, inhibitors of protein synthesis, did not affect wt ER steady-state transcript levels when cells were treated with TPA. These results suggest TPA-dependent down-regulation of ER mRNA steady-state levels is mediated by an untranslated, short-lived RNA molecule which destabilizes ER mRNA or has catalytic activity and, therefore, directly degrades ER mRNA (Ree et al, 1992). Ree et al (1992) also hypothesize that TPA treatment of cells may result in the phosphorylation of a destabilizing protein which forms an RNA-protein complex with a short-lived RNA molecule leading to the observed down-regulation of wt ER transcripts. Therefore, TPA appears to alter the stability of wt ER transcripts.

Keaveney et al (1993) subcloned fragments of the 3'-UTR of the ER gene into CAT expression vectors and found all fragments analyzed reduced CAT expression suggesting an important role for the 3'-UTR in regulating ER expression. The 3'-UTR of the ER gene contains 13 copies of the sequence ATTTA (Keaveney et al, 1993) which is believed to promote instability of transcripts. The AU-rich region in the 3'-untranslated region may act as a substrate for cellular nucleases leading to degradation of transcripts (Wreschner et al, 1981). Although clone 4 contains only part of the wt ER sequence, the LINE-1-like sequences of clone 4 have been sequenced (Dotzlaw

et al, 1992) and an analysis of this sequence shows 4 putative ATTTA motifs are present. This may explain the apparent parallel regulation of two transcripts whose 3'-untranslated sequences are dissimilar. Alternatively, the 3' region of the ER transcript contains a number of sequences which could potentially form stem and loop structures which may affect stability of the ER transcript. This mechanism of post-transcriptional regulation is seen in the regulation of the transferrin receptor (Mullner and Kuhn, 1988). Stem and loop-forming sequences may also be present in the LINE-1-like sequences of clone 4. This remains to be determined.

Another mechanism which has not been studied with respect to wt ER transcript regulation is the effect of TPA on primary transcript processing. The generation of wt ER transcripts is dependent upon the processing of the primary transcript to yield a mature mRNA and shuttling of the transcript to the cytoplasm. Alteration or interference with factors required for this processing would result in a decrease in wt ER steady-state levels. The steady-state levels of clone 4 would also be affected by this modulation of processing if it was derived from the same primary transcript as the wt ER transcript. Further studies are required to examine this possibility more closely.

This study examined the effect of two agents, MPA and TPA, on the steady-state levels of wt ER and clone 4 ER-like mRNAs using RNase P assays and RT-PCR analysis. The rationale for analyzing samples by two different methods was to ensure results obtained by RT-PCR were valid. RNase P was used in a previous experiment to analyze clone 4 transcripts in human breast tumour samples (Murphy et al, 1995). Several days are usually necessary to perform one RNase P assay making it difficult to control for laboratory conditions from experiment to experiment. Furthermore, RNase P requires large quantities of RNA for detection of transcripts. In this experiment, one

source of variability in the RNase P experiments may have resulted from the absence of a loading control in the PAGE step.

RT-PCR is an efficient way to analyze small quantities of transcripts, however, this method is also subject to variability. In RT-PCR experiments, GAPDH was used as a loading control. This decreases the variability between experiments, however, does not eliminate it. Northern analysis suggested that MPA and TPA did not alter GAPDH expression, however, quantitative analysis is required to confirm those conclusions. GAPDH may increase slightly resulting in an underestimation of the decrease in clone 4 and wt ER transcripts or may slightly decrease which would result in an overestimation of the decrease of these transcripts.

Reverse transcription reaction results may vary up to 50% (Ferre et al, 1994). This error may be further compounded by the exponential amplification of the RT products using PCR. For a valid comparison of PCR products within a primer set and also between primer sets, exponential amplification is necessary. During exponential amplification, proportional amplification of the template input concentration should occur, however, primer annealing efficiency may vary from cycle to cycle. Normalization of clone 4 and wt ER to GAPDH products assumes GAPDH and these transcripts are being amplified at the same rate during the time course. This assumption may further contribute to the variability seen between experiments. Another source of experimental variation may result from ethidium bromide staining of the agarose gels. Intercalation of EtBr with the PCR products may vary between samples. Furthermore, a photograph of the EtBr stained gel was scanned. The accuracy of the scan results obtained were limited by the sensitivity of the scanner.

Although variability between the two methods was found, these methods both suggested that clone 4 is regulated in a parallel fashion to wt ER

transcripts by two agents proposed to down-regulate wt ER mRNA by two different mechanisms. These results strongly suggest clone 4 is an alternatively spliced transcript derived from transcription of the ER gene.

Although these studies suggest that wt ER and clone 4 transcripts are regulated in a parallel fashion in T47D5 human breast cancer cells, differences in regulation must occur under certain circumstances since clone 4 is significantly elevated in tumours versus normal mammary tissue (Leygue et al, 1996) and altered ratios have been found amongst breast tumors (Murphy et al, 1995). The altered ratios of clone 4 to wt ER transcripts was related to poor prognosis and lack of endocrine sensitivity (Murphy et al, 1995). The detection of variant transcripts in normal mammary tissue (Leygue et al, 1996; Pfeffer et al, 1995), however, suggest that variants are expressed normally. Furthermore, the ER signalling pathway may involve variant ER-like proteins. Alteration of the ratio of variant proteins to wt ER proteins may offset the balance normally maintained in the cell, thereby contributing to the deregulation of cellular functions and, therefore, the development of hormone resistance in human breast cancer.

In this study I have demonstrated the parallel regulation of the steady-state levels of clone 4 ER-like mRNA relative to wt ER mRNA when T47D5 cells were treated with the agents MPA and TPA which were previously shown to alter wt ER steady-state transcript levels. In conclusion, this thesis provides evidence that the generation of clone 4 ER-like transcripts depends on the transcription of the wt ER gene.

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