MOLECULAR MECHANISM OF ESTROGEN ACTIVATION OF *C-MYC* ONCOGENE EXPRESSION IN HUMAN BREAST CANCER CELLS

by

DONALD DUBIK

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in Partial Fulfillment of the Requirements for the Degree of
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"Exploring is delightful to look forward to and back upon, but it is not comfortable at the time, unless it be of such an easy nature as not to deserve the name"

-Samuel Butler

ABSTRACT

One third of all breast tumors exhibit increased growth when treated with 17β-estradiol. Treatment of these tumors with antiestrogenic drugs attenuates tumor growth and may cause tumor regression. Unfortunately the effect of antiestrogenic drugs is temporary as the tumors eventually become hormone-independent. To understand the mechanism of this hormone-dependent to hormone-independent transition, it is important to elucidate the mechanism of estrogen induced proliferation in these cells. The importance of the *c-myc* proto-oncogene in estrogen regulated growth of breast cancer cells was suggested when hormone depleted, estrogen-responsive, human breast cancer cell lines MCF-7 and T-47D revealed a rapid transient induction of *c-myc* expression after treatment with estradiol. This induction of *c-myc* mRNA was evident 15 min post-hormone addition and was maximal (>10 fold) within 1-2 h. The observation that estrogen receptor negative cell lines MDA-MB-231 and BT-20 exhibited high, hormone-independent expression of *c-myc* further supported *myc's* role in breast cancer and suggested that deregulated *c-myc* expression may be important in the transition to hormone-independency. Analysis of mechanisms that could account for deregulated *c-myc* expression in the human breast cancer cells revealed that increased *c-myc* transcription and post-transcriptional stabilization of the mRNA were responsible. In the estrogen-unresponsive MDA-MB-231 cells, the elevated level of *c-myc* expression resulted from a more stable *c-myc* mRNA, with a half-life of 49 min versus the normal half-life of 15-20 min. Conversely in MCF-7 cells, estradiol had no effect on the *c-myc* mRNA half-life but increased *c-myc* transcription more than 10-fold within 20 min. In addition, pretreatment of the MCF-7 cells with the translational inhibitor cycloheximide did not prevent estradiol induction of the *c-myc* proto-oncogene, indicating that this induction is a primary effect of estrogen, not requiring the synthesis of a new protein intermediate(s). This observation and the knowledge that the estrogen receptor complex is a *trans*-acting factor, suggested that the estrogen receptor complex may be a *trans*-activator of the *c-myc* gene. Therefore this complex must interact with a *cis*-acting estrogen response element (ERE) in the *c-myc* gene. To localize the ERE, constructs containing varying lengths of the *c-myc* 5′ flanking region ranging from -2327 to +25 (relative to the P1 promoter) placed adjacent to the chloramphenicol acetyl transferase reporter gene (CAT) were prepared. They were used in transient expression studies in MCF-7 and HeLa cells cotransfected with an estrogen receptor expression vector. These studies reveal that all constructs containing the P2 promoter region exhibited estrogen regulated CAT expression and that a 116 bp region upstream and encompassing the P2 TATA-box is necessary for this activity. Computer analysis of this 116 bp region failed to identify a *cis*-acting element with sequences resembling the consensus ERE; however, cotransfection studies with mutant estrogen receptor expression vectors

showed that the DNA binding domain of the receptor is essential for estrogen regulated CAT gene expression. We have also observed that antiestrogen receptor complexes can weakly *trans*-activate from this 116 bp region but fail to do so from the ERE-containing ApoVLDLII-CAT construct. These results suggest a new mechanism of estrogen *trans*-activation in the human *c-myc* gene promoter.

LIST OF ABBREVIATIONS

Å	angstrom [10^{-10} meters]
aa	amino acids

Nonpolar Amino Acids:

A	**Ala**	Alanine
V	**Val**	Valine
L	**Leu**	Leucine
I	**Ile**	Isoleucine
P	**Pro**	Proline
F	**Phe**	Phenylalanine
T	**Trp**	Tryptophan
M	**Met**	Methionine

Polar / Uncharged Amino Acids:

G	**Gly**	Glycine
S	**Ser**	Serine
T	**Thr**	Threonine
C	**Cys**	Cysteine
Y	**Tyr**	Tyrosine
N	**Asn**	Asparagine
Q	**Gln**	Glutamine

Acidic Amino Acids:

D	**Asp**	Aspartic acid	[Aspartate]
E	**Glu**	Glutamic acid	[Glutamate]

Basic Amino Acids:

K	**Lys**	Lysine
R	**Arg**	Arginine
H	**His**	Histidine

AAD	acidic activation domain
AMV	avian myelocytomatosis virus (*v-myc* source)
bp	base pairs
BR	basic region
°C	degrees centigrade
C	FBS containing medium
C'	cFBS containing steroid depleted medium
$CaPO_4$	calcium phosphate
CAT	chloramphenicol acetyl transferase
CBP	CAAT binding protein
CEF	chicken embryo fibroblasts
cFBS	charcoal treated FBS
CHX	cycloheximide

cm	centimeter
cpm	counts per minute
CO_2	carbon dioxide
CsCl	cesium chloride
DBD	DNA binding domain
DEX	dexamethasone (glucocorticoid)
DHT	5α-dihydrotestosterone
DMBA	7,12-dimethylbenzanthracene
DMEM	Dulbecco's modified Eagle's medium
DNA / cDNA	deoxyribonucleic acid/ complementary-
DNase	deoxyribonuclease
dpm	disintegrations per minute
DTT	dithiothreitol
E	17β-estradiol
E'	cFBS and E ($10^{-7/-8/-9}$M) containing medium
ER / ER+ / ER-	estrogen receptor/ positive/ negative
ERE	estrogen responsive element
ERR	estrogen receptor related protein
EDTA	ethylenediaminetetraacetic acid
ExoIII	exonuclease III
FBS	fetal bovine serum containing medium (same as C but without insulin)
g / mg / µg / ng	grams/ milli-/ micro-/ nano-
GF / IGF / EGF/ TGF / PDGF/ FGF	growth factor/ insulin-like-/ epidermal-/ transforming-/ platelet derived-/ fibroblast-
GR	glucocorticoid receptor
h *XR* / m *XR* / r *XR*	human *receptor*/ mouse-/ rat-
H_2O	water
HBC	human breast cancer
HBD	hormone binding domain
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HLH	helix-loop-helix
HRE	hormone regulatory element
HSH	helix-span-helix

hsp	heat shock protein
IU	international units
KAc	potassium acetate
kDa	kilodaltons
kb	kilobase
l / ml / µl	liters/ milli-/ micro-
LOH	loss of heterozygosity
LTR	long terminal repeat
M / mM / µM / nM	molar/ milli-/ micro-/ nano-
$MgCl_2$	magnesium chloride
MMTV	mouse mammary tumor virus
MPA	6α-methyl-17α-hydroxy progesterone
MYC	*c-myc* oncoprotein
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NRE	negative response element
NTP / UTP / GTP	nucleotide triphosphate/ uridine-/ guanosine-
PIPES	1,4-piperazine diethanesulfonic acid
R / ER / GR / PR / MR / RAR TR / VDR	receptor/ estrogen-/ glucocorticoid-/ progesterone-/ mineralcorticoid-/ retinoic acid-/ triiodothyronine-/ vitamin D3-
RNA / mRNA / hnRNA /tRNA	ribonucleic acid/ messenger-/ heteronuclear-/ transfer-
RNP	ribonuclear proteins
RA	retinoic acid
*X*RE	*hormone* responsive elements
SV40	simian virus 40
T'	steroid depleted medium containing cFBS and the antiestrogen TAM (10^{-6}M)
TAM	antiestrogen tamoxifen
TF	transcription factor
TK	thymidine kinase
TLC	thin layer chromatography
Tris	tris (hydroxymethyl) amino methane
UEF	upstream element binding factor

Vit	vitellogenin
v/v	volume / volume
Zip	leucine zipper region
$ZnSO_4$	zinc sulfate

LIST OF FIGURES

TABLE OF CONTENTS

INTRODUCTION

A. Endocrinology of the Breast Epithelium:

Normal Breast - Development and Hormonal Influences:

Extensive studies detailing hormonal control of mammary growth and development have been done using various animal models. Studies utilizing human breast tissue have been minimal and most of our knowledge of human breast endocrinology has been extrapolated from animal studies. In humans, the hormones required for mammary growth and secretory activity are not fully defined although both estrogen and progesterone exert key influences.

In the developing embryo there are two dense bands of ectodermal cells, running ventrolaterally from the axilla to the groin, that comprise the "milkline". Thickening of these ectodermal cells in the region of the thorax by 6 weeks post-conception is the first observable sign of the developing breast. This thickening corresponds with epithelial cell proliferation into the underlying mesenchyme and results in a structure known as the primary mammary bud (Moore, 1977). By 24 weeks the primary bud sprouts 15-25 secondary projections that develop into the lactiferous ducts. Studies in fetal mice have indicated that this early development is a result of poorly understood epithelial-stromal interactions and requires little if any hormonal involvement (Balinsky, 1950; Topper and Freeman, 1980). By the third trimester, canalization of the lactiferous ducts commences. At this point in breast development two layers of epithelial cells are distinguishable: an inner secretory layer and an outer myoepithelial layer. Just prior to parturition and for approximately four weeks after birth, fetal prolactin and placental steroids influence limited differentiation of and secretion from appropriate ductal epithelial cells (Friesen, 1973). Subsequently the breast epithelium reverts to an undifferentiated state in which it remains until puberty. At this point there is no histological or functional difference between the human female and male breast (Topper and Freeman, 1980); however, secretion of testosterone from the murine fetal testis midway through gestation inhibits breast epithelial development (Drews and Drews, 1977).

During childhood there is minimal ductal growth and no lobuloalveolar development. At puberty the secretion of estrogen by the ovaries stimulates lactiferous duct proliferation as well as adipose tissue deposition and stromal connective tissue development. This ductal development is dependent on estrogen as seen when quiescent ductal end buds in

ovariectomized mice can be stimulated to grow by systemic addition of estradiol (Bresciani, 1968). Estrogen alone was shown to be unable to stimulate ductal growth in triply operated mice in which the ovaries, pituitaries and adrenals were removed (Lieberman et al, 1978; Lyons, 1958). Partial ductal end bud growth was, however, obtained in these mice by treating them with both estrogen and growth hormone (Nandi, 1958). An indirect and local influence of estrogen on ductal development has also been supported *in vitro* in mouse and human mammary epithelial cells (Stampfer, 1985; Stampfer, 1987) and *in vivo* in various animals where implantation of estrogen-containing pellets into the mammary gland resulted in local, but not distal, end bud growth (Daniel et al, 1988). In some of these *in vivo* studies, estrogen receptor was observed in the ductal epithelium, stroma and luminal cells of the end bud, but not in the proliferative cap cells of the bud. Corresponding developmental studies have not been done in teenage females, but biopsies from normal, nonlactating women have indicated no estrogen receptors in stromal cells while only 7% of epithelial cells contain receptor (Petersen et al, 1987). These ER containing cells are scattered with highest frequency in lobules.

Once the nonlactating breasts have formed, they exhibit only slight growth during each follicular and luteal phase of the menstrual cycle and subsequent size reduction at menses. Dramatic and final maturation (lobuloalveolar development) of the breast occurs during pregnancy and lactation. During pregnancy, the end buds differentiate into alveoli which are clusters of epithelial cells (lobules) organized into spheres (Kaplan and Schenken, 1990). During the last trimester the secretory cells fill with droplets and the alveoli become distended with a proteinaceous, eosinophilic secretion called colostrum and the alveolar epithelium differentiates in preparation for its secretory function during lactation. Several hormones are known to affect mammary development during pregnancy in humans. Prolactin and placental lactogen (hPL) induce mammary growth *in vitro* and *in vivo* in rats (Talwalker and Meites, 1961; Topper, 1970) and are sufficient for mammogenesis in humans (Franks et al, 1977; Nielsen et al, 1979). Estrogen and progesterone are also important. Estrogen indirectly stimulates mammary growth and proliferation of epithelial cells while progesterone is primarily responsible for lobuloalveolar growth and simultaneous suppression of secretory activity (Topper and Freeman, 1980). In humans glucocorticoid levels also increase. Although not essential, glucocorticoids enhance lobule formation (Topper and Freeman, 1980). Studies in other species also indicate that insulin and thyroid hormone may also play a role in mammary development during pregnancy. In addition to these hormones a variety of growth factors including insulin-like growth factor I & II, epidermal growth factor and transforming

growth factor alpha are present in the maternal serum and have been demonstrated to stimulate lobuloalveolar growth (Tonelli and Soroff, 1980; Schreiber et al, 1986).

Lactogenesis, the production of milk, is possible in the pregnant female in the second trimester as the necessary milk proteins and enzymes have been produced. There are two independent stages of lactation: pre- and postpartum. In prepartum lactogenesis, the appearance of essential milk proteins and enzymes coincide with maximal placental lactogen production, suggesting that prepartum lactogenesis is regulated primarily by this hormone (Egli et al, 1961). After parturition an additional surge in lactogenesis occurs. At this point the expulsion of the placenta results in a removal of the prolactin antagonist, placental lactogen, and a decrease in the lactogenic inhibitor, progesterone. Coincidentally the level of estrogen, glucocorticoid, prolactin, oxytocin and prostaglandin $F_{2\alpha}$ increase (Kaplan and Schenken, 1990). Prolactin and glucocorticoid are believed to be the principal hormones involved in postpartum lactogenesis as these hormones are minimally required for milk secretion in triply operated rats in which the ovaries, pituitaries and adrenals have been removed (Lyons, 1958). Once lactation is established, continuous suckling is required for its maintenance. Suckling not only stimulates prolactin release from the pituitary, but it also stimulates release of oxytocin, a hormone that triggers myoepithelial contraction and assists in milk expulsion from the breast. Oxytocin has also been shown to stimulate prolactin release *in vitro* (Lumpkin et al, 1983). Estrogen's role in lactation appears unclear but high levels prepartum are believed to prime the breast for lactation. Estrogen promotes synthesis and release of prolactin, prolactin receptor and prostaglandin which are all essential to lactogenesis; however, estrogen administration postpartum inhibits lactogenesis and as such estrogen levels decline rapidly following parturition.

Breast Cancer - Development and Hormonal Influences:

-Pathology:

Breast cancer is the most common cancer among Canadian women. Approximately one in twelve women will develop the disease in her lifetime. The average annual incidence of breast cancer is approximately 61 cases for every 100,000 women (Haagensen, 1986). In Canada in 1988, 4,480 women and 33 men died from breast cancer (NCI, 1990). Based on accumulated statistics, 80% of future breast cancer deaths will be women 55 years of age or older, 18% between 35 - 54 and 2% will be younger than 35. (Safneck, 1988). Modern surgical, hormonal, chemotherapeutic and radiation therapies have not

decreased breast cancer related deaths which have remained at an annual level of 32-40 per 100,000 women since 1931 (NCI, 1990). Breast cancer research in the past 60 years has led to earlier tumor detection and better tumor characterization which has allowed more effective, tumor-specific treatments and subsequently has increased the disease-free survival period.

Hyperplasia of the lobular/ductal epithelial cells is the primary cause of breast lesions. Most of these lesions are not neoplasms but represent common physiological anomalies either a result of fibrocystic changes or inflammation of the breast. Of all palpable breast lesions only about 10% are diagnosed as breast cancers (Vorherr, 1980). Most breast cancers are carcinomas: 75-80% originating from glandular epithelial cells of ductal origin and 10-20% from epithelial cells of lobular origin (Ramzy, 1990).

All neoplasia originates from hyperplastic cells. In the breast normal hyperplasia occurs at puberty and during pregnancy primarily as a result of hormonal influences. Normally this hyperplasia is regulated and ceases upon removal of the hormonal stimuli. In some instances the hyperplastic glandular epithelial cells may change to resemble apocrine cells. This condition is commonly referred to as apocrine metaplasia and is still not indicative of neoplastic transformation. In other instances proliferating cells will undergo atypical hyperplasia, grossly characterized by pleiomorphism and nuclear enlargement with chromosomal abnormalities. Atypical hyperplasia is a hallmark of neoplasia. Tumors in which atypical hyperplasia are observed but in which the basement membrane of the duct or the lobule have not been transgressed are called ductal or lobular carcinomas-*in-situ*. Conversely, breast neoplasms which have invaded surrounding tissue are called invasive or infiltrating ductal or lobular carcinomas.

About 20% of breast lesions are benign fibroadenomas or intraductal papillomas and 10% are malignant carcinomas. Several factors dictate whether a neoplasm is benign or malignant. These factors include the extent of differentiation and atypical hyperplasia, the rate of tumor growth, the presence or absence of encapsulation and metastasis. In very broad terms, malignant cells tend to not undergo terminal differentiation, to exhibit a higher degree of atypical hyperplasia and to metastasize to local or distal sites. Benign tumors are usually more differentiated, are encapsulated in a fibrous membrane and do not metastasize.

Malignant tumors follow three basic pathways of metastasis. The first pathway is seeding

within a body cavity. In this case, certain tumor cells possess proteolytic mechanisms which enable them to penetrate their supporting basement membranes and eventually enter a natural body cavity. Once in the cavity, the cancer cell can attach to an appropriate host organ and attempt to establish a new tumor mass. The subsequent pathways of tumor metastasis involve the lymphatic and vascular systems. In the case of most carcinomas including those of the breast, the lymphatic system is the preferred means of dissemination. By attaching to the lymphatic basement membrane and enzymatically degrading it, the primary cancer cells can enter the lymphatic drainage and form secondary tumors in lymph nodes. As a result lymph nodes nearest the primary neoplasm are involved in 40% of breast cancer cases (Vorherr, 1980). The axillary nodes are the first to be affected, followed by the nodes along the internal mammary artery and then the supra- and infraclavicular nodes. Using the same proteolytic mechanisms either directly or through numerous interconnections between the lymphatic and vascular systems, vascular invasion and hematogenous spread to almost any organ can occur. The preferred sites of breast metastases are the liver, lung, bone marrow and adrenals as well as brain and skin. In many cases metastases may not be evident for decades following excision of the primary tumor.

- Hormonal Influences:

The potential importance of hormones in breast cancer was first shown in 1896 by the physician George Beatson when he observed temporary regression of the disease in his patients after removing the ovaries (Beatson, 1896). The observation that estrogens are the crucial mitogen in the development of these tumors has been reaffirmed by numerous studies. The frequency of breast cancer in men and in women without functional ovaries is only 1% that of women with intact ovaries (Thomas, 1986). Epidemiological studies of breast cancer in women exposed to radiation from atomic weapons revealed that the incidence of disease was highest in women who were at puberty during radiation exposure (McGregor et al, 1977). At puberty the mammary tissue is very responsive to estrogen. Several risk factors for breast cancer in women are also closely associated with dose and time of exposure to estrogen. An early age at menarche, late age at menopause and a late age at first full-term pregnancy result in prolonged exposure to elevated levels of estrogens and have been associated with an increased risk for breast cancer in women. Similarly obesity is also believed to increase the risk of breast cancer as elevated levels of estrogens result from aromatization of circulating androgens to estrogens in adipose tissue. Since cancerous transformation may involve a series of factors including genetic predisposition, chemical carcinogens, exposure to radiation,

retroviral infection and hormonal involvement, prolonged exposure to estrogen may enhance disease formation in a susceptible population of women. This hypothesis is supported by an observation in rats where the carcinogen 7,12-dimethylbenzanthracene (DMBA) is maximally effective at inducing mammary tumors in estrogen primed animals (Kiang and Kennedy, 1971). Physiological doses of estradiol have also been shown to induce growth of neoplastic breast cells (MCF-7) *in vitro* and *in vivo* in the nude mouse (Engel and Young, 1978; Soule and McGrath, 1980; Katzenellenbogen et al, 1983). Furthermore, whereas 7% of normal epithelial cells express estrogen receptor (ER), 60% of diagnosed breast neoplasms produce receptors to estrogen with 30-60% of these tumors responding to estrogen treatments (McGuire, 1980). Although it is not possible to determine the proportion of early (undetectable) tumors responsive to estrogen, the low occurrence of breast tumors in men and women with dysfunctional ovaries would tend to indicate that most tumors are derived from hormone responsive, ER containing cells and together with McGuire's data above would indicate that some 50% of these tumors eventually progress to a hormone independent state. In hormone responsive tumors significant growth arrest in response to endocrine therapy such as treatment with antiestrogen drugs like tamoxifen or surgical removal of the ovaries, adrenals and/or pituitary further supports the importance of estrogen in the development of breast cancer (Benz and Lewis, 1986).

Although estrogen action on breast tumors is poorly understood it has been proposed to influence development of the disease through a variety of mechanisms. Estrogen has been shown to be involved in the regulation of several genes involved in DNA replication such as DNA polymerase (Edwards et al, 1980), thymidine kinase (Kasid et al, 1986), dihydrofolate reductase (Cowan et al, 1982), thymidylate synthase (Aitken et al, 1985) uridine kinase, carbamyl phosphate synthetase, aspartate transcarbamylase and glucose-6-phosphate dehydrogenase (Aitken and Lippman, 1983; Aitken et al, 1985; Dickson et al, 1987). Estrogen has also been shown to stimulate the expression of a variety of growth factors such as insulin-like growth factors I and II (IGF-I and IGF-II) (Dickson et al, 1986; Yee et al, 1988) and transforming growth factor-α (TGF-α) (Dickson et al, 1987). These growth factors in turn have been shown to be mitogenic for breast cancer cells (Myal et al, 1984; Imai et al, 1982; Lippman and Dickson, 1989). Estrogen has been shown to transcriptionally regulate early response, growth competence genes, *c-myc* (Dubik and Shiu, 1988), *c-fos* (Weisz and Rosale, 1990) and *c-jun* (Weisz et al, 1990). Estrogen also stimulates expression of the progesterone receptor whose presence in breast tumors is postulated to indicate a functional estrogen receptor pathway (McGuire, 1980).

In both humans and the DMBA rat model, estrogen also stimulates expression of prolactin (Kim, 1965; Yen et al, 1974). Since estrogen will not stimulate mammary tumor growth in hypophysectomized DMBA rats (Sterental et al, 1963), its mitogenic action in this system is believed to be indirect through prolactin regulation.

Another potential mechanism of estrogen action in breast cancer development has been proposed to involve hydroxylated intermediates of estradiol that have been shown to be carcinogenic (Fishman, 1983). These hydroxylated intermediates, which can still bind the ER, have been shown to occur at higher levels in women with breast cancer or in women with a predisposition to the disease (Schneider et al, 1982; Bradlow et al, 1986).

Other steroid hormones have also been implicated in breast neoplasia. Progestins, although growth stimulatory in DMBA-induced tumors in rats, are growth inhibitory for human breast cancer cells *in vitro* as are glucocorticoid, retinoids and vitamin D (Lacroix and Lippman, 1980; Vorherr, 1980). Androgens are growth inhibitory in some HBC cell lines and in DMBA-treated rats; however, 85% of breast tumors contain androgen receptors (Lea et al, 1989) and 7% of breast tumors are androgen growth dependent as is the mouse mammary tumor cell line S115 (Hobbs et al, 1974). Thyroid hormones have been shown to be stimulatory in human breast cancer cell lines (Shiu, 1981). The mechanism by which these hormones act on breast cancers are for the most part unknown. The inhibitory actions of progestins and androgen are at least in part due to a down-regulation of the estrogen receptor (Horwitz and McGuire, 1978; Poulin et al, 1989) while retinoids have been described to down-regulate IGF-like binding proteins (Fontana et al, 1991).

The peptide hormones prolactin, growth hormone and insulin have also been implicated in breast cancer. Prolactin, whose receptor is present in 60% of human breast cancers, still plays a controversial role in the human disease. Studies have indicated that prolactin stimulates pathways associated with proliferation in breast cancer cells (Salih, 1972; Simon et al, 1985) but no direct effect on the growth rate itself has been observed (Shiu, 1981). Human growth hormone has prolactin-like effects on breast cancer cells and is believed to act via cross-reactivity with the prolactin receptor (Shiu, 1981). Similarly insulin has been shown to be mitogenic for breast cancer cell lines and for DMBA-induced mammary tumors (Heusen et al, 1972; Barnes and Sato, 1979). In HBC cell lines insulin is believed to act by cross-reactivity with the IGF-I receptor rather than through its own receptor (Myal et al, 1984).

In addition to hormones a variety of locally produced growth factors have also been implicated in breast tumor growth. Included among these are TGF-α (transforming growth factor alpha), EGF (epidermal growth factor), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), IGF-I, IGF-II and TGF-β.

TGF-α and EGF are part of a family of growth factors that function via the EGF receptor. TGF-α or a related family member is produced by breast cancer cells and in rat mammary tumors upon stimulation by estrogen (Bates et al, 1988; Liu et al, 1987). It is known to stimulate breast cancer cell proliferation, with antibodies to TGF-α or the EGF-receptor suppressing MCF-7 cell growth *in vitro* (Bates et al, 1988). A potential role of EGF in mammary tumors was suggested from studies in mouse models where removal of the major source of EGF, the submandibular gland, lowered tumor incidence in the mice, while reinfusion of EGF into these sialoadenectomized mice re-established tumor incidence (Oka et al, 1988). EGF has also been shown to down-regulate estrogen-stimulated progesterone production thereby resulting in a decreased antiproliferative progesterone effect (Nardulli et al, 1988; Cormier et al, 1989). A concrete involvement of EGF in human tumors remains to be established.

IGF-I (somatomedin C) and IGF-II are also mitogenic for human breast cancer cells (Furlanetto and DiCarlo, 1984; Huff et al, 1986b). All breast cancer cell lines examined secrete IGF-I or IGF-I-related proteins (Huff et al, 1986a). In MCF-7 cells expression of the IGF-I-related proteins is stimulated by estrogen, TGF-α, EGF or insulin and inhibited by antiestrogens, TGF-β and glucocorticoids (Dickson and Lippman, 1991). IGF-I and IGF-II receptors have also been detected in HBC cell lines (DeLeon et al, 1988; Furlanetto and DiCarlo, 1984) although IGF-II is believed to potentiate its mitogenic cellular response through the IGF-I receptor. The mechanism of IGF-I and IGF-II induction and their biological role in breast cancer is still unknown.

PDGF and PDGF-related factors are also expressed in many breast cancer cell lines (Dickson and Lippman, 1991). Recognition that PDGF mediates proliferation of stromal cells like fibroblasts *in vitro* together with the lack of PDGF receptor expression in HBC cell lines (Dickson and Lippman, 1991), has lead to speculation that PDGF may influence breast cancer cells by stimulating adjacent fibroblasts to produce and secrete growth factors mitogenic to the cancer cells (Lippman and Dickson, 1989; Karey and Sirbasku, 1988).

FGF or related proteins have been shown to be essential for growth of normal mouse mammary cells in culture and to be present in extracts from human mammary epithelium (Hammond et al, 1984; Smith et al, 1984). There is evidence that FGF can act on both the breast epithelial cells and the surrounding stroma (Dickson and Lippman, 1991). An FGF-like protein has been isolated from HBC cell lines in culture (Halper and Moses, 1983).

TGF-β is a potent inhibitor of normal mammary epithelium *in vivo* in neonatal mice (Silberstein, 1987) and *in vitro* on human breast cancer cells (Wang and Hsu, 1986). TGF-β is expressed in all HBC cell lines studied to date (Dickson and Lippman, 1991). Its expression in HBC cells is inhibited by estrogen and other mitogens, but stimulated by antiestrogens and glucocorticoids (Knabbe et al, 1987). The recent observation in keratinocytes that TGF-β reduces *c-myc* expression through a mechanism involving the tumor suppressor protein RB-1, may explain TGF-β's inhibitory effect in breast cancer cells (Pietenpol et al, 1990).

In addition to these established growth factors, two novel proteins, amphiregulin (an EGF-related protein) and mammastatin have been characterized and shown to be growth inhibitory for several breast cancer cell lines (Plowman et al, 1990; Ervin et al, 1989).

Human Breast Cancer Cell Lines:

The ability to define the growth conditions of cultured human breast cancer cell lines has made this model system pivotal in studying hormonal regulation and gene expression. To date some 50 breast cancer cell lines have been established and characterized to varying extents (Engel and Young, 1978). These cell lines have been established from either solid tumors, or pleural or ascites effusions of patients with breast cancer. The latter source has yielded the majority of cell lines as effusions tend to provide large numbers of disassociated viable tumor cells with little fibroblast contamination.

Breast cancer cell lines offer several advantages over *in vitro* organ culture or whole animal breast cancer model systems such as the 7,12 dimethylbenzanthracene (DMBA)-induced rat mammary tumor and mouse mammary tumor virus (MMTV)-induced tumor model systems: (1) human breast cancer cell lines eliminate interspecies differences associated with animal models, (2) cell culture permits the study of a homogeneous cell population, avoiding the complex multi-cell type scenario associated with breast tumors,

(3) because of the homogeneous population, continuous cell culture permits sequential experiments with the same tissue and (4) since cell culture allows complete control over culturing conditions, it enables sequential study of growth influences on breast tumor cells by specific addition to the growth media of defined cellular components, hormones and growth factors.

There are some important shortcomings of breast cancer cell lines as a model system of the disease. These shortcomings stem from the fact that methods to establish cell lines discriminate against certain malignant cell types (in particular those that grow slowly) and from the fact that strictly defined *in vitro* culture conditions may not adequately represent the environment of these cells *in vivo* lacking cell-cell and cell-matrix influences. As a result of these shortcomings, molecular pathways defined in breast cancer cell lines may differ considerably from the molecular pathways of tumor cells *in vivo*.

Several breast cancer cell lines were utilized in these studies. These cell lines include the estrogen-responsive, estrogen receptor positive MCF-7 and T-47D, and the estrogen receptor negative MDA-MB-231 and BT-20 cell lines. With the exception of BT-20 which was isolated from a solid tumor, all others were established from pleural effusions. Other characteristics of these cell lines are listed in a review of breast cancer cell lines by Engel and Young (1978). In addition to these breast cancer cell lines, we also utilized a immortalized "normal" breast cell line HBL-100 isolated from breast milk (Gaffney, 1982).

B: Estrogen and the Estrogen Receptor:

Estrogen -Chemistry, biosynthesis and metabolism:

Naturally occurring estrogens are synthesized and secreted in large amounts by the theca cells of the ovarian follicles, by the corpus luteum and by the placenta, and in small amounts by the cells of the adrenal cortex and by Leydig cells of the testes. They are synthesized from free or fatty acid esterified cholesterol via androgen precursors. They can also be formed in the circulation by aromatization of androstenedione to estrone by the enzyme aromatase. This enzyme also converts testosterone to estradiol. Most but not all synthesized estrogens are secreted into the circulation where the majority exist complexed to either albumin or steroid-binding globulin. Of the three naturally occurring estrogens (estradiol, estrone, and estriol), estradiol is the most potent and estriol is the

least potent. In the circulation estradiol is in equilibrium with estrone, the precursor to estriol. The small amount of free estrogen (3%) can passively traverse the cellular membranes to interact with a nuclear receptor in target cells. Circulating estrogens are eventually metabolized in the liver where they are converted to glucuronide sulfate conjugates. These metabolites are either secreted into the bile and reabsorbed or excreted in the urine.

Steroids are potent signal transducers that act to coordinate biological functions related to cell development and differentiation. Being simple molecules, their biological effects are potentiated through protein receptors. Unbound, aporeceptors are closely associated with the nucleus as large inactive protein complexes. By binding steroid, the complex dissociates releasing an active transformed receptor. A receptor dimer can interact with specific regulatory elements usually located upstream of the regulated gene which interacts with the RNA polymerase complex to influence expression of the gene. Recent cloning of these steroid receptors has revealed considerable sequence and structural similarity among themselves and non-steroidal thyroid, retinoid and vitamin D3 receptors. Together all these receptors form the steroid and thyroid hormone receptor superfamily.

In recent years extensive research has been done on the structure/function relationship of these receptors and their mechanism of gene regulation. With many fine recent reviews on this topic (Evans, 1988; Beato, 1989; Carson-Jurica et al, 1990) this literature review will focus on more recent studies with emphasis on the estrogen receptor.

Estrogen Receptor:

-The Estrogen Aporeceptor:

In the absence of hormone and under hypotonic conditions, steroid receptors can be isolated from the cytosol of target cells as large complexes (MW ~300,000) that sediment at 8-9S (Sabbah et al, 1989; Sanchez et al, 1990). The significance of these complexes is unknown; however, evidence is mounting to indicate that these complexes are not an artifact of isolation and they may be of biological relevance. All steroid receptor complexes contain untransformed aporeceptor and a 90-kDa heat shock protein (*hsp90*)(Carson-Jurica et al, 1990). A 59 kDa protein is also loosely associated with the complex (Gasc et al, 1990). In addition to *hsp90*, certain receptor complexes may also contain another heat shock protein, *hsp70* (Sanchez et al, 1990), and uncharacterized 56, 54, 50 and 23 kDa proteins (Sanchez et al, 1990; Smith et al, 1990). In all cases receptors

of these oligomeric complexes do not interact with DNA. Treatment with either hormone or salt or metal chelators will liberate transformed receptor that can bind steroid regulatory elements.

Hsp90 proteins are a family of ubiquitous and highly abundant proteins. This family includes two other subgroups: the *hsp70* and *hsp60* proteins (Schlesinger, 1990). They were initially discovered in *Drosophila* cells where they exhibited enhanced expression following heat shock or environmental stress. They were subsequently found to be present and highly conserved in organisms as evolutionarily diverse as bacteria and mammals.

In the current model of ER-protein interaction, the 8-9S ER complex is composed of two receptor proteins and two *hsp90* proteins (Redeuilh et al, 1987) and has been reconstituted *in vitro* (Inano et al, 1990). *Hsp90* is an acidic phosphoprotein possessing an ATP binding site and possibly an intrinsic autophosphorylating activity (Csermely and Kahn, 1991). It has been shown to interact with steroid receptors (but not thyroid, retinoid and vitamin D3 receptors) through a highly charged region near the N-terminus (Dalman et al, 1990). Recently a report has been published that the viral *v-erb-A* oncogene, a virally transduced, altered version of the *c-erb-A*/thyroid receptor locus, forms a complex with *hsp90* (Privlasky, 1991). Since no such complex has been detected with the thyroid receptor (Dalman et al, 1990), the concept that the *v-erb-A/hsp90* complex can be associated with the establishment of the oncogenic phenotype has been proposed. *Hsp90* is primary localized to the cytoplasm of cells; however, considerable amounts of *hsp90* are located in the nucleus with increasing amounts following cell stress (Gasc et al, 1990). In addition to steroid receptors, the *hsp90* proteins transiently bind several kinases. In the case of eIF-α kinase, a kinase that phosphorylates the α-subunit of the translation-initiation factor eIF-2, binding *hsp90* enhances its activity (Hardesty and Kramer, 1989). The *hsp90* proteins also bind cytoskeletal proteins such as actin and tubulin and are believed to stabilize these proteins and their respective cytoskeletal fibers during prolonged heat stress (Schlesinger, 1990).

With steroid receptors, the role of *hsp90* is still speculative. Earlier studies suggested that the *hsp90* simply blocks, through steric interference, a reactive site of the steroid receptors until the receptor binds hormone (Binart et al, 1989). Evidence that *hsp90* may protect the receptor against proteolytic cleavage also exists (Housley et al, 1990). Today these functions of *hsp90* on steroid receptors have been expanded to include their role as

possible "molecular chaperones" (Schlesinger, 1990; Sambrook and Gething, 1989). Although proteins are thought to spontaneously acquire their final tertiary structure, evidence that certain proteins require a secondary "chaperone protein" to obtain their preferred conformation is accumulating. Unlike enzymes, chaperones cannot catalyze covalent bond formation, can remain associated with their target protein for long periods of time and can interact with a wide range of diverse substrates. Such a chaperone molecule *Pap D* of *E. coli* was described to form transient complexes with several proteins of the bacterial pilus, shuttling them to the proper site and assisting in their correct assembly (Holmgren and Branden, 1989). Structural and functional similarities between *Pap D* and the heat shock proteins have led to the hypothesis that certain heat shock proteins also act as chaperone molecules (Rothman, 1989). That the *hsp90* may act as a chaperone molecule to steroid receptors was shown recently in an experiment by Picard et al (1990). By constructing a *hsp90*-difficient strain of *Saccharomyes cerevisiae* which is incapable of forming the 8-9S ER complexes with transfected receptors, they were able to show that upon addition of hormone, transformed receptor complexes formed but these were unable to activate expression of a reporter construct unless very high hormone concentrations were used. This result implied that the *hsp90*-uncomplexed receptors were only partially active. This study complements earlier work by Bresnick et al (1989) who showed that *hsp90* was necessary to maintain a competent, high-affinity steroid binding site on the receptors. Together both studies support the model that *hsp90* may act as chaperones for steroid receptors in order to establish or stabilize the hormone binding domain and provide a highly active transformed receptor for signal transduction.

-Molecular and Protein Structure:

To date the estrogen receptor (ER) genes from human (Walter et al, 1985; Green et al, 1986; Greene et al, 1986), chicken (Krust et al, 1986; Maxwell et al,1987), mouse (White et al, 1987), rat (Koike et al, 1987) and *Xenopus laevis* (Weiler et al, 1987) have been cloned. In all cases a single mRNA of between 6.2-7.5 kb codes for a 589-600 amino acid protein with a molecular weight of about 66.5 kDa. All ER mRNAs contain a large 3'-untranslated region the significance of which is unknown. The estrogen receptors cloned are remarkably similar [Fig. 1A] indicating a strong evolutionary bias towards maintaining the ER unchanged and underlies its importance in the basic development of various cells. All receptors of the superfamily are encoded by one mRNA with no gene locus amplification. The A and B forms of the progesterone receptor resulting from an alternate translation initiation event from a single mRNA (Conneely et al, 1989).

Once transcribed, capped and polyadenylated, the ER mRNA, like most eukaryotic mRNA is transported into the cytoplasm to undergo translation. For some time it was hypothesized that the unbound cytosolic receptor entered the nucleus only upon hormone binding. This hypothesis was shown to be incorrect when careful immunocytochemical analysis using monoclonal ER antibodies and whole cells or nucleoplasts and cytoplasts, revealed predominant nuclear localization of both the hormone-bound and unbound receptors (King and Greene, 1984; Welshons, 1984). Today the consensus is that the majority of the steroid/thyroid hormone superfamily receptors are located in the nucleus in their inactive state. This model suggests that *in vivo* synthesis of the receptors may occur on the outer nuclear membrane with near-immediate nuclear translocation. This nuclear translocation may occur non-specifically by diffusion of the receptors through the nuclear membrane (Paine et al, 1975) or specifically by nuclear pore protein-receptor interactions (for review see Dingwall and Laskey, 1986). This latter mechanism requires that the translocated protein contain a specific nuclear translocation signal sequence (Kalderon et al, 1984). Such a sequence may be present in the ER from amino acids 256-303 (Didier et al, 1990).

Traditional biochemical studies of steroid receptors utilizing proteolytic fragments revealed that these receptors contained distinct functional regions responsible for hormone binding and DNA binding. Near simultaneous cloning of the glucocorticoid, progesterone and estrogen receptors supported these findings and revealed distinct organizational and structural similarities among these receptors. Based on sequencing data, the receptors could be subdivided into regions A-F of high or low amino acid similarity (Krust et al, 1986). Using the highly conserved C-region as a probe, other members of the steroid receptor family were isolated (Petkovich et al, 1987; Giguere et al, 1988). In addition identification of sequence similarities between the ER and the *v-erb-A* oncogene (Green et al, 1986; Krust et al, 1986) lead to the eventual discovery that the host cell progenitor *c-erb-A* coded for the thyroid hormone receptor (Weinberger et al, 1986). Receptors for retinoic acid and vitamin D3 receptors were discovered to be part of this superfamily (Petkovich et al, 1987; Baker et al, 1988) as was the chicken ovalbumin upstream promoter transcription factor (COUP)(Wang et al, 1989) which was subsequently shown to be the dopamine receptor (Power et al, 1991). Also discovered were several receptors such as ERR1, ERR2 and HAP with unknown ligands (Giguere et al, 1988; Dejean et al, 1986; deThe et al, 1987). HAP was subsequently identified as the β-retinoic acid receptor.

Structurally each receptor can be subdivided into six regions A-F based on their amino acid similarity with other receptors. Region A/B (the N-terminal) is a hypervariable region with considerable differences among receptors. The length of this region varies from 25 to 603 residues for the vitamin D3 and mineralocorticoid receptors respectively [Fig. 1B]. Estrogen receptors from different species vary in length only in this region [Fig. 1A]. Region C is the most conserved region. It contains nine cysteine residues, the first eight of which tetrahedrally coordinate two zinc ions, forming the DNA binding domain (DBD). Region D is a short hydrophilic sequence which was initially postulated to act as a hinge between the DBD and the hormone binding domain (HBD) due to the amino acid and size variation (Kumar et al, 1987). Recently this region has been implicated as containing the possible intranuclear localization signal (Carson-Jurica et al, 1990) and part of the *hsp90* binding domain (Chambraud et al, 1990). The remaining C-terminal sequences, comprising regions E and F form the largest region of the receptor and functionally the most diverse. Almost every functional aspect of the receptor including hormone binding, dimerization, *hsp90* binding, and *trans*-activation are associated completely or partly with this part of the receptor.

The genomic organization of the estrogen receptor has revealed that the A/B region and the 5' untranslated sequences are encoded by the first exon, the DNA binding C region is encoded by exons 2 and 3, each exon encoding one of the zinc fingers, while the remaining 3 structural domains and the large 3' untranslated region are encoded by the remaining 5 exons [Fig. 2](Ponglikitmongkol et al, 1988).

-Functional Domains:

Numerous functions have been associated with the estrogen receptor. These include: (1) hormone binding, (2) response element recognition and DNA binding, (3) *trans*-activation, (4) nuclear localization, (5) receptor dimerization, (6) *hsp90* association and (7) interaction with a growing number of transcription factors. Because of the modular nature of the nuclear receptors, attempts have been made to localize the structural regions responsible for these functions. In some circumstances specific functions have been associated with precise regions: the DBD to region C for example. However, most functions appear to involve several interspersed regions of the receptor. Analysis of the above mentioned functions in relation to their association with the estrogen receptor will be reviewed below.

- DNA Binding Domain:

Through immunohistochemical studies, the interaction of steroid receptors with DNA has been known for years. However, our understanding of the mechanisms of protein-DNA interactions has been clarified in the past decade. In this time three structurally distinct types of protein-DNA interactions have been described: the helix-turn-helix, the leucine zipper/helix-loop-helix and the zinc finger models (for reviews see Abel and Maniatis, 1989; Latchman, 1990; Mitchel and Tjian, 1989).

-The helix-turn-helix model:

The first protein-DNA interactions to be studied were those of the bacteriophage λ operon regulatory proteins. X-ray crystallography studies of the tertiary structures of the operon regulatory proteins *cro*, CAP and the lambda repressor revealed a common structural similarity (Anderson et al, 1981; McKay and Steitz, 1981; Pabo and Lewis, 1982). All three proteins contain two α-helical regions involving 8 amino acids each separated by a 3 amino acid turn. These proteins bind DNA in a way such that the first helix binds across the major groove while the second recognition helix fits into the adjacent major groove forming specific chemical interactions between the helix amino acids and nucleotide sidechains. This interaction is not rigidly strict, but rather a custom fit interaction involving hydrogen bonding, van der Waals contacts, interactions with surrounding water molecules and possible adjustments of the DNA phosphate backbone to provide the most stable interaction possible (for review see Brennan and Matthews, 1989).

To date numerous prokaryotic and eukaryotic transcription factors are recognized to contain helix-turn-helix motifs. In *Drosophila melanogaster,* a series of genes which are critical to development have been recognized. These genes are known as homeotic genes. Comparison of their sequences revealed a highly homologous 60 amino acid region subsequently referred to as the homeobox or homeodomain. This region was shown to be critical for DNA binding of these homeotic proteins (Müller et al, 1988). Structural predictions and three-dimensional spectroscopic studies of two of these homeodomains revealed that this region interacts with DNA in a manner analogous to the helix-turn-helix containing proteins (Kissinger et al, 1990; Qian et al, 1989). These studies revealed that the 60 amino acid homeodomain contains three or possibly four α-helical stretches. The first two helices are positioned antiparallel to each other and are located on top and perpendicular to the large 20 amino acid recognition helix. This

recognition helix and the second helix form the helix-turn-helix equivalent motif. The C-terminal end of the recognition helix fits into the major groove of the DNA recognition sequence while helix two, unlike the classical nonrecognition helix makes few contacts with the DNA and is believed to hold the recognition helix tightly in position. A region N-terminal to the first helix contacts residues in the minor groove and stabilizes the DNA-protein interaction. Highly conserved homeobox amino acids Trp-48, Phe-49, Asn-51 and Arg-53 are involved in the actual contact with DNA residues. Although there are distinct similarities in DNA binding mechanisms of helix-turn-helix and homeodomain containing proteins, the actual interactions with DNA are significantly different (Kissinger et al, 1990).

A new class of homeobox containing regulatory proteins including the pituitary specific factor Pit1, the octamer binding factors Oct1 and Oct2 and the nematode gene *unc-86* have been identified (Latchman 1990, and refs within). These proteins contain in addition to the homeobox region itself an adjacent conserved domain known as the POU-specific region. This POU-specific domain can also interact with DNA and contribute in varying degrees to the specific recognition of these proteins with their corresponding regulatory elements (Theill et al, 1989; Sturm and Herr, 1988).

-The leucine zipper / helix-loop-helix model:

Another DNA binding model was recognized recently in the liver transcriptional factor C/EBP, the yeast factor GCN4 and in several oncogene proteins including *c-fos*, *c-jun* and *c-myc* (Abel and Maniatis, 1989). All these protein share an α-helical region containing several evenly spaced leucine residues. The positioning of the leucine residues are such that they occur every two turns along the helix and on the same side of the helix. Two such regions on different molecules were shown to align to form a dimer structure. This type of leucine zipper [Zip] dimer was shown to form between the *c-jun* and *c-fos* proteins. Once the dimer formed, a highly basic region adjacent to the zipper would be capable of interacting with specific DNA elements.

Subsequently a group of DNA-binding proteins, including the muscle regulatory protein MyoD1 that is critical in the conversion of fibroblasts to myoblasts, were found to contain a region homologous to the leucine zipper proteins DNA-binding basic region although they lacked the leucine-containing helix. Instead these proteins possessed a helix-loop-helix structure in which the helices contained charged amino acids evenly arranged on one side of the helix analogous to the leucine residues of the leucine zipper

(for recent reviews see Jones, 1990; Olson 1990). These structures, like the leucine zippers, could participate in dimerization of the protein and facilitate interaction with DNA regulatory elements of the adjacent basic region. Recently the mammalian transcriptional factor AP-2 was shown to be a member of yet another leucine zipper-like family of proteins (Williams and Tjian, 1991). In AP-2 two putative amphipathic α-helices are separated by a very large intervening region. This region has been termed the helix-span-helix (HSH) and as with the Zip and HLH regions, the HSH is responsible for dimerization. Upon dimerization a basic region adjacent to the HSH recognizes a specific response element.

-The zinc-finger model:

Studies in *Xenopus laevis,* which addressed the transcription of the 5S RNA genes by RNA polymerase III, lead to the isolation of an essential transcriptional factor TFIIIA. The factor bound some 50 nucleotides upstream of the 5S gene and required zinc for this interaction. It was subsequently shown that nine zinc molecules were associated with TFIIIA. Once TFIIIA was cloned, nine repeat units of 30 amino acids rich in cysteine and histidine were identified as the most likely regions of zinc binding. Based on the fact that zinc is normally tetrahedrally coordinated with cysteine or histidine in organic compounds, each unit was proposed to form a "zinc finger" (Miller et al, 1985) which bound DNA by interacting with nucleotide side chains in the major groove.

Steroid receptors are also known to associate with zinc ions. Upon cloning these receptors, the highly conserved C region containing nine cysteine residues evidently coordinated zinc ions and formed a finger structure similar to that of TFIIIA. This cysteine rich region of the steroid receptors was shown to be solely responsible for DNA binding (Green and Chambon, 1987). By constructing a chimeric glucocorticoid receptor in which the glucocorticoid receptor cysteine-rich region was exchanged with the corresponding estrogen receptor region, they were able to show that the transformed chimeric receptor specifically bound and activated transcription from the estrogen response element (ERE) and not the glucocorticoid response element (GRE). Subsequently gel retardation studies using receptors lacking region C or with key mutations in this region confirmed its importance in DNA binding (Kumar and Chambon, 1988).

The high conservation of the DNA binding domain (DBD) among different nuclear receptors and their ability to differentiate between closely related response elements lead

to precise studies to identify key amino acids in this recognition. Initial "swapping" experiments involving the N-terminal region of the ER DBD [known as C1: amino acids 185-215] with the respective GR C1 region resulted in an chimeric ER that recognized the GRE (Green et al, 1988). Ensuing studies of the C1 subregion revealed that mutating three key amino acids of the ER in the carboxyl terminal of the first finger could change its specificity from an ERE to a GRE (Mader et al, 1989; Danielson et al, 1989). The mutations responsible were Glu_{203} to Gly, Gly_{204} to Ser and Ala_{207} to Val and were located in a helical region believed to interact directly with the response element nucleotides within the major groove. Interestingly the $Glu_{203}Gly_{204}$ is conserved between the hER, hRAR, hTR and hVDR while the Gly_{203} Ser_{204} is conserved in the hGR, hPR, hMR and hAR. Although recent studies using a peptide comprising just the first zinc finger of the GR have shown that it binds zinc ions and specifically recognizes the GRE (Archer et al, 1990), the second finger has also been shown to be essential to DNA binding in the ER, GR and VDR (Danielson et al, 1989; Hollenberg and Evans, 1988; Hughes et al, 1988). A chimeric first finger GR and ER interfinger/second finger receptor exhibited promiscuity between the GRE and ERE suggesting some dependence on the second finger and interfinger sequences to stabilize the DNA interaction possibly by participating in receptor dimerization (Danielson et al, 1989). Furthermore the five residues from Pro_{221} to Gln_{226} were shown to be important in discriminating the half-site spacing between the ERE [GGTCA-nnn-TGACC] and the TRE [GGTCA-TGACC] (Umesono and Evans, 1989).

Confirmation of the mutational studies addressing the structure of the DBD and its interaction with DNA have been obtained recently for the DBD of both the ER and the GR using two-dimensional ^{1}H-NMR techniques (Schwabe et al, 1990; Hard et al, 1990b) and for the GR-DNA interaction using x-ray crystallography (Luisi et al, 1991). From these studies, the DBD was found to consist of a globular body from which the finger regions extended. Helical regions were confirmed at the "knuckle" of each finger from residues 203 to 212 and 239 to 251 in the ER. The key amino acids Glu_{203}, Gly_{204} and Ala_{207} can interact with the ERE in the major groove, while the basic Arg_{235}, Arg_{236}, Lys_{237} tip of the second finger is in a position to interact with the acidic phosphate backbone of the minor groove. These studies have also confirmed that the ninth Cys and the His in the DBD are not involved in zinc coordination. Finally Pro_{221} to Gln_{225} were shown to be the best candidates for receptor-receptor dimer interactions [Fig. 3].

With the recent tertiary data for the ER and GR DBD and also for another zinc

coordinating DNA-binding factor, Gal4 (Pan and Coleman, 1990), increasing evidence suggests that zinc finger regions are not similar to the original TFIIIA zinc fingers. Although tertiary structure data of the TFIIIA zinc fingers is not available, indirect studies have indicated that finger-like structures are actually formed, with residues in the fingers contacting the DNA regulatory element. For the DBD of the ER and GR, true finger-like structures are absent and the α-helix, just outside of the zinc coordination region, is responsible for the DNA interaction. With Gal4 the coordination of the two zinc ions is totally different from that of TFIIIA, ER or GR, involving six cysteine residues that place the zinc ions at an atomic distance of ~3.5 Å, not ~13 Å as seen in GR and ER. Therefore the zinc finger category of DNA binding proteins has been proposed to be represented more accurately as three distinct groups (Vallee et al, 1991): 1) the classical zinc finger structures like those of TFIIIA, the eukaryotic transcription factor Sp1 (Kadonaga et al, 1987) and the yeast ADR1 protein, 2) the zinc-twist motif of the steroid superfamily and 3) the zinc-cluster structures of Gal4 and metallothionein (Schultze et al, 1988).

In addition to the three groups of DNA-binding proteins discussed above, several factors including the ubiquitous CAAT binding factor [CTF/NF-1] have been described that bind DNA but lack any of the motifs described above. Therefore it is probable that other DNA binding families will be discovered.

-Nuclear Localization Domain:

The nuclear membrane is interspersed with large proteinaceous structures known as nuclear pores. Molecules such as proteins and RNA can enter the nucleus through these pores in a size dependent fashion either through simple diffusion or by active ATP dependent transport. Molecules of less than 4.6 kDa can traverse these pores while larger molecules are restricted proportional to their size [in particular their radius]. To enter the nucleus passively the molecule must not exceed the pore radius of ~45 Å (Paine et al, 1975). Therefore passive diffusion is limited to spherical molecules of less than ~69 kDa.

Active transport through nuclear pores was first shown for the *Xenopus* oocyte nuclear protein nucleoplasmin. When a C-terminal mutated protein was microinjected into cells, the mutated protein was not able to re-enter the nucleus (Paine et al, 1975). Subsequently the nuclear SV40 T-antigen was shown to lose its nuclear localization by a single mutation of Lys_{128} (Lanford et al, 1984). The basic sequence Pro-Lys-Lys_{128}-Lys-Arg-

Lys-Val was recognized as the nuclear localization signal (NLS), which when fused to the β-galactosidase protein was capable of directing it to the nucleus. Sequences resembling the SV40 T-antigen NLS were found in most of the superfamily receptors in the D region immediately adjacent to the DBD. These sequences alone could not direct nuclear localization of a fusion protein. In the GR a second hormone-dependent NLS was identified in the hormone binding domain (HBD)(Picard and Yamamoto, 1987).

For the ER, the SV40 T-antigen-like NLS spans amino acids 256-263: alone it cannot direct a fusion protein to the nucleus. Unlike the GR a secondary NLS in the HBD is not present and nuclear localization is not dependent on hormone binding. By monitoring the cellular localization of various ER mutants, a broader region between amino acids 256-303 was shown to be essential for nuclear localization and was capable of directing β-galactosidase to the nucleus when fused to the C-terminus (Didier et al, 1990). This region of the ER contains three basic stretches: 256-260, 266-271, 299-303. The first two basic stretches are very well conserved among ERs from different species and are most likely critical to nuclear localization of the ER. The importance of the first two basic stretches has been recently suggested by Robbins et al (1991) who have characterized in detail the nucleoplasmin nuclear localization sequence. This NLS is considerably different from that of SV40 T-antigen resembling it only at the carboxyl terminal end. It consists of 16 amino acids with two basic regions at each end separated by a 10 amino acid spacer. The authors were able to show that the basic residues were critical for nuclear localization of nucleoplasmin while mutation of, or a small insertion into, the spacer region had no effect. Analysis of nuclear proteins revealed that the presence of the basic-spacer-basic nuclear localization motif was common. In most cases the SV40 T-antigen-like NLS formed part of the basic-spacer-basic motif. In the ER the nucleoplasmin NLS corresponded to amino acids 256-272 which are the conserved basic sequences of the proposed NLS 256-303.

-Hsp90 Binding Domain:

8-9S *hsp90*-steroid receptor complexes have been shown for the estrogen, glucocorticoid, progesterone and androgen receptors (Sanchez et al, 1985; Catelli et al, 1985; Riehl et al, 1985). The highly conserved nature of the heat shock proteins and the steroid receptors would indicate that the amino acids involved in the interaction are probably conserved. Studies to identify the region of the steroid receptor involved in this interaction were first done for the GR and subsequently for the PR and ER. In the early studies it was shown that by limited trypsinization of the GR receptor, a C-terminal containing 27 kDa steroid

binding fragment was generated. This fragment could form a 8-9S receptor complex and could be immunoprecipitated by an antibody specific for *hsp90* (Denis et al, 1988). Subsequent studies using *in vitro* translated GR and PR deletion mutants further localized the *hsp90*-binding domain between amino acids 480-617 and 568-616 for the hPR-A and the GR respectively (Carson-Jurica et al, 1989; Howard et al, 1990). This part of the HBD also contained a highly conserved ~60 aa subsequence that was suggested to be a candidate *hsp90* binding site. Short deletions in this conserved sequence of the hPR-A were shown not to prevent complex formation (Carson-Jurica et al, 1989). Conversely for the GR, this region was shown to be essential for *hsp90* complex formation, with mutants lacking this region (574-632) forming very unstable *hsp90* complexes and being susceptible to proteolytic cleavage (Housley et al, 1990). A subsequent study revealed that deletion of region 604-659 of the GR totally prevented *hsp90* complex formation (Dalman et al, 1991). Peptide competition studies of this region revealed two distinct contact sites of *hsp90* interaction in the GR: within the nuclear receptor conserved region 574-632 and region 632-659 (Dalman et al, 1991). Analysis of this area of the GR and corresponding regions in other steroid receptors revealed a short hydrophobic region (627-635) which contains a highly conserved proline residue and an adjacent "dipole-plus-cysteine" motif [aa^- -n- aa^+ -n_{5or10}-cys where aa-, aa+ are negatively and positively charged amino acids respectively and n is any amino acid]. This structure is conserved among the steroid receptors and the VDR but is lacking in the non-*hsp90* associating thyroid and retinoic acid receptors (Dalman et al, 1991). This motif is also less well conserved in the ER and may explain why the ER-*hsp90* complex is less stable than the GR, PR, AR and MR complexes. Although this motif is present in VDR, whether a VDR-hsp90 complex can form is unknown.

The most recent study localizing the *hsp90* binding domain of the ER has shown that multiple regions of the ER are involved (Chambraud et al, 1990). This study confirmed the work in the GR and PR indicating that the N-terminal and DNA binding domains are not involved in *hsp90* complex formation. Furthermore it was shown that the HBD was also involved; however, no subregion could be identified. Also identified was a highly positive charged region (251-271) which was involved in the interaction. This region, which is just outside the DBD in the D region of the receptor, overlaps the postulated NLS and although not sufficient alone, was essential for the formation of the 8-9S ER complex. Although the GR and PR *in vitro* mutational studies discussed above indicated that a C-terminal fragment of these receptors was sufficient to generate the *hsp90* complex, other studies by Pratt et al (1988) and Weigel et al (1989) indirectly supported

the involvement of the D region sequence in the *hsp90* interaction. The ER-*hsp90* complex is postulated to be less stable than the corresponding PR, GR complexes and the amino acid 251-271 interaction is critical to stabilizing this complex (Chambraud et al, 1990). Together these studies support the view that the *hsp90* binding domain of steroid receptors encompasses multiple loosely defined regions in the HBD and also the positively charged sequence immediately adjacent to the DBD.

-Dimerization Domain:

In addition to interacting with *hsp90*, most steroid receptors have also been shown to exist in the transformed state as receptor dimers. The large 8-9S ER complex can be dissociated to generate a 5S unit (Sabbah et al, 1989). This 5S unit consists of two 66 kDa proteins and binds two ER antibody molecules indicating a receptor dimer complex (Redeuilh et al, 1987). Further confirmation of a receptor dimer was obtained with DNA-binding (gel retardation) studies utilizing homogeneous and heterogeneous receptor/ mutant receptor complexes (Kumar and Chambon, 1988). The importance of steroid receptor dimers *in vivo* is supported by the dyad axis of symmetry exhibited by most steroid regulatory elements (more later).

Three dimensional structural analysis of the dimer complexes has not yet been done and as such the precise physical interaction between receptor molecules has not been characterized. However extensive receptor mutation studies have illuminated some of the important characteristics of these receptor-receptor complexes. To date the most complete study of dimerization of the ER has been done by Kumar and Chambon (1988). Using *in vitro* gel retardation studies and a host of mutated estrogen receptors, they showed that receptor deletion mutants in the A/B, D or F regions could form dimers both in solution and in the presence of a consensus estrogen response element. Dimerization and stable binding to the ERE of these constructs was observed only in the presence of hormone. Conversely, mutated receptors in the DNA binding C region, receptors consisting of only the HBD and receptors with deletions, insertions or point mutations in the HBD, could not form dimers in solution or in the presence of a consensus ERE. Surprisingly mutant receptors totally lacking the HBD formed dimers in the presence of a consensus ERE in the presence or absence of hormone. These dimers bound weakly or not at all to non-consensus EREs like those found in pS2 and vitellogenin B1. Dimers formed in the presence of a consensus ERE, irrespective of the presence or absence of the A/B region indicated that a weak dimerization domain must be present in the DNA binding C region. This weak dimerization domain most likely involves the five amino

acids between the first two zinc coordinating cysteines of the second zinc finger (Schwabe et al, 1990). In addition Kumar and Chambon showed that estrogen was important in the formation of dimers. By mixing ER types prior to addition of hormone, both hetero- and homodimer receptor complexes formed; however, hormone treatment prior to mixing resulted primarily in receptor homodimers. This result indicates that binding of estradiol to the receptors stabilizes the dimer.

For the ER, the major determinant for dimer formation resides in the HBD with a weaker interaction within the DBD. A recent mutation study aimed at localizing the dimerization domain in the HBD of the mouse ER revealed that residues 507-518 were critical for receptor dimerization and subsequent DNA binding, with a point mutation in this region detrimentally affecting dimerization (Fawell et al, 1990). In the GR, an additional dimerization determinant in the A/B region of the receptor has been identified (Eriksson and Wrange, 1990). Both the ER and GR have been shown to bind individually in a cooperative manner, with the first receptor molecule binding the downstream HRE half-site and then enhancing binding of the second receptor molecule in the dimer to the upstream half-site (Ponglikitmongkol et al, 1990; Tsai et al, 1989; Dahlman-Wright et al, 1990; Hard et al, 1990a). Characterization of this cooperative binding has revealed that varying the distance between the half-sites or changing their relative orientation interferes or abolishes GR dimer formation (Dahlman-Wright et al, 1990). Similarly mutation of the GRE or the five amino acids of the DBD believed to be partially involved in dimer formation also abolishes cooperative DNA binding and subsequent *trans*-activation (Dahlman-Wright et al, 1991).

-Hormone Binding Domain:

To regulate gene expression by interacting with response elements, nuclear receptors must first bind their appropriate ligand. Whether or not ligand binding is essential to receptor DNA interaction has been a controversial topic. Several groups, using DNaseI footprinting and gel retardation studies have shown, that *in vitro* unliganded receptor dimers can associate with DNA as can some truncated receptors (Kumar and Chambon, 1988; Willmann and Beato, 1986). These unliganded receptors, unlike their hormone bound counterparts, interact slowly and poorly with the response element, remain associated with the response element for a shorter time, form no or weak dimers and fail to activate transcription.

Through deletion, insertion and site-directed mutational studies, the hormone binding

domain of nuclear receptors has been shown to encompass the C-terminal 250 amino acids. Selective point mutations throughout this region can greatly impair or eliminate hormone binding and subsequent receptor activity. With three dimensional structural data lacking, affinity-labelling experiments have revealed some of the critical amino acid/ steroid interactions (Harlow et al, 1989). In the hER, using an affinity labeling compound for both an estrogen and an antiestrogen, a covalent interaction was observed with cys_{530}. Since antiestrogens bind the receptor and interact with the ERE but fail to *trans*-activate, this result indicates that the contact point of receptor-hormone interaction is less important than the overall conformational change to the receptor induced by this binding.

As mentioned previously, receptors mutated in the hormone binding domain cannot form dimers, bind ERE weakly or not at all, and cannot *trans*-activate. Conversely receptors lacking this region entirely form weak dimers in the presence of certain EREs and have been shown to constitutively *trans*-activate from certain promoters (Didier et al, 1990). The reason for this phenomenon is still obscure; however, several explanations have been proposed. These explanations are centered around blockage or interference by the HBD itself or some HBD related protein (ie *hsp90*) with either the DNA binding or dimerization domains. Selective mutation throughout the HBD may not prevent this interference, while complete removal of the entire C-terminal region removes this conformational constraint and enables the receptor to constitutively regulate the gene.

As with the DNA binding domain, the HBD is modular in nature. Exchange of the glucocorticoid binding domain with that of the estrogen receptor generated a chimeric receptor that upon binding estradiol activated transcription from a GRE (Klein-Hitpass et al, 1989). Similarly an ER HBD chimeric containing the unrelated Gal4 DNA binding domain was also functional (ten Heggeler-Bordier et al, 1987).

Estrogen Receptor *Trans*-activation:

The ultimate function of the steroid receptors is to control the expression of a regulated gene. Several mechanisms of *trans*-activation have been recognized. These mechanisms involve either direct contact of receptor dimer complexes with appropriate *cis*-acting promoter associated regulatory elements or indirect contact with other promoter associated transcription factors. Once bound, the receptor complex facilitates transcription via a poorly understood mechanism believed to involve either recruitment of essential intermediate transcription factors and/or their activation.

Direct interaction of transformed steroid complexes with specific *cis*-acting promoter related regulatory elements is believed to be the principal mechanism of steroid *trans*-activation. To date three distinct groups of steroid regulatory elements have been recognized: the ERE which binds the ER, the GRE/PRE which binds the GR, PR, AR and MR and the TRE/RRE recognized by the thyroid and retinoic acid receptors [Fig. 4].

Trans-activation by steroid receptors is usually studied by introducing modified receptor expression constructs into receptor-deficient cells together with an appropriate reporter construct. This reporter construct consists of a steroid regulatory element placed upstream of a heterologous promoter and an easily assayable gene, most commonly the *E. coli* chloramphenicol acetyl transferase (CAT) gene. In this manner both *trans*-activation domains of specific receptors and steroid regulatory elements of hormonally regulated genes have been identified.

-The *Trans*-Activation Domain:

Early deletion studies of the ER revealed a distinct independent *trans*-activation domain in the HBD (Webster et al, 1988; Kumar et al, 1987). That this *trans*-activation did not require the A/B or C regions was shown using chimeric GAL4 DBD, ER HBD receptors that in the presence of estrogen bound the GAL4 regulatory element and *trans*-activated the respective reporter gene (Webster et al, 1989). Further dissection of this *trans*-activation region (also known as TAF-2) revealed that no single exon of the five exons encoding the HBD contained the *trans*-activation function (Webster et al, 1989). Alternatively for the GR a *trans*-activation domain has been localized to a 30 amino acid region of the HBD (Hollenberg and Evans, 1988). This acidic region known as $\tau 2$ accounts for a small portion of the total *trans*-activation potential of the HBD as a single $\tau 2$ domain could only activate transcription weakly from an adjacent promoter (Hollenberg and Evans, 1988). However, multiple adjacent $\tau 2$ domains acted synergistically on transcriptional activation from an adjacent promoter. Conversely the corresponding region of the ER could not activate transcription when placed in front of a heterologous promoter (Webster et al, 1989). Therefore the *trans*-activation domain associated with the HBD probably involves several interspersed elements.

In the glucocorticoid receptor, an additional *trans*-activation domain (known as $\tau 1$) was localized to an acidic region of the A/B domain (Webster et al, 1989; Giguere et al, 1986; Danielson et al, 1987). Although such an acidic region is lacking in the estrogen receptor, both hER and chicken PR A/B regions were shown to act cooperatively with the

TAF-2 domain to optimally activate transcription of certain genes (Kumar et al, 1987; Gronemeyer et al, 1987). Initially when tested in HeLa cells, the A/B region alone could only stimulate transcription less than 5% compared to the whole receptor. Subsequently the observation that in chicken embryonic fibroblasts (CEF), but not in HeLa cells, the A/B region alone could *trans*-activate different promoters from 50-100% that of intact ER allowed recognition of a second independent *trans*-activation domain TAF-1 (Tora et al, 1989). TAF-1 unlike TAF-2 could *trans*-activate in the absence of hormone and exhibited cell type specific activation.

A further difference between TAF-1 and TAF-2 was shown by analyzing synergistic interactions of TAF-1 and TAF-2 with themselves, each other, with the upstream element binding factor (UEF) of the adenovirus-2 promoter or acidic activation domains (AAD) like those of Gal4 or VP16 (Tora et al, 1989). These experiments revealed that when the TAF-1 and TAF-2 domains were attached separately to unique DBD and bound in tandem on a promoter, they could act synergistically on the adjacent gene to achieve transcriptional levels greater than that of the intact ER. This synergism is dependent on the position or stereoalignment of each *trans*-activation domain relative to the other. Similarly two TAF-2 domains or a TAF-2 domain and the UEF could also potentiate synergistic transcription but in a stereoalignment-independent manner (Ponglikitmongkol et al, 1990). Conversely TAF-1 could not exhibit synergistic transcription with itself or with the UEF (Tora et al, 1989); however, TAF-1 but not TAF-2 could synergize with the AAD of Gal4 or VP16. This study revealed that the TAF-1 and TAF-2 were distinct from each other and from acidic activation domains.

A subsequent study expanding on this characterization of distinct transcriptional activation domains through their synergistic properties also revealed that the $\tau 1$ and $\tau 2$ acidic domains of the GR were a distinct class of *trans*-activation domains related to the AAD, and that they were contained within ER TAF-1 and TAF-2-like domains (Tasset et al, 1990). This study also distinguished between the various classes of activation domains based on their ability to interfere (squelch) each other's activity supposedly by competing for a limited pool of common intermediate transcription factors. These interference/squelching studies revealed that AADs interact with a factor essential to their ability to *trans*-activate, which is not essential for ER TAF-1 or TAF-2 *trans*-activation, while ER TAF-1 and TAF-2 interact with a factor necessary for both their activation and for activation by acidic *trans*-activation domains.

-Estrogen Regulatory Elements (ERE):

A number of estrogen responsive genes have been identified and their estrogen *trans*-activation studied, revealing several sequence-related *cis*-acting estrogen responsive elements [Fig. 4]. These EREs can be viewed as two palindromic or near-palindromic 5 bp half-sites separated by a variable 3 bp spacer. From the characterized elements an ERE consensus has been proposed $^{5'}$GGTCAnnnTGACC$^{3'}$. Binding of the ER to the consensus sequence has been shown directly by DNaseI footprinting (Klein-Hitpass et al, 1988a) and indirectly by gel retardation studies (Klein-Hitpass et al, 1989; Kumar and Chambon, 1988) and by electron microscopy (ten Heggeler-Bordier et al, 1987).

Properties of the consensus ERE have been studied *in vivo* in transfection assays using both a heterologous promoter (Klein-Hitpass et al, 1988a) and a minimal promoter containing a TATA box region (Ponglikitmongkol et al, 1990). In assays utilizing a heterologous thymidine kinase promoter, the ERE region (-337 to -21) of the Vit-A2 gene was shown to function, albeit somewhat less efficiently, in the reverse orientation and when placed 3' of the promoter (Klein-Hitpass et al, 1988a; Martinez et al, 1987). It was also found to function in either orientation when placed 2.3 kb upstream of the promoter (Klein-Hitpass et al, 1988a); therefore, the HREs exhibit enhancer properties. Point mutation in the consensus ERE could greatly impair or abolish its ability to function as a *cis*-acting element (Klein-Hipass et al, 1987; Berry et al, 1989). Furthermore selective mutations could also convert the ERE to a GRE (Martinez et al, 1987).

The inability of certain functional HREs to activate transcription from a minimal TATA box promoter emphasized the importance of the promoter region as a whole on their activity. In the case of the vitellogenin B1 and ApoVLDL II genes, tandem imperfect EREs are associated with the promoter. Alone these imperfect EREs do not function as *cis*-acting regulatory elements when placed in front of a minimal promoter; however, as a pair they act synergistically to activate transcription as well as the ERE consensus sequence (Martinez et al, 1987; Nunez et al, 1989). This synergism can occur between other hormone regulatory elements (HRE) which has been demonstrated for the dimeric GREs in the rat aminotransferase gene (Jantzen et al, 1988) and between the ERE and PRE in the chicken vitellogenin II gene (Cato et al, 1988). Synergism has also been demonstrated between the ER and GR (Ankenbauer et al, 1988) and between steroid receptors and non-steroidal transcription factors (Schüle et al, 1988; Strähle et al, 1988; Nunez et al, 1989; Brüggemeier et al, 1991). The mechanism of this synergism is not

understood. The current hypothesis is that direct receptor-receptor interactions enhance the time the ER complex is associated with the HRE and the strength of this association. This increased time and strength of binding enhances the probability of the receptor complex associating with and influencing the "transcriptional machinery". In the case of the GR and the PR, binding of one receptor complex enhances binding of the adjacent complex: a process referred to as cooperative binding (Schmid et al, 1989; Tsai et al, 1989). Whether cooperative binding can occur between a dimeric ERE is still uncertain. Studies with the Vit B1 gene initially indicated cooperative binding to the tandem EREs (Martinez et al, 1989). A subsequent study was unable to demonstrate cooperative binding between consensus or non-consensus ERE pairs or between a consensus/non-consensus pair, although cooperative binding of tandem PREs was clearly evident (Ponglikitmongkol et al, 1990). A study attempting to identify the regions involved in synergistic receptor-receptor interactions between the ER and the GR or PR revealed that the amino terminal regions of both the PR and GR are essential for synergism with ER (Cato et al, 1988). Conversely deletion of the ER A/B region did not affect synergism with either GR or PR; however, deletion of the C-terminal region abolished synergism with the GR but not with the PR (Cato et al, 1989). Therefore the specific interactions involved in synergism appear to vary between different *trans*-acting factors.

In addition to considering the regulatory unit itself in a synergistic interaction and in the subsequent *trans*-activation, increasing evidence suggests that attention must be directed equally to the entire promoter region. When the estrogen responsive unit (ERU) from Vit-B1 gene was placed in front of the complex HSV-tk promoter, it was shown to *trans*-activate irrespective of the stereoalignment between the two imperfect EREs (Martinez et al, 1989). When the same region was placed upstream of the minimal TATA promoter, *trans*-activation was dependent on the stereoalignment of the two imperfect EREs (Ponglikitmongkol et al, 1990). This apparent discrepancy has been explained by suggesting that in the complex heterologous promoter, TAF-2/TAF-2 stereoalignment-independent interactions between the ERs and other promoter associated factors predominates in *trans*-activation. In the minimal promoter, the lack of other promoter associated transcription factors necessitates stereoalignment-dependent TAF-1/TAF-2, ER-ER interactions for *trans*-activation. With consensus EREs, tandem elements act synergistically when placed at a distance from the promoter but unlike imperfect EREs, they act additively when placed near the promoter, perhaps indicating a maximal transcriptional induction level (Ponglikitmongkol et al, 1990).

Because of the inherent complexity in studying the mechanism of steroid receptor *trans*-activation in poorly defined *in vivo* systems, attempts have been made to reproduce ER *trans*-activation *in vitro* using ER containing extracts. These early studies have unfortunately proven to be equally difficult to interpret as some extracts like that of frog liver yielded expected hormone-dependent transcriptional activation of the reporter gene (Corthesy et al, 1988) while other extracts like that of a rat liver cell line unexpectedly revealed hormone-independent transcriptional induction (Kaling et al, 1990).

DNA-independent Steroid Hormone Receptor Activation:

The concept that nuclear receptors activate transcription by interacting with specific *cis*-acting promoter associated elements is well accepted. The ability of these receptors to interact directly with other transcription factors has led to speculation that they may influence transcription without binding DNA. DNA-independent transcriptional activation has recently been confirmed in the chicken ovalbumin gene (Tora et al, 1988). This gene contains an expansive regulatory domain spanning several kilobases and is regulated by all four classes of steroid hormones including estrogens (Gaub et al, 1990). The proximal 132 bp region of this promoter contains positive elements and although no ERE consensus is observed, two ERE GGTCA half-sites are present at position -77 and -47 relative to the TATA box (Tora et al, 1988; Gaub et al, 1990; Dierich et al, 1987). The proximal half-site can function as an ERE in CEFs but not in HeLa cells and ER at high concentrations appeared to bind to this proximal ERE (Tora et al, 1988). It was subsequently shown that this proximal ERE did not bind ER at physiological concentrations; however, it did bind the AP-1, *fos / jun* complex (Gaub et al, 1990). The proximal ERE half-site formed part of an AP-1 site, TGGGTCA, which closely resembles the canonical AP-1 site TGAGTCA. By placing this AP-1 site into a reporter transfection vector, cotransfection studies revealed that the ER together with *c-fos* could coactivate transcription from the AP-1 transfection vector. Since the ER did not bind to the AP-1 site, coactivation occurs by either cooperative binding by the ER to a weak adjacent element or a protein-protein interaction of the ER with the *fos-jun* dimer. To test these possibilities, transfection studies were done using an ER mutant lacking the DBD. These studies revealed that the DBD was not necessary for coactivation and therefore coactivation was occurring independently of DNA binding (Gaub et al, 1990). Although a direct ER-AP-1 interaction was not shown, this study does confirm that steroid receptors can activate transcription without binding *cis*-acting elements.

Transcriptional Repression by Steroid Receptors:

Regulation of gene transcription is accomplished through a dynamic array of stimulatory and inhibitory influences involving both *trans*-activating and *trans*-repressing factors. Factors that stimulate transcription can alternately repress transcription depending on the promoter context. The GR, ER, RAR and TR have been recognized to act as *trans*-repressors of specific genes. This repression has been shown to occur by a variety of mechanisms usually involving physical or functional interference with transcription activation factors or with the RNA polymerase transcription unit.

In the case of the proopiomelanocortin gene, repression by the GR has been shown to involve receptor binding to a non-consensus GRE that overlaps the transcriptional activator CAAT binding protein (CBP) regulatory element (Charron and Drouin, 1986; Drouin et al, 1989). Similarly, in the α-subunit glycoprotein gene (Akerblom et al, 1988), the α-fetoprotein gene (Guertin et al, 1988) and the prolactin gene (Sakai et al, 1988) GR represses by binding to a non-consensus GRE overlapping activating regulatory elements. That steric interference with adjacent *trans*-activating sites is the mechanism of repression has been confirmed by showing that GR-DBD alone could not repress the α-subunit glycoprotein gene but GR-DBD/MR-HBD or GR-DBD/β-galactosidase fusion proteins could (Akerblom et al, 1988). Whether the non-consensus overlapping GREs are true negative regulatory elements or simply imperfect GREs whose repression or activation is dictated by the promoter is unclear. Repression through steric interference with adjacent *trans*-activating sites has also been shown in the case of TR repression of the TSHα and TSHβ genes where TR binding to a TRE near the promoter TATA box is believed to prevent RNA polymerase II complex binding (Krishna et al, 1989; Darling et al, 1989).

Evidence suggests that some unliganded receptor can act as *trans*-repressors. The *v-erbA* oncoprotein, a modified TR unable to bind triiodothyronine [T3], can prevent *trans*-activation by TR from the rat growth hormone promoter or the LTR of the Moloney murine leukemia virus (Damm et al, 1989; Sap et al, 1989). Similarly both unliganded TR and RAR have been shown to bind with high affinity to positive promoters where they act as inhibitors (Glass et al, 1989b; Damm et al, 1989; Sap, 1989). However, depending on the promoter context, the reverse can also be true; unliganded TR and RAR can be activators while the liganded receptors can act as repressors. Such is the situation in the EGF gene (Hudson et al, 1990) where T3 and retinoic acid are believed to induce allosteric changes to their receptors resulting in silencing of adjacently bound promoter

trans-activators. Therefore, the ligands regulate whether the EGF gene is activated or repressed.

The ability of various receptors to interact with similar receptor regulatory elements has also been shown to be a mechanism of *trans*-repression. The TR receptor can bind modified TREs $^{5'}$GGTCA-n_{1-6}-TGACC$^{3'}$ which include the ERE (Glass et al, 1989a). When bound to the ERE, the TR acts as a *trans*-repressor of ER *trans*-activation. Similarly the RAR can bind TREs (Umesono et al, 1988) and has lead to speculation that the two receptors may have an agonistic/antagonistic relationship in the regulation of development and morphogenesis (Hudson et al, 1990 and references within). A close regulational relationship between the TR and RAR is further indicated by their ability to readily form heterodimers that can bind a subset of TREs and activate or repress transcription depending on both the promoter context and the ligand status (Glass et al, 1989b). Identification of several forms of TR (*erbAβ-1, erbAβ-2, erbAα-1*) and RAR (RAR-α,-β,-γ) may also be indicative of distinctive *trans*-activating or *trans*-repressing functions among the different receptor types; however, this has yet to be shown (Darling et al, 1989; Vasios et al, 1989 and ref. within). Preliminary studies with the various TR types have shown that they appear to bind and function similarly from the positive rGH or the negative TSHβ promoters (Darling et al, 1989).

Thus far the repression mechanisms described involve repressor-DNA interaction. Repression mechanisms involving repressor-activator protein-protein interaction, independent of repressor-DNA binding have also been observed. Such is the case for Gal80 repression of the Gal4 *trans*-activator and SV40 T-antigen repression of the AP-2 transcription factor (Mitchell et al, 1987; Ma and Ptashne, 1987). DNA-independent protein-protein repression has also been described for members of the steroid receptor family. The ER and GR can inhibit expression of the rPRL gene by interaction with PRL enhancer bound factor called Pit-1 (Adler et al, 1988). This interaction involves the ER D-region [251-314], which can be substituted by the corresponding GR D-region, and does not require the presence of the DBD (Adler et al, 1988). Another example of protein-protein inhibition by GR has also been described recently for the osteocalcin gene and the metalloproteinases: collagenase and stromelysin (Schüle et al, 1990; Yang-Yen et al, 1990; Jonat et al, 1990). These genes are activated by a variety of growth and transcription factors which include vitamin D3 and retinoic acid in the case of the osteocalcin gene. The effects of these activating factors all appear to be potentiated through a common intermediate transcription factor AP-1 [the *c-jun/c-fos* dimer]. Using

mutation, competition and cross-linking experiments, GR repression was shown to involve a direct interaction between the GR-DBD and the *c-jun* (and possibly the *c-fos*) basic DBD. This interaction prevents formation of the intermediate *trans*-activating AP-1 complex, hindering the activity of promoter bound *trans*-activators. In a similar manner, overexpression of the ER was shown to inhibit *trans*-activation by the GR and PR by monopolizing or disrupting a mutual intermediate transcription factor (Meyer et al, 1989).

Enzymatic Functions Associated With the Estrogen Receptor:

In addition to its role as a *trans*-activator, two enzymatic activities have been associated with the the transformed estrogen receptor. Baldi et al (1986) have observed that immunopurified estrogen receptor was able to phosphorylate itself and phospholipids *in vitro*. This ER peptide and phospholipid kinase activity has not been further characterized and as such its relevance *in vivo* is unknown. However, *in vitro* phosphorylation of the ER at a tyrosine, within or near the hormone binding domain, has been shown to stimulate maximal binding of estradiol (Migliaccio et al, 1989). In addition, phosphorylation of a serine has been shown to increase the affinity of the transformed estrogen receptor for the vitellogenin ERE with enzymatic dephosphorylation greatly decreased receptor-ERE binding affinity (Denton et al, 1991).

A chymotrypsin-like proteolytic activity has also been described for the estrogen receptor (Puca et al, 1986). This activity was initially observed with a partially purified ER preparation and has recently been confirmed using immunoaffinity isolated receptor (Molinari et al, 1991). This latter study revealed that this proteolytic activity was exclusive to hormone bound receptor and was not evident with hormone-free or antiestrogen bound receptor (Molinari et al, 1991). Like chymotrypsin, the transformed ER selectively cleaves the carboxyl side of phenylalanine. Although the *in vivo* relevance of this proteolytic activity is unknown, an additional role is suggested for the transformed receptor in gene activation.

C. Proto-oncogenes and Human Breast Cancer:

Oncogenes Associated With Breast Cancer:

Breast cancer is a disease believed to result from multiple somatic mutations which deregulate normal cell growth or provide a growth advantage to the tumor cells (Weinberg, 1989). These mutations affect two broad gene subsets: those that stimulate

cell growth and those that suppress this growth, commonly referred to as proto-oncogenes and tumor suppressor genes respectively.

In the past twelve years some 60 oncogenes and their normal cellular counterparts have been characterized. Many of the proto-oncogenes are growth factors, growth factor receptors, proteins involved in signal transduction or nuclear transcriptional activators. A wide range of genetic alterations resulting in either physical changes to the proto-oncogene itself (point mutations, deletions, insertions) or to the regulation of its expression (promoter insertion, amplification, translocation or viral transduction) have been shown to influence carcinogenesis and have been recognized in a variety of tumors. In breast cancer tumors several oncogenes have been repeatedly associated with tumorigenesis. These oncogenes include the *int-1*, *int-2*, *c-H-ras*, *c-neu* and *c-myc* oncogenes.

int-1, int-2: A highly studied model system for breast cancer has been the mouse mammary tumor virus (MMTV) infected mouse. The MMTV induces mammary tumors in mice 4-9 months after infection, by integrating itself into the mouse genome at specific sites adjacent to critical regulatory genes and placing these genes under the regulation of its promoter/enhancer element. Thus far several genes regulated by MMTV have been identified and are designated as *int-1*, *int-2*, *int-3* and *int-4*.

int-1 is believed to code for an important developmental factor. Although its function in humans is unknown, it is highly conserved among diverse species and in *Drosophila* it is known to be important in the development of wings and legs (Rijsewijk et al, 1987). *Int-2* codes for a basic fibroblast growth factor (FGF)-like protein which may also be important in regulating cell growth and proliferation (Dixon et al, 1989). Expression of both *int-1* and *int-2* have been shown to be modified in certain breast cancers (Ali et al, 1989). Newer members, *int-3* and *int-4*, are not as well characterized and have yet to be associated with human breast tumors.

c-H-ras: The *c-H-ras* p21 protein product has been recognized as a GTP-binding protein involved in signal transduction from membrane bound receptors (McCormick, 1989; Bos, 1989). Oncogenic activation occurs as a result of specific point mutations at amino acids 12-13 or 59-61 resulting in a more active p21 protein that fails to release the activating GTP substrate. Although activated *c-H-ras* oncogene have been reported in DMBA-induced mouse tumors as well as primary and metastatic breast tumors (Dandekar, 1986; Rochlitz, 1989) activation is generally believed to be uncommon in human breast cancer.

Similarly amplification of the *c-H-ras* is rare (Garcia et al, 1989). Nonetheless overexpression of the unmutated *c-H-ras* product is more common in cancerous tissue than in the corresponding normal tissue (Ohuchi et al, 1986). A more important mutation, reported to be correlated with a more aggressive breast cancer, is loss of the *c-H-ras* allele (11q14) (Theillet et al, 1986; Ali et al, 1987; Garcia et al, 1989). This loss of heterozygosity (LOH) may indicate an important stage in the progression of the disease.

In our studies and others, estrogen has had no effect on *c-H-ras* expression in HBC cells (Dubik et al, 1987; Kasid et al, 1985) although overexpression of *v-H-ras* in MCF-7 cells was shown to allow these cells to overcome estrogen dependency (Kasid et al, 1985; Kasid et al, 1987).

c-neu/c-erbB-2/HER-2: The *c-neu* oncogene was initially isolated from chemically induced rat neuroblastomas (Shih et al, 1981). It was subsequently found to encode a 185 kDa, membrane traversing, glycoprotein with inherent tyrosine kinase activity (Schechter et al, 1984; Hunter and Cooper, 1985; Stern et al, 1986). The human homolog known as *c-erbB-2* was found to be related to, but distinctive from, the EGF receptor (Schechter et al, 1985; Bargmann et al, 1986). Numerous breast cancer studies have identified an inverse relationship between EGF receptor and ER levels with high EGF receptor levels correlated with a poorer prognosis (Fitzpatrick et al, 1984; Sainsbury et al, 1985a; Sainbury et al, 1985b; Fekete et al, 1989). Although the natural ligand of the *c-neu* receptor is not known, a point mutation in the transmembrane domain has been associated with oncogenic activation (Bargmann et al, 1986). Overexpression of *c-neu* mRNA has been seen in some primary breast cancers and amplification of the gene and its product has been observed in 20-30% of human breast cancer biopsies (King et al, 1989; Slamon et al, 1987; Borg et al, 1990). Overexpression of the *c-neu* oncoprotein has been correlated with malignant development and a poorer prognosis (Venter et al, 1987; Slamon et al, 1987; Varley et al, 1987; Borg et al, 1990). High *c-neu* expression is primarily associated with ER-negative tumors, while in ER-positive tumors, exposure to physiological levels of estradiol decreases both mRNA and protein levels (Read et al, 1990).

c-myc: Although the *c-myc* oncogene was one of the first oncogenes discovered, the function of its 65 kDa product is still uncertain. Possible functions include gene *trans*-activation and the regulation of DNA synthesis, both processes critical to cellular proliferation and differentiation (Lüscher and Eisenman, 1990). A detailed discussion of

the *c-myc* gene and protein will follow later. In human breast cancer amplification and /or rearrangement of the *c-myc* gene is commonly seen in 20-30% of breast tumors with high levels of *myc* expression (Mariani-Costantini et al, 1988; Garcia et al, 1989; Callahan and Campbell, 1989). No correlations have been observed between *myc* amplification and other clinical parameters (class, grade, ER status) in both primary and metastatic breast tumors (Garcia et al, 1989; Varley et al, 1987). Nonetheless amplification of *c-myc* has been statistically correlated with infiltrating ductal carcinomas a particularly aggressive breast carcinoma with a poor prognosis (Garcia et al, 1989; Varley et al, 1987). A role of *c-myc* in breast cancer is further supported by our studies where we observe estrogen induced *c-myc* expression in ER+ HBC cells and unregulated high constitutive levels in various ER- HBC cell lines (Dubik et al, 1987).

To help ascertain the relevance of each of the above mentioned oncogenes to breast cancer, transgenic techniques were employed directing these oncogenes to the breast epithelium of mice. Targeting and high expression of the oncogenes was accomplished by placing them under the control of the MMTV promoter/enhancer or the murine whey acidic protein promoter (Wap). Mammary tumor formation varied slightly with the different promoters because the Wap promoter is weaker than the MMTV promoter, resulting in fewer mammary non-specific tumors: the Wap/*H-ras* transgenic mice exhibited a very low incidence of mammary tumors when compared to their MMTV/*H-ras* counterparts (Andres et al, 1987). In general both *c-myc* and/or *c-H-ras* transgenic mice developed mammary tumors at varying times, among random epithelial cells expressing the transgene (Stewart et al, 1984; Andres et al, 1987; Sinn et al, 1987; Schoenenberger et al, 1988). The incomplete transformation of all transgenes containing epithelial cells appears to indicate that *c-myc* and *c-H-ras* alone are not sufficient to transform mammary epithelium and that other genetic alterations are also necessary. The development of breast cancer most likely resembles the multi-stage process described for colon cancer (Vogelstein, 1990). With MMTV/*c-neu* transgenic mice, uniform malignant transformations of all mammary epithelial cells was observed (Müller and Sinn, 1988), indicating that *c-neu* alone may be sufficient for malignancy. This result has been disputed (Bouchard et al, 1989). MMTV/*int-1* or *int-2* transgenic mice also exhibited pronounced mammary gland hyperplasia (Tsukamoto et al, 1988; Müller et al, 1990).

A variety of other oncogenes including *N-ras*, K-ras, *N-myc*, *L-myc*, *c-myb*, *v-src* and *c-fos* have also been studied in breast tumors. Genetic mutations or aberrant expression of these oncogenes are more infrequent and as such their relevance to breast tumorigenesis

remains uncertain.

Tumor Suppressor Genes (Antioncogenes):

Certain types of cancers have been recognized to develop as a result of mutation in both alleles of a regulatory gene. The first example of this type of tumor was hereditary retinoblastoma where dual internal mutations in the RB gene or a genetic expulsion of one allele followed by a subsequent mutation in the other resulted in tumorigenesis (Cooper and Whyte, 1989). Another well characterized tumor suppressor gene p53 was initially discovered through its association with oncogene products of DNA tumor viruses. It was believed to be an oncogene because its cellular homolog isolated from normal mouse liver cells could act with the *c-H-ras* oncogene to transform primary rat embryo fibroblasts. Subsequently the p53 gene was discovered to contain a cloning mutation. The unmutated gene not only failed to transform normal cells, but could actually act as a suppressor of other tumor promoters (Lane and Benchimol, 1990).

How these tumor suppressor genes act is still poorly understood. Both the p53 and RB suppressor proteins have been shown to form complexes with transforming regions of a variety of regulatory proteins such as the SV40 T-antigen, the adenovirus protein E1A and the papilloma virus protein E7, indicating that these *trans*-activators may be cellular targets of tumor suppressor proteins (Huang et al, 1990; Vogelstein, 1990 and ref. within). In addition to its ability to complex with other regulatory proteins, the wild-type, but not mutated p53 protein has also been shown to possess a potent transcriptional activating sequence supporting the idea that p53 may also act as a *trans*-activator of genes that suppress cell proliferation (Raycroft et al, 1990; Field and Jang, 1990).

The possible importance of tumor suppressor genes in human breast cancer has been suggested by numerous studies that have identified alterations in both the RB and p53 gene loci in these tumors (Nigro et al, 1989; Malkin et al, 1990; T'Ang et al, 1988; Varley et al, 1989). In addition reconstitution of the normal RB gene into breast cancer cells was shown to suppress the neoplastic phenotype (Huang et al, 1988). Since suppressor gene mutations commonly involve total loss of one allele with a critical mutation or deletion in the other allele, loss of heterozygosity (LOH) within the genome of tumor cells is believed to correspond to regions of the genome harboring tumor suppressor genes (Chen et al, 1989 and refs. within). Analysis of breast tumor DNA for LOH has identified several frequently affected chromosomal regions including 1p, 1q, 3p, 11p, 13q14.1, 17p, 17q and 18q (Chen et al, 1989; Cropp et al, 1990 and refs. within). These results suggest

the possible involvement of several suppressor genes in breast tumorigenesis and support the view that breast cancer develops as a result of multiple somatic mutations, deregulating normal cell growth in a manner parallel to that described by Vogelstein (1990) in colon cancer.

The *myc* Proto-oncogene Family:

The *myc* oncogene family consists of three well defined members *c-, N-* and *L-myc* with several minor members *R-myc*, *P-myc* and *s-myc* also described (DePinho et al, 1987b; Sugiyama et al, 1989). *c-myc* was first discovered in 1979 as the cellular homolog of the avian myelocytomatosis retrovirus transforming gene *v-myc* (Sheiness and Bishop, 1979). Subsequently *N-myc* was identified in human neuroblastomas and *L-myc* was cloned from small cell lung carcinomas through low stringency cross-hybridization with *c-myc* (Kohl et al, 1983; Schwab et al, 1983; Nau et al, 1985). All three genes code for nuclear phosphoproteins which share regions of structural similarity particularly in the carboxyl-terminus (Nau et al, 1985; Kohl et al, 1986). Furthermore all three are probably functionally related as they exhibit similar *in vitro* transforming ability (Schwab et al, 1983; Land et al, 1983; Yancopoulos et al, 1985). Although the precise physiological function of the *myc* protein (*MYC*) is not known, there is overwhelming evidence supporting its importance in some aspect of cellular proliferation and/or differentiation (for a review see Lüscher and Eisenman, 1990). The importance in cellular development and distinct physiological function of each *myc* member is further supported by the high degree of evolutionary conservation among species of the vertebrate phylum (Van Beneden et al, 1986; DePinho et al, 1987b; King et al, 1986).

-Structural Characteristics of the *myc* Gene Family:

The *c-myc*, *N-myc* and *L-myc* genes are distinct genes mapping to chromosomes 8, 2 and 1 respectively (Taub et al, 1982; Neel et al, 1982; Schwab et al 1984; Nau et al, 1985). They share a three exon, two intron organization with the major coding domain within the second and third exons [Fig. 5a]. The gene structure of the *myc* family has been extensively studied not only in humans but in many other species. The *c-myc* gene initially identified in humans has also been characterized in cat, mouse, rat, chicken, frog and in trout (Stewart et al, 1986; Bernard et al, 1983; Hayashi et al, 1987; Watson et al, 1983; King et al, 1986; Van Beneden et al, 1986). In the frog, *Xenopus laevis,* two *c-myc* genes, *c-mycI* and *c-mycII*, have been identified which differ primarily in 5' and 3' untranslated regions (Vriz et al, 1989). These modifications are believed to regulate differential expression of the two *c-myc* genes from either the maternal or zygote genome

at different stages of oogenesis (Vriz et al, 1989).

Alignment of the known *c-myc* sequences reveals sequence similarity near 80% at the nucleotide level and 90% at the amino acid level (Vriz et al, 1989). This degree of sequence similarity is also seen for the *N-* or *L-myc* genes among vertebrates (DePinho et al, 1987). When compared to each other, the *c-*, *N-* and *L-myc* genes differ considerably in exon 1 and the 5' and 3' untranslated regions. These regions are well conserved for any given *myc* class across species (DePinho et al, 1987a; Bernard et al, 1983; DePinho et al, 1986). In the translated regions of the *myc* genes, there is substantial sequence and amino acid similarity. When conserved amino acid changes are considered, comparisons of the *c-*, *N-*, *L-* and *v-myc* proteins reveal an overall similarity from 52-84% with similarities of 85-97% in the exon 3 encoded areas. This similarity in the translated regions further supports a common function among the *myc* proteins, while the conservation across species in exon 1 and the 5' and 3' regions for each type of *myc* gene reaffirms that these regions are most likely responsible for differential regulation.

Transcriptional initiation also varies for the different *myc* genes. The human *c-myc* gene is transcribed primarily from two promoters P1 and P2 (Bernard et al, 1983). P2 is within exon 1 and is 150 bp downstream of P1 [Fig. 5a]. In all vertebrates studied, with the exception of *c-mycII* in *Xenopus*, P2 appears to be the principal promoter for transcriptional initiation. The minor *Xenopus c-mycII* transcript has an upstream start site designated as P' (Vriz et al, 1989). This site appears to resemble the 5% of *c-myc* transcripts in human B cell lymphomas that initiate from an upstream promoter P0, 550-650 bp from P1 (Bentley and Groudine, 1986a). These P0 transcripts exhibit staggered start sites and are more stable than the P1 or P2 initiated transcripts, indicating that 5' upstream sequences in addition to the sequences in the 3' end are important in post-transcriptional regulation of the *c-myc* mRNA (Bentley and Groudine, 1986b; Jones and Cole, 1987; Pei and Calame, 1988). For *c-myc* the polyadenylated mRNA produced from the P0, P1 and P2 promoters are approximately 3.1, 2.4 and 2.2 kb respectively. Under normal conditions the P1 and P2 mRNA are extremely unstable with half-life values around 20 minutes (Dani et al, 1984; Ikegaki et al, 1989). S1 nuclease mapping and primer extension studies of the *N-myc* gene have revealed multiple transcription initiation sites throughout exon 1 (Kohl et al, 1986). The *N-myc* mRNA produced is approximately 3.0 kb long, but depending on the cell type, incomplete processing of the heteronuclear *N-myc* RNA results in a range of transcripts revealed by Northern analysis (Murphy et al, 1987; Schwab et al, 1985). In the *L-myc* gene there appears to be a single transcriptional

initiation site but alternate splicing of the introns and the use of different polyadenylation signals result in a series of different size mRNA (Ikegaki et al, 1989). The principal transcripts are a 3.9 kb complete mRNA that may contain the short first intron and a 2.2 kb truncated transcript that contains exon 1, 2 and part of intron 2. This latter transcript produces a truncated 206 amino acid protein. As illustrated in Fig. 4, the principal difference in the *myc* mRNAs are variable 3' untranslated regions of 350, 900 and 1900 bp in the *c-*, *N-* and *L-myc* respectively. Contained throughout this 3' untranslated region are multiple AU-rich regions that are believed to account for the short half-life of these mRNAs (Shawn and Kamen, 1986).

-The *myc* Protein (*MYC*) -Structure Characteristics:

The human *c-myc* mRNA codes for two proteins, a 439 residue product initiating from the classical AUG codon in the second exon and a minor 455 amino acid protein initiating from a CUG codon in the first exon (Hann et al, 1988). The functional significance, if any, of the additional 16 amino acids is not known. The human *N-myc* mRNA codes for a single protein of 464 amino acids while the full length *L-myc* transcript codes for a much smaller 364 amino acid protein (Kohl et al, 1986; DePinho et al, 1986; DePinho et al, 1987a). The dramatic shortness of the *L-myc* protein is primarily due to the lack of approximately 80 amino acids in a nonconserved region of exon 2 in *c-* and *N-myc* (DePinho et al, 1987a). As with the normal *myc* transcripts, the *myc* proteins also have very short half-lives (Ramsay et al, 1984; DeGreve et al, 1988).

Since the discovery of *c-myc* extensive studies have been directed at identifying the biological function of this protein and subsequent family members. To date this function remains elusive; however, these studies have identified regions of *MYC* that could be important in its function. Key structural characteristics of the *c-myc* proteins are illustrated in Fig. 5b and are shared by the *N-* and *L-myc* proteins as well.

-Nuclear localization motif:

As mentioned earlier the *myc* proteins are nuclear proteins and as such require specific nuclear targeting sequences to facilitate their transport through the nuclear pore. This sequence has been identified in both the *c-myc* and *N-myc* proteins (Dang and Lee, 1988; 1989). In the *c-myc* protein *(C-MYC),* the removal of residues 320-328 (PAAKRVKLD) resulted in its distribution in both the nucleus and cytoplasm (Dang and Lee, 1988). Furthermore when these residues were fused to the pyruvate kinase protein, the fusion protein was directed into the nucleus (Dang and Lee, 1988). A similar sequence

PPQKKIKS in *N-myc* from residues 345-352 was also shown to be essential for nuclear localization (Dang and Lee, 1989). These nuclear localization motifs are highly conserved and resemble the nuclear translocation signal in the SV40 large T antigen and steroid receptors described earlier. In *L-MYC* a nuclear localization motif similar to that of *C-MYC* or *N-MYC* is not present; however, there is a potential nucleoplasmin basic-spacer-basic NLS [HPK-pvssntevt-KRK] from residues 269-284.

-Helix-loop-helix / leucine zipper motif:

These motifs (described previously, see ER pg. 15) were recognized as regions of structural similarity in a widespread group of transcriptional activators (for reviews see Olson, 1990; McKnight, 1991). Subsequently the *myc* proteins were observed to share structural similarity with these transcriptional regulators in that they possessed these motifs. What is particularly interesting about the *myc* proteins is that the basic DNA binding domain is followed by both the helix-loop-helix [HLH] and the leucine zipper [Zip] motif. This peculiar dual motif arrangement has subsequently been recognized in a variety of transcription factors including TFE3, TFEB, USF and AP4 (Beckman et al, 1990; Carr and Sharp, 1990; Gregor et al, 1990; Hu et al, 1990). Deletion studies in the HLH-Zip region of USF and AP4 indicate that both regions are needed for dimer formation and DNA binding (Gregor et al, 1990; Hu et al, 1990). For *c-myc* and *N-myc,* mutation of either region can greatly impair or eliminate their cotransformation abilities, their negative regulation of preadipocytes and their autonegative regulation (Stone et al, 1987; Penn et al, 1990a; Freytag et al, 1990). Thus the carboxy-terminal 90 amino acids of *MYC*, containing the BR-HLH-Zip motif appear to be critical to its function and would suggest that the *myc* proteins form dimeric complexes that may act as transcriptional regulators. The precise nature of the *MYC* dimeric complex is still unknown. At high concentrations purified *C-MYC* has been shown to form homodimers and tetramers; however, *in vitro* translated *C-MYC* does not appear to form these complexes (Dang et al, 1989b; Smith et al, 1990). *C-MYC* also fails to dimerize with other HLH or leucine zipper proteins (Lüscher and Eisenman, 1990).

Recently by screening a cDNA expression library with a probe corresponding to the *c-myc* HLH-Zip region, a novel protein called *Max* was isolated (Blackwood and Eisenman, 1991). This protein forms a heterodimeric complex with the *c-*, *N-* and *L-myc* proteins, but not with other HLH or Zip containing proteins. *Max* is a 151 amino acid protein, with an electrophoretic mobility on SDS-PAGE of 21 kDa. It is encoded by a 2.1 kb mRNA and contains the BR-HLH-Zip motif. The mouse equivalent of *Max*

known as *Myn* has also been identified as a 160 amino acid, 18 kDa protein (Prendergast and Ziff, 1991). Deletion studies with both *MYC* and *Myn* indicate that dimerization is dependent on the BR-HLH-Zip motif with mutation of the Zip motif sufficient for loss of DNA-binding activity. A time course of *Myn* expression reveals that it is expressed maximally four hours after mitogen stimulation and this expression requires *de novo* protein synthesis. Both the *MYC-Max* and *MYC-Myn* recognize the putative *c-myc* recognition element, GACCACGTGGTC. Methylation of this recognition site inhibits complex binding and as such methylation changes associated with growth, differentiation and transformation have been proposed as a possible regulatory mechanism of *MYC* action *in vivo* (Prendergast and Ziff, 1991).

-Phosphorylation of MYC

The *myc* proteins are all phosphoproteins. The significance of this phosphorylation is unknown. However, deletion of phosphorylated regions affects the transformation phenotype of these proteins (Ramsay and Hayman, 1982). Since phosphorylation appears to be a major mechanism used by cells to regulate the activity of its proteins, phosphorylation of *myc* proteins has been studied (Sibley et al, 1988; Zajac-Kaye and Levens, 1990; Saksela et al, 1989). Of the known kinases only two, casein kinase II and glycogen synthetase kinase II, have been shown to phosphorylate *myc in vitro* at sites corresponding to those *in vivo* (Lüscher et al, 1989). Casein kinase II appears to phosphorylate serine or threonine residues in the 249-252 and 347-348 regions, while glycogen synthetase kinase II phosphorylates residues between 45-55 (Lüscher et al, 1989; Lüscher and Eisenman, 1990).

-DNA binding of MYC:

Non-specific and specific DNA binding has been associated with the *myc* proteins. Non-specific DNA binding has been observed for years and the *myc* proteins have been proposed to participate in DNA replication (Gutierrez et al, 1988; Ariga et al, 1989). The region of the *c-myc* protein involved in this DNA binding was localized to amino acids 265-318 and surprisingly did not involve the highly conserved basic region associated with the HLH-Zip motif (Dang et al, 1989a). Comparisons of *c-myc* proteins from other species indicate that residues 265-271 and 290-318 are highly conserved. Transformation studies using *myc* mutants lacking this region yielded mixed results, with some mutants exhibiting normal cotransformation activities and others unable to transform (Dang et al, 1989b).

Despite the inability of the HLH-Zip DNA binding basic region [BR] to bind the sequence used to identify the non-specific DNA binding site, domain swapping experiments in which the E12-basic DNA-binding region was replaced with the corresponding *c-myc* region, revealed binding of the E12-*c-myc*-BR homodimeric complex to a CA--TG consensus (Prendergast and Ziff, 1991). Alternate approaches have also recognized CACGTG as the *myc* specific binding site (Blackwell et al, 1990). This element coincides with part of the binding sites described for the human transcription factors USF and TFE3 (Kerkhoff et al, 1991). Most recently the *MYC-Myn* complex was shown to bind this core sequence CCACGTGA preferentially when preceded by a GA or AG at the 5' end and followed by a TC or CT at the 3' end (Prendergast et al, 1991). Therefore the basic region associated with the HLH-Zip in the *myc* protein appears to bind a specific DNA element as do the corresponding regions of other HLH and/or zipper proteins.

-Potential functions of MYC:

Stimulation of cell growth by mitogens can be separated into two phases: competence and progression. Competence, the earlier phase, is characterized by cellular enlargement, increased RNA content, synthesis of novel proteins, responsiveness to further activation signals and activation of early-response cell cycle genes (Pledger et al, 1977; Baserga, 1984). *C-myc* is an early-response, cell cycle competence gene. *MYC* can transform embryo fibroblasts when expressed together with the p21 *c-H-ras* protein (Land et al, 1983) or when cotransformed with a variety of other oncogenes including *c-raf-1* and *bcl-2* (Pfeifer et al, 1989; Strasser et al, 1990). For this reason *MYC* is suspected to play a major role in cellular proliferation and differentiation. The mechanisms by which *MYC* potentiates these actions remains obscure; however, characterization of the *myc* protein itself, identification of several *MYC*-regulated genes and detailed descriptions of *myc* expression in various cells have yielded some insight into the complex functions associated with this protein.

Perhaps the most important observation regarding the *myc* proteins was the recognition that they are members of the helix-loop-helix (HLH) and leucine zipper (Zip) gene family. This observation suggested that *MYC* is a transcription factor. Using *trans*-activation assays, a small group of genes were identified to be regulated both positively and negatively by *MYC*. Included in this group are the *hsp70* and E4 promoters, the plasminogen activator inhibitor-1 (PAI-1) and most recently the α-prothymosin gene, all of which are activated by *MYC* (Kingston, 1984; Onclercq et al, 1988; Prendergast and

Cole, 1989; Eilers et al, 1991). Conversely the metallothionein promoter appears to be suppressed by the *myc* protein as are the leukocyte function antigen (LFA-1) receptor, the neural cell adhesion molecule (N-CAM), variant histones H1 and $H1^{0}$ and the major histocompatibility complex (MHC) cell surface proteins (Kaddurah-Daouk et al, 1987; Versteeg et al, 1989; Akeson and Bernards, 1990; Bernard et al, 1986; Cheng and Skoultchi, 1989). The mechanisms of *myc* regulation of these genes are poorly understood and appear to vary considerably. In the case of the *hsp70*, E4 promoter and α-prothymosin, a direct effect on transcription appears probable, although there is no direct evidence of a *MYC* complex with these promoters. In the case of the LFA-1, a tetrameric protein, *MYC* is suspected of decreasing α_1-chain mRNA expression via a post-transcriptional mechanism involving α_1-chain RNA processing or transport (Inghirami et al, 1990). Post-transcriptional mechanisms are also believed to be involved in the induction of PAI-1 (Prendergast and Cole, 1990). Suppression of MHC cell surface proteins by *MYC* is thought to result from a post-translational modification of the H2K enhancer *trans*-activator H2TF1 (Lüscher and Eisenman, 1990).

In addition to regulating the aforementioned promoters, *MYC* has been shown to down-regulate its own expression (Penn et al, 1990b). Negative autoregulation of *myc* expression was first observed in Burkitt's lymphomas where translocation of the *c-myc* gene placed it under the regulation of the strong immunoglobulin regulatory region (Leder et al, 1983; Bernard et al, 1983). This enhanced, deregulated *c-myc* expression prevented *c-myc* transcription from the other, untranslocated *c-myc* allele. Subsequently transfection with a deregulated *myc* construct was shown to inhibit expression of the endogenous *myc* gene even if the endogenous gene was a different family member (Rapp et al, 1985; Clynes et al, 1988; Morse et al, 1986). This autoregulation also appears most pronounced in non-transformed cells suggesting that transformed cells may have acquired a growth advantage through a disruption in this mechanism (Grignani et al, 1990). Although the mechanism of autoregulation is not known, other *trans*-acting factors are believed to be involved. The *c-myc* gene in NIH 3T3 cells, normally resistant to autoregulation, can be made autoregulatory by fusion with the responsive Rat-1 cell line (Grignani et al, 1990). Mutational studies of the *myc* protein have revealed two regions that if mutated can interfere with the autoregulatory function. One of these regions is the BR-HLH-Zip area; the other is a region spanning amino acids 105-143. Incidentally both these regions are also essential for cotransforming activity, indicating a link between transformation and autoregulation.

Observations that cells constitutively expressing high levels of *MYC* grow faster and enter the DNA synthesis S-phase of the cell cycle sooner, while terminally differentiated cells exhibit down-regulation in *MYC* expression and a corresponding cessation in DNA synthesis, have suggested that *MYC* is involved in DNA replication. This hypothesis is supported by the observation that cells expressing high levels of *MYC* appear to support transfected SV40 DNA replication more efficiently (Classon et al, 1987). There have also been studies suggesting an origin of repliction 5' of the *c-myc* gene with which the *c-myc* protein interacts (Iguchi-Ariga et al, 1987; 1988). These latter studies have been difficult to confirm and remain controversial. Studies directed at blocking genomic or SV40 transfected DNA synthesis in cells by interfering with the *myc* protein have yielded conflicting results also. Whereas studies using anti-*myc* antibodies have failed to indicate a direct effect of *MYC* on DNA synthesis (Gutierrez et al, 1988), the use of antisense *myc* oligomers has prevented mitogenically stimulated cells from entering the DNA synthesizing S-phase of the cell cycle but has allowed progression from the G^0 to G^1 (Heikkila et al, 1987). Although a direct interaction of the *myc* protein with the replication machinery at the replication fork may be possible, current evidence supports an indirect effect. The more probable hypothesis is that *MYC* may *trans*-activate genes in early G^1 necessary for progression through the cell cycle, thereby providing a connection between its potential role as a *trans*-activator and its potential role in DNA synthesis.

Deregulated Expression of the *c-myc* Gene:

Deregulated *myc* expression can occur by numerous mechanisms and has been associated with many tumors. These deregulatory mechanisms can be separated into two groups: 1) those mechanisms affecting the physical gene structure, such as retroviral insertion or transduction, chromosomal translocation and gene amplification/rearrangement and 2) mechanisms affecting the expression of mature *myc* transcripts.

-Structural Modification of the *c-myc* Gene:

-Viral insertion and transduction:

The discovery that viruses can transform normal cells by influencing the expression of critical regulatory genes has provided great insight into the mechanism of tumorigenesis. By integrating into the host genome, the virus utilizes host cellular "machinery" to proliferate. Successful proliferation depends on the site of integration into the host genome. There is considerable evidence to indicate that this integration is by no means random, and in many instances has been found to occur near the important regulatory

protooncogenes (for review see Peters, 1990). An erroneous assembly process in some viruses during their evolution has resulted in the incorporation of part of the host genome into their own genome. Where this erroneous assembly has provided the virus with a genetic advantage, the host sequences have been retained in the viral genome throughout its evolution. Transduction is the process of transferring these host sequences from one host to another. In the case of the avian myelocytomatosis virus (AMV) its genome contains a fusion gag-*myc* gene. The *myc* portion was most likely obtained from the avian genome and corresponds to the coding exons 2 and 3 of the *c-myc* gene. During the viral evolution, the *v-myc* gene has acquired further mutations to enhance its activity over its cellular homolog *c-myc* (Papas and Lautenberger, 1985). These advantageous mutations, together with its viral promoter regulated expression, have provided the AMV retrovirus with an acute transforming ability.

Tumors deriving from viral integration differ from those of oncogene transducing viruses in that they grow slowly and represent clonal outgrowths derived from a single virally infected cell (Varmus, 1982; Nusse, 1986; Ramsay et al, 1990). In general, tumors derived from viral integration are much more common than virally transduced tumors (Peters, 1990). Viral insertion and transduction have been shown to occur with *c-myc* and *N-myc* (Peters, 1990).

-Chromosomal translocation:

Another important mechanism identified to deregulate *myc* expression was observed from tumors first described in African children in 1958 as a "sarcoma" of the jaw (Burkitt, 1958). These tumors have subsequently been reclassified and are known as Burkitt's lymphomas (for a review, see Magrath, 1990). Burkitt's lymphomas are a series of tumors involving a genetic translocation of the immunoglobulin heavy or light chain loci with the *c-myc* locus. The original translocation event characterized, involved movement of the *c-myc* locus from 8q24 to a region upstream of the immunoglobulin heavy chain enhancer on 14q32 (Dalla-Favera et al, 1983). Subsequently reciprocal translocations, designated as 8:22 and 2:8, were also observed where the λ- and κ- Ig light chain gene loci at 22q11 and 2p11-12 were translocated to positions distal to the *c-myc* locus on chromosome 8 (Magrath, 1990 and refs. within). Although the precise site of translocation varies in Burkitt's lymphomas, the coding region of *c-myc* and the constant region of the immunoglobulin chains are never disrupted.

The importance of *myc* associated chromosome translocation in tumorigenesis is further

supported by homologous translocation observed in mouse plasmacytomas and rat immunocytomas (Magrath, 1990 and refs. within). Murine plasmacytomas are induced by injecting susceptible mouse strains with pristane, while rat immunocytomas occur spontaneously. In both instances the 8:14 equivalent translocation predominates [mouse 12_{myc}:15_{IgH}, rat 7_{myc}:6_{IgH}].

-Gene amplification and rearrangement:

Amplification and to a lesser extent rearrangement of the *myc* locus has been recognized in many tumors. These tumors include human leukemias, a variety of carcinomas, small cell carcinomas, glioblastomas, neuroblastomas and retinoblastomas (Wong et al, 1986; Varley et al, 1987 and refs. within). Amplification in mammary carcinomas and human breast cancer cell lines has been reported for *c-myc* and on rare occasion *L-myc* (Wong et al, 1986; Varley et al, 1987 and refs. within). Many of these reports are clinical studies looking for *myc* gene anomalies in primary breast tumors. Reports of *c-myc* amplification in 20-35% of the biopsies are common. Rearrangement although detected sporadically is much less common, while anomalies involving the *N-myc* gene in breast tumors or breast cancer cell lines have not been observed.

-Regulation of *c-myc* mRNA Expression:

Regulation of eukaryotic mRNA expression can and has been shown to occur at any step in the biogenesis of mRNA. This process includes the initiation and elongation of transcription, splicing of hnRNA, mRNA processing [5'-7 methyl G capping and poly A tailing], transport from the nucleus or degradation of mRNA. In the most general terms *c-myc* mRNA accumulation is known to increase in mitogenically stimulated, actively proliferating cells, and to decrease in cells committed to terminal differentiation. Studies addressing these aspects of *c-myc* expression have revealed an array of regulatory mechanisms. These mechanisms can be subdivided into two groups: 1) transcriptional mechanisms involving either initiation or elongation/termination of new transcripts or 2) post-transcriptional mechanisms principally involving mRNA stability.

-Transcriptional regulation:

Although most eukaryotic cells derived from the same individual contain the same genetic information, there is preferential expression of different genes in different cell types. Two basic factors are critical to determining which genes will be expressed in any given cell type: first, the presence of an essential milieu of transcription factors in the cell that can interact with the gene and activate its expression and second, the

accessibility of the gene itself to these factors.

In eukaryotic cells, the genomic DNA and thereby the genes comprising this DNA, is organized into compact structures known as chromatin. In chromatin, a small segment of DNA (~140 bp) is wrapped about a protein core composed of two copies of each of 4 histone proteins, H2A, H2B, H3 and H4, forming structures referred to as nucleosomes. A fifth histone H1 may also be associated with the nucleosomes primarily in the more tightly bound, inactive chromatin. Nucleosomes are subsequently wound into fibers and ultimately into higher order structure chromatin in which the DNA has been packaged approximately a thousand fold. Where genes on the chromatin are accessible to the transcriptional machinery, the chromatin is referred to as active chromatin. The converse is inactive chromatin.

Early studies of chromatin structure revealed a distinctive difference between active and inactive chromatin: active chromatin was more susceptible to pancreatic DNaseI digestion (Reeves, 1984). This increased sensitivity to DNase I is believed to represent a more loosely packed chromatin structure and DNaseI hypersensitive sites are commonly associated with promoters of actively transcribed genes. How or why active chromatin is more sensitive to DNaseI is not fully understood. Several distinctions between active and inactive chromatin have been made that may explain active chromatin's increase in DNaseI sensitivity: 1) in active genes the level of cytosine methylation is considerably lower than in inactive genes (Weisbrod, 1982), 2) histone H1 has been shown to differentially bind active and inactive chromatin (Weintraub, 1984), 3) post-translational modification (acetylation, phosphorylation and others) of the histone proteins that can influence protein-DNA interaction have been described (Vidali et al, 1978) and 4) topoisomerase I, an enzyme responsible for releasing torsional strain on unwinding DNA, is more readily associated with active chromatin where it may function to permit accessibility to the polymerases or transcription factors (Gross and Garrod, 1988).

Once accessibility to the gene regulatory region has been obtained, precise regulation of the gene is dependent on the transcriptional activator milieu of the cell and the precise structure in terms of responsive elements present in the regulatory and promoter regions. Traditionally activators were characterized by their ability to bind *cis*-acting regulatory elements and simultaneously to interact with adjacent promoter bound proteins to stimulate expression of the associated gene. More recently certain activators have been shown to not bind DNA but to still be essential for transcription (Gaub et al, 1990). Therefore a distinction should be made between DNA binding *trans*-activators and non-

DNA binding activators. To date many prokaryotic and eukaryotic activators/*trans*-activators have been identified revealing several generalized characteristics (Ptashne, 1986; Ptashne, 1988): 1) these proteins are modular in structure with distinctive regions associated with specific functions. Corresponding regions between two factors are readily interchangeable, 2) for *trans*-activators, the DNA binding domains are highly specific while the *trans*-activating domains are more flexible in their interaction, 3) activator/*trans*-activators are functional in diverse eukaryotic cell types if appropriate DNA elements and auxillary factors are present, 4) most of these factors act cooperatively to stimulate transcription and 5) t*rans*-activators with strong DNA binding and *trans*-activating sites function more readily at greater distances from the activated gene promoter and interact with promoter bound factors by "looping out" intervening DNA sequences.

To potentiate their effects *trans*-activators must bind specific *cis*-acting elements. These elements are usually located upstream of the gene promoter region. In the *c-myc* gene, several lines of evidence point towards the 5' flanking region, exon1 and possibly intron1 as the gene regulatory region: 1) these regions are commonly disrupted in cells exhibiting deregulated *myc* expression, 2) although these regions of the *myc* gene vary considerably among species, there are several pockets of high sequence similarity and 3) all of the major DNaseI hypersensitive sites are associated with this region as are many of the *trans*-activator binding elements.

-DNase I hypersensitive sites in the c-myc gene:

DNase I hypersensitive sites have been resolved in both the human and mouse *c-myc* genes (Siebenlist et al, 1984; Mango et al, 1989). There are five consistent DNaseI hypersensitive regions in both *c-myc* genes, all located in the 5' flanking region and exon 1 [Fig. 6A]. Although the arrangement of these sites is similar in human and mouse *c-myc*, the positions and sequences differ somewhat when corresponding regions are aligned (Mango et al, 1989). Nonetheless sequences encompassing or immediately adjacent to these hypersensitive sites in the *c-myc* genes exhibit high conservation.

In the transcriptionally inactive germline *c-myc* gene in Burkitt's lymphoma BL31 cells, hypersensitive site I is very intense with the remaining 4 sites undetectable (Siebenlist et al, 1984). This pattern also appears in germline *c-myc* in plasmacytomas (Stanton et al, 1983) and retrovirally-induced tumor cells (Mango et al, 1989). It has been proposed that the hypersensitive site I encompasses a repressor binding site; however, the relevance of

hypersensitive site I for *c-myc* repression has been questioned by at least three independent groups (Mango et al, 1989; Dyson et al, 1985; Fahrlander et al, 1985).

-Positive and negative regulatory regions in c-myc:

In different model systems, a variety of growth factors and mitogens have been shown to stimulate *c-myc* gene expression by increasing initiation of new transcripts (Asselin et al, 1989; Lang et al, 1988 and refs. within). In many systems, effects on transcriptional initiation have been shown to involve signal transduction pathways in particular, the cAMP-dependent protein kinase and protein kinase C-dependent pathways (Simpson et al, 1989; McCaffrey et al, 1987; Ran et al, 1986; Spangler et al, 1991). In other systems a direct interaction of the mitogen with regulatory elements in the *c-myc* gene regulatory region appear responsible (Dubik and Shiu, 1988). Regulatory elements are believed to influence both initiation (Hall, 1990; Peitenpol et al, 1990; Marcu, 1987) and elongation (Miller et al, 1989) of transcription. For this reason much effort has been directed to identifying the *c-myc* regulatory factors and the *cis*-acting negative and positive elements within the *c-myc* locus.

Systematic transfection studies using MYC-CAT constructs have been employed to identify transcriptionally active domains within the *myc* regulatory region. These studies have revealed both enhancer and repressor regulatory regions [summarized in Fig. 6B]. Whitelaw et al (1988) has defined two enhancer regions, one encompassing the P1 & P2 promoters and the other region far upstream in the DNase I hypersensitive site I between -2300 bp to -1980 relative to P1. In addition they have also defined two repressor regions NRE1 and NRE2 encompassing hypersensitive regions II_1 and II_2 respectively. NRE1 mapped between -1527 bp to -1246 while the strong regulatory region NRE2 mapped between -1052 bp to -607 (Marcu, 1987). Other groups have also reported negative regulatory elements (Chung et al, 1986; Hay et al, 1987; Lipp et al, 1987; Remmers, 1986). Most groups have extended this region to include sequences up to -428 bp (Hay et al, 1987; Lipp et al, 1987; Remmers, 1986). More careful dissection of this region in mouse *c-myc* has revealed that the 5' and 3' ends possess the dehancer function (Marcu et al, 1988). In general reported enhancer and dehancer regions appear to overlap. Variation may be a result of differences in human and murine *c-myc* regulatory sequences or differences in the transfection system and constructs used. As an example of this variation, Marcu et al (1988) has observed that the dehancer -615 bp to -424 when placed upstream of the SV40 enhancer/promoter in a CAT construct reduced CAT activity up to 50 fold; in contrast the entire dehancer region -1140 bp to -424 with the

intact *myc* promoter reduced CAT activity only 3 fold.

-Minimal sequence requirements for promoter usage:

In addition to identifying positive and negative regulatory regions, MYC-CAT transfection studies have also identified minimal sequences necessary for P1 and P2 promoter usage. In murine *c-myc*, 109 bp upstream of the P1 promoter are sufficient for P1 usage (Asselin et al, 1989). This finding coincides well with reports that in human *c-myc* -60 bp to -37 relative to P1 are essential for its activity (Nishikura et al, 1986). Similarly sequences upstream of P2 from -7 bp to 116 appear essential for P2 usage in both the mouse and human *c-myc* (Asselin et al, 1989; Lipp et al, 1987). Within this region is a positive *cis*-acting element ME1a1 that if removed results in preferential usage of the weaker P1 promoter (Asselin et al, 1989).

-Trans-acting factors and cis-acting elements:

Within the positive and negative regulatory regions of the *c-myc* gene are a number of *cis*-acting elements. In many cases the corresponding *trans*-acting factors binding these elements have also been characterized. A listing of the known *trans*-acting factors and *cis*-acting elements is provided in Fig. 6C. The *cis*-acting elements 5' Mf, 5'Mg1, 5'Mg2, 5'Mg3, ME1a1 and ME1a2 were identified in mouse *c-myc* (Asselin et al, 1989). The corresponding human sequences are very similar particularly for ME1a1, ME1a2 , 5'Mg3 and 5'Mf. DNA-protein complexes formed with these elements and extracts from various cells were found to be competed by DNA corresponding to the SV40 early promoter region (Asselin et al, 1989). This region of the SV40 early promoter contains multiple GC-rich motifs that bind a common transcription factor Sp1 (Briggs et al, 1986). Competition binding studies using purified Sp1 showed that this factor bound with a high affinity to 5'Mg1 and 5'Mg3 and with a low affinity to the ME1a sites (Asselin et al, 1989). Therefore Sp1 appears to be involved in the regulation of *c-myc*. This result is somewhat intriguing to our studies as we have shown an estrogen-regulated CAT activity from a SV40-CAT construct. We have localized this effect to the SV40 early promoter which lacks a traditional estrogen regulatory element indicating a novel mechanism of estrogen activation that may involve Sp1.

DNaseI protection studies using partially purified nuclear extracts and the *c-myc* regulatory region revealed multiple protected sites (Whitelaw et al, 1988). Using purified preparations of Sp1 and CAAT transcription factor (Kadonaga and Tjian, 1986) one of these protected sites was attributed to Sp1 and two to CTF (Lang et al, 1988). The

nuclear factor κ B (NF-κB) family, first identified to *trans*-activate in B and T lymphocytes (Sen and Baltimore, 1986), also binds multiple sites at the 5' end of NRE2 (Duyano et al, 1990). Nearer the core promoter region, a *cis*-acting DNA sequence element has been identified that binds a ribonucleoprotein (RNP)(Davis et al, 1989). Several transcriptional repressors have also been identified with binding sites overlapping activator binding sites (Lobanenkov et al, 1990). Other ubiquitous transcription factors including NF-1 (Siebenlist et al, 1984), AP-2 (Imagawa et al, 1987), E2F(Hiebert et al, 1989), AP-1 and octomer binding factor (Takimoto et al, 1989) have all been shown to bind the *c-myc* gene and as such may be involved in the complex regulatory mechanism of this gene.

-Non-coding antisense c-myc transcripts:

In vitro nuclear runoff assays were used to determine the rate of transcriptional initiation in the *c-myc* gene and also revealed that the noncoding strands of the unrearranged human and murine *c-myc* genes were transcribed (Bentley and Groudine, 1986b; Nepveu and Marcu, 1986; Kindy et al, 1987). The antisense transcripts initiated at multiple sites throughout the non-coding DNA strand, were not translated and were transcribed independently from the sense transcripts. Whereas the sense transcripts increased upon mitogen stimulation and decreased in cells induced to differentiate, the corresponding antisense transcripts appeared to remain constant (Nepveu and Marcu, 1986; Kindy et al, 1987). The role of these antisense transcripts in the regulation of *c-myc* is still uncertain.

Evidence that antisense transcripts could play a regulatory role was first shown for plasmid replication in *E. coli* where antisense RNA complimentary to ompF gene RNA inhibits the expression of this gene (Mizuno et al, 1984). For the *myc* protooncogene, many groups have shown that expression of the endogenous gene can be suppressed by either transfected plasmids carrying antisense human *myc* DNA (Yokoyama and Imamoto, 1987) or by short antisense oligomers spanning the translation initiation sequences (Wickstrom et al, 1988; Griep and Westphal, 1988; Watson et al, 1991). In these instances, the exogenous *c-myc* is at much higher molar concentrations than the normal transcripts. How critical the endogenous antisense transcripts are to the overall regulation of the *c-myc* gene remains unknown.

-Transcriptional attenuation / termination:

Whereas many mitogenic compounds stimulate initiation of *c-myc* transcription, *c-myc* expression is depressed in terminally differentiating cells. Although differentiating cells

exhibit a decrease in *c-myc* initiation, the majority of transcripts initiate properly from the P1, P2 promoters but elongate poorly and ultimately terminate prematurely, generating highly unstable, untranslatable RNA (Bentley and Groudine, 1986b).

Transcriptional attenuation and premature termination are important regulatory mechanisms of many prokaryotic and eukaryotic genes (Kerppola and Kane, 1988 and refs. within). Among these genes are the *c-fos* and *c-myb* protooncogenes. In the *c-myc* gene, multiple attenuation sites are observed with transcriptional termination occurring preferentially at two thymidine stretches, T1 and T2, near the exon1/intron1 boundary (Bentley and Groudine, 1988). These T stretches are insufficient to block a heterologous gene, although a region encompassing T1, T2 and 125 bp upstream were able to regulate the attenuation processes (Almendral et al, 1988). The mechanisms regulating transcriptional attenuation / termination are not known but there is evidence that this process involves *trans*-acting factors as removal of the ME1a1 site in mouse *c-myc* abolishes the transcriptional block (Asselin et al, 1989; Miller et al, 1989).

Post-Transcriptional *c-myc* Regulation:

-Regulation of *c-myc* mRNA Stability:

Treatment of quiescent cells with mitogens or growth factors stimulates progression through the cell cycle by regulating early response genes. These genes, of which *myc* is a member, share common characteristics in that they all encode unstable mRNAs which are rapidly and transiently expressed upon mitogenic stimulation and are superinduced upon treatment with protein synthesis inhibitors such as cycloheximide (Almendral et al, 1988). When stimulated by mitogens, expression of these early genes increase; however, in most systems, transcriptional initiation alone cannot account for the levels of mRNA observed and therefore post-transcriptional mechanisms must be involved.

The first indications that post-transcriptional *myc* mRNA stabilization may play a critical role in its regulation came from studies of the translocated *c-myc* gene products of murine plasmacytomas and Burkitt's lymphomas. In these tumors, the deregulated *c-myc* mRNA is ten times more stable with a half-life of several hours instead of 20-30 min (Eick et al, 1985; Piechanczyk et al, 1985; Rabbitts et al, 1985). As most of these translocated *myc* genes lack 5' flanking and exon 1 sequences, it was hypothesized that these regions contained sequences essential to regulating *c-myc* mRNA stability. A subsequent study by Jones and Cole (1987) was aimed at confirming this hypothesis. They prepared a series of human and mouse *c-myc* constructs spanning all regions of the

gene, stably transfected the constructs into mouse fibroblasts and monitored the stability of the cytoplasmic mRNA produced from these constructs. They found that removal of the 5' flanking and exon 1 sequences had only a small effect on *c-myc* mRNA stability. Conversely, removal of a 140 bp AU-rich region from the 3' untranslated end of the *c-myc* mRNA greatly increased *myc* mRNA stability. The destabilizing effects of the AU-motif may be due to its signaling a shorter poly A tail or to its recognition by specific endonucleases (Brewer and Ross, 1988; Nielsen and Shapiro, 1990). When the 3' ends of stable genes were replaced by the AU-rich region, the heterologous transcripts produced by these genes were unstable. Even so, the AU-rich region is not solely responsible for *c-myc* mRNA stability as some mutant *myc* constructs containing only a mutation in the 5' flanking region / exon1 and not in the AU-motif also produced stable mRNA (Jones and Cole, 1987). This result indicates that certain 5' sequences can overcome AU-motif destabilization in *c-myc*.

In addition to the AU-motifs, there is also evidence of an additional secondary destabilization mechanism coupled to translation (Wisdom and Lee, 1990). A relationship between translation and mRNA stability has been described for many mRNAs. The β-globin, μ-heavy chain and triose phosphate isomerase mRNA appear to be stabilized by translation, as shown by a mutation that causes premature termination which also results in unstable transcripts (Wisdom and Lee, 1991 and refs. within). Conversely other transcripts including the early response genes, β-tubulin, histone and transferin receptor are destabilized by translation and these transcripts are stabilized by translational inhibitors and mRNA mutations that disrupt translation (Wisdom and Lee, 1991 and refs. within). The molecular determinants of *c-myc* essential to this translational destabilization have been localized to the region of the mRNA coding for amino acids 335-439 (Wisdom and Lee, 1991). This region can impart translational destabilization to a heterologous gene. Whether the destabilization signal is present in the mRNA or the nascent *c-myc* peptide is unknown. It has been suggested that the helix-loop-helix region 360-396 may be interacting with another protein resulting in a transduction signal for mRNA degradation (Wisdom and Lee, 1991). This type of mechanism was shown for β-tubulin mRNA turnover (Yen et al, 1988).

To summarize, we see that regulation of the *c-myc* gene, and most likely of all early response genes, is a highly complex process. Whereas differentiated cells exhibit decreased *c-myc* transcript initiation and premature termination, most mitogen stimulated cells enhance *c-myc* expression by a combination of increased transcriptional initiation

and post-transcriptional *myc* mRNA stabilization. The corresponding increase of *myc* protein then influences cellular proliferation through poorly understood mechanisms most likely involving transcriptional activation of key regulatory genes responsible for progression through the cell cycle. Anomalies in this critical regulatory process can disrupt normal growth and differentiation processes; therefore, deregulated *myc* expression is commonly associated with tumor cells.

D: Research Objectives:

One-third of all breast tumors are composed of cells responsive to and dependent on estrogen to proliferate. In the hundred years since the discovery that estrogens are important in breast cancer, few advances have been made in controlling this disease. There are many reasons for this lack of progress in the past century. Among these reasons is a generally poor understanding of the regulatory mechanism governing cellular proliferation and differentiation and an even poorer understanding of the specific functions of estrogen in these tumors. From the outset of this project, the objective of our lab was to expand the knowledge relating to the latter point: the specific function of estrogen in hormone responsive breast cancers. With the knowledge that the estrogen-receptor complex was a *trans*-acting factor, we used human breast cancer cell lines as a model system to identify specific estrogen-regulated genes. Based on the essential requirement of hormone responsive tumors for estrogen, we surmised that the estrogen-receptor complex must act on a gene critical to cellular proliferation. Therefore the objectives of this study were:

1) To identify estrogen regulated proto-oncogene expression in human breast cancer cells.

2) To characterize the molecular mechanism of this regulation by estrogen.

MATERIALS AND METHODS

A. Cell Culture:

The human breast cancer cell lines MCF-7, T-47D, MDA-MB-231, BT-20 and the partially transformed "normal" cell line HBL-100 were obtained from various sources described by Lima and Shiu (1985). HeLa epithelial cervical carcinoma cells were purchased from American Type Culture Collections Rockville, Maryland. Stock cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (4 mM), glucose (4.5 g/liter), penicillin/streptomycin (100 IU/ml and 100 μg/ml, respectively), bovine insulin (10 μg/ml), and 5 or 10% (v/v) fetal bovine serum. This media is referred to as complete media (C). Trypsin/EDTA in Hank's balanced salt solution was used to detach cells for passage. All reagents were obtained from Flow, Gibco or Sigma laboratories. The cells were kept in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Currently, DMEM without the slightly estrogenic pH indicator phenol red is used (Berthois et al, 1986); however, earlier experiments were performed in medium containing phenol red.

B. Hormones:

17β-estradiol (E), 5α-dihydrotestosterone (DHT), 6α-methyl-17α-hydroxy progesterone acetate (MPA), dexamethasone (DEX) and retinoic acid (RA) were obtained from Sigma Chemical Co. The antiestrogens tamoxifen and ICI 164,384 were provided by Dr. Allen E. Wakeling of ICI Pharmaceuticals. Stock solutions of 0.5 X 10^{-2}M in ethanol were prepared and stored in the dark at -20°C.

C. cDNA Probes:

The *c-myc* probe used for Northern analysis is a 0.5 kb cDNA probe corresponding to the *PstI* fragment of the second exon of the human *c-myc* gene and was generously provided by Dr. W. S. Hayward (Saito, 1983). The genomic *c-myc* probe used for the Southern hybridization analysis is a 8.2 kb *HindIII-EcoRI* fragment containing the entire human *c-myc* gene with 2.3 kb of 5' flanking sequences. This clone was described previously by Favera et al (1982). The *c-H-ras* probe is a 0.7 kb cDNA probe corresponding to the *SstI-PstI* fragment of the *v-H-ras* gene and was purchased from ONCOR, Inc., Gaithersburg, MD.

D. Plasmid Constructs:

i) MYCCAT constructs:

All MYCCAT constructs were derived from two principle MYCCAT construct designated as Cla#12 and Nsi#4 (Fig. 7A). These constructs were prepared by removing the *ClaI-XbaI* and *NsiI-XbaI* fragments from the genomic human *c-myc* construct described above, adding *HindIII-ClaI* linkers to the ends and subcloning into the *HindIII* site of pSVCAT. pSVCAT is a pBR322 based vector containing the SV40 early promoter (*HindIII-BglII* fragment) adjacent to the *E. coli* chloramphenicol acetyl transferase gene and the SV40 polyadenylation region. It was kindly provided by Dr. P. Cattini in our department (Cattini and Eberhardt, 1987). The single promoter P1 containing constructs, ClaXho and NsiXho, were derived from Cla#12 and Nsi#4 respectively by removing the *XhoI-BglII* fragment and blunt-end ligating the vector. Cla#12(-) and Nsi#4(-), both lacking the SV40 early promoter, were similarly constructed by removing the *SstI-BglII* fragment and blunt-end ligating the vector.

A series of 3' deletion constructs were made from Nsi#4 using exonuclease III digestion. Exonuclease III catalyzes the stepwise 3' to 5' removal of 5' mononucleotides from double stranded DNA containing a 3'-OH end. The enzyme requires a 5' protruding end or a blunt end to function and will not affect 3' protruding ends or d-S-NTP blocked ends. Therefore Nsi#4 was: 1) cut with *BglII*, 2) blocked with d-S-GTP, 3) cut with *NsiI* which generates a 3' overhang, 4) klenow filled to blunt this end and 5) ExoIII digested for various times from 15 seconds to 4 minutes. The Exo III digestion was carried out in 80µl of ExoIII buffer (66mM Tris-HCl pH 8.0, 0.66mM $MgCl_2$) at 35°C using 8µg of the appropriately prepared Nsi#4. At an enzyme concentration of 4 U/µl under these conditions 150-200 bp were removed every 30 sec. At appropriate times 9.5µl aliquots were removed and the reaction was then immediately stopped. The remaining single stranded DNA was digested by adding 28.5µl of S1 nuclease buffer (40 mM KAc, 300 mM NaCl, 1.3 mM $ZnSO_4$, 7% glycerol and 0.25 U/µl of S1 nuclease). The deleted vector was then blunt-end ligated and transformed into competent *E. coli* strain RR1 bacteria. Using this procedure a series of MYCCAT 3' deletion constructs were obtained. These constructs were sequenced using the Sanger dideoxy method (Sanger et al, 1977) across the MYC - pSVCAT junction. Deletion constructs used in this study include 3'#20 (MYCCAT[-667→+141]), 3'#19 (MYCCAT[-667→+202]), 3'#14 (MYCCAT[-667→+250]), 3'C#1 (MYCCAT[-667→+396]), 3'B#1 (MYCCAT[-667→+557]), 3'B#2 (MYCCAT[-667→+662]) and 3'B#3 (MYCCAT[-667→+701]). Using 3'#19 and 3'#14, constructs MYCCAT[-1255→+202], MYCCAT[-1255→+250] and MYCCAT[-2327→+202], MYCCAT[-2327→+250] were prepared by replacing the *HindIII-XhoI* in

3'#19 and 3'#14 with the corresponding region of Cla#12 and the genomic *c-myc* construct. The constructs MYCCAT[-667→+202{-P1} or +250{-P1}] were prepared by removing the *SmaI-NotI* fragment from MYCCAT[-1255→+202 or +250]. Similarly MYCCAT[+25→+202 or +250] was prepared from MYCCAT[-1255→+202 or +250] by removing the *HindIII-NotI* fragment. For a summary see Fig. 7B.

(ii) Human estrogen receptor expression constructs:

The human estrogen receptor expression vector HEO and several mutant receptors HE4 (lacking part of the DBD and D regions), HE11 (lacking the entire DBD), HE19 (lacking the N-terminus A/B region) and HE21(lacking most of the C-terminus) were used in this study. These constructs were all generously provided by Dr. Pierre Chambon (Kumar et al, 1987). Also see Fig. 8 for more details.

(iii) Constructs used to identify an ERE in the SV40 early promoter:

The vectors pSVCAT (described above), pCAT (pSVCAT without the SV40 promoter) and pUCTKCAT (a pUC based vector containing the thymidine kinase promoter) were provided by Dr. Peter Cattini (Cattini and Eberhardt, 1987) and are further described in Fig. 9. Constructs designated pE-CAT have had the 952 bp *Eco47III* fragment from the pBR322 backbone removed. The pTKCAT construct was made by removing the *HindIII-BglII* SV40 early promoter fragment from pSVCAT and replacing it with the *HindIII-BglII* TK promoter of the pUCTKCAT. pESV[1-209] was prepared by removing the *HindIII-NsiI* fragment of pESVCAT and blunt-end ligating the vector. Similarly the pESV[1-132] was prepared from pESV[1-209] by removing the *SfiI-BglII* fragment [see Fig. 9].

(iv) ERE containing constructs used as positive controls:

Two control ERE-containing vectors were used. The first ApoVLDLII-CAT was provided by Dr. Roger Deeley and contains region -465 bp to +48 of the ApoVLDLII gene upstream of the CAT reporter gene. The second construct VitTKCAT was provided by Dr. Chambon and contains the upstream 337 bp of the vitellogenin A2 gene adjacent to the TK promoter and CAT gene (Klein-Hitpass et al, 1986) [also see Fig. 9]. ApoVLDLII contains two imperfect adjacent EREs while vitellogenin A2 contains a single consensus ERE.

E. Growth Response Studies:

For growth studies, cells were plated at a density of 1 X 10^3 cells/cm^2 in insulin-free

medium containing FBS (C) and left for 48h. The medium was then changed to either C, C' (containing charcoal treated FBS (cFBS)) or T' (C' with 10^{-6}M tamoxifen). After 48 h, estradiol (final concentration, 10^{-7}M) was added to the C' and T'. These conditions were then denoted as E' and TE', respectively. The cells were then allowed to grow for a further 8-10 days without medium change. After this time the cells were trypsinized, were dispersed through an 18-gauge needle and the cell number was determined using an electronic cell (Coulter) counter.

F. RNA Isolation and Northern Hybridization Analysis:

Stock cells were plated at 0.7 X 10^4 cells/cm^2 in medium C and were left to attach for 48 h. The earlier experiments were done with phenol red containing media: the later experiments were done with phenol red free media. Where phenol red was present maximal cell growth inhibition was obtained only in the presence of C' with tamoxifen (10^{-6}M). In the absence of phenol red C' alone was sufficient for maximal cell growth inhibition. Cells were maintained in the hormone depleted medium for 48 h and then estradiol (10^{-7}M) was added for varying times depending on the experiment. At the appropriate time after estradiol addition, cells were harvested for RNA isolation.

RNA isolation was done by the guanidinium isothiocyanate/CsCl method (Chirgwin et al, 1979). Northern analysis of formamide and formaldehyde denatured RNA was performed as outlined by Maniatis et al (1982) and was described previously (Dubik and Shiu, 1987). All probes used in Northern hybridizations were nick-translated cDNA probes. Hybridizations and washes of the nitrocellulose blots were done under stringent conditions (Dubik and Shiu, 1987).

Dot blot analysis was done according to the procedure of White and Bancroft (1982). For both dot blot analysis and Northern analysis, quantitation of the relative amounts of specific RNA transcripts was performed by densitometric scanning of the autoradiographs.

G. DNA Isolation and Southern Analysis:

DNA was isolated from appropriate breast cancer cell lines according to the protocol of Davis et al (1986). To analyze the genomic DNA for *c-myc* amplification or rearrangement, the isolated DNA was cut with the restriction endonuclease *PstI*, fractionated on a 0.8% agarose Tris-borate-EDTA buffer gel and transferred to nitrocellulose. Once the DNA was fixed to the nitrocellulose by baking for 2h, the blot

was hybridized with a nick-translated 8.2 kb *HindIII-EcoRI* genomic *c-myc* probe for 24h and then washed as described previously (Dubik and Shiu, 1987). All blots were also subsequently reprobed with a nick-translated *c-mos* cDNA probe. Amplification of *c-myc* relative to *c-mos* was determined by densitometric scanning of the autoradiographs.

H. *In Vitro* Nuclear Run-off Transcription Assay:

Nuclei from hormone depleted or estradiol treated cells (see E above) were isolated and nascent RNA transcripts elongated in the presence of [^{32}P]-UTP using a procedure described in detail previously (Dubik and Shiu, 1988). RNA from the cytoplasmic supernatant was also isolated using the guanidinium / cesium chloride procedure and used for Northern analysis. The nascent radioactive labeled transcripts produced in the *in vitro* transcription reaction were then isolated and the relative amounts of key mRNA was determined by hybridizing these labeled transcripts to an excess of denatured pBR322, *c-myc* (exon 2), pS2 and chicken β-actin DNA that had been fixed onto nitrocellulose strips (Dubik and Shiu, 1988). Following hybridization, the strips were washed and exposed to Kodak XAR film for several days at -70°C with an intensifying screen. Bound RNA was then quantitated by densitometric scanning of the autoradiographs.

I. RNA Stability Study Using Actinomycin D:

Human breast cancer cell line MCF-7 and MDA-MB-231 were maintained in hormone depleted medium with tamoxifen (10^{-6}M) for 48 h. These cells were then treated with estradiol (10^{-7}M) for 45 min to stimulate *c-myc* mRNA accumulation. The cells were then treated with the RNA polymerase inhibitor actinomycin D (5μg/ml). At various times from 0-120 min after actinomycin D addition, the cells were harvested and the total RNA was isolated. The amount of *c-myc* mRNA transcripts contained within this RNA was determined by Northern hybridization using the nick-translated second exon PstI fragment as a probe. Quantitation of the autoradiograph by densitometric scanning provided the data necessary to plot the rate of *c-myc* mRNA degradation in both MCF-7 and MDA-MB-231 cells.

J. Transient Transfection and Chloramphenicol Acetyl Transferase (CAT)Assays:

Cells used for transient transfection were primarily HeLa cervical carcinoma cells; however, in some experiments MCF-7 human breast cancer cells were used. These cells were plated at a density between 0.7-1.0 X 10^6 cells per 10 cm dish, in insulin-free medium containing fetal bovine serum (C). To study the effect of estrogen on the transfected constructs, it was necessary to deplete the endogenous levels of estrogens in

both the cells and the medium. This depletion was done by culturing the cells for 48 h in phenol red free medium containing 5% steroid-depleted (charcoal treated) fetal bovine serum (C') 24 h after plating. The cells were then transfected with the appropriate construct (10-15 μg) and in some instances co-transfected with an estrogen expression vector (2.5-5 μg) using the calcium phosphate precipitate method. In this method, 0.5 ml of 2X calcium chloride-DNA solution [0.25M $CaCl_2$, with 20-30 μg construct DNA] was added dropwise to 0.5 ml of 2X HEBS [280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, pH 7.1 ± 0.05] through which air was bubbled. The mixed solution was then allowed to stand for 30-45 min to allow a fine $CaPO_4$-DNA precipitate to form. Equal amount of this precipitated solution (450 μl) was then added to two identical dishes of hormone depleted cells containing 7 ml of fresh C' medium. Four hours later, DNA uptake by the cells was enhanced by removing the medium from the dishes and treating the cells with a 20% glycerol DMEM solution for 8-10 min. Following this treatment the cells were washed with DMEM, 10 ml of fresh C' medium was added and one of the matched plates was treated with estradiol (final concentration 10^{-7}M). The cells were then incubated for 36-42 h in order to express the CAT gene. After this incubation cells were isolated using trypsin/EDTA and resuspended in 120 μl of 0.25M Tris-HCl pH 7.5. Cellular extracts were obtained using a rapid-freeze-thaw procedure. This procedure entailed rapid freezing (in an ethanol dry ice slurry) and thawing of the cells several times with gentle agitation at every thawing. The cellular debris was pelleted by microcentrifugation for 5 min. To remove any endogenous acetylases that may degrade the acetylated products, the supernatant was heated at 65°C for 10 min and recentrifuged. The supernatant was then isolated and the concentration of proteins within it was determined using a BioRad (Bradford) protein assay. Extracts containing equal amounts (30-70 μg) of protein were assayed for the CAT enzyme by measuring CAT acetylation of [^{14}C]-chloramphenicol. The assay was done by bringing the volume of extracts to 100 μl with 0.25M Tris-HCl pH 7.5 and adding 62 μl of CAT Buffer (40 μl H_20, 20 μl Tris-HCl pH7.5, 2 μl [^{14}C]-chloramphenicol) and 10 μl of acetyl coenzyme A (70 mg/ml), the source of the acetyl group. Acetylation of the labeled chloramphenicol was allowed to proceed for 4-6 h at 37°C. After the incubation, acetylated products were extracted with ethyl acetate (600 μl) and lyophylized. Acetylated [^{14}C]-chloramphenicol was isolated from the mixture by thin layer chromatography in a chloroform/methanol (19:1) mixture and visualized by autoradiography 24-96 h later. Acetylated chloramphenicol product was quantitated by scraping the isolated 3-mono-acetylated [^{14}C]-chloramphenicol product from the TLC plate which was then scintillation counted. Significant differences in CAT expression between hormone treated and untreated transfected cells were confirmed by statistical

analysis of multiple experiments.

K. Quantitative Polymerase Chain Reaction (PCR) Analysis:

To determine the extent of *c-myc* gene amplification in the various human breast cancer cell lines, both Southern analysis (described in section G) and quantitative PCR were employed. Using quantitative PCR the *c-myc* gene copy number was determined relative to the copy number in the normal lymphocyte genome after standardization to the *c-mos* gene. Both the *c-myc* and *c-mos* genes are localized to the same arm of chromosome 8. The PCR analysis procedure used in this study was established by Dr. P.H. Watson in our laboratory (Watson et al, 1992). Genomic DNA (250 ng) isolated from HBC cell lines as described previously (see section G) was subjected to PCR in the presence of 200 μM dGTP, dATP, dCTP and 400 μM dUTP and 1 unit uracil N-glycosylase, 4 mM $MgCl_2$, 5% DMSO, 1.5 units of *Taq* polymerase in 1X PCR buffer (Promega), and 1 μM of the appropriate primers in a total volume of 50 μl overlaid with mineral oil. Pre-PCR incubation for 10 min at 20°C followed by 7.5 min at 95°C was used to allow the glycosylase to eliminate any potential contaminating dUTP containing sequences. The *c-myc* and *c-mos* products (204 bp and 300 bp respectively) were then amplified using the following PCR profile [95°C, 60 sec; 56°C, 60 sec and 72°C, 90 sec] for 35 cycles. Ethidium bromide stained products were localized by gel electrophoresis. The product bands were photographed and then quantitated using densitometric scanning. The primers used were as follows; 5'*c-myc* $^{5'}$GCCACAGCAAACCTCCTCACA$^{3'}$, 3'*c-myc* $^{5'}$CGTTGTGTGTTCGCCTCTTGA$^{3'}$ [posn. 6753 and 6957 respectively] and 5'*c-mos* $^{5'}$GTCTCTTCATTCACTCCAGC$^{3'}$, 3'*c-mos* $^{5'}$ACTTGTTCACTTGCTTTATGG$^{3'}$ [posn. 194 and 494 respectively].

RESULTS

A. Estrogen-Induced *c-myc* Expression in Human Breast Cancer Cells:

Protooncogenes have been recognized as being important in cancerous proliferation and transformation (Bishop, 1983; Bishop, 1987; Varmus, 1984). Amplification, rearrangement and translocation of the *c-myc* proto-oncogene or variations in *c-myc* mRNA levels have repeatedly been observed in various tumors, differentiating cell lines, and mitogenically stimulated cell lines (Blanchard et al, 1985; Escot et al, 1986; Kelly et al, 1983; Lachman et al, 1985; Wong et al, 1986). In breast carcinomas, amplification and rearrangement of *c-myc* has been recognized (Escot et al, 1986) and in some of these carcinomas, these changes have been associated with enhanced *c-myc* mRNA expression. Clinically, about one third of all breast tumors are estrogen-responsive, in that estrogen stimulates their proliferation (Mourisden et al, 1978). To better understand the action of estrogen in HBC we chose to investigate the effects of this steroid hormone on *c-myc* mRNA expression in several HBC cell lines.

-Cell Response to Estrogen and Tamoxifen:

In order to maximize the effects of estradiol on HBC cells, the endogenous levels of estrogen present in both cells and growth medium were depleted. This depletion was achieved by using charcoal-treated, fetal bovine serum (cFBS) in the medium and by adding a growth inhibitory, non-toxic, concentration of the antiestrogen tamoxifen (10^{-6} M) to offset the effects of any residual estrogens. The growth response data for the ER+ cell lines T-47D, MCF-7 and the ER- cell line MDA-MB-231 are summarized in Fig. 10. In the ER- MDA-MB-231 cells the presence or absence of estradiol or tamoxifen had no effect on cell growth. Conversely in the ER+ cell lines partial growth inhibition was observed in estrogen-depleted medium (C') and was further suppressed on addition of tamoxifen (T') when compared with cells growing in the presence of untreated FBS (C) which contains endogenous estrogens. This growth inhibition was reversed by estradiol (10^{-7}M). Although Fig. 10 shows cell growth 10 days after various treatments, we have also measured the growth response in the first 48 hours, the time interval in which we subsequently examined *c-myc* expression. In T-47D at 48 hours, the fold increase in cell number for the five groups was: C-2.0, C'-1.3, T'-1.2, E'-1.8, TE'-1.6. This data corresponds well with the results in Fig. 10.

-*c-myc* Expression in ER+ Cells:

Fig. 11 depicts the time course of *c-myc* and *c-H-ras* mRNA accumulation in tamoxifen-inhibited MCF-7 cells "rescued" by estradiol. An increase in *c-myc* mRNA accumulation was observed after 15 min of estradiol rescue, with maximal levels (>10 fold) achieved between 60-90 min. The level of *c-myc* expression gradually declined for the next 10 h and remained at a level 3 fold above tamoxifen-treated cells for at least 54 h thereafter [Fig. 12]. Fig. 12 also depicts a similar pattern of induction in another ER+ cell line T-47D although the maximal accumulation of *c-myc* mRNA occurred around 2 h after estradiol rescue. In contrast, the expression of *c-H-ras* was unaffected by either estradiol or tamoxifen [Fig. 11]. Increased *c-myc* mRNA results in a corresponding increase in *c-myc* protein as depicted by Western blot analysis in Fig. 13.

-Comparison of *c-myc* and c-*H-ras* Expression in ER+ and ER- HBC Cells:

The patterns of expression of *c-myc* and *c-H-ras*, 90 min after estradiol rescue, in MCF-7 (ER+) and MDA-MB-231 (ER-) cells are depicted in Fig. 14. Whereas tamoxifen inhibited and estradiol stimulated *c-myc* expression in MCF-7, they had no effect on *c-myc* expression in MDA-MB-231 cells. It is of interest to note that the MDA-MB-231 cell line expressed a higher level of *c-H-ras* mRNA which was unaffected by tamoxifen and estradiol.

Using dot blot analysis, the estrogen nonresponsive BT-20 tumor line and the nontumorous HBL-100 line showed no regulation of *c-myc* expression by either estradiol or tamoxifen in contrast to MCF-7 and T-47D cells [Fig. 15]. These estrogen nonresponsive cell lines, similar to the MDA-MB-231 cell line, also exhibited high, estrogen-independent, constitutive expression of *c-myc* mRNA.

-Effects of Steroid Hormones on *c-myc* Expression:

We were interested in determining whether the estrogen regulated expression of *c-myc* seen in ER+ HBC cell lines was unique to the estrogen receptor complex, or could also be seen with other steroid receptor complexes. To study the possibility that other steroid receptor complexes may also influence *c-myc* expression we cultured MCF-7 cells in a hormone-depleted medium for 48 h and then treated individual dishes with a physiological concentration (2×10^{-9}M) of various steroid hormones, whose receptors are known to be present in this cell line.. The cells were exposed to hormone for 90 min and then total RNA was isolated and *c-myc* mRNA was quantitated by Northern analysis. The results of this experiment shown in Fig. 16 clearly indicate that within the 90 min time frame only estradiol significantly stimulates *c-myc* expression in these cells while the

glucocorticoid, dexamethasone, the progesterone derivative, medroxyprogesterone acetate and the androgen, dihydrotestosterone exhibit no effect on *c-myc* mRNA expression. The results from two independent experiments suggest that retinoic acid may slightly increase *c-myc* expression; however, insufficient data was collected to confirm this observation statistically. This experiment also shows that estrogen regulates *c-myc* expression even at physiological concentrations, reaffirming the importance of estrogen regulated *c-myc* expression to proliferation of breast cancer cells.

-Amplification and Rearrangement of the *c-myc* Gene in HBC cells:

To determine whether this high level of *c-myc* expression in the estrogen nonresponsive cells was a result of gene rearrangement or amplification, Southern analysis and quantitative PCR of the *c-myc* gene was performed. The result of this study, shown in Fig. 17, indicates no rearrangement of the *c-myc* gene in any of the breast cancer cell lines or in the nontumorous breast cell line HBL-100. Two-fold amplification of the *c-myc* gene is seen in MCF-7 and BT-20 cells while a five-fold amplification is observed in HBL-100.

B. Transcriptional Regulation of *c-myc* Expression in Estrogen Responsive Breast Cancer Cells:

The *c-myc* oncogene has been recognized as a cell cycle competence gene essential for cell growth (Kaczmarek et al, 1985). Its expression is activated by many peptide growth factors such as PDGF, FGF, EGF and growth hormone among others (Kelly et al, 1983; Kaibuchi et al, 1986; Ran et al, 1986; Murphy et al, 1987). The activation of *c-myc* expression by most peptide growth factors is primarily at a post-transcriptional level by stabilization of the *c-myc* messenger RNA (Kelly and Siebenlist, 1986; Blanchard et al, 1985; Kindy and Sonenshein, 1986; Greenberg and Ziff, 1984). This knowledge of *c-myc* regulation has been derived primarily from studies of peptide hormones that initiate their actions by binding to cell surface receptors on target cells. Little is known about the mechanism of regulation of *c-myc* by steroid mitogens, whose biological actions are mediated by nuclear-bound receptors.

Estradiol, an ovarian steroid hormone, is a potent mitogen for human breast cancer cells (Darbre et al, 1983). We have shown that the estradiol-stimulated proliferation of human breast cancer cells is accompanied by a rapid accumulation of *c-myc* mRNA. The action of estrogen, unlike that of peptide growth factors, is mediated by a nuclear estrogen receptor which is a nuclear transcriptional activator (Yamamoto, 1985). For this reason

estradiol may regulate *c-myc* oncogene expression at the transcriptional level. We provide evidence that in receptor-positive, estrogen-responsive human breast cancer cells, estradiol activates the *c-myc* oncogene solely by a transcriptional mechanism. In receptor-negative, estrogen-independent breast cancer cells, there is an elevated level of *c-myc* mRNA, primarily due to increased *c-myc* mRNA stability. This study indicates that different mechanisms of regulation of *c-myc* oncogene expression exist in estrogen-responsive and estrogen-nonresponsive human breast cancers.

-Analysis of *c-myc* Transcription:

Addition of 10^{-7}M estradiol to hormone depleted MCF-7 cells results in a rapid accumulation of *c-myc* mRNA, followed by cell proliferation. In the estrogen-nonresponsive human breast cancer cells MDA-MB-231, constitutive *c-myc* expression is high and unaffected by either the antiestrogen tamoxifen or estradiol. An *in vitro* transcriptional assay was used to determine whether the estrogen-induced *c-myc* mRNA accumulation was a consequence of transcriptional regulation. In this assay nuclei isolated from MCF-7 cells, tamoxifen-inhibited [T] or tamoxifen-inhibited but rescued by estradiol [TE], were incubated *in vitro* in the presence of [^{32}P]-UTP to allow elongation of *in vivo*-initiated nascent RNA transcripts. This labeled RNA was isolated and hybridized to *c-myc* cDNA as well as other specific gene probes fixed onto nitrocellulose. These latter genes included pS2, an estrogen regulated gene described by Masiakowski et al (1984), β-actin, a gene whose expression is not influenced by tamoxifen or estradiol within our experimental time-frame [Fig. 18A] and plasmid pBR322 DNA, to monitor the extent of background hybridization. The results of experiments examining *c-myc* transcription at various times after estradiol rescue of MCF-7 cells are shown in Fig. 18. A very rapid (within 5 min) transcriptional induction of the *c-myc* gene, with a maximal >10 fold increase within 20 min was observed [Fig. 18B, 18C]. This level of transcription was similar to that of *c-myc* mRNA produced by estradiol treatment [Fig. 18A, 18C]. As described previously (Brown et al, 1984), the pS2 gene was also transcriptionally regulated [Fig. 18B]. Neither the rate of transcription of the β-actin gene nor the level of β-actin mRNA was affected by estradiol. Detailed time-course studies revealed the peak of *c-myc* transcriptional activity occurs at approximately 20 min while the maximal level of *c-myc* mRNA occurs 1 h after estrogen treatment [Fig. 18C].

-Effects of Translational Inhibition on Estrogen Regulated *c-myc* mRNA Accumulation:

We pretreated MCF-7 cells for 2 h with the protein synthesis inhibitor cycloheximide (50 μM) prior to addition of estradiol, to determine whether estradiol induction of *c-myc* transcription required the synthesis of an additional regulatory factor. Fig. 19 shows that cycloheximide treatment did not abolish estradiol-induced *c-myc* expression, indicating that the activation of *c-myc* was a primary action of estradiol. The enhanced level of *c-myc* mRNA [Fig. 19A] in the presence of cycloheximide is not due to increased transcription but is due to increased stability of the untranslated mRNA as described by Wisdom and Lee (1991). Additionally, the estradiol-induced transcription of the pS2 gene was also not affected by cycloheximide [Fig. 19B].

-Analysis of *c-myc* mRNA Stability:

Tamoxifen-treated, hormone-independent MDA-MB-231 breast cancer cells expressed approximately 5-fold more *c-myc* mRNA than did similarly treated MCF-7 cells [see Fig. 20A]. To determine whether this enhanced *c-myc* expression in MDA-MB-231 was a result of increased transcription, *in vitro* transcription studies were carried out in these cells. The results indicated that the rate of *c-myc* transcription in MDA-MB-231 cells was not affected by either estradiol or tamoxifen, and was similar to that in MCF-7 cells inhibited by tamoxifen [Fig. 20B]. Therefore, the enhanced constitutive expression of *c-myc* mRNA in MDA-MB-231 is not a result of increased transcription of the *c-myc* gene, but may be due to an increased *c-myc* mRNA stability in this cell line. To test this hypothesis, actinomycin D was used to inhibit the synthesis of new mRNA molecules, and the degradation of existing *c-myc* mRNA in the two cell lines was compared. Because *c-myc* mRNA levels were very low in tamoxifen-treated MCF-7, we were unable to determine the half-life of the *c-myc* mRNA in these cells. However, in estradiol-stimulated MCF-7 cells the half-life of *c-myc* mRNA was determined to be 18 ± 7 min [Fig. 21]. This result is very similar to the half-life of *c-myc* mRNA found in other "normal" quiescent cells (Dani et al, 1984) and differs significantly from the half-life of >120 min in cells stimulated by peptide growth factors (Blanchard et al, 1985; Kindy and Sonenshein, 1986). Therefore, we conclude that estradiol has no influence on *c-myc* mRNA stability in MCF-7 cells. In MDA-MB-231 cells the *c-myc* mRNA half-life was not influenced by the absence or presence of estradiol. However, the mRNA was 3 times more stable than that of MCF-7 cells, with a half-life of 49 ± 3 min [Fig. 21]. Therefore the higher constitutive expression of the *c-myc* gene in MDA-MB-231 can in part be accounted for by a more stable *c-myc* mRNA.

C. Identifying the Estrogen Response Element in the *c-myc* Proto-oncogene:

The estrogen receptor complex is a known *trans*-acting factor that regulates transcription of specific genes through an interaction with a specific estrogen responsive *cis*-acting element (ERE), in the gene regulatory region. The previous studies have shown that in MCF-7 cells the estrogen receptor complex activates *c-myc* expression at a transcriptional level and does not require the synthesis of a new protein intermediate. Therefore it is probable that an ERE is present in the *c-myc* gene regulatory region. To localize the ERE containing region, constructs containing varying lengths of the *c-myc* 5' flanking region ranging from -2327 to +25 (relative to the P1 promoter) adjacent to the chloramphenicol acetyl transferase reporter gene (CAT) were prepared and used in transient transfection studies. This study revealed that the P2 promoter region was essential to estrogen regulated CAT expression of the MYCCAT constructs and that a minimal region approximately 116 bp 5' to this promoter contained the ERE. This 116 bp region lacks a *cis*-acting element related to any known ERE; however, cotransfection studies with mutant estrogen receptor expression vectors revealed that the DNA binding domain is essential for estrogen regulated CAT gene expression. Therefore the results suggest that a novel ERE must be contained in the 116 bp region upstream of the P2 promoter of the *c-myc* gene.

-Estrogen regulated CAT activity from MYCCAT constructs:

To localize the region of the *c-myc* gene essential for estrogen regulated transcriptional activity, a series of MYCCAT constructs were made [Fig. 7]. These constructs incorporated the known *c-myc* regulatory regions including exon 1, upstream 5' flanking sequences (2327 bp) and part of the first intron (the first 616 bp) and contained at least one of the three *c-myc* promoters P0, P1 and P2.

Identical dishes of HeLa cells were cotransfected with 10 μg of each of these MYCCAT constructs and 5 μg of the estrogen expression vector HEO using the $CaPO_4$-DNA precipitation method as described in MATERIALS AND METHODS. One of the paired dishes was then treated with estradiol (10^{-7}M) and 36-42 h later cells were isolated, standardized in terms of total proteins and assayed for CAT activity. Representative results for several of these MYCCAT constructs are summarized in Fig. 22 and indicate that although the level of acetylation is low relative to the positive control ApoVLDLII-CAT, there is a consistent ~5 fold induction of CAT activity in the hormone treated cells when compared with the hormone untreated cells. The activation is dependent on the

estrogen receptor since receptor-free transfections produced only background levels of acetylated product [see Fig. 23, no HEO]. Similar transfection studies of MYCCAT in MCF-7 cells yielded similar results [Fig. 24].

-The P2 Promoter is Necessary for Estrogen Regulated CAT Activity in MYCCAT Constructs:

To test the importance of the three *c-myc* promoters for estrogen regulated CAT activity, MYCCAT constructs were made containing various combination of these promoters. MYCCAT[-667→+202]{-P1} contains the P0 and P2 promoter regions but lacks the P1 containing *SmaI-NotI* region; MYCCAT[+25→+202] contains only the P2 promoter; MYCCAT[-667→+67] contains P0 and P1 but lacks P2. These constructs were cotransfected with HEO into HeLa cells, hormone treated and assayed for CAT activity in a manner analogous to that described above. The results summarized in Fig. 25 show that the P2 promoter region is essential for hormone induced CAT activity as the P0, P1 containing construct MYCCAT[-667→+67] does not induce CAT activity upon hormone treatment while MYCCAT[+25→+202] containing only the P2 promoter does show estrogen regulated CAT activity.

A further construct MYCCAT[-667→+141] containing the P2 promoter and TATA box region but lacking the P2 transcription initiation site at bp 172 also consistently exhibits estrogen induced CAT activity [Fig. 23]. Therefore the estrogen responsive element must be contained in the 116 bp region of the *c-myc* gene between +25 and +141.

-Analysis of the 116 bp *c-myc* P2 region for consensus EREs:

In steroid responsive genes, *trans*-activation occurs through direct interaction of the hormone-bound receptor complex with specific *cis*-acting DNA elements in the regulatory region of the gene (for a review see Beato et al, 1989). Based on the responsive elements of several estrogen-regulated genes, a palindromic consensus element $^{5'}$GGTCA-nnn-TGACC$^{3'}$ has been described [Fig. 4]. Variations of this sequence result in less effective EREs; however, several adjacent imperfect EREs can act collectively to impose estrogen responsiveness on a downstream gene (Martinez et al, 1987; Nunez et al, 1989). Using the consensus ERE $G_{73}G_{100}T_{91}C_{82}A_{100}$-nnn-$T_{100}G_{100}A_{64}C_{100}C_{91}$ described in Fig. 4 and permitting considerable variation at nonconserved base pairs [those less than 100] computer analysis of the 116 bp ERE containing region of *c-myc* revealed that there are no sequences resembling any known perfect or imperfect EREs in this DNA fragment. Therefore we believe that the *c-myc*

gene either contains a novel uncharacterized ERE near the P2 promoter, or that the receptor complex can activate transcription in an unconventional manner, perhaps through a protein-protein interaction.

-The DNA Binding Domain of the Estrogen Receptor is Essential for Estrogen Regulated CAT Expression from MYCCAT[+25→+202]:

Recent studies of estrogen regulation in the ovalbumin gene have revealed that the estrogen receptor potentiates its effect not through a direct interaction with a promoter associated *cis*-acting element but through an interaction with the regulatory region bound AP-1 *(fos-jun)* complex (Gaub et al, 1991). In this study it was also shown that a mutant estrogen receptor lacking the DNA binding domain was still effective in regulating ovalbumin expression.

To determine the domains of the estrogen receptor necessary for estrogen regulation of the *c-myc* gene, CAT assays were done with HeLa cells cotransfected with a series of estrogen receptor mutants expression vectors [Fig. 8] and MYCCAT[+25→+202]. The results of these experiments are shown in Fig. 25 as are the results of the same experiments done with the estrogen regulated positive control ApoVLDLII-CAT. Clearly mutants in the DNA binding domain and the hormone binding domain (HE4 and HE21 respectively) do not exhibit an estrogen regulated induction. The N-terminal deletion mutant HE19 supports estrogen regulated CAT expression from MYCCAT but not from ApoVLDLII suggesting that the A/B regions of the receptor are not essential to hormone regulation in MYCCAT. The absence of estrogen receptor gives only background CAT activity from the CAT constructs.

-Estrogen but not other steroid hormones specifically activate *c-myc* mRNA accumulation and CAT expression:

Northern analysis was used to determine *c-myc* mRNA levels in steroid depleted MCF-7 cells after 90 min of treatment with either 10^{-7}M dexamethasone, dihydrotestosterone, medroxyprogesterone acetate or retinoic acid. This experiment was done to determine whether the *c-myc* mRNA accumulation was specific to estradiol treatment and not to other steroid hormones. The result shown in Fig. 26A clearly indicates that within the 90 min time frame these steroids have little influence on *c-myc* mRNA accumulation.

Treatment of MYCCAT[+25→+202] and HEO cotransfected HeLa cells with these same steroid hormones yielded results consistent with those of the Northern analysis in that enhanced CAT expression was observed only from estradiol treated cells (Fig. 26B).

-CAT expression in MYCCAT[+25→+202] is activated by the antiestrogen-receptor complex:

The realization that estrogen is a potent mitogen for breast tumor cells has led to antiestrogen therapies based on antagonizing the interaction of the steroid with the estrogen receptor. We tested the ability of antiestrogenic drugs to antagonize estrogen regulated CAT expression from MYCCAT[+25→+202] by exposing estrogen treated MYCCAT[+25→+202] and HEO cotransfected HeLa cells to the antiestrogen ICI 164,384. As expected ICI 164,384 did antagonize estrogen regulated CAT activity, however, this "pure" antagonist alone also stimulated CAT expression to approximately 32% that of estradiol stimulated cells [Fig. 27]. Another antiestrogen tamoxifen also stimulated CAT expression from MYCCAT[+25→+202]. Both ICI 164,384 and tamoxifen failed to activate CAT expression from ApoVLDLII-CAT and HEO cotransfected HeLa cells [Fig. 27]. The stimulatory effects of the antiestrogens appear to be specific for the MYCCAT constructs as endogenous *c-myc* mRNA accumulation in MCF-7 is not stimulated by antiestrogens (Dubik et al, 1987).

D. Estrogen-Regulated Gene Expression from the SV40 Early Promoter:

In the course of studying estrogen regulation of the MYCCAT constructs we observed that a pSVCAT construct unexpectedly exhibited considerable estrogen-regulated CAT gene expression when cotransfected with the estrogen receptor into HeLa cervical carcinoma cells. Transfection without HEO or the addition of other steroid hormones resulted in no hormone-inducible CAT expression. Replacement of the SV40 early promoter of pSVCAT with the thymidine kinase (TK) promoter resulted in a construct, pTKCAT, that had no estrogen-regulated CAT expression. This result indicates that the SV40 early promoter confers estrogen-responsiveness to the pSVCAT. Similar to the 116 bp region upstream of the *c-myc* promoter, the SV40 early promoter does not contain sequences resembling known EREs. Furthermore as with the *c-myc* 116 bp region, cotransfection studies of pSVCAT with estrogen receptor expression mutants revealed that the DNA binding domain of the estrogen receptor is essential to hormone regulation of the pSVCAT construct. Therefore the novel estrogen-regulatory mechanism seen in the *c-myc* gene may be shared by the SV40 early promoter region in the pSVCAT construct.

-HeLa Cells Cotransfected with pSVCAT and the Estrogen Receptor Expression Construct HEO Exhibit Estrogen-Regulated CAT Expression:

The pSVCAT vector used in this study [Fig. 9] was constructed by replacing the *SphI - EcoRI* fragment of pBR322 with a corresponding fragment containing the SV40 promoter, the CAT gene and the SV40 polyadenylation region. The 209 bp SV40 promoter contains sequences 79 bp downstream of the origin of replication, the TATA box and Sp1 binding region, and 26 bp of the enhancer, a region referred to as the P-element. Although the P-element contains an AP-1 binding site (Lee et al, 1987a; Lee et al, 1987b), it alone cannot enhance transcription from the SV40 promoter (Zenke et al, 1986).

When HeLa cells were co-transfected with 10 μg of pSVCAT and 5 μg of HEO (estrogen receptor expression vector) and treated with 10^{-7} M estradiol, a hormone-dependent induction of CAT expression was observed [Fig. 28]. The activation was dependent on estrogen receptor since HEO-free transfections produced no acetylated product [Fig. 28, no HEO]. Predictably, HeLa cells transfected with the promoterless construct pCAT also revealed no CAT activity [Fig. 28].

-Potential Estrogen Responsive Elements (EREs) Upstream of the SV40 Promoter are not Responsible for the Estrogen-Regulated CAT Expression:

Using the ERE consensus described in Fig. 6, computer analysis of pSVCAT was done. Three potential imperfect EREs were identified all within 830 bp upstream of the SV40 promoter [Fig. 29]. The proximity of these EREs to the SV40 promoter suggested a potential influence on the promoter that could subsequently influence expression from the CAT gene. To test this possibility we removed the ERE-containing *Eco47III* fragment from pSVCAT generating pESVCAT. In addition we also removed the *HindIII-NsiI* pBR322 fragment immediately adjacent to the early promoter to generate pESVCAT[1-209] [Fig. 9]. Cotransfection of HeLa cells with either pESVCAT or pESVCAT[1-209] and HEO supported CAT expression similar to that of pSVCAT [Fig. 28]. This result shows that the ERE-like sequences upstream of the SV40 promoter are not responsible for the estrogen-regulated CAT expression of pSVCAT.

-Replacement of the SV40 Early Promoter with the Thymidine Kinase (TK) Promoter Abolishes Estrogen-Regulated CAT Expression:

To determine the importance of the SV40 promoter to the estrogen-regulated CAT expression in pSVCAT, this promoter was replaced with a TK promoter, as described in MATERIALS AND METHODS, to generate pTKCAT [Fig. 9]. The TK promoter was unable to support estrogen-dependent CAT expression [Fig. 28]. This finding demonstrates a specific requirement for the SV40 early promoter in estrogen-regulated

CAT expression of pSVCAT.

-Estrogen-Regulated CAT Expression in pSVCAT is Localized to 132 bp of the SV40 Promoter and is Specific for Estrogen:

The pSVCAT vector contains 209 bp of SV40 promoter sequence. We have shown that removal of the entire promoter region eliminates CAT gene expression [see pCAT Fig. 28]. Similarly others have shown that the GC-rich early promoter region is essential for *in vitro* and *in vivo* SV40 promoter activity (Everett et al, 1983). To decide whether sequences downstream of the TATA box are necessary for estrogen-regulated CAT expression in pSVCAT, we removed the *SfiI - BglII* fragment from pESVCAT[1-209] [see Fig. 9], leaving only 132 bp of the SV40 promoter, upstream of the origin of replication (ori). Transfection with the pESVCAT[1-132] gave results identical with the intact pSVCAT [Fig. 26].

We also treated HeLa cells, transfected with pESVCAT[1-132] and HEO, with 10^{-7}M dexamethasone, dihydrotestosterone, medroxyprogesterone acetate and retinoic acid, and assayed for CAT activity. The result clearly demonstrated that only estradiol can activate CAT expression [Fig. 30]. Similar results (not shown) were obtained with pSVCAT transfected into MCF-7 human breast cancer cells.

-Comparison of CAT Expression by Various Constructs in HeLa and MCF-7 Cells:

Several pSVCAT constructs were also tested in the human breast cancer MCF-7 cell line. The CAT assay results obtained with MCF-7 cells [Fig. 31] were very similar to those of HeLa cells, with the following exceptions: (1) estradiol-regulated CAT expression in HeLa cells is dependent on cotransfection with estrogen receptor HEO whereas that in MCF-7 cells is not (since MCF-7 cells contain endogenous receptor) and (2) pTKCAT consistently exhibited higher constitutive CAT expression.

-Estrogen-Regulated CAT Activity in pSVCAT is Dependent on an Estrogen Receptor Containing an Intact DNA Binding, Zinc Finger Region:

The lack of consensus ERE related sequences in the estrogen responsive 132 bp region of the SV40 early promoter led us to suspect a novel mechanism of estrogen regulation. To determine which regions of the estrogen receptor are essential for this activity, a series of estrogen receptor mutants were used [see Fig. 9] in transient cotransfection studies with pESVCAT[1-132]. The results of this study shown in Fig. 32 clearly indicate that only the intact receptor HEO and the N-terminal deletion mutant HE19 can support estrogen regulated CAT expression from the SV40 early promoter region. Receptors disrupted in

the DNA binding region alone [HE11] or in the DNA binding region and D regions [HE4] did not support estrogen-regulated CAT expression of pESVCAT[1-132]. Similarly the hormone binding domain deletion mutant HE21 did not support estrogen-regulated CAT expression from the 132 bp SV40 early promoter region. These results indicate that unlike the recently reported DNA binding region-independent estrogen regulation of the ovalbumin promoter (Gaub et al, 1990), hormone regulation of the SV40 early promoter requires the zinc finger region and conceivably involves a conventional DNA-receptor interaction.

-The Effects of the Antiestrogens Tamoxifen and ICI 164,384 on Estrogen-Regulated CAT Expression of pESVCAT[1-132]:

To confirm that the estrogen receptor complex is responsible for the estrogen-regulated CAT expression seen in pESVCAT[1–132] and HEO cotransfected HeLa cells, we attempted to antagonize estrogen-regulated CAT expression by treating the cells with the antiestrogen ICI 164,384. ICI 164,384 is believed to act by preventing the estrogen receptor from binding DNA by interfering with receptor dimerization (Fawell et al, 1990). It has been reported to be a pure estrogen antagonist, lacking any residual agonistic effects characteristic of other antiestrogens, most notably tamoxifen. To test its effect on our pESVCAT[1–132], HEO cotransfected HeLa cells, we treated these cells with either estradiol (10^{-8}M) or a combination of estradiol (10^{-8}M) and ICI 164,384 (10^{-7}M) and compared the level of CAT activity in both groups of cells. As anticipated the results clearly indicate that ICI 164,384 could antagonize estradiol stimulated CAT expression [Fig. 33, E compared with EI]. Unexpectedly, however, there is a distinct and reproducible induction of CAT expression in cotransfected cells treated with the antiestrogen ICI 164,384 (10^{-7}M) alone [Fig. 33, I]. A similar result is observed with the antiestrogen tamoxifen (10^{-6}M) [Fig. 33, T]. Antiestrogen-ER inductions range from approximately 15-40% that of estradiol regulated induction and indicates that in our transfection system, ICI 164,384 has a distinctive agonistic effect. Specific activation by the antiestrogen-receptor of the SV40 ERR is indicated from identical experiments with ApoVLDLII-CAT, HEO cotransfected HeLa cells. In these cells the antiestrogen-receptor complex does not influence CAT gene expression, although it does antagonize estradiol induced CAT expression [Fig. 33].

DISCUSSION

A. Stimulation of *c-myc* oncogene expression associated with estrogen-induced proliferation of human breast cancer cells:

We have shown that an induction (>10 fold) of *c-myc* mRNA occurs between one and two hours after addition of estradiol to ER+ HBC cells which have been growth retarded by culturing in steroid depleted medium. This time course is similar to *c-myc* induction by estradiol in the rat uterus (Murphy et al, 1987) or by serum in hamster lung fibroblasts (Blanchard et al, 1985) and aortic smooth muscle cells (Kindy and Sonenshein, 1986). The higher level of *c-myc* expression in cells grown in medium containing untreated FBS [C: Fig 11, 12] is due to endogenous estrogens present in the FBS. Besides the estrogens present in FBS, the pH indicator phenol red used in the growth medium has been reported to have a weak estrogenic effect on ER+ cells (Berthois et al, 1986). Due to the commercial inavailability of phenol red free medium during our earlier studies, we added the antiestrogen tamoxifen to the medium to offset the phenol red effects and that of residual estrogens associated with the cells. This antiestrogen addition resulted in a 30-50% decrease in cell growth when compared to that of cells grown in phenol red containing medium supplemented with cFBS alone [Fig 10]. We had also observed that *c-myc* induction was less marked if the cells were plated at more than 2.1 X 10^4 cells / cm^2. One possible explanation is that ER+ HBC cells normally, or as a result of cell-cell contact, produce internal or external factors that, when present and at a sufficiently high concentration, can overcome the effects of estrogen depletion. Therefore cell plating concentrations were routinely maintained below 0.7 X 10^4 cells / cm^2.

Estradiol-induced gene expression in HBC cells appears relatively specific for *c-myc*, in as much as expression of *c-H-ras* [24; Fig 12, 14] and *c-sis* (result not shown) was not similarly affected. Furthermore early induction of *c-myc* gene expression is specific for estradiol as other steroid hormones, dexamethasone, medroxyprogesterone acetate, dihydrotestosterone and retinoic acid did not significantly stimulate *c-myc* mRNA levels within 90 min of their addition to steroid depleted cells [Fig. 16]. Since only a single time point of 90 min was selected, it is conceivable that a time course study with these other steroid hormones could reveal an earlier transient, or later *c-myc* mRNA induction.

Two interesting observations were made by comparing *c-myc* expression in ER+ versus ER- cell lines [Fig.14-15]. First, neither the removal or addition of estradiol, nor the

presence of tamoxifen, had an effect on cell growth or *c-myc* expression in ER- cells. Second, high constitutive expression of *c-myc* mRNA occurred in all three ER- cell lines we studied. Southern analysis revealed that in MDA-MB-231 this induction is not due to amplification of the *c-myc* gene, whereas gene amplification appears to be able to account for the increased *c-myc* mRNA in BT-20 cells and in the nontumorous breast cells HBL-100 [Fig. 17]. The steady-state levels of *c-myc* mRNA in the various breast cell lines [Fig. 15] correspond well with the gene copy number. These observations suggest the possibility that in HBC cells the ability to express unregulated high levels of *c-myc* may be a prerequisite for estrogen-independent cell growth. Thus, estradiol regulation of *c-myc* expression may represent a necessary step in the mechanism by which estrogen enhances the growth of ER+ human breast cancer cells.

B. Transcriptional Regulation of *c-myc* Oncogene Expression by Estrogen in Hormone-Responsive Human Breast Cancer Cells:

Studies addressing the molecular mechanisms involved in mitogenic activation of *c-myc* expression have indicated two principle pathways: 1) enhanced transcription or 2) stabilization of existing *c-myc* mRNA (Kelly and Siebenlist, 1986; Blanchard et al, 1985; Kindy and Sonenshein, 1986; Greenberg and Ziff, 1984). We addressed both possibilities by *in vitro* transcription run-off studies and actinomycin D mRNA stability studies to determine which pathway was responsible for enhanced *c-myc* expression in our system. These studies revealed distinct mechanisms responsible for hormonally induced *c-myc* expression in estrogen-dependent MCF-7 cells and for constitutive high *c-myc* expression in estrogen-independent MDA-MB-231 cells. In estrogen-responsive MCF-7 cells, enhanced transcription appears to be the major cause of *c-myc* mRNA accumulation as estradiol activates (independent of new protein synthesis) *c-myc* transcription within minutes while it does not alter the stability of the *c-myc* mRNA [Figs. 18, 19, 21]. Conversely in estrogen-independent MDA-MB-231 cells, post-transcriptional mechanisms appear to play a major role, as a 2-3 fold increase in *c-myc* mRNA stability was observed with no effect on transcriptional initiation [Figs. 20, 21].

In designing the *in vitro* transcription run-off study, we were interested in identifying newly initiated full length *c-myc* transcripts and not newly initiated transcripts whose transcription was subsequently blocked in the first exon. Therefore we chose to use the double stranded *c-myc* second exon *PstI* fragment to quantitate *in vitro* synthesized [^{32}P]-UTP labeled *c-myc* transcripts. This probe enabled us to show that estradiol stimulated *c-myc* transcription within 20 min of hormone addition; however, we failed to address two

important questions: (1) Does estradiol regulate elongation of exon 1 attenuated transcripts? (2) Is the increase in [^{32}P]-UTP labeled transcripts coded by the sense and not the antisense strand of the *c-myc* gene?

Although not as commonly studied as transcriptional initiation and mRNA stabilization, attenuation or blockage of newly initiated *c-myc* transcripts is recognized as an important mechanism in *c-myc* gene regulation (Bentley and Groudine, 1986b; see also INTRODUCTION). In our nuclear run-off study, quantitation of first exon transcripts in addition to full length transcripts would have allowed us to determine whether the estrogen receptor acts on the *c-myc* gene to relieve the transcriptional block associated with the AT-rich region of the first exon. Although in our subsequent study the isolation of an ERE in the 116 bp region 5' to the P2 confirms the induction of *c-myc* transcription by the estrogen receptor complex we can not rule out the possibility that this complex also affects the transcriptional block associated with the *c-myc* gene.

The possibility that the transcriptional induction seen in the nuclear run-off experiments may be due to antisense transcripts is probably less significant. Although several groups have confirmed that *c-myc* antisense transcripts are readily formed (Bentley and Groudine, 1986b; Nepveu and Marcu, 1986; Kindy et al, 1987) these groups have also shown that these low level transcripts remain constant in both actively proliferating and growth arrested cells. Therefore although it is possible that the hormone-dependent transcriptional increase seen in the nuclear run-off experiments are a result of second exon antisense transcripts, there is no evidence in the literature supporting regulated expression of *c-myc* antisense transcripts. As such the transcriptional increase we have observed in the *c-myc* gene is most likely a result of sense transcripts.

C. Localization of an Estrogen Response Element to a 116 bp Region 5' to the P2 Promoter:

The previous studies showed that in MCF-7 HBC cells estradiol stimulates *c-myc* oncogene transcription and that this induction does not require the *de novo* synthesis of protein intermediates. In addition, estradiol was shown not to alter stability of the *c-myc* mRNA in these cells. Together these data suggests that the estrogen receptor complex may act as a *trans*-activator of the *c-myc* gene by interacting with a *cis*-acting estrogen response element associated with the gene. As the 5' flanking region, exon 1 and intron 1 have been implicated previously with *c-myc* gene regulation, a search for an ERE was begun, concentrating on these areas of the gene. Transient transfection studies with a series of MYCCAT constructs spanning this area revealed that MYCCAT[-1255→+202]

yielded an average 5-fold induction of CAT activity upon estradiol treatment [Fig. 22]. [Although not shown, larger constructs with 3' *c-myc* sequences extending beyond +396 lost this hormone induced CAT expression. This loss of hormone-regulated CAT expression may be due to a negative response element distal to +396 or may simply be due to an excessive physical distance between the P2 promoter region and the CAT gene in these constructs.] Further dissection of the [-1255→+202] region producing shorter MYCCAT constructs revealed that the P0 and P1 promoter regions are not essential to estrogen-regulated induction of CAT expression [Fig. 25]. A region of the *c-myc* gene from +25 to +141 is sufficient for estrogen regulation of the adjacent CAT gene and as such must contain the ERE.

From Northern analysis of MCF-7 cells treated with a variety of steroid hormones, we were able to show that steroids other than estrogen could not increase *c-myc* mRNA accumulation 90 min after hormone addition [Fig. 26 A]. Furthermore the estrogen induction from the *c-myc* estrogen response region was dependent on the presence of an intact estrogen receptor, as transfection of MYCCAT[+25→+202] into HeLa cells without HEO cotransfection resulted in no CAT expression [Fig. 27]. The DNA binding domain (DBD) of the ER must also be intact for estrogen regulated reporter gene expression in MYCCAT[+25→+202] as indicated by cotransfection with the ER mutant HE4 [Fig. 27]. Although the requirement of an intact DBD would suggest a direct interaction of the E-ER complex with specific DNA sequences, the 116 bp *c-myc* sequences lack sequences related to any of the characterized estrogen response element. Additional evidence that the *c-myc* estrogen response region differs from other well characterized estrogen response regions like those of the ApoVLDLII and vitellogenin A2 genes is shown by its ability to *trans*-activate with the antiestrogen receptor complexes tamoxifen-ER or ICI 164,384-ER to approximately 32-52% that of the E-ER complex [Fig. 27]. This antiestrogen-ER *trans*-activation was observed for the transfected MYCCAT constructs but the endogenous *c-myc* gene was not activated by antiestrogens (Dubik et al, 1987). The lack of consensus ERE related sequences, and the responsiveness to antiestrogen receptor complexes suggests that the estrogen response region in *c-myc* is structurally distinct from that of other characterized estrogen response regions.

How might the estrogen receptor complex potentiate its effect on the *c-myc* gene? Knowing that the estrogen receptor DNA binding domain must be intact for estrogen regulated expression, a direct interaction between the hormone receptor complex and

DNA is indicated. However, studies of various estrogen regulated genes in different species have consistently localized sequences within the estrogen response region closely resembling the ERE consensus GGTCA-nnn-TGACC (Walker et al, 1984; Waterman et al, 1988; Berry et al, 1989; Slater et al, 1990). The absence of such a sequence in the *c-myc* 116 bp estrogen response region would support an alternate mechanism of E-ER action.

A recent report that the DNA binding domain of the retinoic acid receptor can interact with *c-jun* to prevent *trans*-activation of the human collagenase gene provides evidence that the zinc finger region of these receptors, in addition to binding DNA, can participate in a protein-protein interaction (Schüle et al, 1991). Similarly the estrogen receptor complex may regulate *c-myc* transcription by interacting with and inactivating a *c-myc* specific *trans*-repressor or by forming an active *c-myc trans*-activating complex by binding a ubiquitous cellular factor. The antiestrogen-receptor complex would mimic the E-ER complex; however, the protein-protein interaction would be less optimal, resulting in only partial *trans*-activation. Estrogen regulation of the ovalbumin gene has also been shown to involve a potential interaction of the E-ER complex with *c-fos* of the AP-1 complex or with an AP-1 inhibitor (Gaub et al, 1990). The mechanism of estrogen regulation in ovalbumin is distinctive from that in *c-myc* since an ER mutant lacking the DNA binding function fails to activate from the 116 bp *c-myc* region but coactivates with an efficiency similar to that of the wild-type receptor from the ovalbumin promoter.

Another potential mechanism to describe how the estrogen receptor may potentiate its effect from the *c-myc* estrogen response region comes from recent characterization of the rat creatine kinase B (CKB) gene. The CKB estrogen response region contains an ERE half-site adjacent to a GC-rich, Sp1 binding region (Pentecost et al, 1990). The authors proposed that a weak interaction of the E-ER complex with the ERE half-site was stabilized by an interaction with an adjacent bound Sp1 or Sp1-like factor (Wu et al, 1991). A similar ERE half-site and a GC-rich region are present in the 116 bp *c-myc* estrogen response region and may suggest a related mechanism of estrogen regulated gene activation in the CKB and *c-myc* genes [Fig. 34].

Preliminary gel retardation studies, using the estrogen receptor and the +25 to +141 sequence were undertaken to further characterize the ERE. These initial attempts using MCF-7 cytoplasmic extract as the source of ER did not yield satisfactory gel retardation and it became evident that considerable time and effort would have to be expended to

establish favorable retardation conditions.

In our transfection studies with both the MYCCAT and SV-CAT constructs, failure to monitor transfection efficiency has limited our ability to compare relative levels of CAT expression from different MYCCAT constructs. Although we did attempt to monitor transfection efficiencies by cotransfecting with a β-galactosidase expression vector CH110 we consistently experienced poor β-gal expression even though CAT expression was excellent. We currently believe that the freeze-thaw method used to isolate our extracts may have inactivated the β-galactosidase while having no effect on the chloramphenicol acetyl transferase. Recently by using an alternate method of cellular extract isolation involving gentle dispersion of the transfected cell pellet in a Tris-pH 7.8 / Triton X-100 (0.1M/0.1% v/v) buffer, incubation on ice for 15 min followed by removal of cell debris by centrifugation, consistently high β-gal activities have been obtained in transfected ZR-75-1 HBC cells.

Nonetheless even without standardization for transfection efficiencies our experiments do permit us to compare CAT expression of paired identically transfected dishes, of which one was treated with hormone and the other was not. Under these conditions, in a given experiment changes in CAT expression between paired dishes can only be accounted for by the presence or absence of estradiol in the growth medium as plates of identically cultured HeLa cells were used, equal amounts of the same $CaPO_4$-DNA precipitate were added to both dishes and the same experimental procedure was exercised to yield the CAT assay results. Variations in CAT activities in separate experiments where seemingly identical MYCCAT constructs and experimental procedures were used are most likely due to differences in the $CaPO_4$-DNA precipitates and in differences in the growth characteristics intrinsic to the HeLa cells.

During the transfection experiments, we have also observed that several experimental conditions improved both the transfection efficiency and the consistency of hormone induction of the regulated MYCCAT constructs. First, CAT activity was greatly improved when HeLa cells were plated such that they did not reach or exceed confluence upon harvesting and second, maximal CAT activity was seen between 36-42 h post $CaPO_4$-DNA addition, and an extended glycerol shock of 8-10 min with 20% glycerol in DMEM greatly increased the transfection efficiency of our HeLa cells. However, even with these transfection enhancements, MYCCAT constructs still consistently revealed low levels of CAT expression when compared with the control constructs ApoVLDLII-

CAT and VitTKCAT or even the SV-CAT constructs [Figs. 22,23,24]. Possible explanations for the low CAT expression of the MYCCAT constructs include: (1) The MYCCAT constructs can not efficiently transfect the HeLa cells under our experimental conditions possibly due to some intrinsic tertiary or electrostatic structure associated with these plasmids that is lacking in the ApoVLDLII, VitTKCAT and pSVCAT constructs, (2) The MYCCAT constructs may not sufficiently mimic the chromatin structure of the same region of the intact gene and as such may not be regulated as efficiently in this artificial system, (3) HeLa cells may possess low levels or totally lack a crucial intermediate factor necessary for maximal CAT expression of the MYCCAT constructs [although repeating the transfections in MCF-7 cells did not improve CAT expression, Fig. 24] and (4) The ERE localized in this study may represent a weak estrogen dependent enhancer or a response element that interacts cooperatively with adjacent, *myc* gene associated, *trans*-acting factors whose response elements are missing in the MYCCAT constructs.

Recently in the literature there is growing evidence supporting unconventional estrogen regulated gene expression as CAT transfection studies have identified seemingly weak ERE lacking consensus sequences. Such was the case in transfection studies addressing the 20-30 fold stimulation of *c-fos* mRNA by estrogen in rat uterus (Hyder et al, 1991). This group had initially localized a weak ERE (2-fold CAT induction) between bases -278 to -135. Subsequently a stronger ERE (5-fold induction) was recognized in a 1.5 kb region 3' of the *c-fos* gene. Since our study has concentrated only on the region of the *c-myc* gene from -2327 to +1180 it is conceivable that an additional ERE could be present in the remaining 5 kb of *c-myc* sequences. Analysis of this region of the *c-myc* is most certainly warranted. Further examples of unconventional mechanisms of estrogen receptor transcriptional activation are seen in estrogen regulated *c-myc* expression in rat uterus (Dickerman, personal communication) and studies of estrogen regulated TGF-α (El-Ashry et al, 1991) and rat creatine kinase B [CKB] (Pentecost et al, 1990; Wu et al, 1991). In an independent study to localize the ERE in the *c-myc* gene in rat uterus by Herbert Dickerman's group an ERE was localized to a region between -56 (relative to P1) and the P2 promoter (personal communication). By cloning this region of *c-myc* into a CAT vector they observed low levels of acetylation in their transfection studies and a dependence on the P2 promoter for hormone regulated CAT expression. In estrogen regulation studies of the human TGF-α gene (El-Ashry et al, 1991) an estrogen responsive element has been localized to a 58 bp region (-241 to -194) of the TGF-α gene by inserting this region into a CAT expression vector. This region lacks a consensus

ERE but does contain two adjacent imperfect palindromic sequences and yields a 2-fold estrogen regulated CAT induction in MCF-7 HBC cells. Interestingly this same construct when transfected into monkey kidney cells (COS-7) resulted in a 35-fold estrogen dependent CAT induction. In the rat creatine kinase B gene estrogen regulation was localized to a region from -560 to -520 (Wu et al, 1991). This region contains a sequence GGTCA-nnn-CACCC loosely resembling a consensus ERE adjacent to two potential GC boxes (Sp1 binding sites) leading the group to propose that estrogen responsiveness of CKB is due to complex motif consisting of a weak ERE adjacent to a Sp1 binding site. This result is particularly interesting because the 116 bp *c-myc* ERE containing region and the SV40 early promoter region, both contain an estrogen response element half-site adjacent to potential Sp1 binding sites (Kadonaga et al, 1986; Asselin et al, 1987; Briggs et al, 1986) [Fig. 34]. Wu et al have also reported that the "pure" estrogen antagonist ICI 164,384 stimulates CAT expression from CKB-CAT transfected HeLa cells but not from those transfected with ERE-TKCAT. Similar results were seen with MYCCAT[+25→+202] and with pESVCAT[1-132] [Figs. 27 & 33]. These result clearly indicates that antiestrogens can antagonize estrogen induced CAT expression but, in the absence of estradiol they have a agonistic effect on CAT expression similar to that seen in CKB-CAT transfected cells.

When considering all the data on estrogen regulated gene expression it would appear that at least three distinct mechanisms of estrogen receptor gene regulation have been described. These mechanisms include: (1) Direct interaction of the estrogen receptor complex with consensus ERE or closely related sequences as seen in the vitellogenin and ApoVLDLII genes, (2) DNA-independent interaction of the estrogen receptor complex with regulatory region bound *trans*-activators as seen in the ovalbumin gene and (3) DNA-binding domain dependent, non-consensus ERE interactions as seen in *c-myc* and rat creatine kinase B genes. The regulatory elements of these latter genes also appear to be activated by the antiestrogen-receptor complex.

D. Detection of a Novel Estrogen Response Element in the SV40 Early Promoter:

The observation that MYCCAT construct Cla#12 exhibited estrogen regulated CAT expression and that this regulated CAT induction could be prevented by removal of the SV40 early promoter region, led us to study the role of the SV40 early promoter in estrogen regulated CAT expression. This study has shown that the estrogen-receptor complex can function as a *trans*-activator of pSVCAT when pSVCAT is transiently

transfected into HeLa or MCF-7 cells. This *trans*-activation is unique to the estrogen- or antiestrogen-receptor complex and cannot be mimicked by uncoupled receptors or other steroid hormone receptor complexes. The inactivity of ERE-like sequences in the pBR322 backbone upstream of the SV40 promoter and the inability of the substituted TK promoter to exhibit estrogen-regulated CAT expression has led us to suggest that this estrogen-receptor *trans*-activation is mediated by the SV40 early promoter. SV40 promoter sequences 132 bp upstream of the origin of replication are sufficient for this effect. As with the 116 bp ERE containing MYCCAT constructs but not with ApoVLDLII-CAT, N-terminal estrogen receptor mutant HE19 was still effective at regulating CAT expression from the SV40-CAT constructs. With all estrogen-regulated constructs, whether MYCCAT, ApoVLDLII or SV40-CAT, the DNA binding domain of the estrogen receptor is essential to hormone regulated CAT expression. This necessity would argue against an ER, DNA-independent interaction as reported in the estrogen-regulated ovalbumin gene (Gaub et al, 1991).

E. Future Studies:

With no functional consensus EREs within or upstream of the SV40 promoter and 116 bp *c-myc* ERE containing region, how does the hormone-receptor complex potentiate its effect? Studies with the estrogen receptor mutant expression vectors indicate that the DBD of the ER is essential to estrogen regulation thereby supporting a DNA-dependent non-consensus ERE interaction and not a DNA-independent interaction analogous to that in the ovalbumin gene. Although a novel nonconsensus ERE is possible it seems unlikely as several EREs from various species have been characterized and all share considerable sequence similarity with the consensus sequence GGTCA-nnn-TGACC [Fig. 4]. Although the receptor complex is not regulating expression of a new *trans*-acting factor, as hormone-regulated *c-myc* expression is unaffected by the translational inhibitor cycloheximide, it is possible that the receptor complex may be involved indirectly in this regulation by influencing an existing intermediate factor. Discovery of a kinase and protease activity associated with the estrogen receptor complex (Baldi et al, 1986; Puca et al, 1986) supports this possibility. Another possibility is a DNA-independent, receptor *trans*-activator / *trans*-repressor (protein-protein) interaction involving the DNA-binding domain. In such a situation, the estrogen-receptor complex could cooperatively interact with a promoter bound *trans*-activator or interact with a *trans*-repressor preventing its negative effect. This latter possibility seems unlikely as the DBD has not been shown to have any function other that DNA binding and minor

stabilization of receptor dimer formation.

Future studies will be directed at clarifying the mechanism of estrogen regulation by the 116 bp *c-myc* ERE containing region and the SV40 early promoter. Using these regions and purified estrogen receptor it should be possible to show whether the receptor complex binds directly to these regions or to factors associated with these regions. If a hormone-dependent gel retardation is observed, the novel ERE could be identified using DNase I footprinting. Lack of a direct interaction of the estrogen receptor complex with the *c-myc* 116 bp and SV40 promoter regions would support an indirect mechanism of estrogen regulation in *c-myc* and SV40, possibly involving the enzymatic modification of a crucial transcription factor.

In addition to these studies it would be interesting to explore any involvement of the estrogen receptor complex with transcriptional attenuation of *c-myc* transcripts. This experiment would be done by repeating the nuclear run-off experiments using single-stranded first and second exon probes so as to be able to differentiate newly initiated transcripts from attenuated transcripts in which the transcription block has been removed.

In conclusion, our study suggests a novel mechanism of gene *trans*-activation by the estrogen receptor in both the *c-myc* and SV40 promoters. Characterization of this mechanism may reveal a mode of ER regulation common to many estrogen-responsive mammalian genes.

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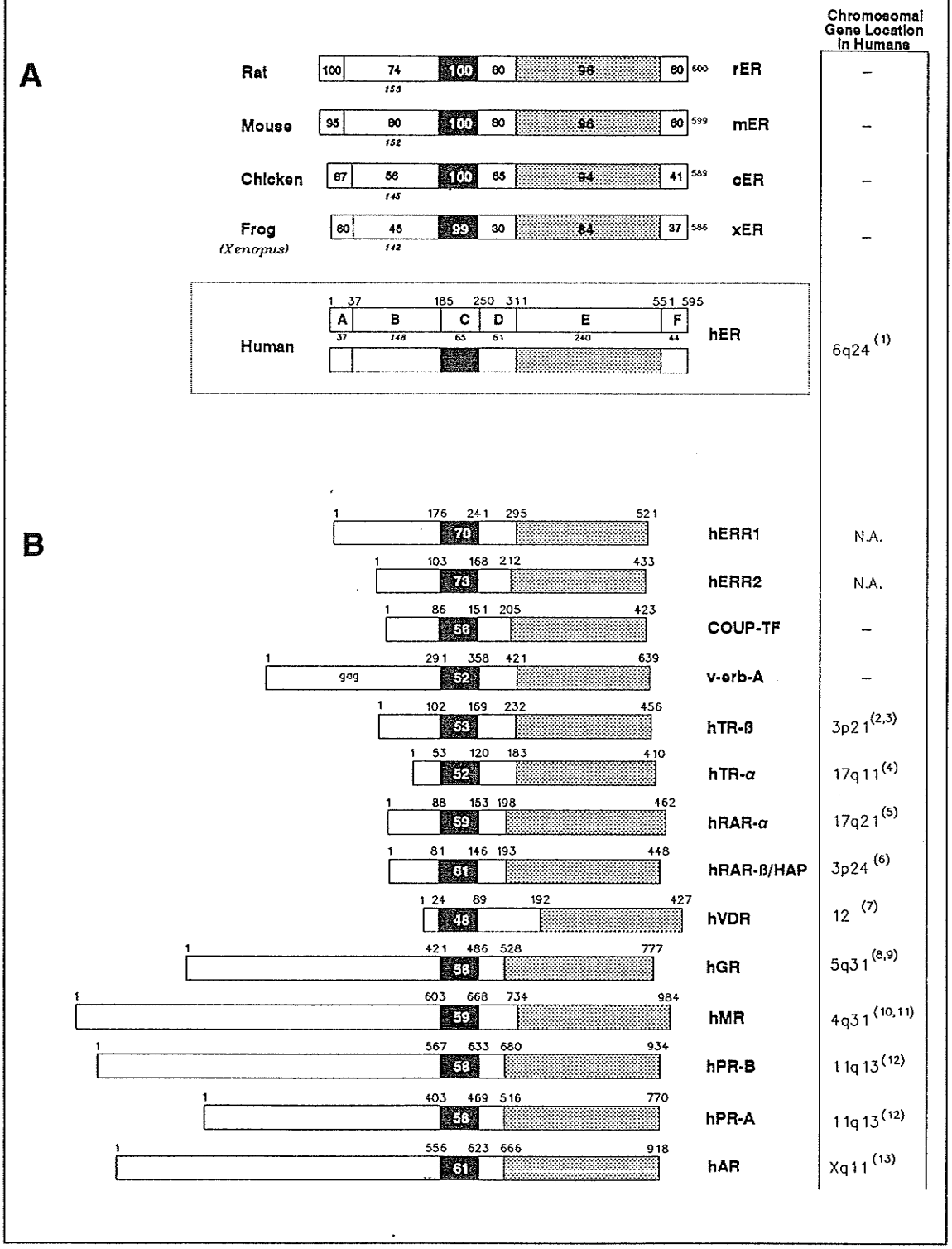
Zhang, X.-Y., Supakar, P.C., Wu, K., Ehrlich, K.C., Ehrlich, M. (1990). An MDBP site in the first intron of the human *c-myc* gene. *Cancer Res.* **50**, 6865-6869.

Fig. 1 

Fig. 1 Domain structure of the steroid receptor and related proteins. (A) Homology of the human estrogen receptor (hER) with ERs from other species. *Numbering within the boxes* represents amino acid similarity with corresponding hER. *Numbering in the right hand column* indicates total number of amino acids in each receptor. The B region varies in length: the numbering below this region indicates the corresponding length of this region for each of the ERs. **(B) Domain comparison of the hER with other steroid receptor family members.** *Numbering within the C-domain* indicates amino acid similarity with the hER. *Numbering above* indicates the amino acid span of each region A through F. COUP-TF is a chicken transcription factor, *v-erb A* is the viral homolog of the thyroid receptor. All abbreviations are described in LIST OF ABBREVIATIONS at the beginning of the thesis. Data has been accumulated from Carson-Jurica et al, 1990; White et al, 1987; Evans, 1988; Weiler et al, 1987. Refs. for chromosome locations are: (1) Ponglikitmongkol et al, 1988, (2) Weinburger et al, 1986, (3) Drabkin et al, 1987, (4) Thompson et al, 1987, (5) Mattei et al, 1988a, (6) Mattei et al, 1988b, (7) Faraco et al, 1989, (8) Gehring et al, 1985, (9) Giuffra et al, 1988, (10) Arriza et al, 1987, (11) Buetow et al, 1989, (12) Law et al, 1987, (13) Liao et al, 1989.

Fig. 2 Organization of the human estrogen receptor (hER) gene. The schematic at the top of the figure represents the 140 kb of chromosome 6 containing the eight exons encoding the hER mRNA. The hER mRNA contains an 5' untranslated region from nucleotides 1-233 and a large 3' untranslated region spanning bases 2020-6322. The region of the protein encoded by each exon is indicated by the numbers above the schematic representing the hER protein at the bottom of the figure. *Numbers below* the hER represent the amino acid boundaries of each region A through F. (Ponglikitmongkol et al, 1988).

Fig. 2 ⟹


A
B
Chromosomal Gene Location in Humans
Rat
Mouse
Chicken
Frog
(Xenopus)
Human
rER
mER
cER
xER
hER
6q24 (1)
hERR1
N.A.
hERR2
N.A.
COUP-TF
v-erb-A
hTR-β
3p21 (2,3)
hTR-α
17q11 (4)
hRAR-α
17q21 (5)
hRAR-β/HAP
3p24 (6)
hVDR
12 (7)
hGR
5q31 (8,9)
hMR
4q31 (10,11)
hPR-B
11q13 (12)
hPR-A
11q13 (12)
hAR
Xq11 (13)

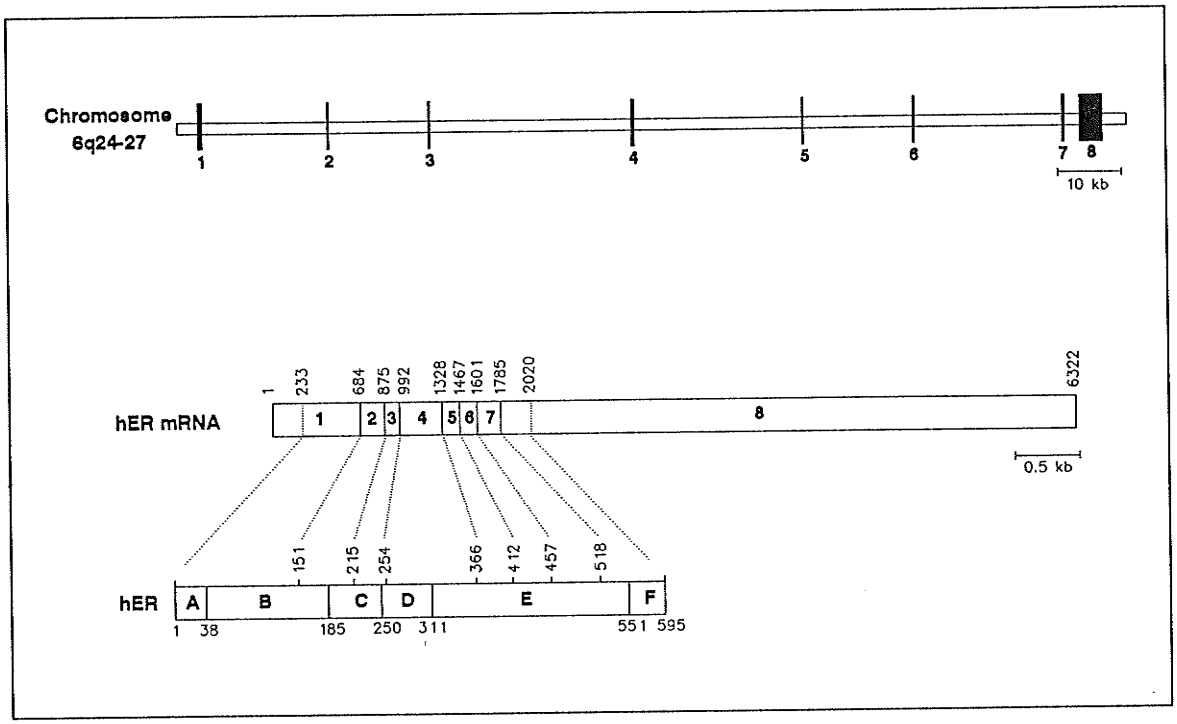

Chromosome
6q24-27
1
2
3
4
5
6
7
8
10 kb
hER mRNA
1
233
684
875
992
1328
1467
1601
1785
2020
6322
1
2
3
4
5
6
7
8
0.5 kb
151
215
254
366
412
457
518
hER
A
B
C
D
E
F
1
38
185
250
311
551
595

Fig. 3 Model of the dimeric complex between the estrogen receptor DNA binding domain (C-domain) and the estrogen response element (ERE). The lightly shaded rectangle represents the α-helix at the "knuckle" (bp 203-212) of the first zinc finger and the dark shaded rectangle corresponding to the α-helix of the second zinc finger "knuckle" (bp 239-251). The glutamic acid (E_{203}), glycine (G_{204}) and alanine (A_{207}) involved in the specific recognition of the ERE are indicated. Also labeled are amino acids 221 to 226 which participate in dimerization and are important in discriminating between the half-site spacing of the ERE and thyroid response element (TRE). The basic arginine, arginine, lysine at the tip of the second zinc finger are represented with "+". This basic region is in close proximity with the phosphate backbone of the minor groove with which it interacts to stabilize the dimer-ERE binding. The zinc ions are represented as a circle with Zn. The A,B,D,E,F domains are represented by a letter. Adjacent to the diagram is the ERE sequence showing the approximate location of the nucleotides relative to the diagram. In B-DNA each nucleotide is separated by 0.34 nm with 10 nucleotides per turn. **Inset:** A schematic representation of the estrogen receptor zinc fingers indicating the functionally important amino acids described above and in the text.

Fig. 3 

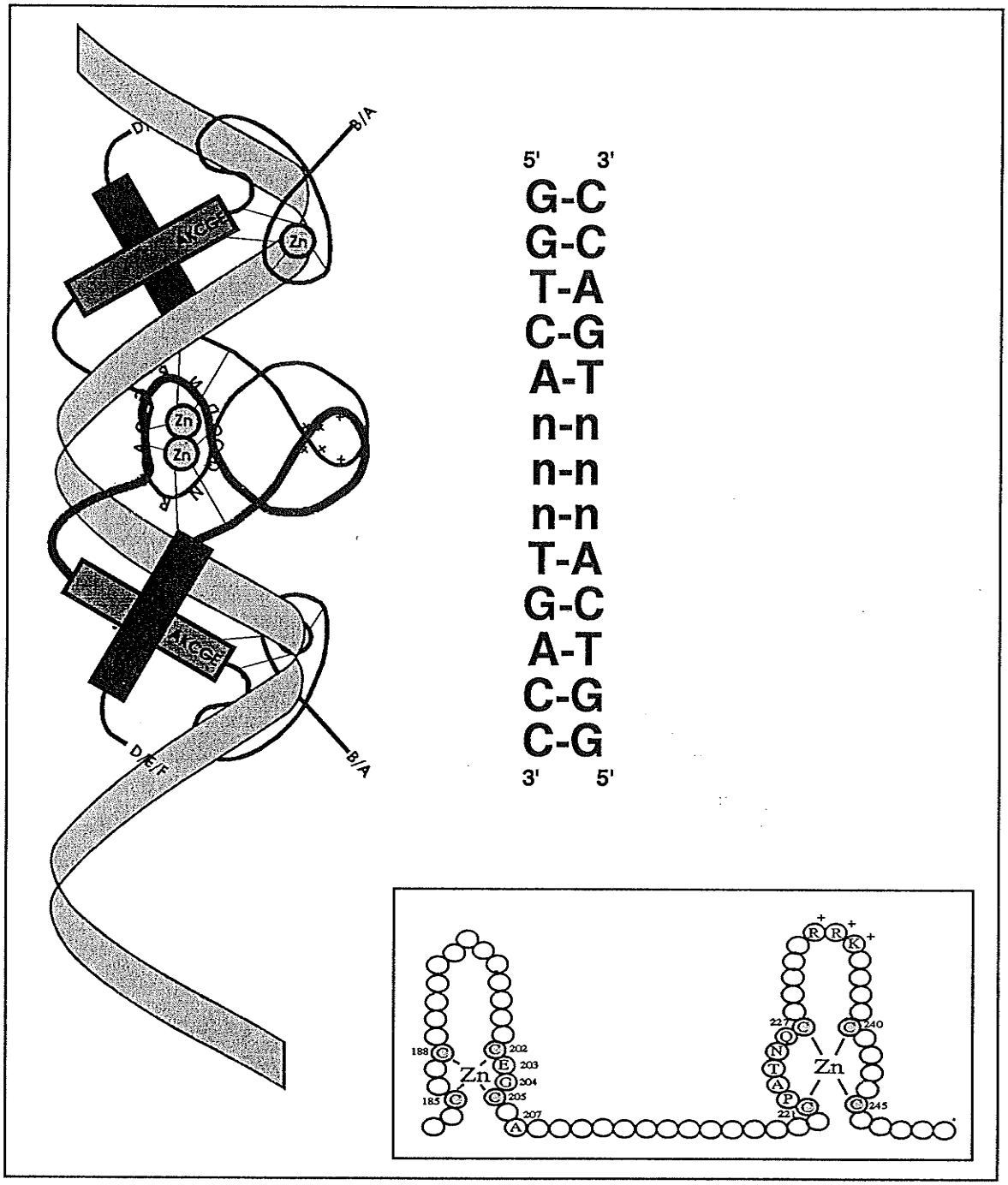

D'
B/A
Zn
Zn
Zn
D/E/F
B/A
5' 3'
G-C
G-C
T-A
C-G
A-T
n-n
n-n
n-n
T-A
G-C
A-T
C-G
C-G
3' 5'
Zn
Zn

Fig. 4 Steroid receptor response elements (A) Consensus sequence for hormone response elements. Shown are the consensus response elements for the glucocorticoid, progesterone, androgen, mineralocorticoid (GRE/PRE/ARE/MRE), estrogen (ERE), and triiodothyronine and retinoic acid (TRE, RRE) receptors. **(B) Characterized estrogen response elements.** References shown on the right correspond to the following: (1) Walker et al, 1984, (2) Klein-Hitpass et al, 1988a, (3) Martinez and Wahli, 1989, (4) Waterman et al, 1988, (5) Shupnik et al, 1989, (6) Berry et al, 1989, (7) Richard and Zingg, 1990, (8) Slater et al, 1990. **(C) Consensus ERE derived from characterized EREs described in (B).** The numbering below each base represents the percentage of specific base conservation.

Fig. 4 

A

GRE/PRE/ARE/MRE	G/T G/T TAC A/C - nnn- T G T T C T
ERE	GGTCA -nnn- TGACC
TRE/RRE	GGTCA-TGACC

B

Gene	Species	Characterized Element	Refs.
Vitellogenin A1	(*Xenopus*)	$^{-334}$GGTCA -nnn- TGACC $^{-322}$	(1,2)
Vitellogenin A2	(*Xenopus*)	$^{-330}$GGTCA -nnn- TGACC $^{-318}$	(1,2)
Vitellogenin B1a	(*Xenopus*)	$^{-314}$AGTTA -nnn- TGACC $^{-302}$	(1,3)
Vitellogenin B1b	(*Xenopus*)	$^{-334}$AGTCA -nnn- TGACC $^{-322}$	(1,3)
Vitellogenin II	(chicken)	$^{-625}$GGTCA -nnn- TGACC $^{-613}$	(1,2)
ApoVLDL II	(chicken)	$^{-166}$GGTCA -nnn- TGACT $^{-154}$	(1)
Prolactin	(rat)	$^{-1581}$TGTCA -nnn- TGTCC $^{-1569}$	(4)
β-LH	(rat)	$^{-1173}$GGACA -nnnnn- TGTCC $^{-1159}$	(5)
pS2	(human)	$^{-405}$GGTCA -nnn- TGGCC $^{-393}$	(6)
Oxytocin	(human)	$^{-164}$GGTGA -nnn- TGACC $^{-152}$	(7)
Uteroglobin	(rabbit)	$^{-264}$GGTCA -nnn- TGCCC $^{-252}$	(8)

C

Consensus ERE $G_{73}G_{100}T_{91}C_{82}A_{100}$-nnn-$T_{100}G_{100}A_{64}C_{100}C_{91}$

Fig 5. (A) Genomic organization of the *myc* family proto-oncogenes. Each of the *myc* family oncogenes *c-*, *N-* and *L-myc* have a 3 exon, 2 intron arrangement. In the figure, the exons are represented as rectangles. Shaded areas of the exons represent untranslated regions. In the *c-myc* mRNA, translation can also commence from a CUG at the end of the first exon (light dots). The arrows indicate transcription initiation sites. The *c-myc* promoters P0, P1 and P2 are indicated. The numbering above represents the amino acids encoded by each translated exon. Lettering below indicates the nucleotide sequence at the intron/exon boundary.

(B) Organization of the *c-myc* protein (*MYC*). Two *c-myc* protein products are possible depending on the translational initiation site used, either CUG or AUG resulting in a 454 or 439 aa protein. Several functional domains are indicated. **Trans/autoreg** is a region important in the cotransformation and negative autoregulatory functions of *MYC*, **ns DNA** is a nonspecific DNA binding region, **NLS** is the nuclear localization sequence, **BR** is the basic DNA binding region and **H-L-H** and **ZIP** are the helix-loop-helix and leucine zipper dimerization motifs respectively. Suspected phosphorylation sites are indicated with a circled P. For more information refer to pages 40-43.

Fig. 5 

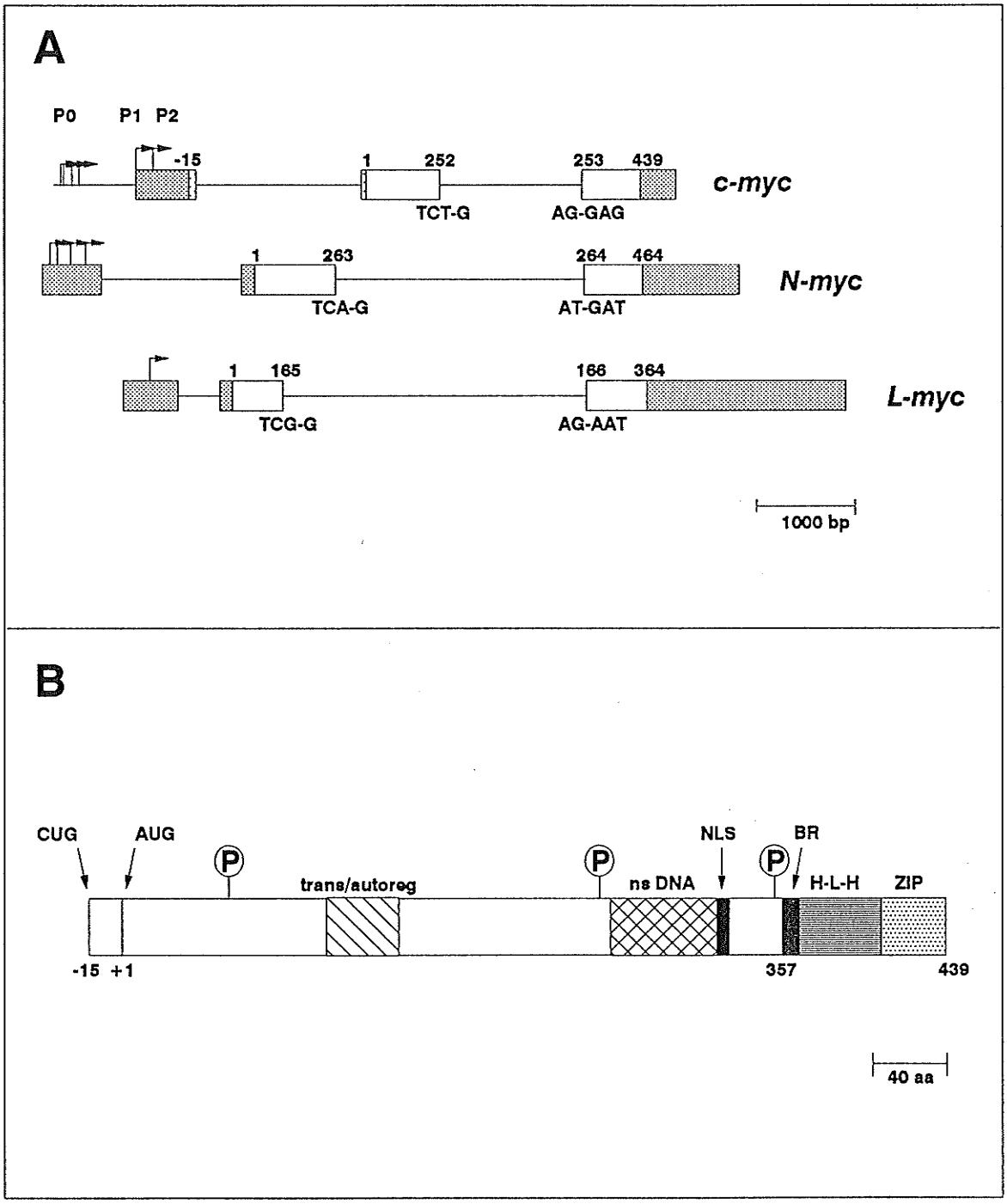
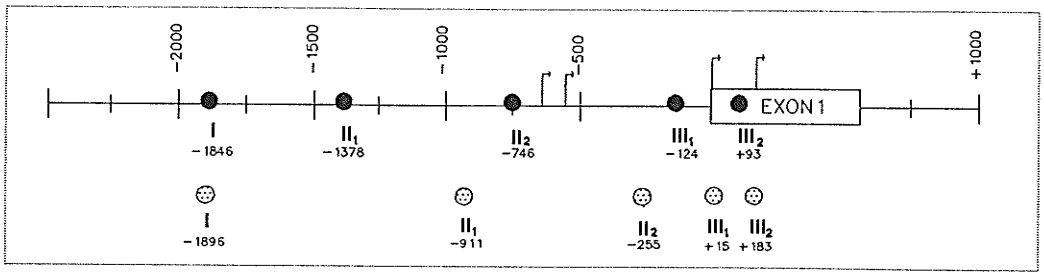

A
P0
P1 P2
-15
1
252
253
439
c-myc
TCT-G
AG-GAG
1
263
264
464
N-myc
TCA-G
AT-GAT
1
165
166
364
L-myc
TCG-G
AG-AAT
1000 bp
B
CUG
AUG
P
trans/autoreg
P
ns DNA
NLS
P
BR
H-L-H
ZIP
-15
+1
357
439
40 aa

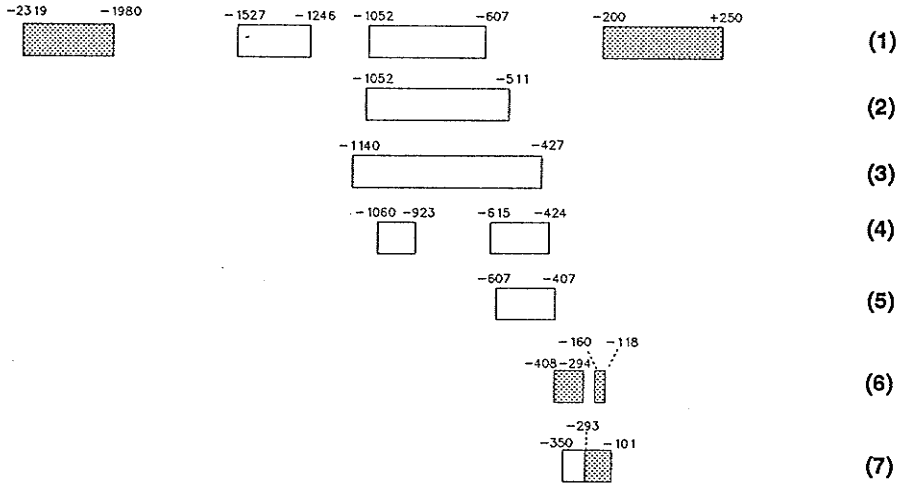
Fig 6. Characterized regulatory regions and transcription factors potentially important in the regulation of the *c-myc* gene. (A) DNaseI hypersensitive sites located in the activated *c-myc* gene. The major *c-myc* hypersensitive sites I, II_1, II_2, III_1, III_2 as described by several groups (Siebenlist et al, 1984; Dyson et al, 1985; Fahrlander et al, 1985; Boles and Hogan, 1987) are indicated as black circles on the schematic representation of the 5' flanking region and exon1 of the *c-myc* gene. These hypersensitive sites are approximately 50 bp in length and are centered about the nucleotide indicated by the numbers below each circle. All numbering is relative to P1. The lighter circles below are hypersensitive sites in a mouse cell line P388D1 as described by Mango et al, 1989. **(B) Positive and negative response regions identified in the *c-myc* gene.** The shaded boxes represent enhancer regions, the white boxes, dehancer regions. Due to the complex nature of the *c-myc* regulatory region and variation in experimental design, there are some inconsistencies. The span of each region is indicated. **(C) Characterized *trans*-acting factors that interact with the *c-myc* gene.** In most studies, the human *c-myc* gene was analyzed but in some studies the mouse or chicken *c-myc* gene was used; these genes are indicated as {M} or {C} respectively. Incompletely characterized response elements were found to bind known *trans*-acting factors, which are indicated in square brackets [ ]; high or low refers to the binding affinity of Sp1. References, given as numbers in brackets (#), correspond with the following: (1) Lang et al, 1988, (2) Lipp et al, 1987, (3) Remmers et al, 1986, (4) Marcu et al, 1988, (5) Chung et al, 1986, (6) Postel et al, 1989, (7) Hay et al, 1987, (8) Asselin et al, 1987, (9) Kadonaga et al, 1986, (10) Imagawa et al, 1987, (11) Davis et al, 1989, (12) Siebenlist et al, 1984, (13) Takimoto et al, 1989, (14) Riggs et al, 1991, (15) Kakkis and Calame, 1987, (16) Lobanenkov et al, 1990, (17) Duyao et al, 1990, (18) Hiebert et al, 1989, (19) Zajac-Kaye and Levens, 1990, (20) Zhang et al, 1990. Abbreviations can be found in the LIST OF ABBREVIATIONS at the beginning of the thesis. $\mathbf{RNP}_{\text{proteins}}$ are proteins factors identified along with the RNP.

Fig. 6 ⟹

A



B

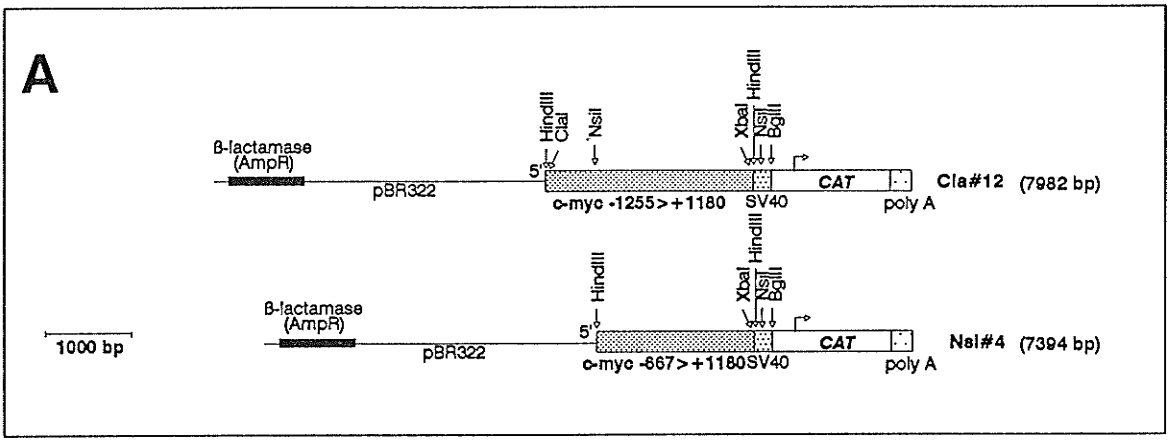


C

Factor	Binding Site	Refs.	Factor	Binding Site	Refs.
5'Mf1	-264 > -248	{M}(8,16)	**AP-2** (multiple sites)	-142 > -115	(6,10,11)
	-242 > -226		**RNP**	-150 > -117	(11)
5'Mg1 [$Sp1_{high}$]	-188 > -157	{M}(8,9)	**RNP** proteins	-143 > -121	
5'Mg2 [$Sp1_{low}$]	-129 > -107	{M}(8,9)	**NF-1**	-1339 > -1325	(12)
	-110 > -88			-705 > -692	
5'Mg3 [AP-2,	-53 > -25	{M}(8-10)	**AP-1**	-337 > -329	(13)
$Sp1_{high}$]	-53 > -25			-329 > -322	
ME1a2 [$Sp1_{low}$]	+58 > +78	{M}(8,9)	**Oct-1**	-335 > -326	(13)
	+62 > +82		**CF1**	around -260	{M}(14)
ME1a1 [$Sp1_{low}$]	+97 > +118	{M}(8,9)	**PRF**	around -290	{M}(15)
	+101 > +122		**CTCF**	-230 > -180	{C} (16)
PuF	-137 > -131	(6)	**NF-κB**	-1101 > -1081	(17)
	-128 > -122		**E2F**	+119 > +129	(18)
MDBP	intron 1	(19,20)		+98 > +105	

Fig. 7. Construct used to determine the ERE containing region of the *c-myc* gene. (A) Principle MYCCAT constructs Cla#12 and Nsi#4. These two constructs were the precursors for all the MYCCAT constructs. In Cla#12 the human genomic *c-myc* fragment from bp -1287 to +1180 was inserted into the *HindIII* site in front of the SV40 early promoter of pSVCAT while in Nsi#4 the genomic *c-myc* fragment from bp -667 to +1180 was inserted into the same site. The *c-myc* sequences are indicated as a dark shaded box. Also shown is the pSVCAT vector. Although drawn as linear molecules, all constructs are circular plasmids and are further described in MATERIALS AND METHODS. **(B) MYCCAT constructs.** At the top of the diagram is a schematic representation of the genomic *c-myc*. The first exon is shaded and the location of the promoters P0, P1 and P2 are indicated. Only the *c-myc* sequences are shown: pSVCAT sequences are omitted. *Note also that only Cla#12 and Nsi#4 contain the SV40 early promoter*. Along the right hand column are the common names of the constructs. These names are listed for reference purposes only and in the thesis MYCCAT names will be used (eg. ClaXho will be MYCCAT[-1255→+67] while Cla#12(-) will be MYCCAT[-1255→+937]). See MATERIALS AND METHODS for further information on these constructs.

Fig. 7 ⟹


A
β-lactamase (AmpR)
pBR322
HindIII
ClaI
NsiI
XbaI
NsiI
BglII
HindIII
5'
c-myc -1255>+1180
SV40
CAT
poly A
Cla#12 (7982 bp)
HindIII
XbaI
NsiI
BglII
HindIII
β-lactamase (AmpR)
pBR322
5'
c-myc -667>+1180
SV40
CAT
poly A
Nsi#4 (7394 bp)
1000 bp

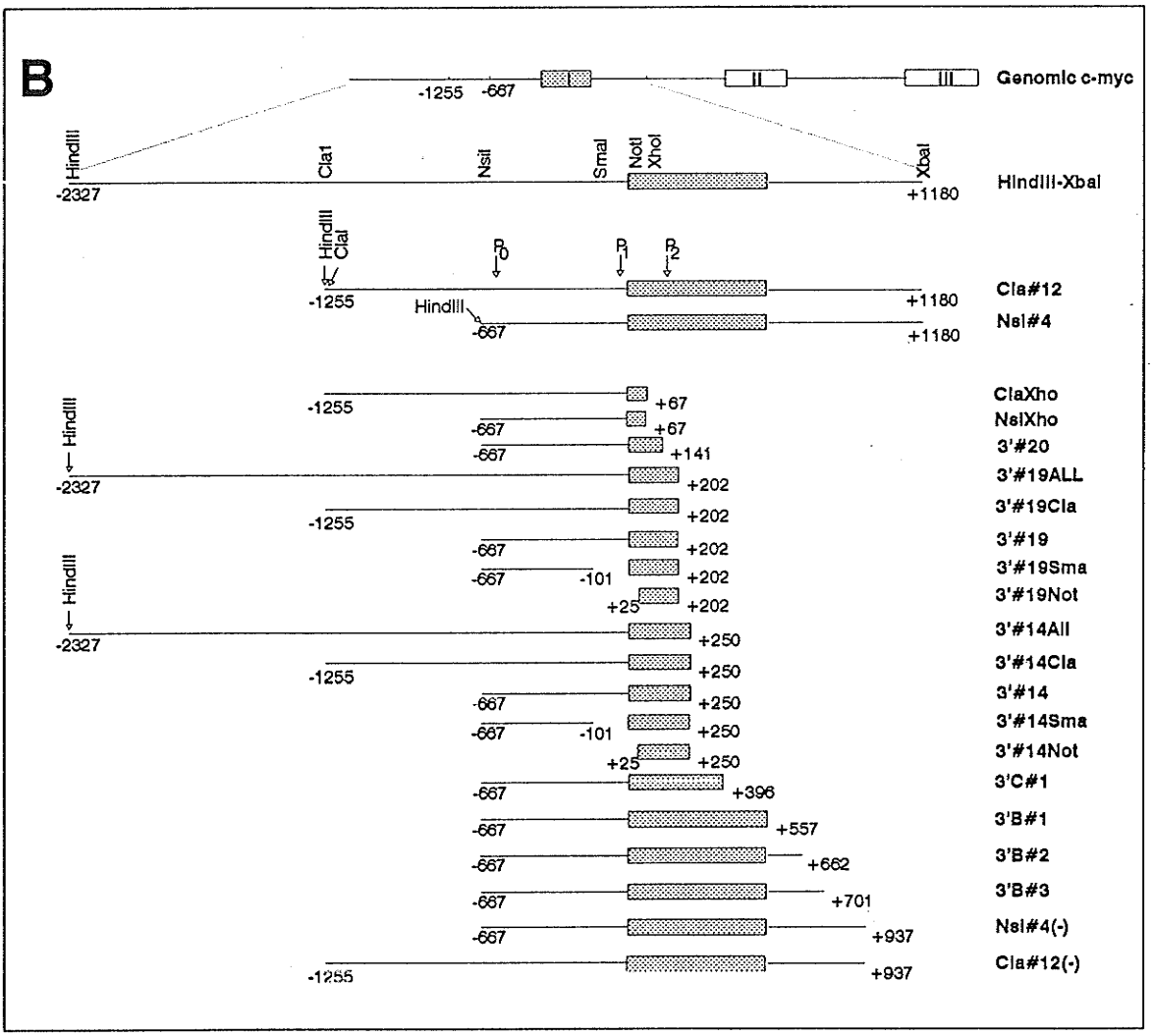

B
Genomic c-myc
-1255
-667
I
II
III
HindIII
ClaI
NsiI
SmaI
NotI
XhoI
XbaI
-2327
+1180
HindIII-XbaI
HindIII
ClaI
P0
P1
P2
-1255
+1180
Cla#12
HindIII
-667
+1180
NsiI#4
-1255
+67
ClaXho
-667
+67
NsiXho
-667
+141
3'#20
HindIII
-2327
+202
3'#19ALL
-1255
+202
3'#19Cla
-667
+202
3'#19
-667
-101
+202
3'#19Sma
HindIII
+25
+202
3'#19Not
-2327
+250
3'#14All
-1255
+250
3'#14Cla
-667
+250
3'#14
-667
-101
+250
3'#14Sma
+25
+250
3'#14Not
-667
+396
3'C#1
-667
+557
3'B#1
-667
+662
3'B#2
-667
+701
3'B#3
-667
+937
NsiI#4(-)
-1255
+937
Cla#12(-)

Fig. 8 (A) Receptors produced by the estrogen receptor expression vectors HEO, HE4, HE11, HE19, HE21. hER is a diagrammatic representation of the structural regions of the estrogen receptor. The numbering represents amino acids at the junction of each region. The protein products produced by the various estrogen receptors are indicated below. The C and E domains are represented by dark and lighter shaded boxes respectively. Amino acids at the junction of deleted regions in HE4, HE11, HE19 and HE21 are shown. The "*" represents an artifactual cloning mutation introduced into the vectors such that the amino acid Val appears instead of Gly at position 400 (Tora et al, 1989b). **(B) Localization of the estrogen receptor functional domains.** For addition information see pages 15-27.

Fig. 8 ⟹

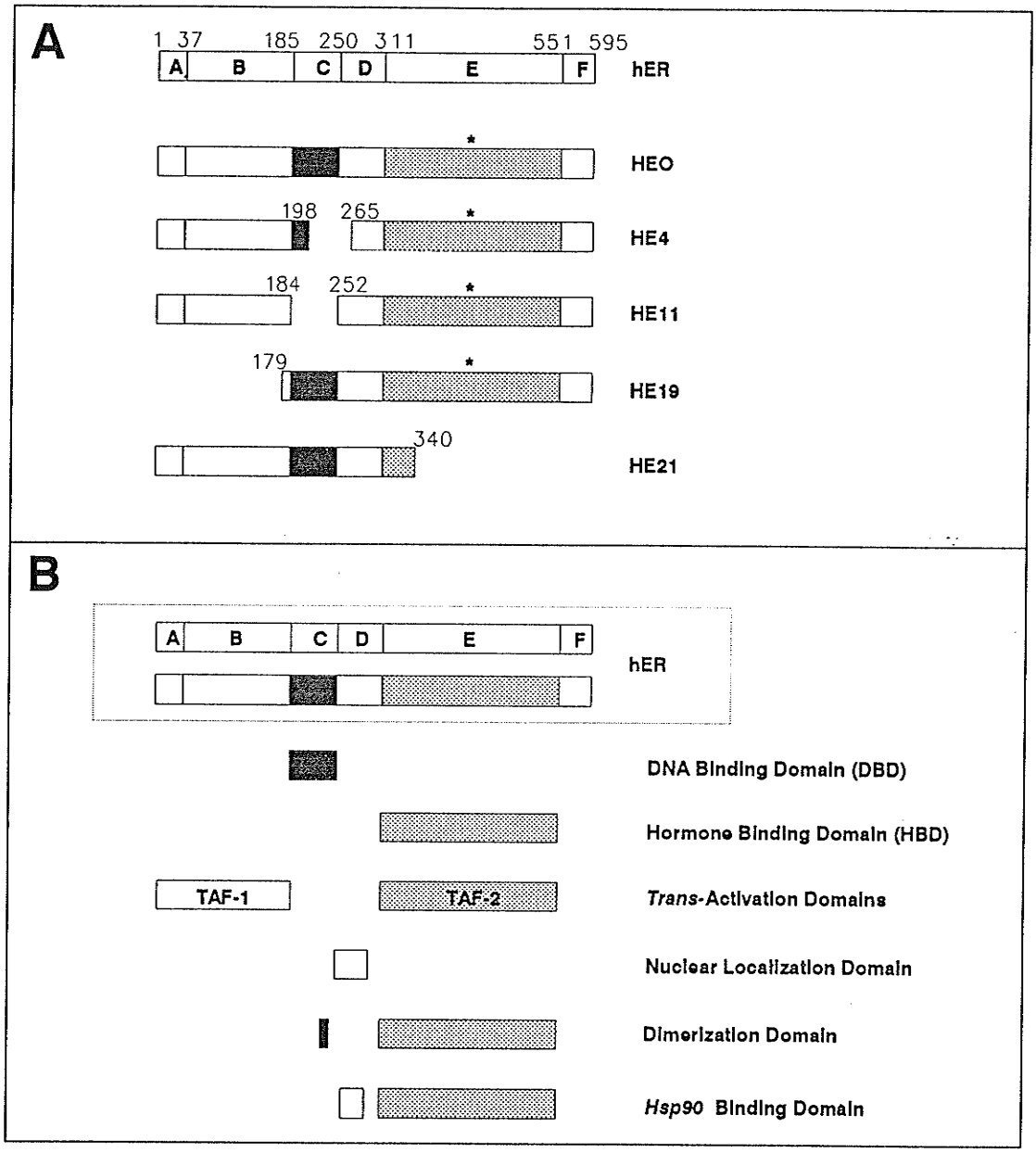

A
1 37
185 250 311
551 595
A
B
C
D
E
F
hER
HEO
198 265
HE4
184 252
HE11
179
HE19
340
HE21
B
hER
DNA Binding Domain (DBD)
Hormone Binding Domain (HBD)
TAF-1
TAF-2
Trans-Activation Domains
Nuclear Localization Domain
Dimerization Domain
Hsp90 Binding Domain

Fig. 9 Constructs used in determining the ERE containing region in pSVCAT. The expanded diagram at the top represents the *HindIII-BglII* SV40 early promoter cassette. The first 90 bp from *HindIII* to *NsiI* are pBR322 sequences. The remaining 209 bp are SV40 promoter sequences representing the $SphI_{(132)}$ to $HindIII_{(5171)}$ SV40 viral DNA fragment. A *BglII* linker (>*BglII*) has been inserted into the $HindIII_{(5171)}$ site. Similarly a *HindIII* linker (>*HindIII*) was inserted into the $SalI_{(651)}$ site of pBR322. The open box represents 26 bp of the enhancer encompassing the P-region. All constructs are circular plasmids and are further described in MATERIALS AND METHODS. [Note: pESVCAT differs from pESVCAT[1→209] by the 90 bp *HindIII-NsiI* pBR322 fragment]

Fig. 9 ⟹

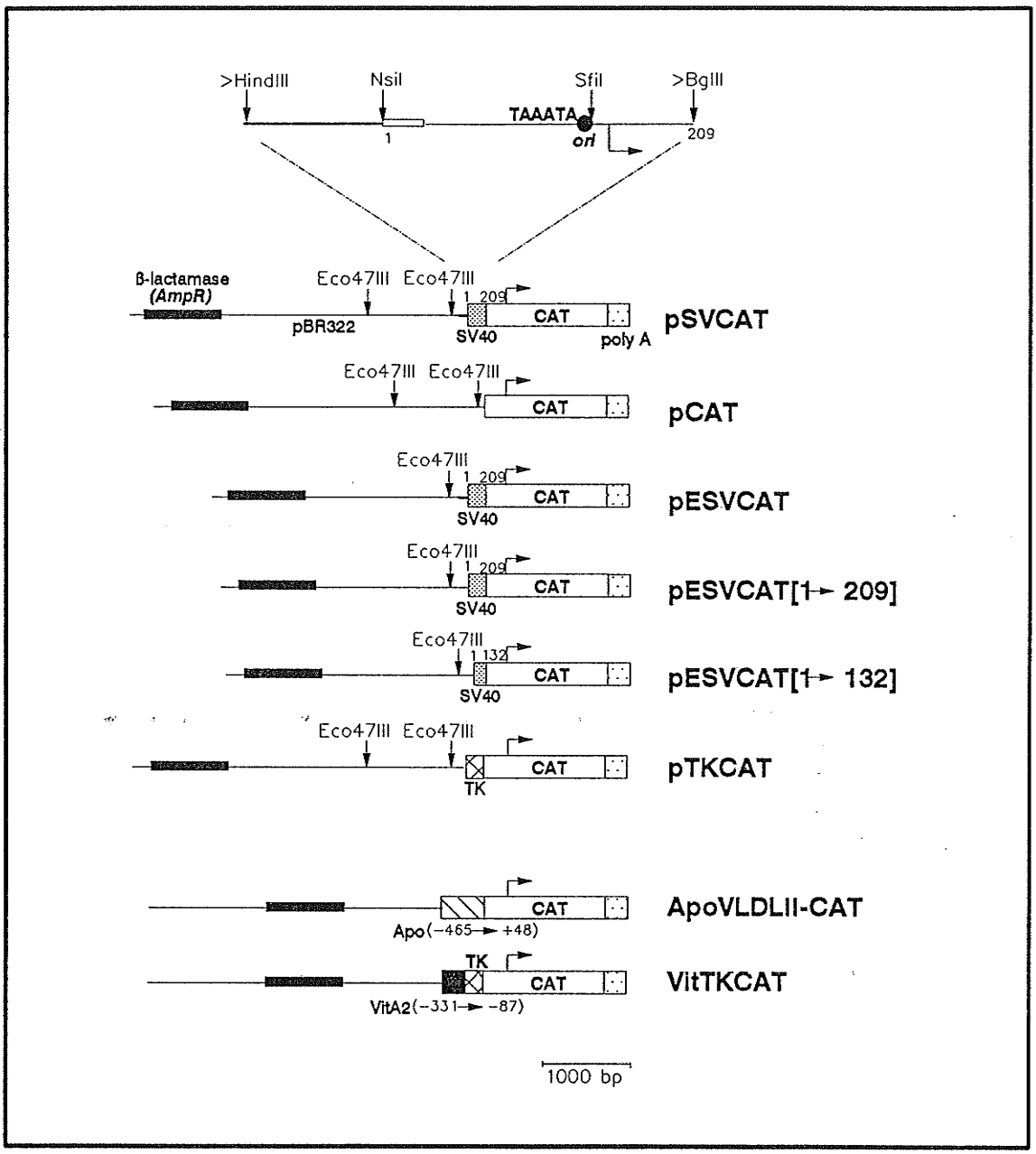

>HindIII
NsiI
SfiI
>BglII
TAAATA
1
ori
209
ß-lactamase
(AmpR)
Eco47III Eco47III
1 209
pBR322
CAT
SV40
poly A
pSVCAT
pCAT
pESVCAT
pESVCAT[1→ 209]
1 132
pESVCAT[1→ 132]
TK
pTKCAT
ApoVLDLII-CAT
Apo(−465→ +48)
VitTKCAT
VitA2(−331→ −87)
1000 bp

Fig. 10 Growth response of estrogen-receptor-positive (ER+) and ER- human breast cancer cells to tamoxifen and estradiol. ER+ MCF-7 and T-47D cells and ER- MDA-MB-231 were cultured and hormone treated as described in MATERIALS AND METHODS. Conditions E' and TE', are cells grown in C' and T' medium for 48 h, then treated with estradiol (10^{-7}M). The delayed addition of estradiol accounts for the apparent incomplete rescue of cell growth when compared to cells grown continuously in medium containing untreated FBS (C). Cell number was determined 10 days after estradiol addition and all dishes were subconfluent. Results are mean values with SD (*bars*) of three experiments normalized to cell growth in medium C.

Fig. 10 

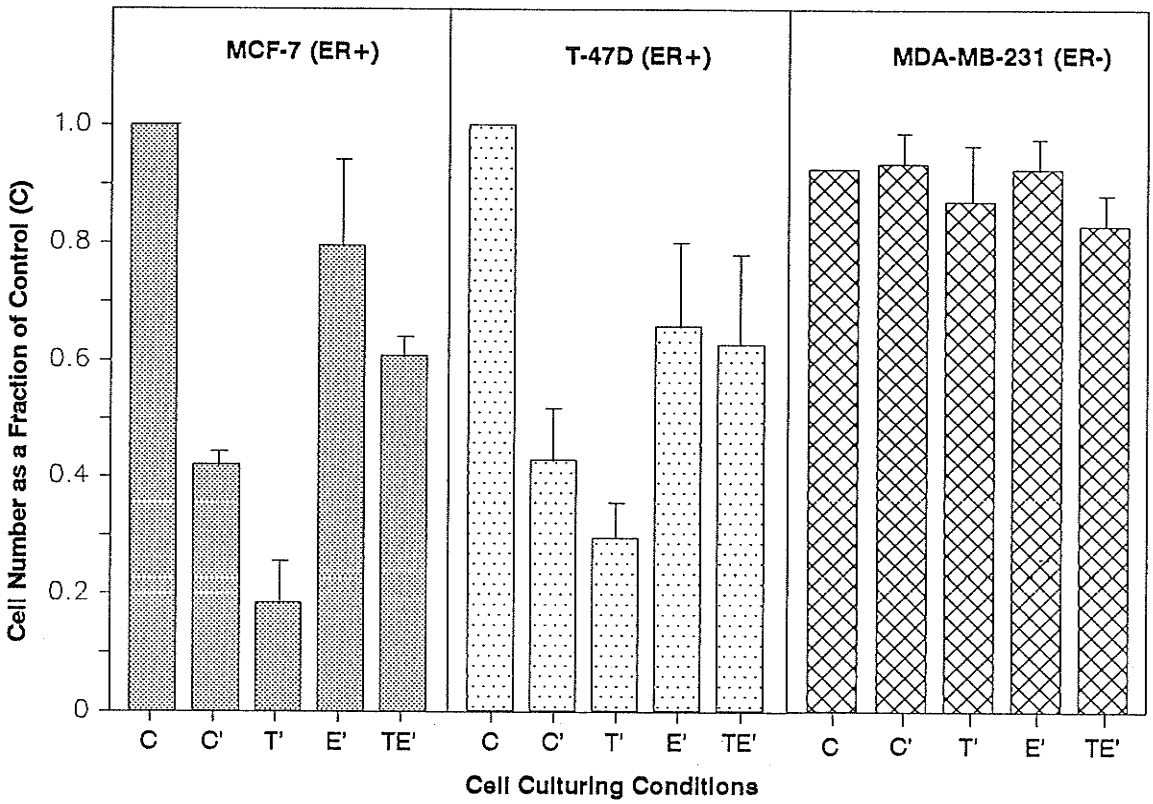

MCF-7 (ER+)
T-47D (ER+)
MDA-MB-231 (ER-)
Cell Number as a Fraction of Control (C)
1.0
0.8
0.6
0.4
0.2
0
C
C'
T'
E'
TE'
C
C'
T'
E'
TE'
C
C'
T'
E'
TE'
Cell Culturing Conditions

Fig. 11 Effects of estradiol on the accumulation of *c-myc* and *c-H-ras* mRNA in MCF-7 cells. Tamoxifen-inhibited cells were exposed to estradiol (10^{-7}M) for 0 to 12 hours. At the times indicated cells were harvested and total RNA isolated for Northern analysis. Cells in medium "C" were harvested at time 0 h. For each sample, 50 μg of RNA was analyzed. **Top panel:** [**RNA**] Ethidium bromide staining of total RNA . [***c-myc***] Hybridization of nitrocellulose blot to *c-myc* insert probe (3 X 10^6 cpm / ml; 1 day film exposure). [***c-H-ras***] Reprobing with *v-H-ras* insert probe (3 X 10^6 cpm / ml; 5 day film exposure). **Bottom panel:** Quantitation of relative amounts of *c-myc* and *c-H-ras*, normalized to levels present in tamoxifen-inhibited cells, prior to estradiol addition (t=0). The straight horizontal line represents *c-myc* expression in cells growing in FBS-containing medium at time 0.

Fig. 11 

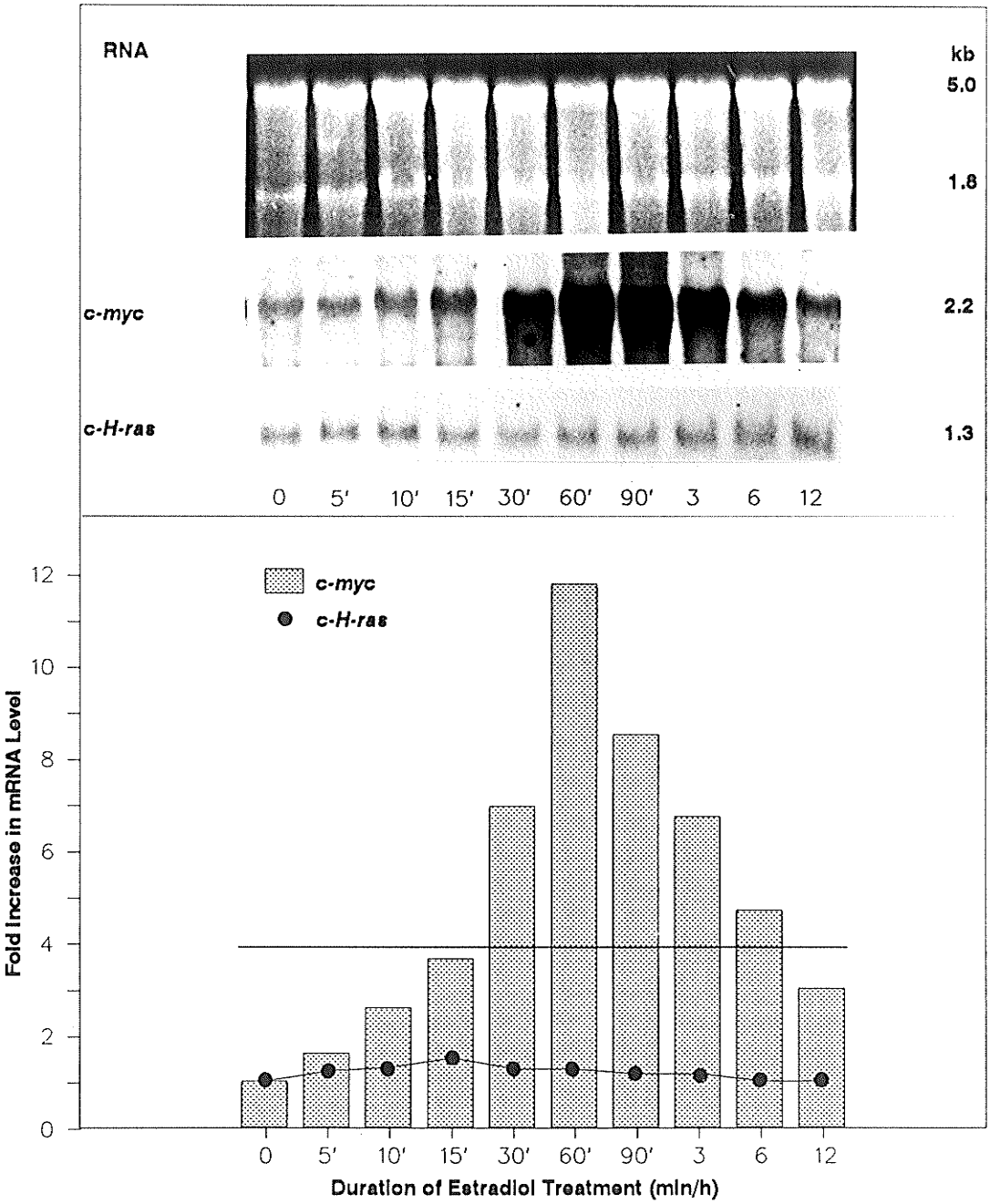
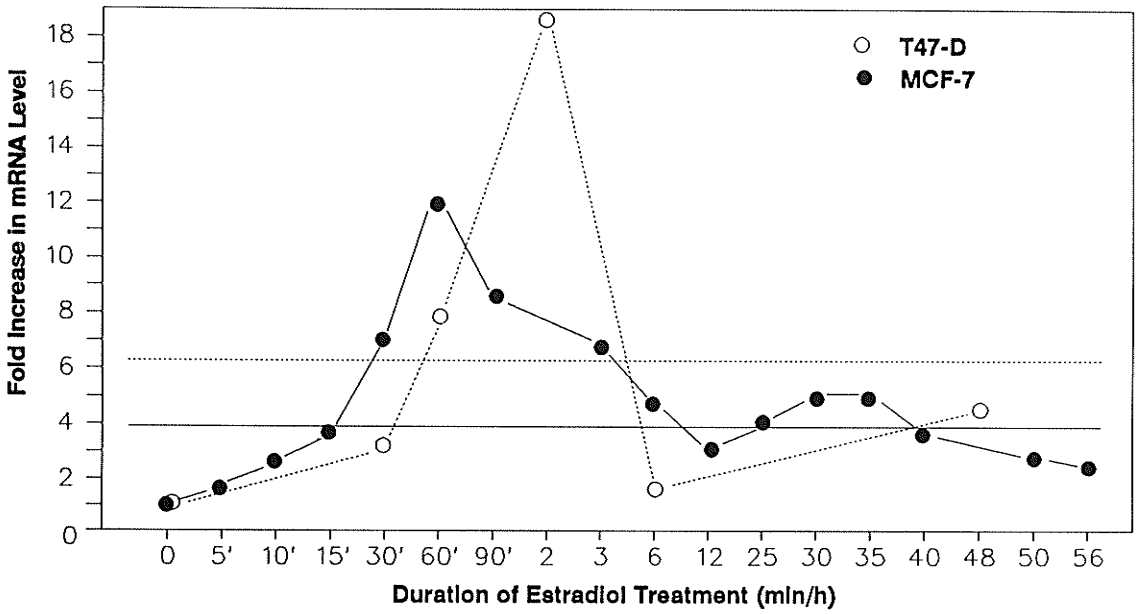

RNA
kb
5.0
1.8
c-myc
2.2
c-H-ras
1.3
0 5' 10' 15' 30' 60' 90' 3 6 12
c-myc
c-H-ras
Fold Increase in mRNA Level
0 2 4 6 8 10 12
0 5' 10' 15' 30' 60' 90' 3 6 12
Duration of Estradiol Treatment (min/h)

Fig. 12 Time course of the effect of estradiol on the accumulation of *c-myc* mRNA in ER+ MCF-7 and T-47D cells. Results are normalized in relation to tamoxifen growth-arrested cells prior to addition of estradiol (t=0). Horizontal lines (----) and (____) represent *c-myc* levels in T-47D and MCF-7 cells grown in FBS-containing medium [C] at time 0.

Fig. 13 Time course of *c-myc* protein expression in estradiol treated MCF-7 cells. This Western blot analysis is presented with permission of Dr. Peter H. Watson. Materials and methods are described elsewhere (Watson et al, 1991).

Fig. 12 ⟹

Fig. 13 ⟹


Fold Increase in mRNA Level
18
16
14
12
10
8
6
4
2
0
T47-D
MCF-7
0
5'
10'
15'
30'
60'
90'
2
3
6
12
25
30
35
40
48
50
56
Duration of Estradiol Treatment (min/h)

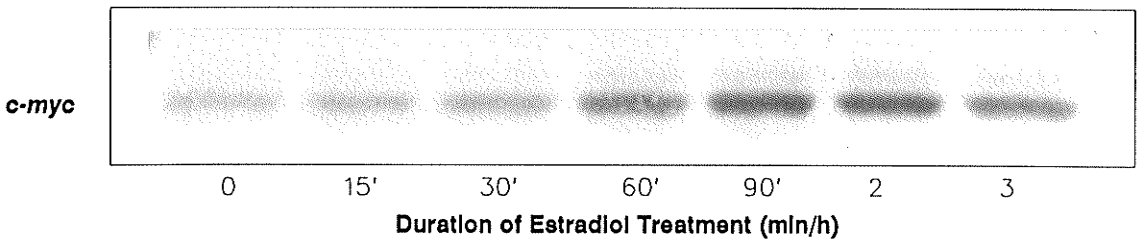

c-myc
0
15'
30'
60'
90'
2
3
Duration of Estradiol Treatment (min/h)

Fig. 14 Comparison of *c-myc* and *c-H-ras* expression in ER+ MCF-7 and ER- MDA-MB-231 cells. Total RNA (50 μg) was applied to each lane. Conditions C, C', T', E', and TE' are as described in the legend to Fig. 10. Exposure to estradiol (10^{-7}M) was 1 hour. The blot was hybridized to *c-myc* and *v-H-ras* probes and exposed to film as described in the legend to Fig. 10. Also shown is the quantitation of the relative amounts of *c-myc*, normalized to levels present in tamoxifen-inhibited MCF-7 cells (T'*).

Fig. 14 

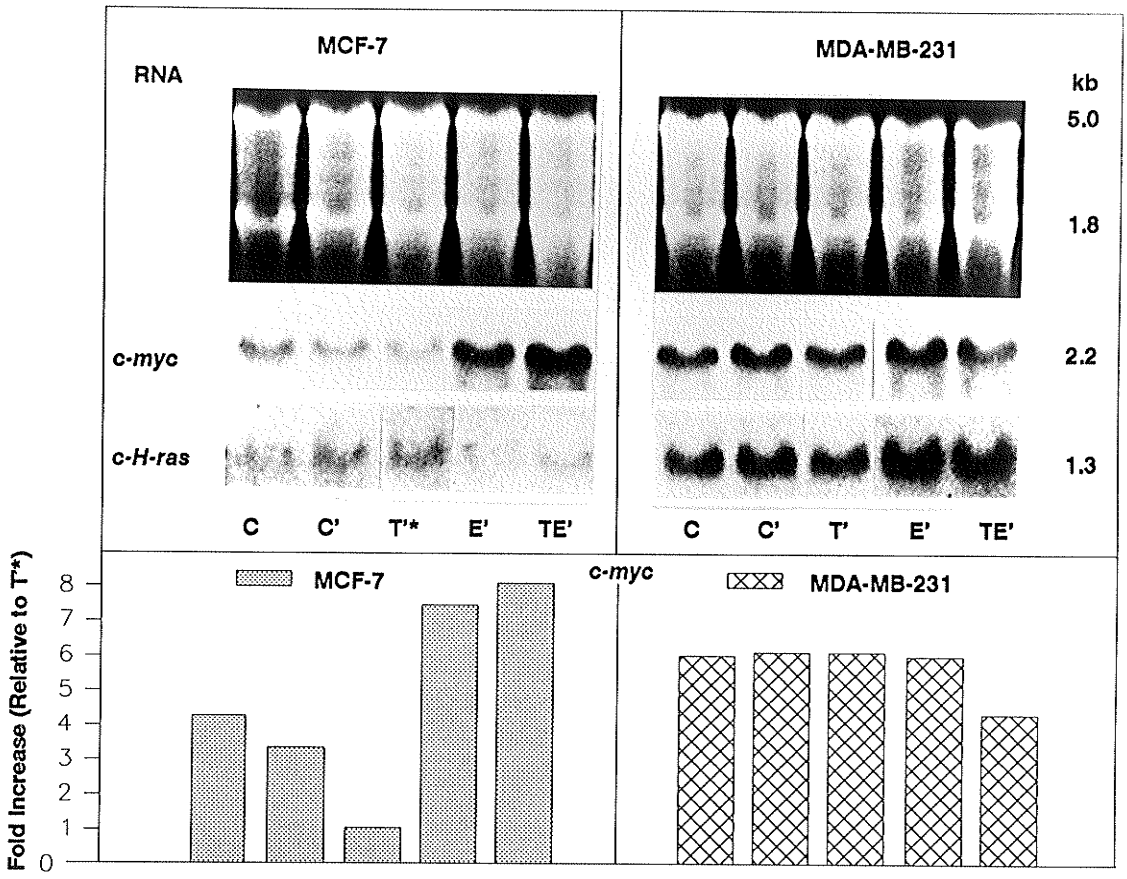

MCF-7
MDA-MB-231
RNA
kb
5.0
1.8
c-myc
2.2
c-H-ras
1.3
C
C'
T'*
E'
TE'
C
C'
T'
E'
TE'
c-myc
MCF-7
MDA-MB-231
Fold Increase (Relative to T*)
0
1
2
3
4
5
6
7
8

Fig. 15 Dot blot analysis of regulation of *c-myc* mRNA expression in human breast cell lines. Cells were grown and RNA isolated as described in MATERIALS AND METHODS. Growth conditions and estradiol treatment were identical to those in Fig. 14. The blot was hybridized to *c-myc* insert probe (1 X 10^6 cpm / ml; film exposure was 14 h). For clarity, hybridizations to only one concentration of RNA (0.625 μg) are shown.

Fig. 15 

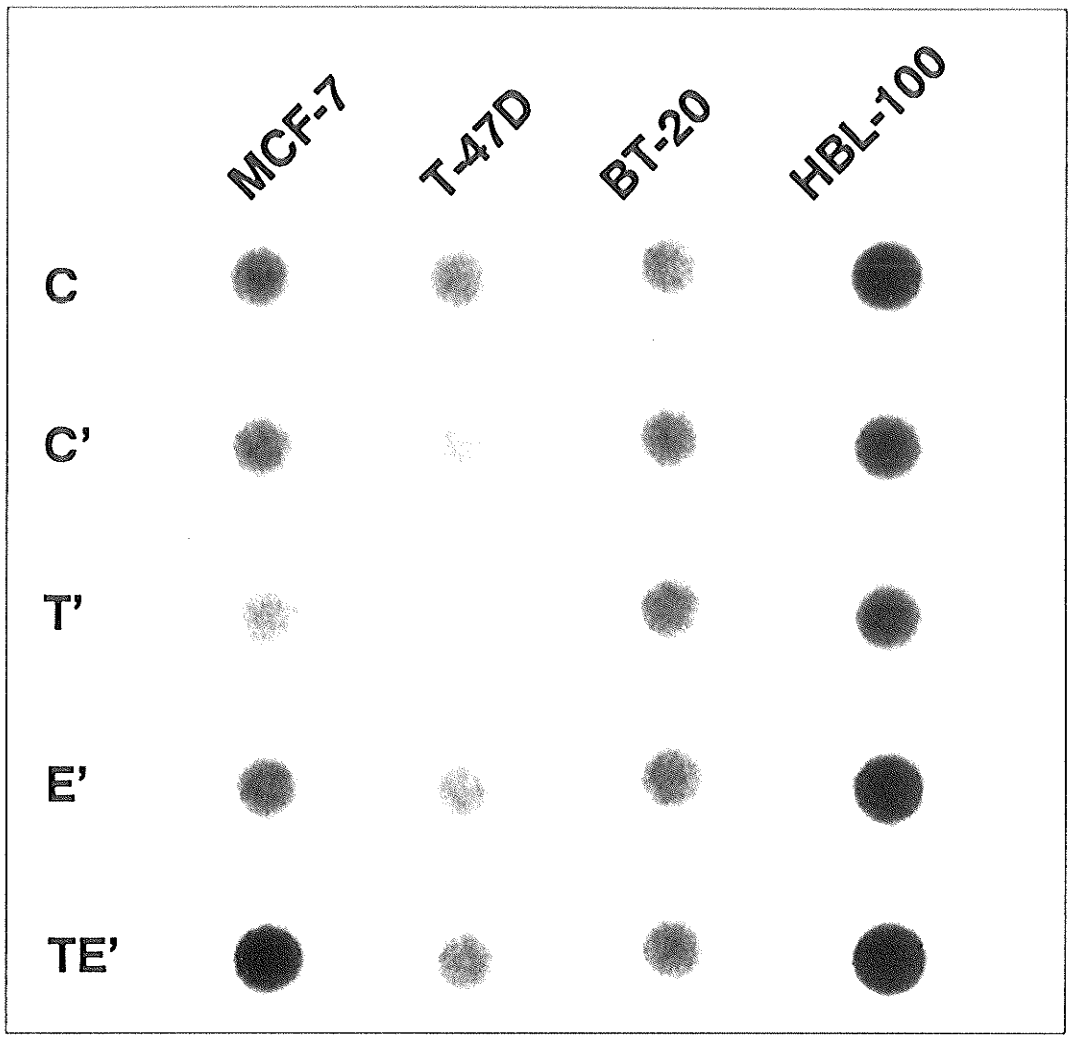

MCF-7
T-47D
BT-20
HBL-100
C
C'
T'
E'
TE'

Fig. 16 Effects of steroid hormones on *c-myc* expression in MCF-7 HBC cells. MCF-7 cells were cultured for 48 h in hormone depleted medium without phenol red as described in MATERIALS AND METHODS. Individual dishes were then treated for 90 min with either no hormone [-], retinoic acid [RA], estradiol [E], dexamethasone [DEX], dihydrotestosterone [DHT] or medroxyprogesterone acetate [MPA] at a physiological concentration of 2 X 10^{-9}M. Total RNA was isolated and Northern analysis of *c-myc* expression was done. **Top panel:** Northern analysis with *c-myc* insert probe. **Bottom panel:** Quantitation of relative amounts of *c-myc* mRNA relative to the level in hormone untreated cells [-].

Fig. 16 

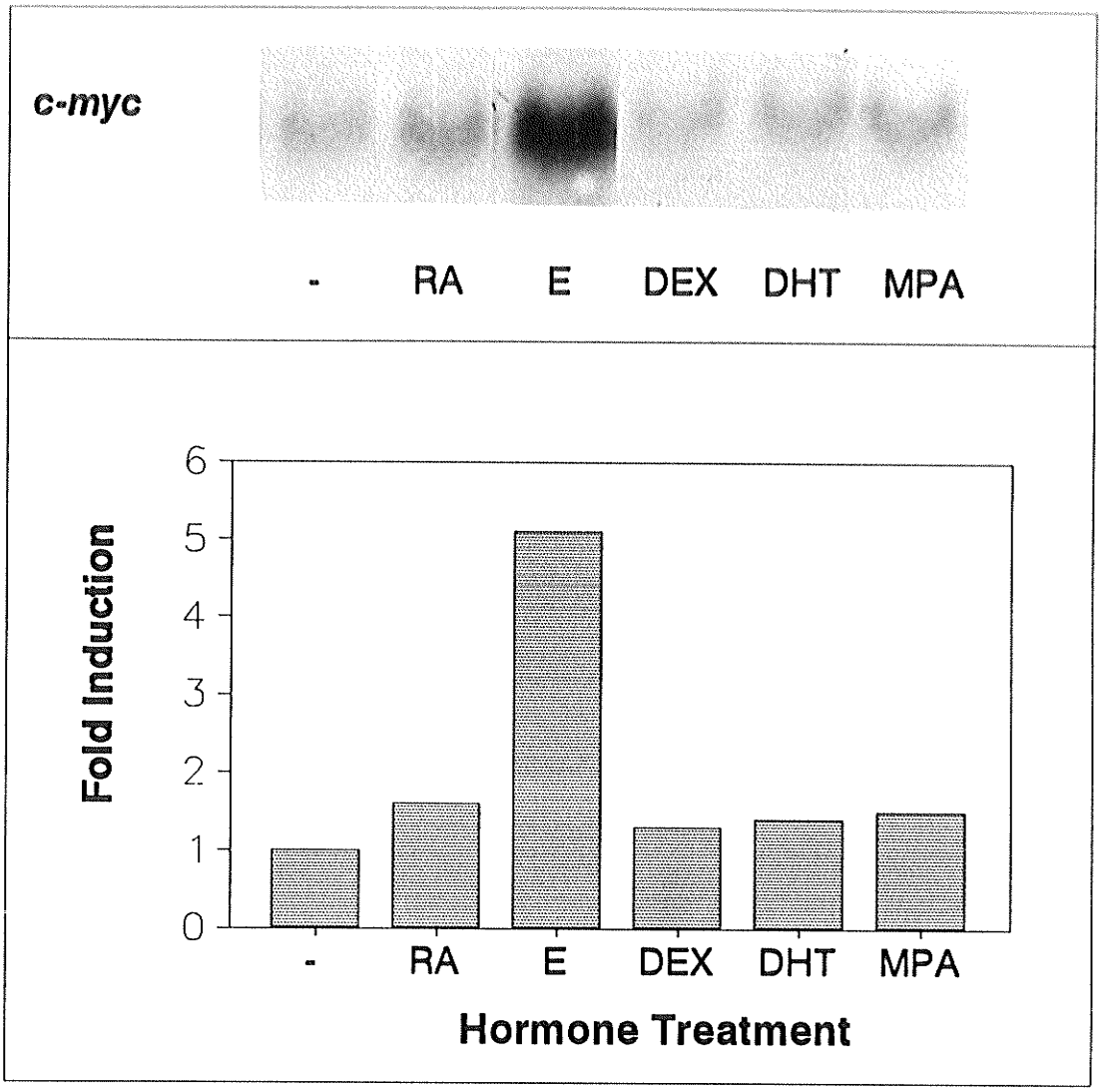
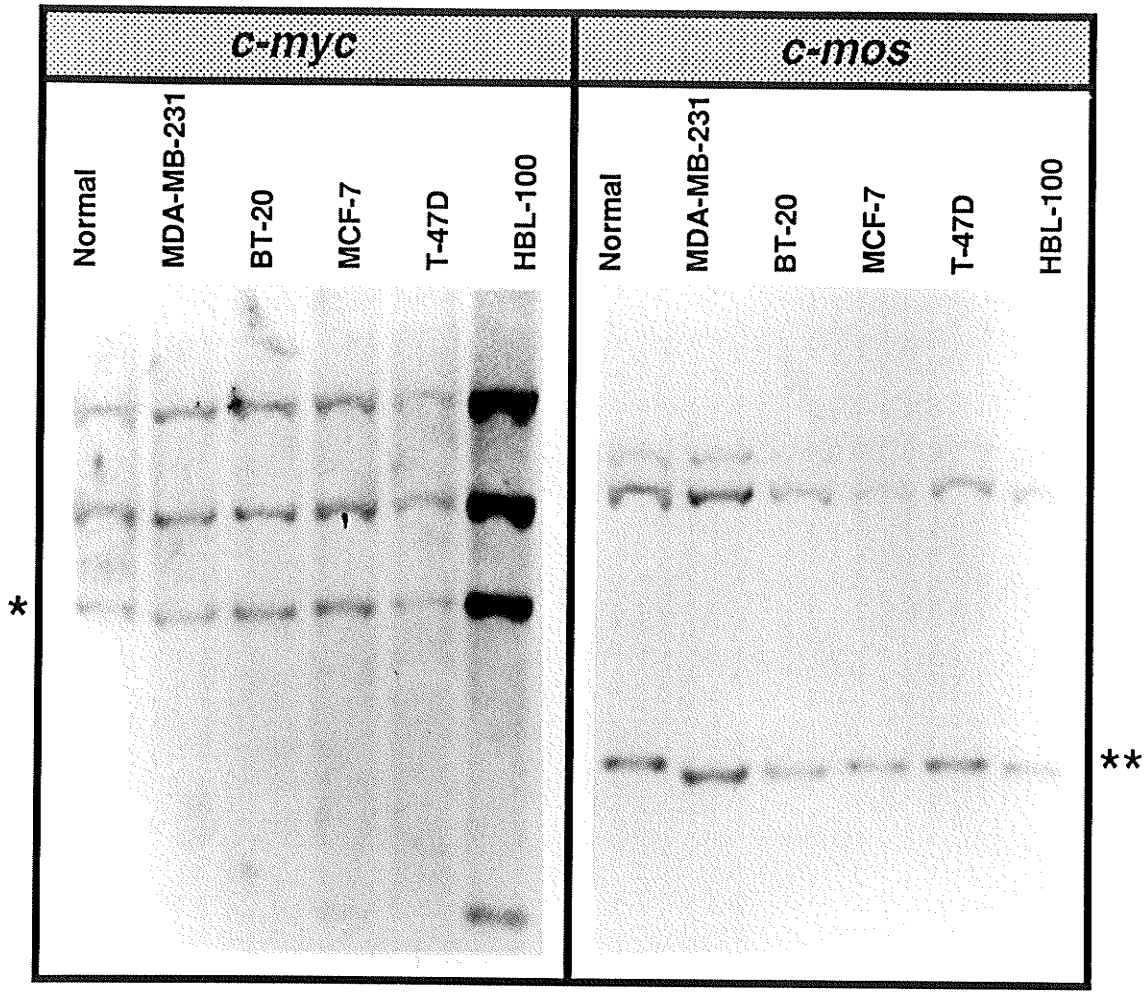

c-myc
-
RA
E
DEX
DHT
MPA
Fold Induction
0
1
2
3
4
5
6
-
RA
E
DEX
DHT
MPA
Hormone Treatment

Fig. 17 Analysis of amplification/rearrangement of the *c-myc* gene in a panel of breast cell lines. (A) Genomic Southern analysis of *c-myc* and *c-mos*. A Southern blot containing 10 μg of *Pst1* cut DNA from several breast cell lines was probed with a genomic *c-myc* probe (~3 X 10^6 cpm / ml; film exposure 24 h). The same blot was then stripped and reprobed with a *c-mos* cDNA probe (~3 X 10^6 cpm / ml; film exposure 24 h). The copy number of the *c-myc* gene was determined relative to that present in normal lymphocyte DNA [**Normal**], using densitometric scanning of one of the *c-myc* bands (*). DNA loading was standardized with relation to one of the *c-mos* bands (**). **(B) The *c-myc* gene copy number in various breast cell lines.** The mean *c-myc* gene copy numbers derived from several (indicated by n=) Southern or quantitative PCR analyses are tabulated together with their standard deviations. All *c-myc* copy numbers are relative to the level in the genome of normal lymphocytes and are standardized relative to the *c-mos* gene.

Fig. 17 ⟹

A

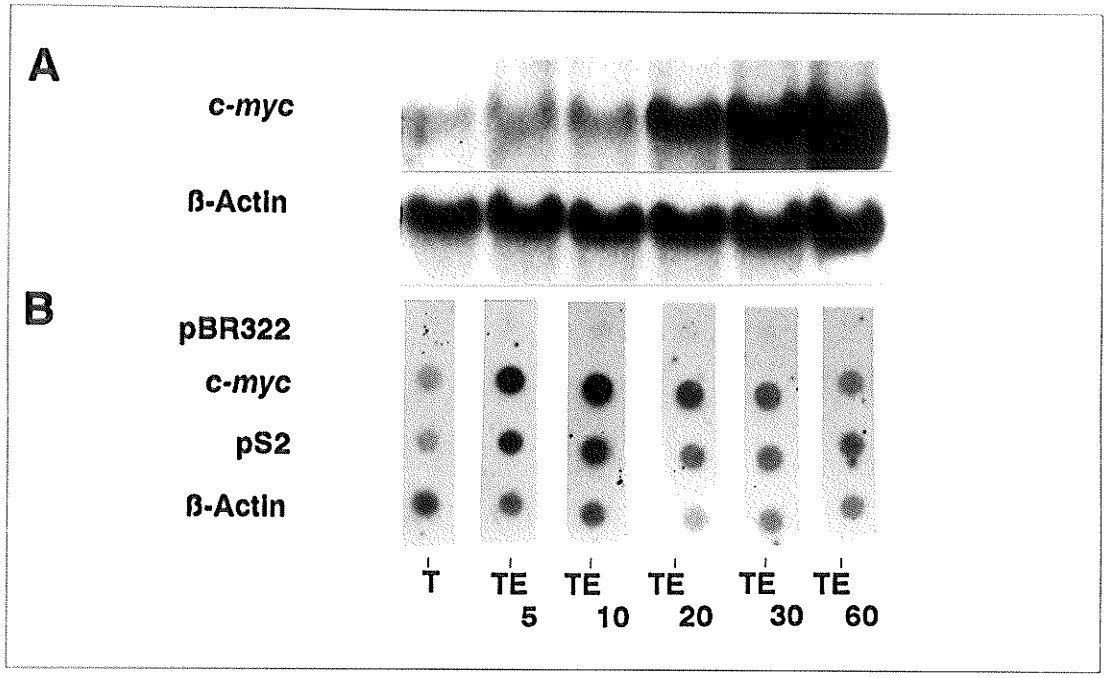


B

c-myc Copy Number Based on Southern Analysis			*c-myc* Copy Number Based on Quantitative PCR Analysis		
MDA-MB-231	1.1±0.3	n=3	MDA-MB-231	1.4±0.5	n=6
BT-20	2.4±0.8	n=3	BT-20	2.6±0.9	n=4
MCF-7	1.7±0.1	n=4	MCF-7	2.0±0.5	n=6
T-47D	0.8±0.4	n=4	T-47D	1.1±0.1	n=4
HBL-100	5.1±2.0	n=4	HBL-100	5.0±1.5	n=6

Fig. 18 Transcription of the *c-myc* gene in estradiol treated MCF-7 cells. Cells arrested by tamoxifen [T] were rescued by the addition of estradiol-17β (10^{-7}M). At various times (min) after estradiol addition [TE], cells were harvested for RNA and nuclei as described in "MATERIALS AND METHODS". **(A) Northern analysis of *c-myc* and β-actin mRNA.** Approximately 3 X 10^6 cpm/ml of *c-myc* [second exon, *Pst1* fragment] (Saito et al, 1983) and chicken β-actin (Cleveland et al, 1980) probes were used. **(B) Results of an *in vitro* transcription assay.** Northern analysis and *in vitro* transcription data for *c-myc* from two separate experiments were quantitated by densitometry and presented in **Fig. 18C**. Due to slight variations of hybridization among the nitrocellulose strips, the β-actin signals were assigned a value of unity, and the hybridization signals for the other genes were normalized with respect to actin.

Fig. 18 ⟹


A
c-myc
ß-Actin
B
pBR322
c-myc
pS2
ß-Actin
T
TE 5
TE 10
TE 20
TE 30
TE 60

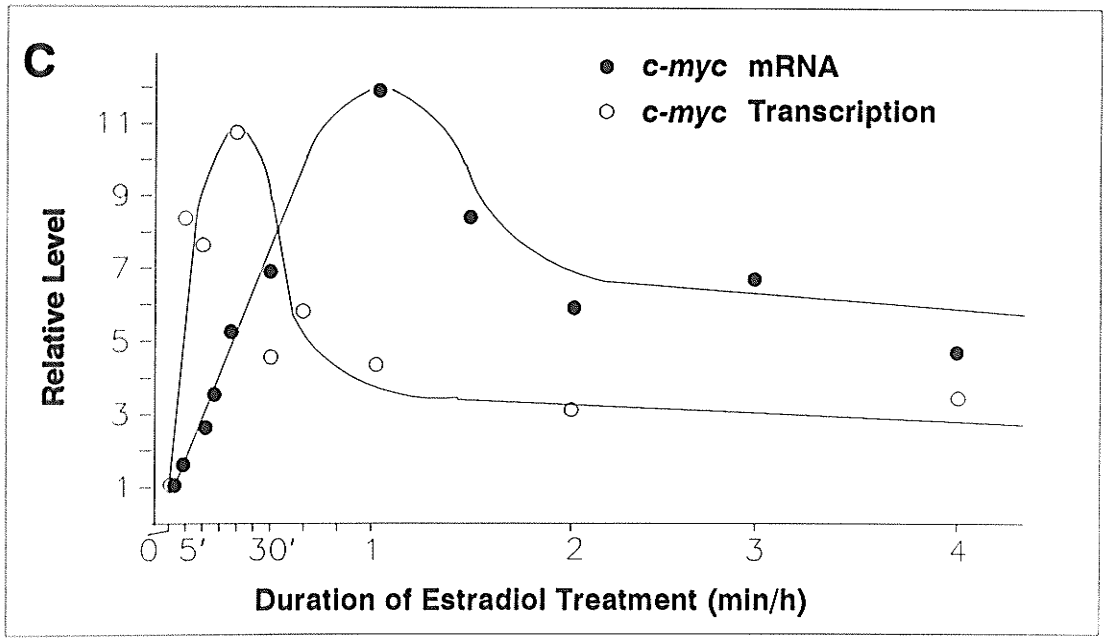

C
c-myc mRNA
c-myc Transcription
Relative Level
1
3
5
7
9
11
0
5'
30'
1
2
3
4
Duration of Estradiol Treatment (min/h)

Fig. 19 Effects of the translational inhibitor cycloheximide on *c-myc* mRNA accumulation and transcription of MCF-7 cells. Tamoxifen [T]-treated cells were exposed to 50 μM cycloheximide (CHX) for 2 h prior to the addition of estradiol (10^{-7}M). Twenty minutes after estradiol addition, RNA and nuclei were isolated. **(A) Northern analysis of *c-myc* and actin mRNA. (B) In vitro transcription assay** (8 X 10^6cpm/ml) of labeled RNA used for each hybridization. **(C) Quantitation by densitometric scanning of *c-myc* mRNA accumulation and transcription;** the quantitation of transcriptional signals for *c-myc* were normalized with respect to actin.

Fig. 19 

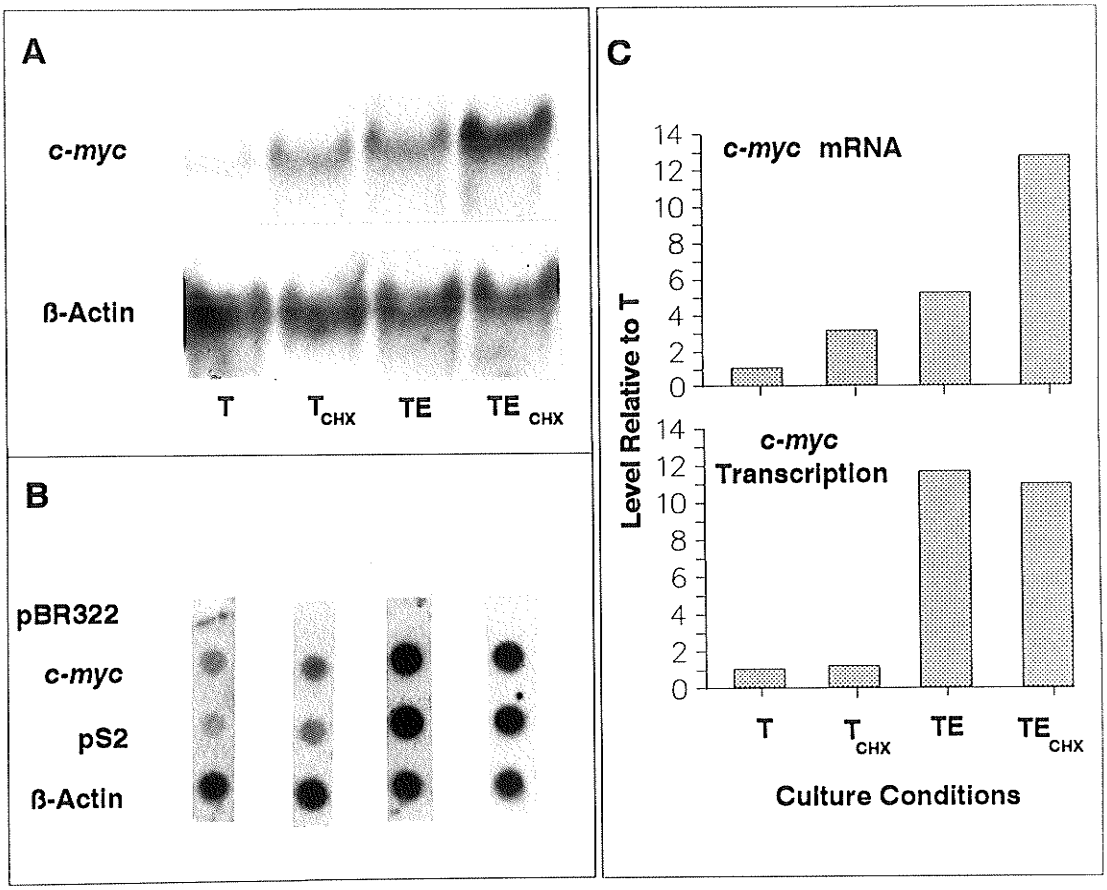

A
c-myc
ß-Actin
T
T CHX
TE
TE CHX
B
pBR322
c-myc
pS2
ß-Actin
C
c-myc mRNA
c-myc Transcription
Level Relative to T
0
2
4
6
8
10
12
14
T
T CHX
TE
TE CHX
Culture Conditions

Fig. 20 Comparison of *c-myc* transcription in MCF-7 and MDA-MB-231 cells. Tamoxifen-treated [T] MDA-MB-231 and MCF-7 cells were rescued with estradiol (10^{-7}M) for 20 min [TE]. RNA and nuclei were isolated from these cells as described under MATERIALS AND METHODS. **(A) *c-myc* mRNA accumulation (20 min post-estradiol treatment). (B) Corresponding *in vitro* transcription assay,** using 8 X 10^6 cpm / ml of labeled RNA for each hybridization.

Fig. 20 ⟹

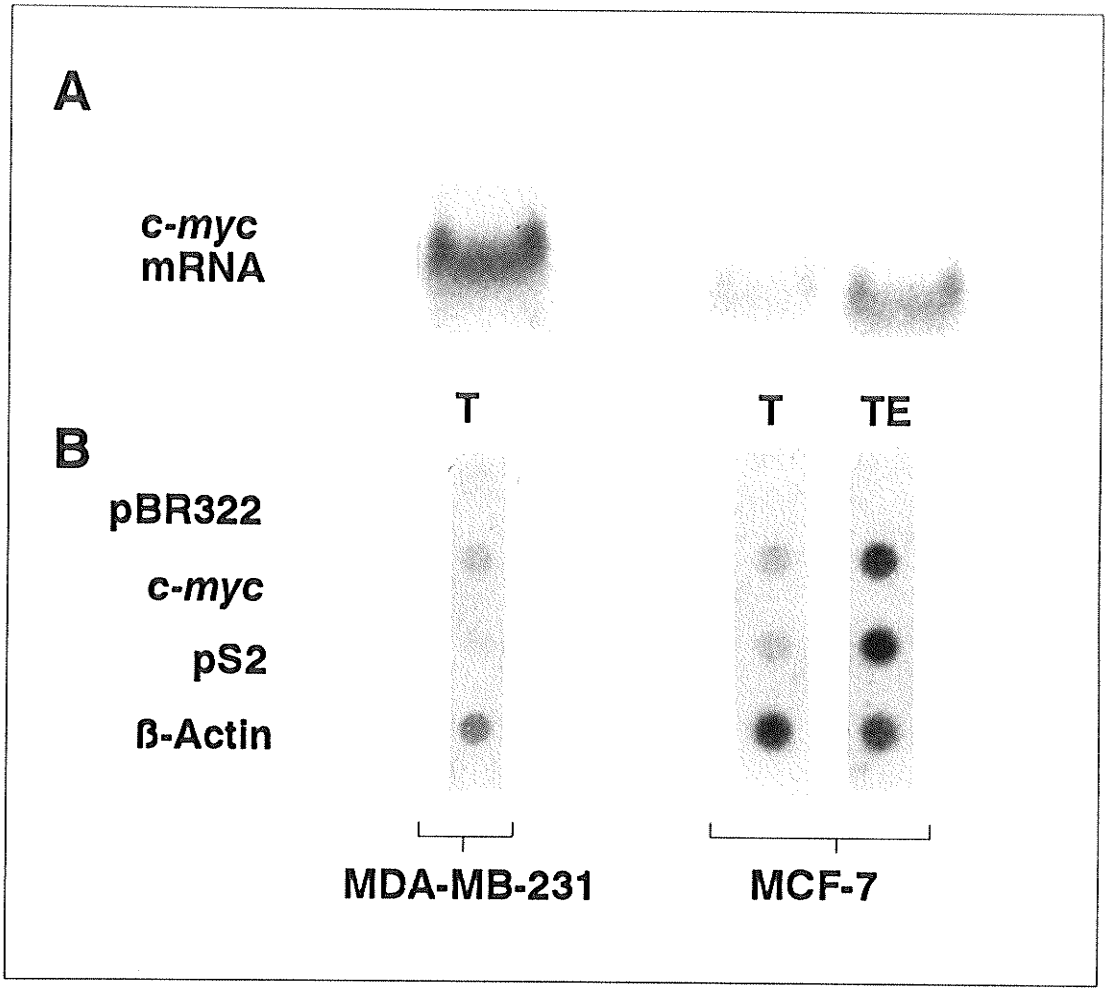
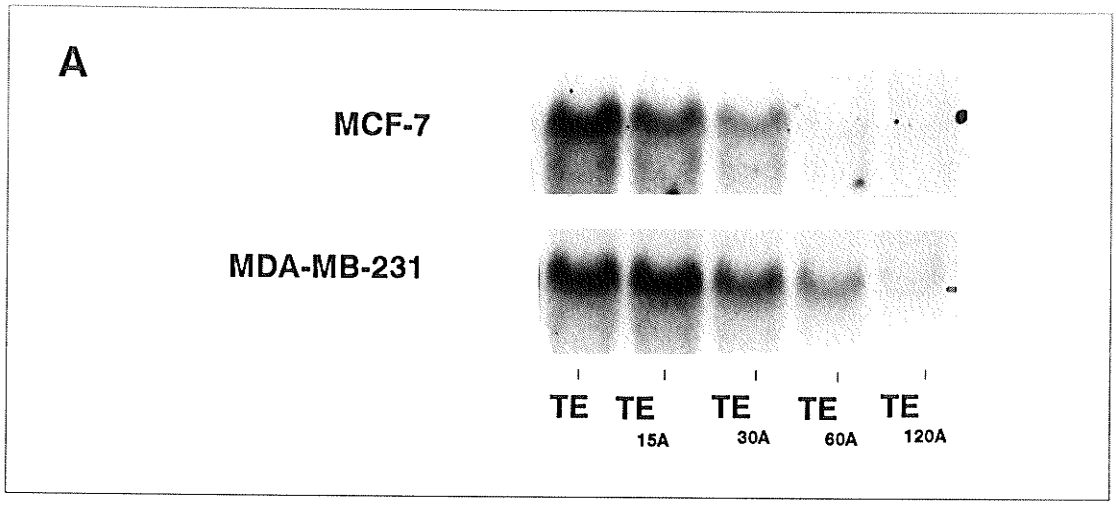

A
c-myc
mRNA
T
T
TE
B
pBR322
c-myc
pS2
ß-Actin
MDA-MB-231
MCF-7

Fig. 21 *c-myc* mRNA stability in MCF-7 and MDA-MB-231 cells. Estradiol-rescued cells (TE, 45 min) were treated with the RNA polymerase inhibitor actinomycin D [A] (5 μg/ml) for the times (min) indicated. RNA was isolated and the amount of *c-myc* mRNA was determined by Northern analysis using the *c-myc* probe. Each lane contains 50 μg of total RNA. This result is shown in Fig. 20A. Quantitation of three of these autoradiographs is shown in Fig. 20B. The half-life of the *c-myc* mRNA is 18.0 ± 7.1 min and 49.2 ± 3.3 min for MCF-7 and MDA-MB-231, respectively. MCF-7, open circles; MDA-MB-231, closed circles.

Fig. 21 ⟹


A
MCF-7
MDA-MB-231
TE
TE 15A
TE 30A
TE 60A
TE 120A

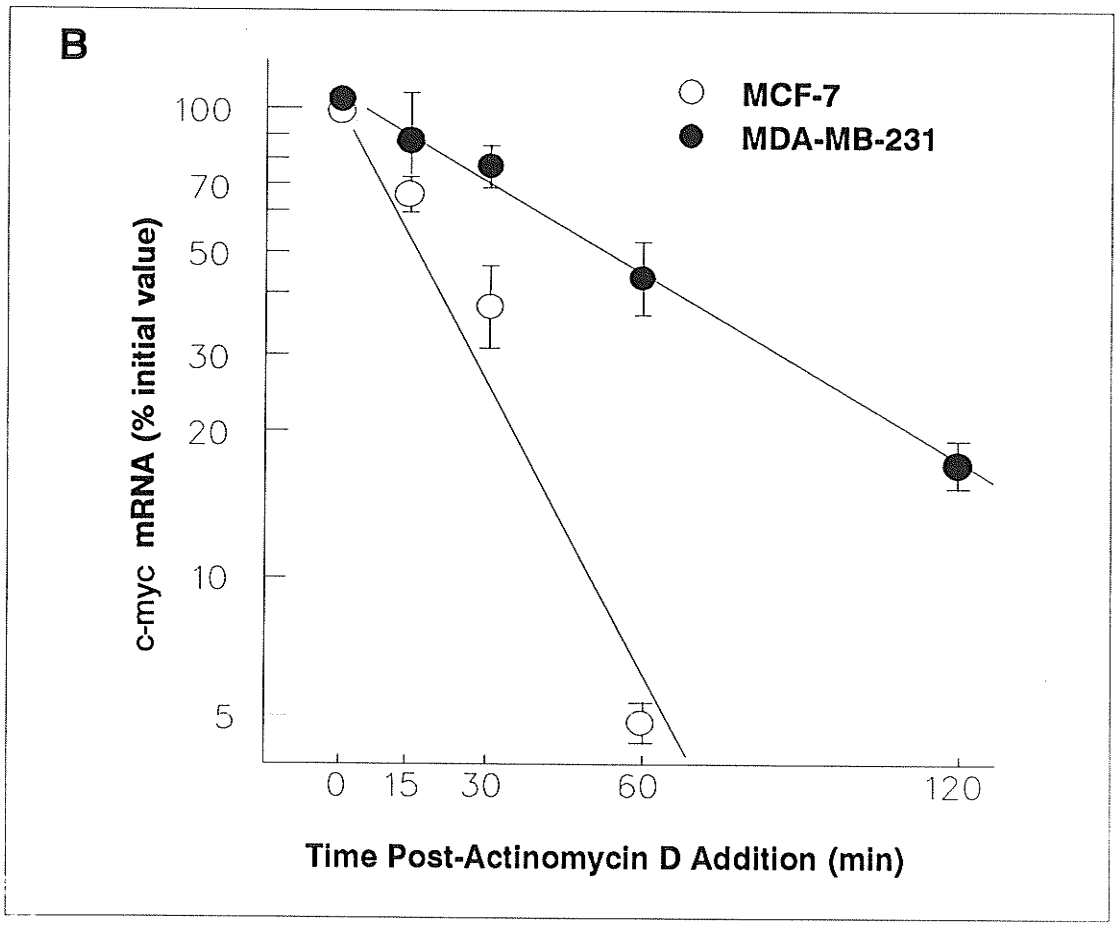

B
MCF-7
MDA-MB-231
c-myc mRNA (% initial value)
100
70
50
30
20
10
5
0
15
30
60
120
Time Post-Actinomycin D Addition (min)

Fig. 22 Hormone regulated CAT activities of representative MYCCAT constructs. The vectors listed were cotransfected with the estrogen receptor expression vector HEO into HeLa cells as described in **MATERIALS AND METHODS**. Transfected cells were subsequently grown in the presence [+] or absence [-] of estradiol (10^{-7}M) for 36 h. Each +/- pair represents identical dishes of HeLa cells transfected with an equal volume (450 µl) of the same $CaPO_4$-DNA precipitate. Following the 36 h incubation, total cellular proteins were isolated and 35 µg was assayed for CAT activity. Acetylated chloramphenicol products were visualized using thin layer chromatography (TLC) followed by autoradiography. The 3-acetyl chloramphenicol dot was scraped from the TLC plate and scintillation counts measured. For the experiment shown these counts "**DPM**" were used to calculate the "**FOLD INDUCTION**" of CAT expression by estradiol. Composite data of several experiments [**n**=*x*] is shown as "**AVERAGE INDUCTION**". Constructs exhibiting a statistically significant greater than 2-fold induction ($p \leq 0.0024$) are indicated with a star [*]. pCAT is the promoterless MYCCAT backbone vector and ApoVLDLII-CAT is an estrogen-regulated vector used as a positive control.

Fig. 22 

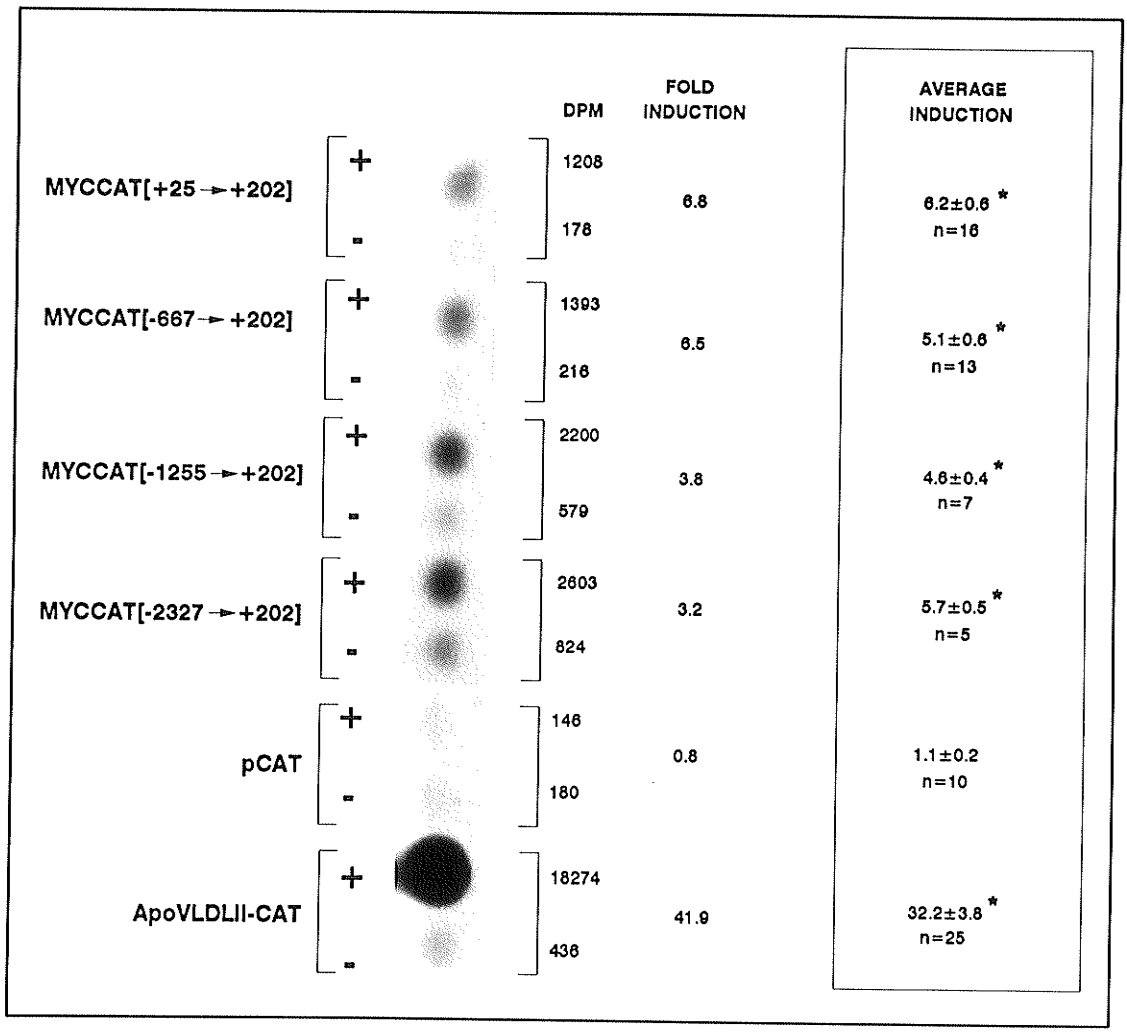

DPM
FOLD INDUCTION
AVERAGE INDUCTION
MYCCAT[+25→+202]
+
-
1208
178
6.8
6.2±0.6 *
n=16
MYCCAT[-667→+202]
+
-
1393
216
6.5
5.1±0.6 *
n=13
MYCCAT[-1255→+202]
+
-
2200
579
3.8
4.6±0.4 *
n=7
MYCCAT[-2327→+202]
+
-
2603
824
3.2
5.7±0.5 *
n=5
pCAT
+
-
146
180
0.8
1.1±0.2
n=10
ApoVLDLII-CAT
+
-
18274
436
41.9
32.2±3.8 *
n=25

Fig. 23 CAT expression from MYCCAT[+25→+202] and ApoVLDLII-CAT when cotransfected with the estrogen receptor expression construct HEO and receptor deletion mutant construct HE4. MYCCAT[+25→+202] or ApoVLDLII-CAT (10 μg per dish) was transfected alone or together with the listed estrogen receptor expression vector (5 μg) into HeLa cells using the $CaPO_4$-DNA precipitation method [**MATERIALS AND METHODS**]. Matched transfected dishes were subsequently incubated in medium containing [+] or lacking [-] 17β-estradiol (10^{-7}M) for 36 h. Cellular proteins were then isolated from all dishes and assayed for CAT activity.

Fig. 23 ⟹

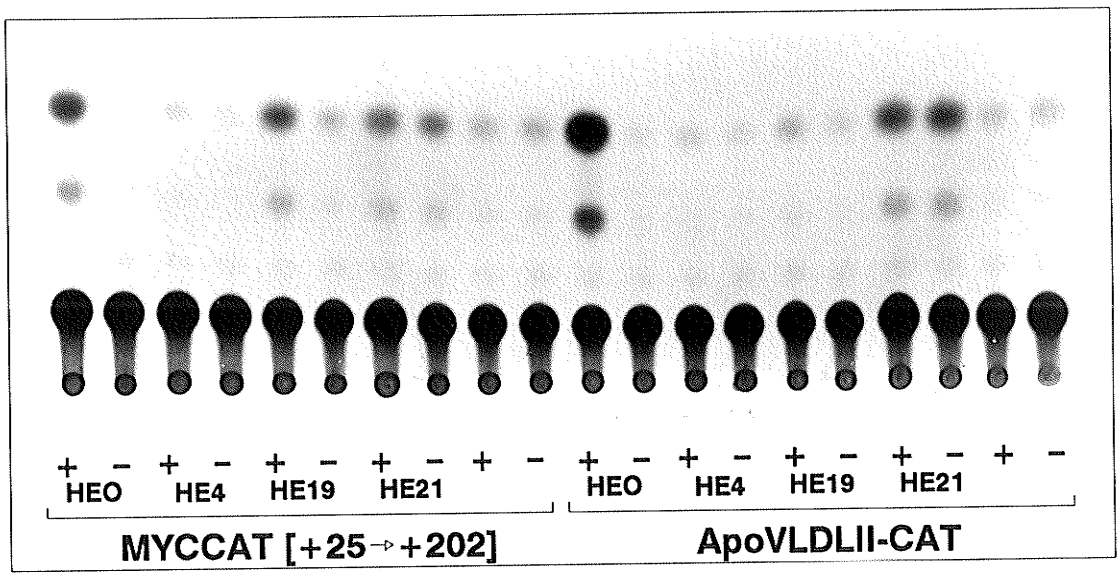

+ − + − + − + − + −
HEO HE4 HE19 HE21
+ − + − + − + − + −
HEO HE4 HE19 HE21
MYCCAT [+25→+202]
ApoVLDLII-CAT

Fig. 24 Comparison of CAT expression of representative MYCCAT constructs transfected into MCF-7 human breast cancer cells and HeLa cervical carcinoma cells. The constructs listed were cotransfected with HEO into MCF-7 or HeLa cells as indicated, using the procedure described in **MATERIALS AND METHODS**. Cellular proteins were isolated from all dishes and 40 μg was assayed for CAT activity 36 h post-estradiol addition [+]. Multiple experiments revealed that the estrogen responsive construct ApoVLDLII-CAT consistently exhibited more complete substrate acetylation in MCF-7 cells than in HeLa cells.

Fig. 24 

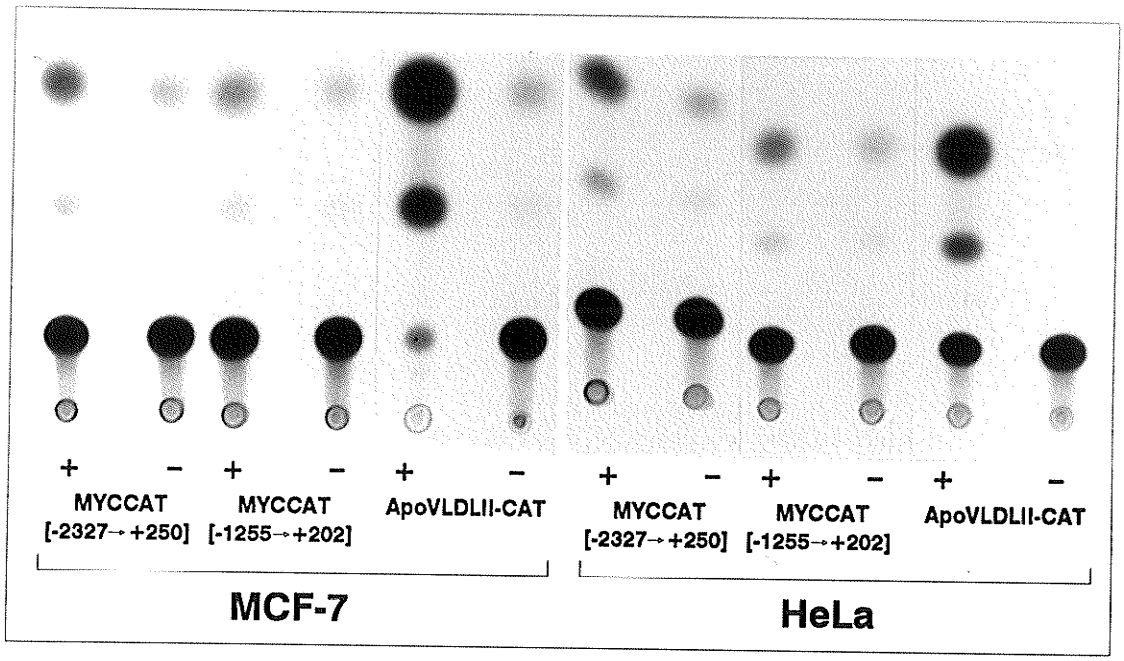

+
−
+
−
+
−
+
−
+
−
+
−
MYCCAT
[-2327→+250]
MYCCAT
[-1255→+202]
ApoVLDLII-CAT
MYCCAT
[-2327→+250]
MYCCAT
[-1255→+202]
ApoVLDLII-CAT
MCF-7
HeLa

Fig. 25 The presence of the P2 promoter in the MYCCAT constructs is required for hormone regulated CAT activity. The four MYCCAT constructs listed, containing varying combinations of the P0, P1 and P2 promoters, were cotransfected together with HEO into HeLa cells as described in **MATERIALS AND METHODS**. The level of estradiol dependent induction of CAT expression was determined for a number of experiments (**n**=*x*) as described in the figure above. Statistically significant, greater than 2-fold induction ($p \leq 0.026$) was seen for all constructs containing at least the P2 promoter [indicated by "*"]. The diagrams represent the *c-myc* promoter regions contained in each construct and specific promoters present [+] or absent [-] in each construct are listed within the brackets "{}".

Fig. 25 

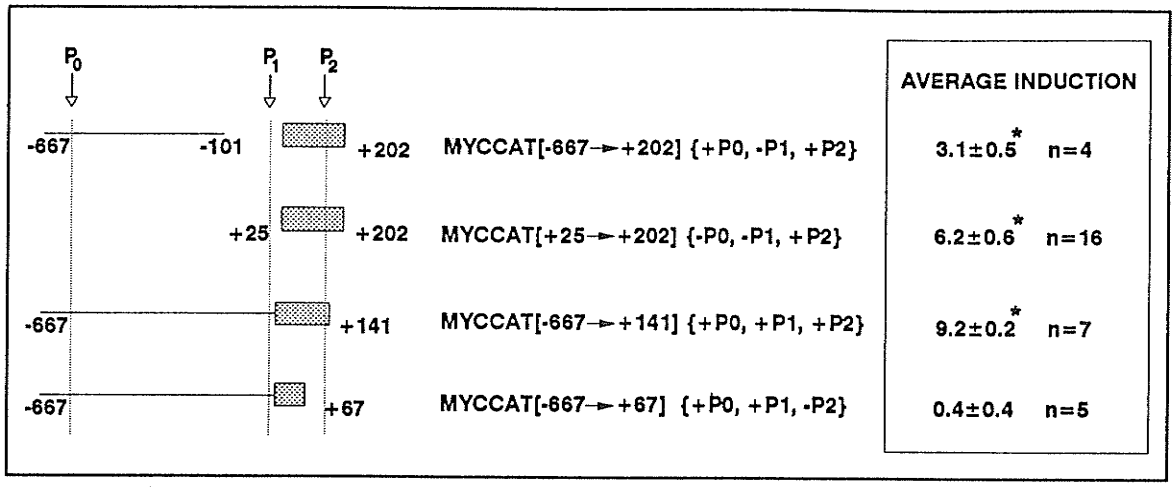

P0
P1
P2
-667
-101
+202
MYCCAT[-667→+202] {+P0, -P1, +P2}
+25
+202
MYCCAT[+25→+202] {-P0, -P1, +P2}
-667
+141
MYCCAT[-667→+141] {+P0, +P1, +P2}
-667
+67
MYCCAT[-667→+67] {+P0, +P1, -P2}
AVERAGE INDUCTION
3.1±0.5* n=4
6.2±0.6* n=16
9.2±0.2* n=7
0.4±0.4 n=5

Fig. 26 (A) Effects of steroid hormones on *c-myc* expression in MCF-7 HBC cells. MCF-7 cells were cultured for 48 h in hormone depleted medium without phenol red as described in **Materials and Methods.** Individual dishes were then treated for 90 min with either no hormone [-], retinoic acid [RA], estradiol [E], dexamethasone [DEX], dihydrotestosterone [DHT] or medroxyprogesterone acetate [MPA] at a physiological concentration of 2 X 10^{-9}M. Total RNA was isolated and Northern analysis of *c-myc* expression was done. **(B) 17β-estradiol, but not other steroid hormones, specifically stimulates CAT expression in MYCCAT[+25→+202] and HEO cotransfected HeLa cells.** Matched dishes of HeLa cells cotransfected with the same $CaPO_4$-DNA precipitate containing MYCCAT[+25→+202] (10 μg) and HEO (5 μg) were cultured in the presence of the steroid hormones indicated for 36 h post-transfection. In all instances hormone concentrations were 10^{-7}M. Cellular proteins were then isolated and 50 μg were assayed for CAT activity. The top CAT result marked [-] represents cotransfected cells maintained in steroid depleted charcoal-treated medium and is representative of background CAT activity. Also shown is the ApoVLDLII-CAT positive control.

Fig. 26 ⟹

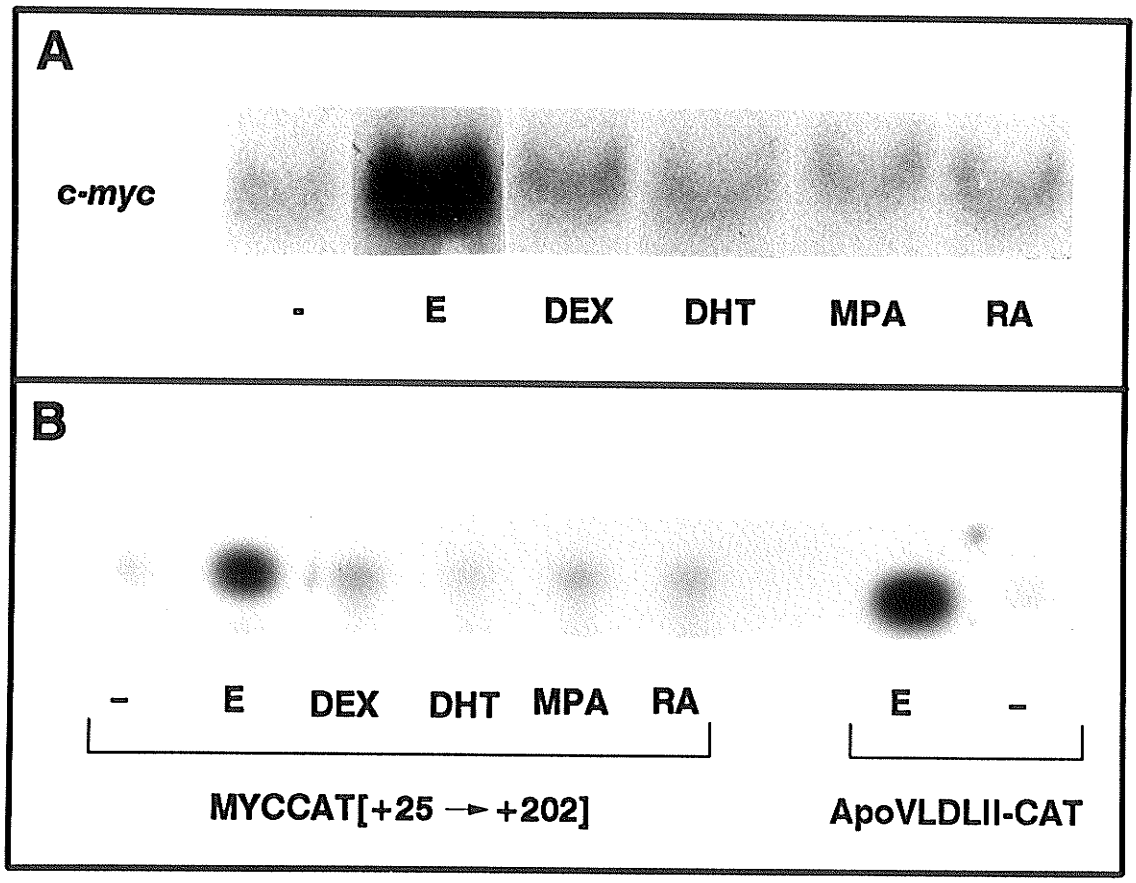

A
c-myc
-
E
DEX
DHT
MPA
RA
B
-
E
DEX
DHT
MPA
RA
E
-
MYCCAT[+25 → +202]
ApoVLDLII-CAT

Fig. 27 Effects of the antiestrogens tamoxifen and ICI 164,384 on estrogen regulated CAT expression from CAT constructs MYCCAT[+25→+202] or ApoVLDLII-CAT. HeLa cells were transfected with either MYCCAT[+25→+202] or ApoVLDLII-CAT (10 μg / dish) alone or together with HEO (5 μg / dish) as described in **Materials and Methods.** Following transfection appropriate dishes were treated with medium depleted of steroids [-] or containing the hormones indicated: **[E]** 17β-estradiol (10^{-8}M), **[I]** the antiestrogen ICI 164,384 (10^{-7}M), **[T]** the antiestrogen tamoxifen (10^{-6}M) or **[EI]** estradiol and ICI 164,384 (10^{-8}M and 10^{-7}M respectively). *Percentage CAT induction relative to [E 10^{-8}M]* was determined by densitometric scanning of the autoradiograph. Background CAT expression (none) was subtracted from all hormone treated samples.

Fig. 27 

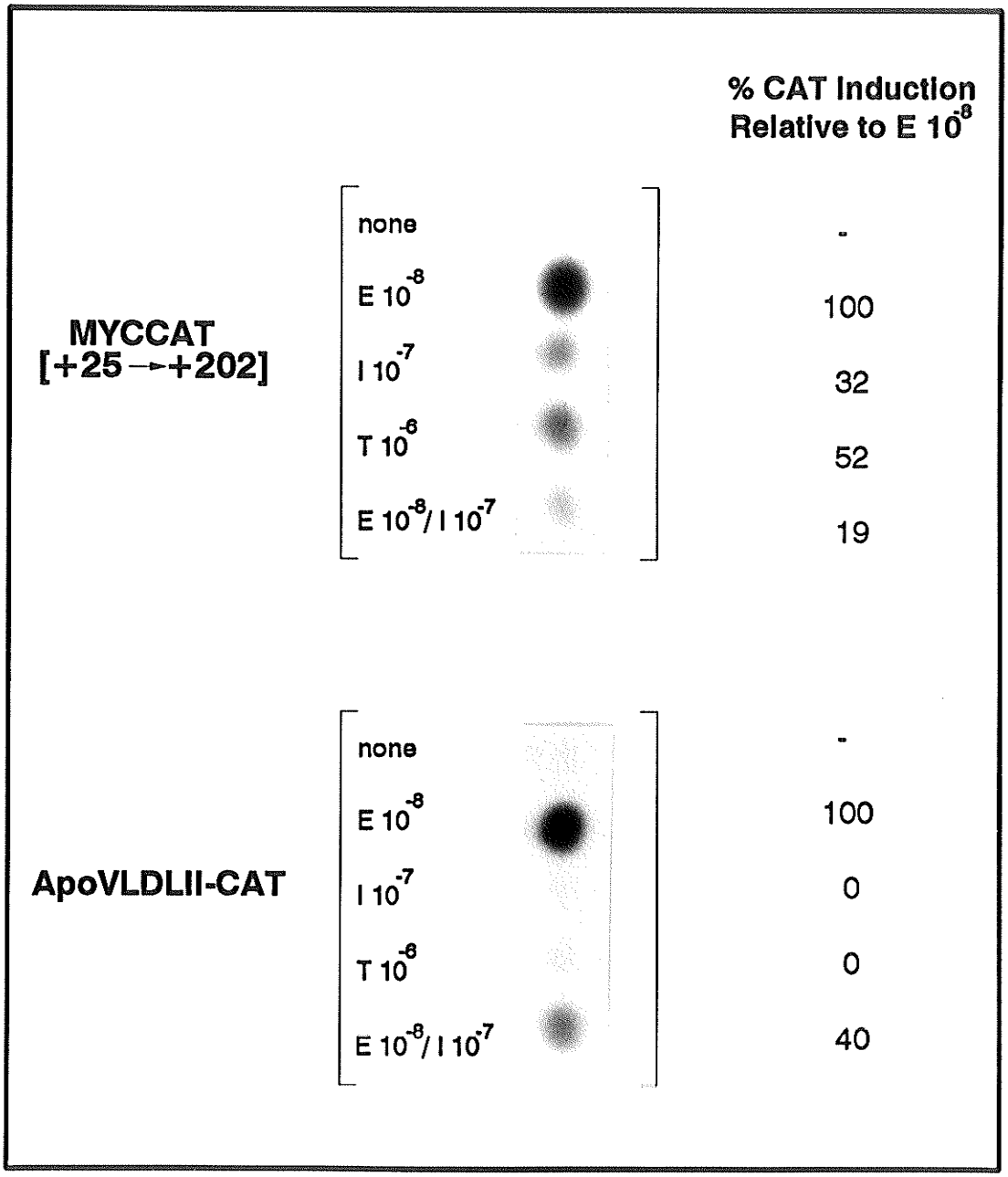

% CAT Induction
Relative to E 10-8
MYCCAT
[+25 → +202]
none
E 10-8
I 10-7
T 10-6
E 10-8/ I 10-7
-
100
32
52
19
ApoVLDLII-CAT
none
E 10-8
I 10-7
T 10-6
E 10-8/ I 10-7
-
100
0
0
40

Fig. 28 CAT expression of pSVCAT related constructs and appropriate control constructs. The constructs indicated were transfected into HeLa cells as described in **MATERIALS AND METHODS**. Except where indicated by "**no HEO**" all constructs were cotransfected with the estrogen receptor expression vector HEO. Each +/- pair represents identical dishes of HeLa cells transfected with an equal volume (450 μl) of the same $CaPO_4$ vector-DNA precipitate. The +/- pairs differ only in the presence (+) or absence (-) of estradiol (10^{-7}M) in their growth medium. **DPM, FOLD INDUCTION** and **AVERAGE INDUCTION** are described in the legend to Fig. 22. Statistically significant hormonal inductions are indicated by the star [*]. Since the data shown is combined from two independent experiments the [1] preceding the construct name is used to distinguish one experiment from the other.

Fig. 28 

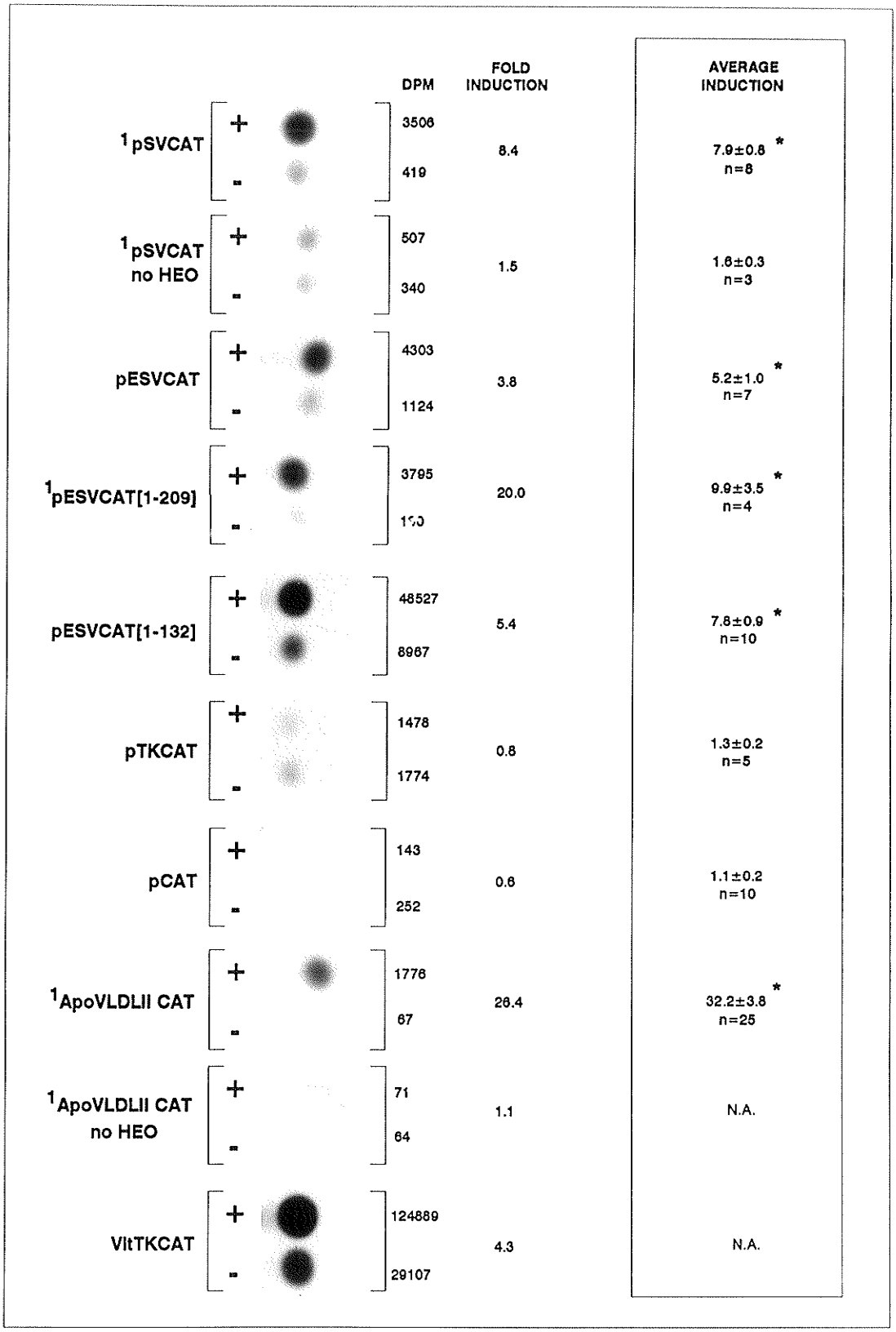
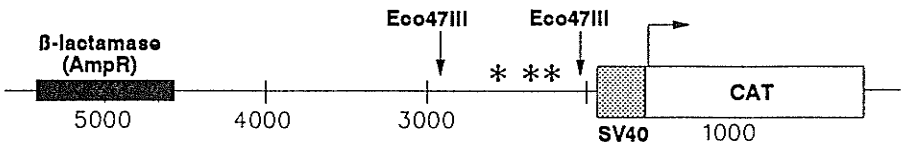

DPM
FOLD INDUCTION
AVERAGE INDUCTION
1pSVCAT
+
-
3506
419
8.4
7.9±0.8 *
n=8
1pSVCAT no HEO
507
340
1.5
1.6±0.3
n=3
pESVCAT
4303
1124
3.8
5.2±1.0 *
n=7
1pESVCAT[1-209]
3795
8.0
20.0
9.9±3.5 *
n=4
pESVCAT[1-132]
48527
8967
5.4
7.8±0.9 *
n=10
pTKCAT
1478
1774
0.8
1.3±0.2
n=5
pCAT
143
252
0.6
1.1±0.2
n=10
1ApoVLDLII CAT
1776
67
26.4
32.2±3.8 *
n=25
1ApoVLDLII CAT no HEO
71
64
1.1
N.A.
VltTKCAT
124889
29107
4.3
N.A.

Fig. 29 Potential EREs located within the pSVCAT construct. Computer analysis using the consensus ERE revealed three potential EREs within the *Eco47III* fragment of the pSVCAT construct. The sequence of these potential EREs at positions 2221, 2338 and 2350 are listed. All three EREs are located upstream of the SV40 early promoter and could conceivably influence expression of the CAT gene.

Fig. 29 ⟹



pSVCAT

2221	GGcgA-nnn-TGgCC	Potential EREs
2338	GGcCA-nnn-TGtCC	
2350	GGaCA-nnn-TGgCC	
	GGTCA-nnn-TGACC	Consensus ERE

Fig. 30 17β-estradiol, but not other steroid hormones, specifically stimulates CAT expression in pESVCAT[1→132] and HEO cotransfected HeLa cells. Matched dishes of HeLa cells cotransfected with the same $CaPO_4$-DNA precipitate containing pESVCAT[1→132] (10 μg) and HEO (5 μg) were cultured in the presence of the steroid hormones indicated for 36 h post-transfection. In all instances hormone concentrations were 10^{-7}M. Cellular proteins were then isolated and 50 μg of proteins were assayed for CAT activity. The top CAT result marked [-] represents cotransfected cells maintained in steroid depleted charcoal-treated medium and is representative of background CAT activity. [E=estradiol, DEX=dexamethasone, DHT=dihydrotestosterone, MPA= medroxyprogesterone acetate, RA=retinoic acid]

Fig. 30 ⟹

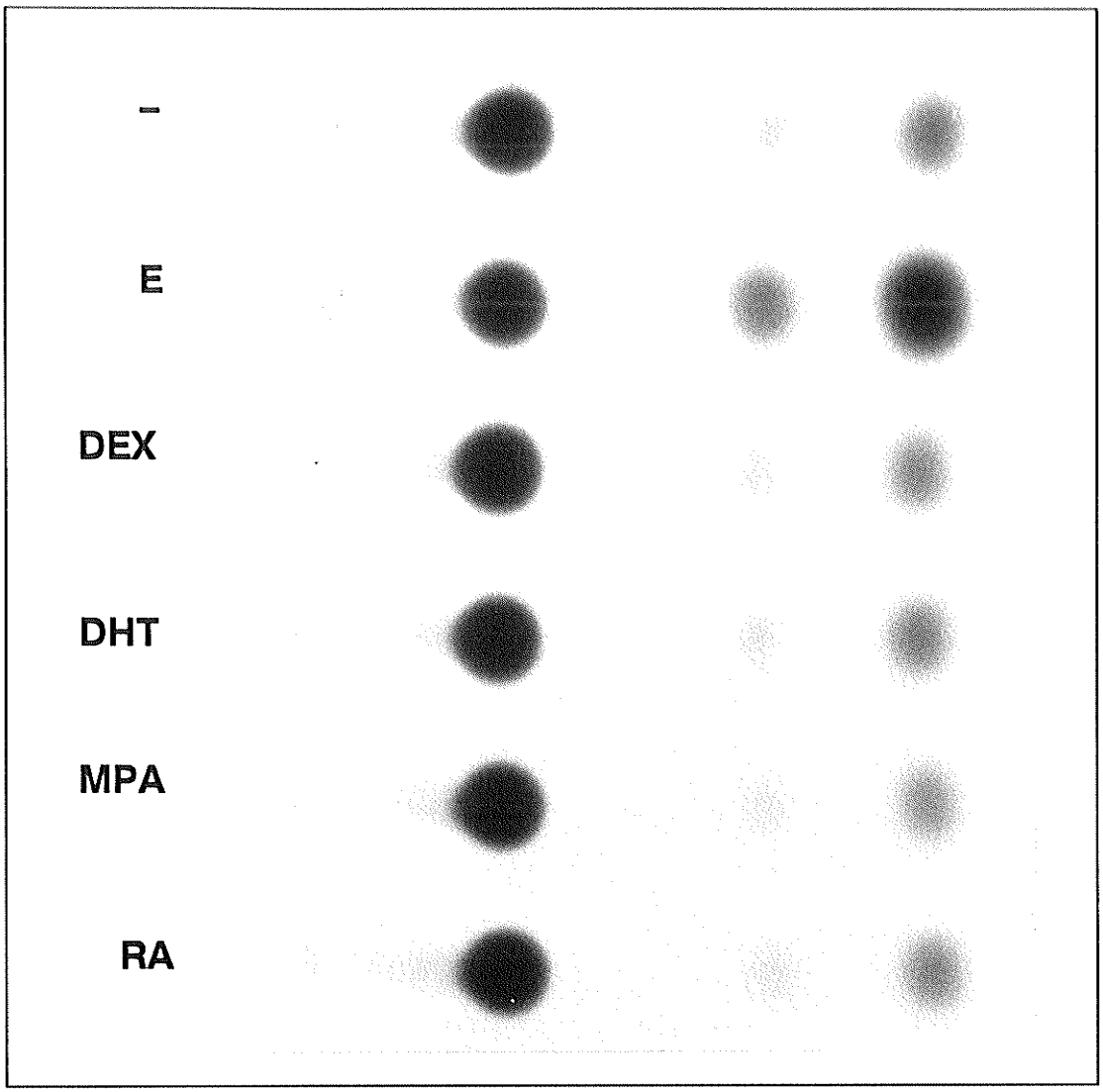

–
E
DEX
DHT
MPA
RA

Fig. 31 Comparison of CAT expression of pSVCAT and related constructs in MCF-7 human breast cancer cells and in HeLa cervical carcinoma cells. The constructs listed were transfected into MCF-7 or cotransfected with the estrogen receptor expression vector into HeLa cells as indicated, using the procedure described in **MATERIALS AND METHODS**. [Note: pSVCAT, no HEO does not contain HEO] Cellular proteins were isolated from all dishes and 40 μg of proteins were assayed for CAT activity 36 h post-estradiol addition [+]. See **MATERIALS AND METHODS** and Fig. 9 for a description of the constructs.

Fig. 31 

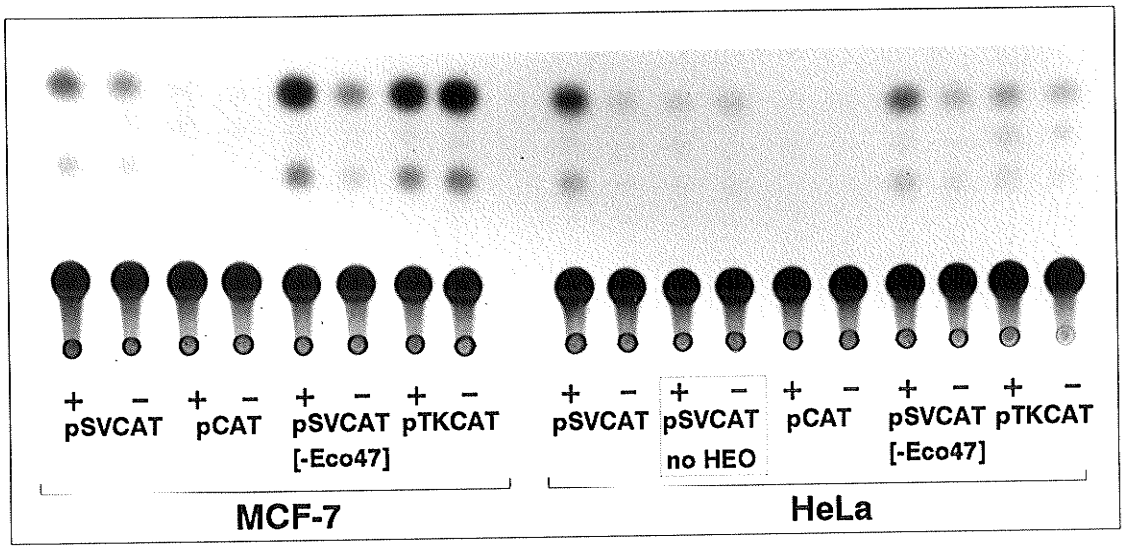

+ − + − + − + −
pSVCAT pCAT pSVCAT pTKCAT
[-Eco47]
MCF-7
+ − + − + − + − + − + −
pSVCAT pSVCAT pCAT pSVCAT pTKCAT
no HEO [-Eco47]
HeLa

Fig. 32 Effects on CAT expression of pESVCAT[1→132] when cotransfected with estrogen expression construct mutants. pESVCAT[1→132] (10 μg per dish) was cotransfected with the listed estrogen receptor expression vector (5 μg) into HeLa cells using the $CaPO_4$-DNA precipitation method [**MATERIALS AND METHODS**]. Matched transfected dishes were subsequently incubated in medium containing [+] or lacking [-] 17β-estradiol (10^{-7}M) for 36 h. Cellular proteins were then isolated from all dishes and assayed for CAT activity. The estrogen expression vectors are described in Fig. 8. Also shown is the CAT assay result of the estrogen responsive construct ApoVLDLII-CAT (10 μg / dish) cotransfected with HEO (5 μg / dish) [also see Fig. 23].

Fig. 32 ⟹

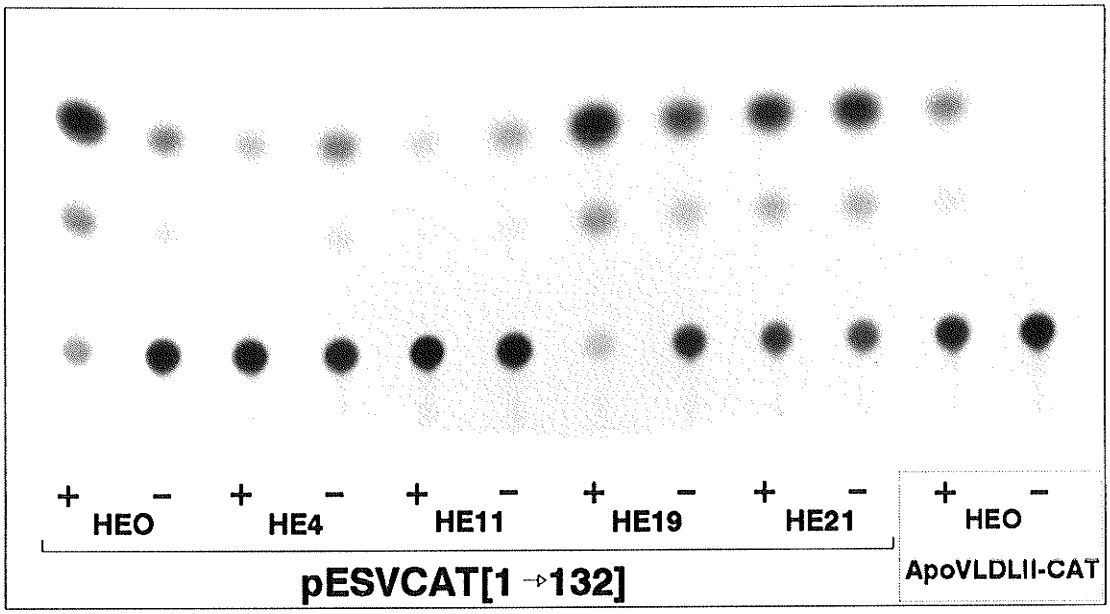

+ HEO −
+ HE4 −
+ HE11 −
+ HE19 −
+ HE21 −
pESVCAT[1 → 132]
+ HEO −
ApoVLDLII-CAT

Fig. 33 Effects of the antiestrogens tamoxifen and ICI 164,384 on estrogen regulated CAT expression from CAT constructs pESVCAT[1→132] or ApoVLDLII-CAT. HeLa cells were transfected with either pESVCAT[1→132] or ApoVLDLII-CAT (10 μg / dish) alone or together with HEO (5 μg / dish) as described in MATERIALS AND METHODS. Following transfection appropriate dishes were treated with medium depleted of steroids [-] or containing the hormones indicated: [**E**] 17β-estradiol (10^{-8}M), [**I**] the antiestrogen ICI 164,384 (10^{-7}M), [T] the antiestrogen tamoxifen (10^{-6}M) or [**EI**] estradiol and ICI 164,384 (10^{-8}M and 10^{-7}M respectively). The 3-acetylchloramphenicol dots were scraped from the TLC plate and scintillation counted yielding the **DPM** indicated. The **FOLD INDUCTION** relative to hormone-depleted HEO cotransfected cells is also shown. In this experiment 39 μg of total cellular proteins were assayed for CAT.

Fig. 33 ⟹

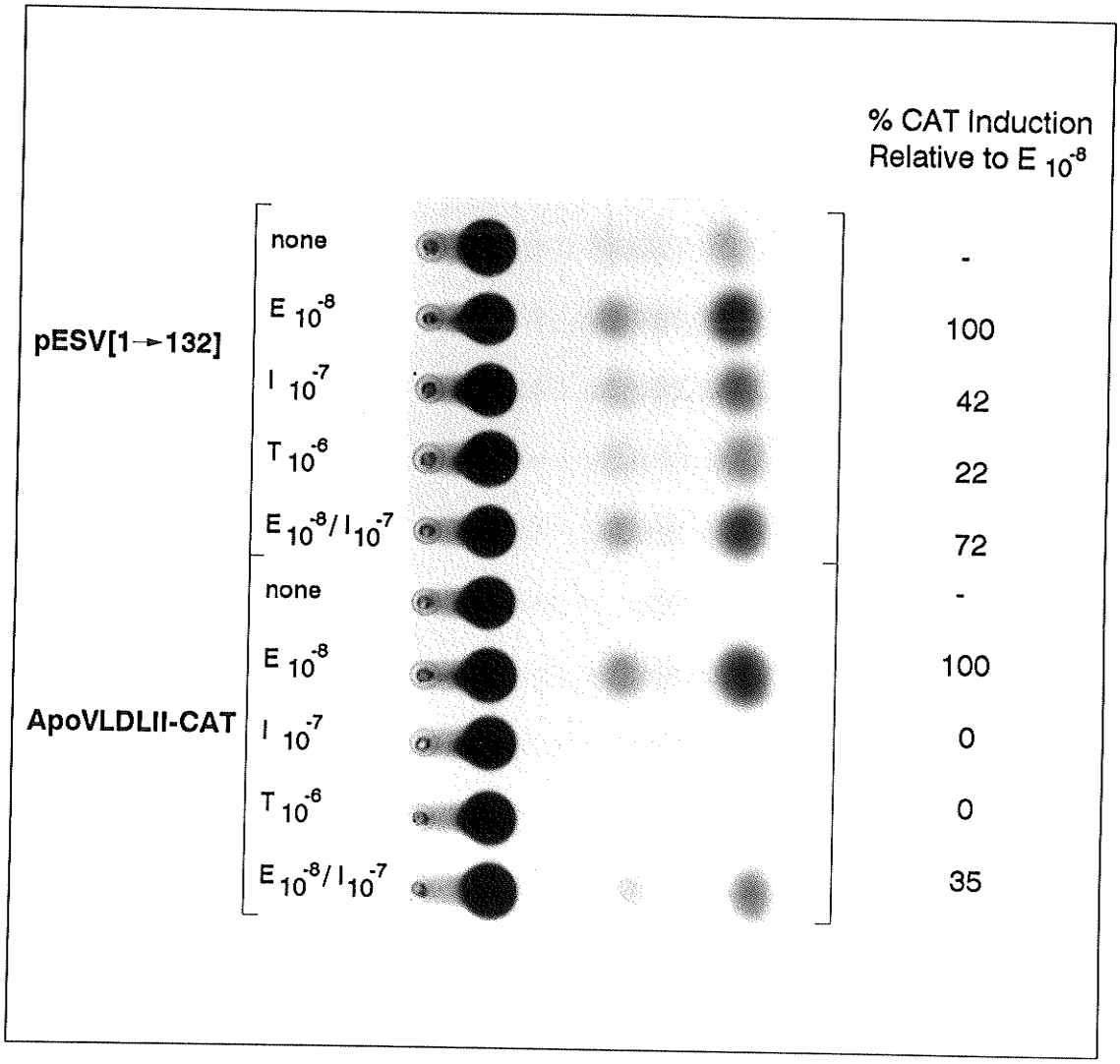
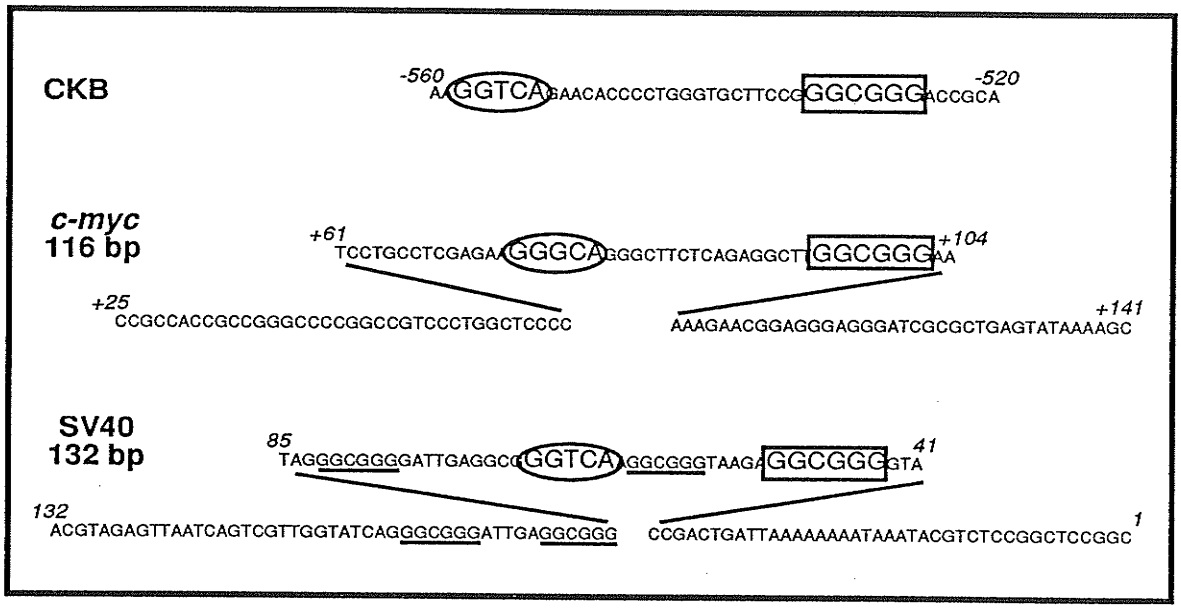

% CAT Induction
Relative to E 10-8
pESV[1→132]
none
E 10-8
I 10-7
T 10-6
E 10-8/I 10-7
-
100
42
22
72
ApoVLDLII-CAT
none
E 10-8
I 10-7
T 10-6
E 10-8/I 10-7
-
100
0
0
35

Fig. 34 The estrogen responsive regions of the SV40 early promoter, and the *c-myc* and rat creatine kinase B (CKB) genes contain a common ERE half-site / Sp1 binding site motif. The ERE half-site is indicated by an oval and the Sp-1 binding site by a box. Also indicated by a line are the additional Sp1 binding sites of the SV40 early promoter.

Fig. 34 


CKB
-560
AAGGTCAGAACACCCCTGGGTGCTTCCGGGCGGGACCGCA
-520
c-myc
116 bp
+61
TCCTGCCTCGAGAAGGGCAGGGCTTCTCAGAGGCTTGGCGGGAA
+104
+25
CCGCCACCGCCGGGCCCCGGCCGTCCCTGGCTCCCC
+141
AAAGAACGGAGGGAGGGATCGCGCTGAGTATAAAAGC
SV40
132 bp
85
TAGGGCGGGGATTGAGGCGGGTCAAGGCGGGTAAGAGGCGGGCGTA
41
132
ACGTAGAGTTAATCAGTCGTTGGTATCAGGGCGGGATTGAGGCGGG
CCGACTGATTAAAAAAAATAAATACGTCTCCGGCTCCGGC
1