MULTI-STEROID REGULATED EXPRESSION OF THE PIP/GCDFP-15/SABP GENE IN HUMAN BREAST CANCER.

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"All the conditions of happiness are realized in the life of the man of science. He has an activity which utilizes his abilities to the full, and he achieves results which appear important not only to himself but to the general public, even when it cannot in the smallest degree understand them." *Bertrand Russell*

ABSTRACT

The growth and differentiation of the mammary gland is a result of the complex interactions of a number of endocrine hormones which act alone and in combination to promote the normal function of the breast. The development of breast cancer is also strongly influenced by hormonal status and one third of all breast tumors proliferate in the presence of estrogen. The effects of other hormones, such as androgens and retinoic acid, are not as well documented, yet a growing body of evidence suggests that these other hormones may also influence the development and growth of breast cancer. The PIP/GCDFP-15/SABP (prolactin-inducible protein/gross cystic disease fluid protein/secretory actin-binding protein) gene is induced by androgen and retinoic acid and inhibited by estrogen and has been proposed to be a marker of apocrine differentiation. We have used the multihormonally regulated PIP/GCDFP-15/SABP gene as a model to study the interaction of the nuclear receptors corresponding to these hormones. Initial studies focussed on the characterization of the PIP cDNA and gene. The sequence, when compared to other known DNA sequences, was shown to be identical to GCDFP-15, a protein found in fluid from gross cystic disease of the breast, and SABP, a protein isolated from seminal plasma based on its affinity for actin. As well the PIP cDNA sequence revealed similarity to the sequence of a cDNA cloned from the androgen-dependent tissue, mouse submaxillary gland. Androgen treatment of T-47D or ZR-75-1 human breast cancer cells results in an induction of PIP mRNA within 24 hours, reaching maximal levels at 5 days. Retinoic acid treatment of these cells results in an induction of PIP mRNA within 30 minutes, reaching maximal levels at 3 hours and slowly declining thereafter. Estrogen inhibits the induction of PIP mRNA by both androgen and retinoic acid at 24 hours of hormone treatment. Using transient transfection assays we identified an area of the 5' flanking region of the PIP gene which appears to confer both androgen and retinoic acid responsiveness to a reporter gene, chloramphenicol acetyl transferase (CAT). This region contains a consensus estrogen response element (ERE) as well as two consensus androgen response element (ARE) half-sites. A purified GST fusion protein

containing the androgen receptor DNA-binding domain and an estrogen receptor translated and transcribed *in vitro* from an estrogen receptor expression vector (pT7βhER; a gift of Dr. S. Tsai, Houston, Texas) were used to further elucidate the interaction of the hormone receptors with the PIP gene. Using gel mobility shift assays, we have shown that increasing amounts of estrogen receptor can decrease the binding of the androgen receptor to an area of the 5' flanking region of the PIP gene. Furthermore, we have demonstrated that androgen receptor and estrogen receptor bind to overlapping sites within this fragment using DNase I footprinting assays. Estrogen inhibits PIPCAT gene expression induced by dihydrotestosterone through competition for a complex hormone responsive element in the 5' flanking region of the PIP gene. The human PIP/GCDFP-15 gene provides an excellent model with which to study the interaction of nuclear hormone receptors on transcriptional regulation.

LIST OF ABBREVIATIONS

A/mA	amperes/milliamperes
AAD	acidic activation domain
ADH	alcohol dehydrogenase
AIS	androgen insensitivity syndrome
APL	acute promyelocytic leukaemia
ApoD	Apolipoprotein D (GCDFP-24)
AR	androgen receptor
ARE	androgen response element
bp	basepair
°C	degrees centigrade
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyl transferase
CO_2	carbon dioxide
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CRE	cAMP responsive element
CS	chorionic somatomammotropin
DBD	DNA binding domain
DEX	dexamethasone
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone-sulfate
DHT	dihydrotestosterone
DMBA	7,12-dimethylbenz-(a)-anthracene
DNA/cDNA	deoxyribonucleic acid/complementary-
DNase	deoxyribonuclease
DRE	vitamin D response element

E	17β-estradiol
EC	embryonal carcinoma
EDTA	ethylenediamine tetraacetic acid
EGF/EGF-R	epidermal growth factor/- receptor
EMA	epithelial membrane antigen
ER/ER+/ER-	estrogen receptor/positive/negative
ERE	estrogen response element
ExoIII	exonuclease III
FBS	fetal bovine serum
g/mg/μg/ng	gram/milligram/microgram/nanogram
GCDFP-15	gross cystic disease fluid protein 15
GnRH	gonadotropin releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HBC	human breast cancer
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HGK	human glandular kallikrein
HRE	hormone responsive element
hsp	heat shock protein
IGF-I	insulin-like growth factor I
IPTG	isopropylthiogalactoside
kb	kilobase
kDa	kilodalton
L/mL/μL	litre/millilitre/microlitre
LB	laminin B1
LBD	ligand binding domain
LH	luteinizing hormone
M/mM/μM	molar/millimolar/micromolar
MMTV-LTR	mouse mammary tumor virus-long terminal repeat

MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NAF	nuclear accessory factor
NGF	nerve growth factor
NLS	nuclear localization signal
NMU	N-methylnitrosurea
NTS	nuclear translocation signal
ODC	ornithine decarboxylase
OH-FLU	hydroxyflutamide
PEG	polyethylene glycol
PIP	prolactin inducible protein
PL	placental lactogen
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
PRE	progesterone response element
PSA	prostate-specific antigen
PSBP	prostatic steroid binding protein
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBF	retinoic acid receptor binding factor
RE	restriction endonuclease
RPBF	RP binding factor
RXR	retinoid X receptor
RXRE	retinoid X response element
SABP	secretory actin binding protein
Slp	sex-limited protein
SBMA	spinal and bulbar muscular atrophy

SV40	simian virus 40
TAF	transcriptional activation function
Tfm	testicular feminized
TGFβ	transforming growth factor β
tk/TK	thymidine kinase
TR	thyroid hormone receptor
TRAP	thyroid hormone receptor accessory protein
TRE	thyroid hormone response element
TRH	thyroid hormone releasing hormone
V	volts
VDR	vitamin D receptor
VIP	vasoactive intestinal peptide
WAP	whey acidic protein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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INTRODUCTION

THE NORMAL BREAST

Development and structure

The mammalian breast consists of tubuloalveolar glands derived from modified sweat glands. In the mammalian embryo, paired lines extending from the future axillary to inguinal regions between the limb buds are referred to as "milk lines". Paired glands develop along these "milk" lines. In males, the mammary glands develop very little postnatally and remain rudimentary. The nonpregnant female breast consists mostly of adipose tissue and an immature duct system. Hormonal stimulation from the ovary at puberty initiates growth of the mammary gland. The breast consists of 15 to 20 lobes of irregularly branched tubuloalveolar glandular tissue, fibrous tissue which connects the lobes and adipose tissue between the lobes. The lactiferous duct of each lobe opens onto the nipple and forms a network of progressively smaller ducts which end in lobules. These lobules constitute the milk producing structures and contain clusters of saclike alveoli lined with epithelial cells (Romrell and Bland, 1991).

Hormonal influences on normal breast development

The ovarian steroids, estrogen and progesterone, stimulate the mammary glands to grow at puberty. The cyclic increase of these hormones with menarche causes further ductal development and the formation of lobules. Estrogen is responsible for the initiation of ductal development and causes an increase in the number of estrogen and progesterone receptors in the mammary epithelial cells. Progesterone is responsible for lobular development and causes the differentiation of the epithelial cells. Progesterone also reduces estrogen binding in the epithelium and limits the proliferation of the ductal system. The peptide hormone prolactin increases the number of estrogen receptors and acts synergistically with estrogen and progesterone in their respective functions. Prolactin also contributes to the development of the adipose tissue and is required in combination

with growth hormone and cortisol for epithelial growth and development (Keller Wood and Bland, 1991).

Normal breast development in pregnancy

During pregnancy, the mammary glands proliferate and differentiate to develop a structure capable of milk production. This proliferation and differentiation is influenced by estrogen and progesterone from the corpus luteum and placenta, as well as pituitary prolactin, placental chorionic somatomammotropin and adrenal corticoids. Proliferation of the ductal epithelium, myoepithelial cells and the stromal cells of the breast parenchyma are stimulated by estrogen while the formation of secretory acinar components in the lobules is initiated under the influence of both estrogen and progesterone. These changes in the glandular tissue are accompanied by a decrease in the connective and adipose tissue components of the breast.

The development of the lobuloalveolar structure of the mammary gland begins in the third week of pregnancy and by the first trimester, the glandular structures arborize and begin to form multiple alveoli. In the second trimester, placental estrogen and progesterone and pituitary prolactin stimulate the further development of ductal elements as the alveolar epithelium proliferates and differentiates to become actively secretory. In the third trimester, under the influence of prolactin, stem cells form presecretory alveolar cells and myoepithelial cells (Keller Wood and Bland, 1991). The mammogenic actions of prolactin also require cortisol, insulin, growth hormone and EGF (epidermal growth factor). Chorionic somatomammotropin may also be involved. Near the end of pregnancy, the alveolar cells and ductular lumen accumulate secretory products and cell proliferation declines. Any further enlargement is caused by the hypertrophy of the alveolar cells.

Lactogenesis and lactation

Lactogenesis is stimulated primarily by prolactin in late pregnancy and postpartum. The milk producing cells of the breast are fully differentiated and the components of milk and

the enzymes required for milk production are synthesized under the influence of prolactin and chorionic somatomammotropin. Prolactin synthesis and release increases throughout pregnancy as high levels of estrogen cause the lactotrophs to undergo hypertrophy and hyperplasia. Lactation is prevented during the end of pregnancy by placental steroids that antagonize the stimulatory action of prolactin on milk secretion. Estrogen causes a decrease in the number of prolactin receptors and progesterone inhibits the biosynthesis of milk products. At parturition, the loss of placental estrogen and progesterone initiates lactation. Prolactin, in the presence of insulin, cortisol, growth hormone and thyroxine, stimulates synthesis of the milk proteins, casein and α-lactalbumin. Prolactin also allows induction of galactosyl transferase and lactose synthetase.

Milk contains water, triglycerides, lactose, milk proteins, vitamins, calcium and phosphate. During the early part of lactation, the first milk, called colostrum, has a lower fat and lactose content but is higher in proteins, specifically lactoferrin (which has bacteriocidal activity) and immunoglobulins. The alveolar epithelial cells synthesize and secrete milk into the alveolar lumen. The milk is then transported to the surface of the nipple through the lactiferous duct. The secretion of milk is dependent on prolactin's action on the alveolar epithelium, but milk ejection is controlled by the neurohypophyseal hormone, oxytocin which is released by suckling. The absence of suckling results in a gradual cessation of milk secretion and mammary gland regression. After menopause, the levels of ovarian hormones diminish and the secretory cells of the alveoli degenerate and disappear. The mammary gland atrophies although some ducts remain (Keller Wood and Bland, 1991).

HUMAN BREAST CANCER

Demographics

Breast cancer is the most common malignant neoplasm occurring in women in North America, accounting for 28% of the total. At present, one in nine women will be diagnosed with breast cancer in their lifetime (Fisher, 1992). During every year of this decade it is predicted that 180,000 North American women will be diagnosed with invasive breast cancer and 30% of them will ultimately die of this disease. Presently, breast cancer accounts for 18% of cancer deaths in women, second in mortality only to lung cancer. For the past half century, mortality rates for breast cancer have remained unchanged.

Risk factors

Recognized demographic determinants for breast cancer rates include sex (females are at 100 fold higher risk than males), age (risk increases up to menopause, then increases more slowly, levels off or decreases), race (Japanese women are at less risk than American women) and social status (women of higher social status are at greater risk).

A variety of risk factors have also been recognized. These risk factors encompass: 1) risk factors associated with family and medical history including a relative with bilateral premenopausal breast cancer (less risk is involved with unilateral breast cancer), a family history with one first degree relative with breast cancer and the presence of a first primary breast cancer increases the risk of developing a second primary cancer; 2) some risk is associated with the presence of benign fibrocystic disease and primary ovarian and endometrial cancer; and 3) risk factors associated with menstrual or reproductive history including an early onset of menarche and an early establishment of regular cycles. A protective effect is associated with early menopause, early first term birth, high parity and lactation. A protective effect of early oophorectomy has also been observed. Other risk factors including ionizing radiation (leading to concerns about the value of mammography) and body build (an increased risk associated with obesity in

postmenopausal women) have also been noted (Mant and Vessey, 1991).

Also described in the literature are several disputable risk factors including: 1) factors in diet (high alcohol, fat or meat consumption); 2) exogenous steroid hormones (oral contraceptive use before the first pregnancy, the use of injectable contraceptives or hormone replacement therapy); and 3) the pattern of use of mammography. These risk factors are controversial as exemplified by a four year followup study involving 89,000 women that failed to show a correlation between dietary fat and subsequent breast cancer development (Willett *et al*, 1987).

Prognostic indicators

Traditional prognostic indicators for breast cancer are: 1) nodal status (the number of histologically involved axillary nodes); 2) the size and grade of the primary tumor; 3) the growth rate and invasive status of the tumor; 4) the nuclear grade (which reflects the degree of differentiation); 5) multicentricity and histological type of the tumor; and 6) the presence of estrogen and progesterone receptors. In addition to these indicators, several other prognostic measures are used today: 1) the tumor cell proliferative activity as measured by thymidine labelling index; 2) DNA ploidy; 3) the fraction of cells in S-phase; and 4) overexpression of EGF receptor, protease cathepsin D and stress response proteins (Leis, 1992).

Recently, the measurement of oncogene overexpression (*int-2*, *c-myc* and *c-erbB2/neu*) has been correlated to increased breast cancer nodal status (reflecting metastasis) and DNA aneuploidy (Bonadonna, 1992 and references therein) but is of unproven clinical value as of yet. One exception is the oncogene *Her-2/neu* which has been observed to be overexpressed in node positive breast cancer (McGuire *et al*, 1990) and is an independent predictor of both disease free survival and overall survival in these patients.

There is also growing evidence that development of cancers is associated with a loss of critical gene expression (Volgelstein, 1990). A loss of heterozygosity on chromosome 1,

3, 11, 13, 17 and 18 has been observed commonly in breast cancer (UCLA Colloquium, 1990: Callahan and Smith) and may prove to be of prognostic benefit in the future. Similarly the degree of tumor heterogeneity in a primary tumor is being considered as a prognostic determinate of treatment selection (Bonadonna, 1992 and references therein).

HORMONES AND HUMAN BREAST CANCER

The recognition that ovarian function is involved in mammary carcinogenesis has been derived from the observation that removal of the ovaries and therefore, removal of the ovarian sex steroids, is beneficial to breast cancer patients (Beatson, 1896). Hormones other than the sex steroids may also be involved, as adrenalectomy (Dao and Huggins, 1955) and hypophysectomy (Pearson and Ray, 1959) are also beneficial.

Estrogen

In postmenopausal patients, women with breast cancer have been observed to have a higher endogenous estrogen levels than normal women. Estrogen receptor (ER) status has been used as a prognostic factor independent of axillary node status. Estrogen and progesterone receptor (PR) positivity correlate with a better prognosis and better response to chemotherapy with or without use of antiestrogens. Over 60% of ER positive tumors and over 80% of tumors positive for both ER and PR will respond to adjuvant hormonal therapy. Receptor positive status has been associated with less aggressive tumors accompanied by a better prognosis and longer survival rates. Receptor negative status is seen with poorly differentiated cancers with a poor prognosis.

Tamoxifen

Tamoxifen is a nonsteroidal antiestrogen which functions by a competitive inhibition of estradiol binding to the estrogen receptor (Skidmore *et al*, 1972). Tamoxifen is now the antihormonal adjuvant therapy of choice for advanced breast cancer in postmenopausal node positive women partly due to its low incidence of side effects (Lerner and Jordan, 1990 and references therein). Since tamoxifen has been observed to prevent DMBA-induced mammary cancer in rodents (Jordan, 1974), its use as an approach in prevention may be feasible. Prevention strategies include treatment of healthy women at increased risk for breast cancer with tamoxifen (Bonadonna, 1992).

Androgen

The risk of breast cancer has been positively associated with testosterone and

dehydroepiandrosterone-sulfate (DHEAS) in the serum and testosterone and androstenediol in the urine of patients. Pre- and post- menopausal breast cancer patients and women with an mammary epithelial hyperplasia excrete increased testosterone and androstanediol in their urine.

Androgen receptor (AR) is positive in 30 to 35% of patients with breast cancer (Allegra *et al*, 1979; Miller *et al*, 1985). No correlation is observed with location and size of the tumor, state of progression, age of the patient, menopausal stature or lymph node metastasis. AR is however correlated with ER and PR. Eighty-five percent of primary breast tumors have been found to be AR positive (more than 10 fmol/mg protein: average 66 fmol/mg protein) compared to the 71% and 67% of breast tumors which are ER (average 87 fmol/mg protein) and PR (average 85 fmol/mg protein) positive respectively (Lea *et al*, 1989).

Retinoic acid

The pattern formation and morphogenesis of the developing embryo is influenced by retinoids through their regulation of homeobox gene expression (Dollé *et al*, 1990). The normal cellular differentiation of the epithelial tissues from which many human cancers can arise depend on retinoids. Cytoskeleton constituents such as keratins which are a measure of differentiation, and polypeptide growth factors that influence cell proliferation and differentiation, are also controlled by retinoids (for review see: Mant and Vessey, 1991). These functions have led to the suggestion that retinoids may be potentially useful in cancer prevention. Accordingly, an increased risk for breast cancer in women of 55 years of age or older has been correlated with a decreased intake of foods containing vitamin A, a precursor for retinoic acid (RA) (Groham *et al*, 1982).

Most retinoids are stored in the liver and can potentially cause hepatotoxicity (Moon *et al*, 1979). Synthetic retinoids have been found to be less toxic than naturally occurring retinoids and are therefore preferentially used in therapy (Sporn *et al*, 1976). Retinoids

have been shown to be potentially effective mammary cancer chemopreventive agents; however, investigations of retinoid-induced inhibition of growth in established tumors has met with less success (Nettesheim, 1980). Retinoids, alone or in combination with ovariectomy, antiestrogens or selenium, have been shown to inhibit initiation and promotion of DMBA or NMU (N-methylnitrosurea) induced mammary tumors in rodents (Moon and Mehta, 1990). Retinoids also inhibit growth of HBC cells alone (Fontana *et al*, 1988; Marth *et al*, 1985) or synergistically with antiestrogen (Fontana *et al*, 1987; Wetheral and Taylor, 1986; Koga and Sutherland, 1991). Synergistic growth inhibition of HBC cell lines has also been shown between RA and interferon (Marth *et al*, 1986). Studies have shown 13-*cis* retinoic acid (RA) to be effective in chemoprevention and chemotherapy alone, and in combination with α-interferon, exhibiting fewer side effects than conventional cytotoxic chemotherapy (Gudas, 1992).

Prolactin

The prolactin receptor is found in 50% of breast tumor biopsies and in human breast tumor cultures; however, clinical studies have failed to establish a role for prolactin in human breast cancer. Alternatively, in the rodent mammary gland, responsiveness to prolactin is retained in neoplastic tissue and the carcinogen-induced mammary tumors of rats are dependent on prolactin (Welsch, 1985). The latent period of these tumors is decreased by prolactin administration. Furthermore, the tumors of hypophysectomized rats regress but recur with prolactin administration. Finally, prolactin promotes the metastasis of cancer cells in spontaneous rat mammary carcinomas (Fisher and Fisher, 1963).

Human breast cancer cell lines

Many cultured human breast cancer cell lines have been established and characterized to date and these cell lines have defined an important model system to study hormonal regulation of gene expression. Several advantages are offered by the breast cancer cell line model system including: the elimination of interspecies differences between human breast cancer and animal mammary carcinoma model systems, and the ability to control

culture conditions such that the growth influences on the breast cancer cells are specifically defined by addition to the culture medium. The major disadvantage of this model system is that the defined *in vitro* culture conditions may lack the *in vivo* cell-cell and cell-matrix interactions and may not adequately describe the environment of the *in vivo* cell. Nonetheless, a number of breast cancer cell lines have been characterized for their expression of steroid and peptide hormone receptors and have been used to study the effects of these hormones on cell growth and gene expression.

Two breast cancer cell lines were used in my studies. The ZR-75-1 (Engel *et al*, 1978) and T-47D (Horwitz *et al*, 1978) human breast cancer cell lines have been characterized as containing functional receptors for estrogen, androgen, progesterone, glucocorticoid, retinoic acid (Roman *et al*, 1992) and prolactin (Shiu, 1979).

The ZR-75-1 human breast cancer (HBC) cell line is an estrogen sensitive human malignant mammary epithelial cell line (Engel *et al*, 1978). The androgens, dihydrotestosterone (DHT) and EM139 are potent inhibitors of the proliferatory effect of estradiol on ZR-75-1 cells (de Launoit *et al*, 1991). These androgens act by increasing the duration of the cell cycle. The inhibitory action of androgen on cell growth is additive to that of antiestrogens. Androgens also exert an inhibitory effect on breast cancer cell growth independent of estrogen inhibition through a decrease in the estrogen receptor (de Launoit *et al*, 1991).

Retinoids are known to inhibit growth of human breast cancer cell lines (Marth *et al*, 1985; Wetherall and Taylor, 1986; Fontana, 1987; Fontana *et al*, 1988; Koga and Sutherland, 1991). Therefore, a number of HBC cell lines were tested for the expression of the retinoic acid receptors (RARs) (Roman *et al*, 1992). The T-47D human breast cancer cell line expresses all three RARs (RARα, RARβ and RARγ) (Krust *et al*, 1989). In these cells, RA decreases the level of progesterone receptor mRNA transcription maximally at three hours suggesting that the RARs are functional (Clarke *et al*, 1990).

Additionally, RARα and RARγ levels have been shown to be decreased by progesterone in these cells.

THE PROLACTIN-INDUCIBLE PROTEIN/GROSS CYSTIC DISEASE FLUID PROTEIN-15 (PIP/GCDFP-15)

The PIP protein

Prolactin is necessary in the normal growth and development of the mammary gland and influences the production of milk. It is vital for the growth of tumors in the rat mammary gland (Welsch, 1985). Its role in human breast cancer, however, is unclear. In order to study prolactin's role in breast cancer, a model to study the mechanism of action of prolactin was sought. The T-47D human breast cancer cell line was chosen as a model cell line because of the eleven human breast cancer cell lines tested, it had the highest number of prolactin receptors (Shiu, 1979). In response to prolactin or human growth hormone, in the presence of hydrocortisone, T-47D cells exhibited a change in cell shape, loss of adhesion to a plastic substrate and increased lipid synthesis (Shiu and Paterson, 1984). They also secreted three unique proteins (11, 14 and 16 kDa) which were named the PIPs or prolactin-inducible proteins (Shiu and Iwasiow, 1985). The two larger proteins were found to be glycosylated forms of the 11 kDa protein. Antibodies to these proteins were raised and used to screen a λGT11 cDNA library made from the mRNA of T-47D cells treated with prolactin and hydrocortisone (Murphy *et al*, 1987a). A PIP cDNA was cloned and sequenced (Murphy *et al*, 1987a) revealing a cDNA of 577 base pairs with an open reading frame corresponding to a protein with 146 amino acids and a calculated molecular weight of 16.5 kDa. A glycosylation signal (Asn-X-Thr) is located at amino acids 105 to 107. The hydrophobicity plot and consensus sequences derived from many signal sequences indicated a cleavage site for PIP at amino acids 23 to 25. The sequence of the PIP cDNA was used to search a DNA sequence database (GENBANK) and indicated 51% sequence identity to a mouse salivary gland protein (Windass *et al*, 1984). This similarity increased to 67% at the protein level when conservative amino acid changes were allowed (Murphy *et al*, 1987a).

GCDFP-15

The cloning and sequencing of the PIP cDNA revealed PIP to be identical to a protein

found in the fluid of gross cystic disease of the breast (Murphy *et al*, 1987a; Haagensen and Mazoujian, 1986). This protein, called gross cystic disease fluid protein (GCDFP-15), is found in normal apocrine tissues such as axilla, perineum, Moll's glands of the eyelids, the ceruminous glands of the ear canal, serous cells of the salivary gland, minor bronchial glands and accessory lacrimal glands (Haagensen *et al*, 1990). It is a component of saliva (Murphy *et al*, 1987b) and is also immunologically identical to a component of human milk (Haagensen *et al*, 1979).

PIP/GCDFP-15 is not found in normal breast ducts or lobules, or in the plasma of normal women (Haagensen *et al*, 1990). It is, however, found in all breast cysts and is elevated in the plasma of women with gross cystic disease (Collette *et al*, 1986) and in the serum of 35-45% of patients with metastatic breast carcinoma (Haagensen, 1986; Murphy *et al*, 1987b). It is found in 60% of breast adenocarcinomas, especially lobular carcinoma (Le Doussal *et al*, 1985).

PIP/GCDFP-15 has also been localized to the apocrine metastatic epithelium lining breast cysts and in epithelium undergoing apocrine metaplasia and atypical lobular epithelial hyperplasia. Therefore, GCDFP-15 has been postulated to be a marker of apocrine metaplasia in breast epithelium. Seventy three percent of tissue explants of breast cancers release GCDFP-15 into the medium. This percentage is correlated to the degree of apocrine differentiation of the carcinoma and its androgen and progesterone receptor content (Miller *et al*, 1988). PIP/GCDFP-15 has also been correlated to epidermal growth factor (EGF) and epithelial membrane antigen (EMA) in breast cyst fluid (Collette *et al*, 1986). Furthermore, breast tumor cells immunoreactive for GCDFP-15 have been correlated to cytosolic progesterone receptor content (Le Doussal *et al*, 1985). Finally, a positive correlation exists between PIP and estrogen receptor, a major prognostic indicator in breast cancer (Murphy *et al*, 1987b).

The observation that GCDFP-15 content of plasma is significantly less when the plasma

is clotted has led to the observation that GCDFP-15 has a high binding affinity to fibrinogen (Haagensen *et al*, 1990). GCDFP-15 binding does not interfere with the clotting process and does not induce clotting of fibrinogen, and the digestion of fibrinogen by plasmin is not blocked by GCDFP-15. The significance of this binding to fibrinogen has not yet been shown.

SABP

Recently, a protein has been isolated from human seminal plasma by actin affinity chromatography and has been called secretory actin-binding protein (SABP) (Akiyama and Kimura, 1990). This protein has been sequenced and has been found to be identical in sequence with the mature PIP (Schaller *et al*, 1991). SABP is found in saliva, submandibular gland extracts and seminal vesicles but not in prostate, mammary gland, liver, pancreas, spleen or kidney. Actin binding proteins have been shown to cross link actin filaments into higher order structures and form orthogonal networks (Yin and Hartwig, 1988). Some actin binding proteins have been shown to act as bridges linking bundles of cytoplasmic actin filaments to membrane spanning integrins at sites of focal adhesion of tissue culture cells to an extracellular matrix (Burridge *et al*, 1990). The functions of actin-binding proteins appear to be intracellular, yet SABP is a secreted protein. Additionally, SABP is different from other actin binding proteins in that it has a different and unique tissue distribution and does not contain the common amino acid motifs found in actin and other actin binding proteins as characterized by Tellam *et al* (1989). The significance of the actin-binding function of PIP/SABP is questionable in view of the lack of characteristics common to other actin-binding proteins. The relevance of this possible function of PIP/SABP remains to be explored.

Hormonal regulation of the PIP/GCDFP-15/SABP

The PIP cDNA was used to study the time course of induction of the PIP mRNA by prolactin/human growth hormone and hydrocortisone in T-47D cells. The PIP mRNA is a single species approximately 800 base pairs in size (Murphy *et al*, 1987a). In these

studies, prolactin and human growth hormone were found to be equipotent in inducing the PIP mRNA; therefore, human growth hormone was used interchangably with prolactin (Murphy *et al*, 1987a). Hydrocortisone or human growth hormone individually caused an induction of 4 and 5 fold in PIP mRNA while the combination of the hormones caused an induction of 12 fold with a maximal stimulation time of 5 days (Murphy *et al*, 1987a). The stimulatory effect was specific to the lactogenic hormones: ovine growth hormone, insulin, transferrin, epidermal growth factor and basic fibroblast growth factor had no effect on PIP mRNA accumulation. The androgen, dihydrotestosterone (DHT), was found to be the most potent steroid. Using the nuclear run-on transcription assay, DHT was found to increase PIP transcription 4 fold (Murphy *et al*, 1987a). The glucocorticoids, dexamethasone and hydrocortisone, and the progestins, R5020 and ORG2058, were approximately 4 orders of magnitude less potent than DHT (Murphy *et al*, 1987a).

Extensive studies of the hormonal regulation of PIP in ZR-75-1 human breast cancer cells were done by Simard and colleagues (Simard *et al*, 1989) and the results are summarized as follows. Estradiol does not increase PIP/GCDFP-15 mRNA levels or protein secretion but can cause an inhibition of basal levels of PIP/GCDFP-15 synthesis. This effect can be abolished by hydroxyflutamide (OH-FLU). This estradiol mediated inhibition can be abolished by the antiestrogen, LY156758, suggesting that this effect is mediated through the estrogen receptor.

DHT causes an increase in both mRNA accumulation as well as protein secretion. High concentrations of dexamethasone, a synthetic glucocorticoid, causes a slight stimulation of both mRNA accumulation and protein secretion. Dexamethasone stimulates PIP/GCDFP-15 to a lower level than DHT; however, this DEX stimulation is probably mediated through the glucocorticoid receptor because DEX can increase PIP/GCDFP-15 induction above that of the maximum DHT induction. Estradiol can inhibit this DEX induction (Simard *et al*, 1989).

In T-47D cells, both DHT and R5020, a synthetic progestin, cause an increase in both PIP/GCDFP-15 mRNA levels (Murphy *et al*, 1987a) and protein secretion (Chalbos *et al*, 1987). This stimulation is thought to be mediated predominantly through the androgen receptor because the effect of both hormones can be inhibited by the antiandrogen, flutamide (Chalbos *et al*, 1987).

The progestin, R5020, causes a 2 to 3 fold stimulation of PIP/GCDFP-15 expression in these cells in the presence of estradiol; however, this stimulation is mainly abolished by the antiprogestin, RU486, and is only slightly inhibited by OH-FLU. In the absence of estradiol, R5020 has no effect on the expression of PIP/GCDFP-15 (Dauvois *et al*, 1990). In ZR-75-1 cells, progesterone receptor expression is dependent on estradiol (Poulin and Labrie, 1986) whereas T-47D cells have a high progesterone receptor content independent of estradiol (Nardulli and Katzenellenbogen, 1988; Wei *et al*, 1988). These results suggest that the majority of the effect of R5020 is mediated by the progesterone receptor with a small component mediated by the androgen receptor (Simard *et al*, 1989).

These steroidal effects on PIP/GCDFP-15 are inversely correlated with their effects on cell growth. Estradiol is a potent mitogen in ZR-75-1 cells, whereas androgens and to a lesser extent, progestins, in the presence of estradiol, and glucocorticoids cause an inhibition of cell growth (Poulin *et al*, 1988). Both DHT and R5020 can inhibit the estradiol stimulation of cell growth and these effects can be reversed by the appropriate antisteroid, OH-FLU and RU486, respectively (Simard *et al*, 1989; Dauvois *et al*, 1990). This inhibition of estradiol stimulated cell growth by DHT and R5020 can be explained by androgen's down regulation of the estrogen receptor in these cells (Poulin *et al*, 1989) and by progesterone's down regulation of its own receptor (Nardulli and Katzenellenbogen, 1988; Wei *et al*, 1988; Alexander *et al*, 1989). PIP/GCDFP-15 expression in breast cancer is correlated with ER content (Murphy *et al*, 1987b) and PR and AR content (Miller *et al*, 1988). Retinoic acid has been shown to inhibit growth of human breast cancer cells in culture (Fontana *et al*, 1988; Marth *et al*, 1985). According

to Labrie and coworkers (pers. comm.), retinoic acid increases PIP mRNA levels. The mechanism of this increase has not been identified. This opposite effect of the steroid hormones and retinoic acid on PIP/GCDFP-15 expression and cell proliferation has led Labrie and coworkers to suggest that PIP/GCDFP-15 could be involved in cell growth inhibition or regression (Simard *et al*, 1989; Dauvois *et al*, 1990).

The above studies of steroidal regulation of the PIP gene have been performed at the level of PIP mRNA and protein expression. The molecular mechanism by which hormones, specifically androgen, estrogen and retinoic acid, affect the transcription of the PIP gene has not been elucidated. The goal of this thesis is to characterize the effects of androgen, estrogen and retinoic acid on the PIP gene and to identify the regions of the PIP gene required for these effects. In order to accomplish this goal, the prior cloning of the PIP gene (Myal *et al*, 1991) was essential.

Cloning of genomic PIP

The PIP cDNA was used as a probe to clone the PIP gene from a human lymphocyte genomic library (Myal *et al*, 1991). The PIP gene is approximately 7 kilobases long and is comprised of four exons and three introns. The transcription start site has been confirmed by both S1 nuclease protection assay and primer extension analysis, and putative CAT and TATA boxes have been identified in the promoter region of the gene (Myal *et al*, 1991). The PIP gene has been localized to chromosome 7q32-26 by *in situ* hybridization (Myal *et al*, 1989).

PIP/GCDFP-15 does not at present have a defined function. Since androgen treatment of patients with breast carcinoma has led to increased plasma levels of PIP/GCDFP-15 (Haagensen *et al*, 1981; Dilley *et al*, 1983; Dilley *et al*, 1986), PIP/GCDFP-15 may be a good marker to follow the hormone responsiveness of breast cancer to hormone treatment. Additionally, since PIP/GCDFP-15 is regulated multihormonally, it may be a good model gene to study the interaction of different hormones in gene transactivation.

PROLACTIN AND PROLACTIN RECEPTORS

Human prolactin is a 22 kDa peptide (198 amino acids) which is synthesized and secreted from the lactotrophs of the anterior pituitary. Prolactin has a 16% amino acid identity with human growth hormone and 13% identity with human placental lactogen. The primate prolactin/growth hormone family consists of prolactin, growth hormone and placental lactogen (see review: Nichol *et al*, 1986). In primates, placental lactogen, also known as chorionic somatomammotropin, is a variant of growth hormone and is localized to the same gene locus. Primate growth hormone is unique compared to growth hormones from other species because it has lactogenic as well as somatogenic activity. The rat prolactin family consists of a group of proteins with structural but not necessarily functional similarity to pituitary prolactin (Nicoll *et al*, 1986). The rat prolactin, placental lactogen-II and prolactin-like proteins A, B, and C have all been localized to chromosome 17; whereas rat growth hormone is localized to chromosome 10 (Cooke *et al*, 1986).

Actions of prolactin

The actions of prolactin have been studied primarily in the rat mammary gland and corpus luteum. Prolactin stimulates growth and development of the mammary glands and in association with insulin and glucocorticoids stimulates milk protein synthesis. It also stimulates postpartum lactation (Forsyth, 1988). Prolactin is required for the extension of the functional lifespan of the corpus luteum during the rat estrous cycle (Morishige and Rothchild, 1974). Prolactin's luteotrophic actions include the stimulation of the growth of the ovarian follicle, stimulation of an increase in the number of LH (luteinizing hormone) receptors in the ovary during formation of the corpus luteum, stimulation of steroidogenesis in the corpus luteum and an increase in high and low density lipoprotein binding in the corpus luteum membranes (Armstrong *et al*, 1970; Morris and Saxena, 1980). The actions of the members of the prolactin family can be defined as: mammotrophic, the stimulation of mammary gland growth; lactogenic, the stimulation of the synthesis of milk proteins by the mammary gland; luteotrophic, the stimulation of corpus luteum function; and luteolytic, the inhibition of corpus luteum function.

During pregnancy, prolactin secretion increases and prolactin, in cooperation with estrogen, progesterone, human placental lactogen, insulin and cortisol, promotes the development of the breast in preparation for milk production (see review: Vonderhaar, 1984). The elevated levels of progesterone and estrogen cause cellular proliferation of the distal portion of the ductal tree into alveoli (proliferation of epithelial cells and enlargement of individual cells). These alveolar lobules increase in size and number and new lobules form from terminal ends and lateral walls of the ductules. The mammary fat pads regress and vascularization of the gland increases. The high levels of estrogen during pregnancy inhibit prolactin secretion, but the postpartum decrease in estrogen allows prolactin to initiate lactation.

Prolactin gene expression

The prolactin, growth hormone and placental lactogen (or chorionic somatomammotropin) genes are thought to have been derived through gene duplication from a common ancestral gene (Niall *et al*, 1971). Prolactin and growth hormone are expressed primarily in the anterior pituitary of vertebrates and placental lactogen is expressed in the placenta (Kelly *et al*, 1991). Prolactin is also expressed in lymphomas, in lymphoblastoid cell lines and in the decidua (DiMattia *et al*, 1988; Gellersen *et al*, 1989; DiMattia *et al*, 1990). Primate decidual prolactin is identical to pituitary prolactin (Hwang *et al*, 1974; Golander *et al*, 1979); however, the desidual prolactin mRNA is longer in the 5' untranslated region (Gellersen *et al*, 1989; DiMattia *et al*, 1990).

Regulation of transcription is tissue specific. Binding sites for the estrogen receptor and the pituitary specific factors, Pit-1/GHF-1 (a homeodomain protein) and LSF-1 (lactotroph-specific factor), are found in the 5' flanking region of the prolactin gene (Day *et al*, 1990; Harvey *et al*, 1991). This region directs cell specific expression to the lactotrophs and somatolactotrophs of the anterior pituitary (Frawley, 1989). TRH (thyroid hormone releasing hormone), estradiol, VIP (vasoactive intestinal peptide) and dopamine antagonists cause an increase in secretion of prolactin, and dopamine agonists cause a

decrease in prolactin secretion. The response of prolactin to TRH is suppressed by glucocorticoids and thyroid hormone (Ben-Jonathan *et al*, 1989). Although maternal circulating prolactin increases dramatically with pregnancy, the expression of prolactin mRNA in the anterior pituitary does not significantly change (Linzer and Talamantes, 1985). Therefore, this regulation is not at the level of prolactin biosynthesis, but is a result of prolactin release from the lactotroph.

The prolactin receptor

Studies in rats have shown that the highest level of prolactin binding is found in the liver. The role of prolactin in the liver is unclear; however, prolactin binding has been associated with regulation of ornithine decarboxylase (ODC), somatomedin, synlactin, insulin-like growth factor I (IGF-I) and a liver lactogenic factor (Francis and Hill, 1975; Richards, 1975; Mick and Nicoll, 1985; Murphy *et al*, 1988; Hoeffler and Frawley, 1987). In the rat liver, prolactin receptor levels are increased by estrogen administration (Posner *et al*, 1974). Inversely, in the prostate, prolactin receptor levels are increased by testosterone and decreased by estrogen (Kledzik *et al*, 1976). In the rat mammary gland, prolactin receptor levels increase early in lactation (Djiane *et al*, 1977). Furthermore, increased expression of the rat prolactin receptor gene has been observed in established DMBA tumors (Jahn *et al*, 1991a). Both prolactin and growth hormone can upregulate prolactin receptor (Posner *et al*, 1975; Baxter *et al*, 1984).

In the rat, two forms of the prolactin receptor have been observed. The shorter form is expressed primarily in the liver (Boutin *et al*, 1988) and mammary gland (Jahn *et al*, 1991b) and is encoded by a 1.8 kb transcript. The prolactin receptor has been observed to be differentially regulated in the liver and mammary gland at both the level of the mRNA and the protein. The longer form of the receptor is expressed primarily in the ovary and is encoded from three longer transcripts which result from alternative splicing of the gene (Shirota *et al*, 1990). The prolactin receptor gene in the rat is a single gene of at least 11 exons spanning 70 kb (Ali *et al*, 1991).

In the rabbit, four transcripts encoding the long form of the receptor have been observed. These transcripts are identical in all organs tested and differ only in the 5' and 3' untranslated regions (Dusanter-Fourt *et al*, 1991). In humans, three transcripts encoding the long form of the receptor have been observed in T-47D HBC cells and hepatoma cells (Boutin *et al*, 1989). In the mouse, two slightly different short forms have been identified, with cytoplasmic domains of 39 and 50 amino acids respectively (Davis and Linzer, 1989). Differential splicing of the prolactin receptor gene accounts for the variety of mRNAs encoding both long and short forms of the prolactin receptor. In the rat and mouse, both translated and untranslated exons are affected by this differential splicing, while in the rabbit and human, only the untranslated regions are affected.

The structure of the prolactin receptor from the rat liver has been deduced from the cDNA sequence (Boutin *et al*, 1988). This receptor is 291 amino acids long and contains a 210 amino acid extracellular region, a 24 amino acid transmembrane region and a 57 amino acid cytoplasmic region. The extracellular region contains five cysteines and three potential N-linked glycosylation sites, two which have been confirmed by amino acid sequence analysis (Boutin *et al*, 1988). The rabbit mammary gland prolactin receptor is comprised of 592 amino acids and is similar in structure to the rat liver receptor except that the cytoplasmic domain is 358 amino acids long (Edery *et al*, 1989). The human prolactin receptor is similar to the rabbit receptor and is 598 amino acids long (Boutin *et al*, 1989). The mouse liver contains two forms of the prolactin receptor with cytoplasmic domains of 39 and 50 amino acids (Davis and Linzer, 1989). Human and rabbit tissues express only the long form of prolactin receptors. The rat liver primarily expresses the short form while the ovary expresses the long form (Shirota *et al*, 1990). In the Nb2 pre-T cell rat lymphoma cell line that is dependent on lactogenic hormones for growth, the prolactin receptor (Ali *et al*, 1991) is 393 amino acids long. A mutation in the Nb2 prolactin receptor gene results in a loss of 594 base pairs in the region encoding the cytoplasmic domain of the long form and produces a truncated version of the long form of the receptor.

According to amino acid sequence, placental lactogens have a higher similarity to pituitary and placental growth hormones then they do to prolactin. However, in primates and rodents, placental lactogens are primarily lactogenic: they are somatogenic in other mammals (Carr and Friesen, 1976). Both PL-I and PL-II can interact with prolactin receptors in mice (Harigaya *et al*, 1988).

Human growth hormone is lactogenic (Forsyth *et al*, 1965) and binds to both the growth hormone receptor and the prolactin receptor. Human growth hormone binding to the prolactin receptor is modulated by zinc ions (Cunningham *et al*, 1990). A 44 kDa receptor from fetal and maternal ovine liver which specifically binds ovine placental lactogen but not prolactin or growth hormone has been identified (Freemark and Comer, 1989). A human growth hormone mRNA with an exon 3-specific deletion has been identified in the placenta and is the only form of hGH receptor in the placental villi (Urbanek *et al*, 1992). The distribution of the exon 3 containing or deleted forms of the hGH receptor mRNA is tissue specific. This exon 3 encoded region is present in all of the cloned GH receptors and is absent in the cloned prolactin receptors, which suggests a role for this region in differentiating lactogenic receptors from somatogenic (Urbanek *et al*, 1992). The characterization of the exon 3 deleted hGH receptor isoform suggests that this receptor may have a lactogenic specificity and is potentially a placental lactogen receptor (Urbanek *et al*, 1992).

Prolactin receptors are members of a large hematopoeitic cytokine receptor protein superfamily (Bazan, 1990; Cosman *et al*, 1990). Each receptor contains a similar extracellular domain and differs in the cytoplasmic domain. The characteristics of this family are a 14 to 25% amino acid identity over a 200 amino acid region. Two pairs of cysteines in the N-terminal region are thought to form a ligand binding pocket for the specific ligand (Rozakis-Adcock and Kelly, 1991; Rozakis-Adcock and Kelly, 1992). The C-terminus contains a WSxWS (tryptophan-serine-amino acid-tryptophan-serine) motif which is conserved in all members of the gene family except for the growth hormone

receptor which contains conservative changes in those positions. The extracellular region is predicted to form two homologous immunoglobulin-like subdomains of 100 amino acids, each containing seven antiparallel β-strands (Thoreau *et al*, 1991).

Prolactin receptor acts at the cell surface and must depend on a secondary messenger to mediate prolactin's effects. No clear signal transduction pathway has been identified with the prolactin receptor. G proteins (Too *et al*, 1990), phospholipases A2 and C (Ofenstein *et al*, 1987), protein kinase C (Buckley *et al*, 1986; Buckley *et al*, 1988), intracellular calcium influx (Buckley *et al*, 1986) and receptor associated tyrosine kinases (Stredt *et al*, 1990) have all been associated with prolactin receptor cellular transduction. In addition, cAMP can affect prolactin-induced mitogenesis of Nb2 rat lymphoma cells (Larsen and Dufau, 1988) although prolactin does not modulate cAMP concentrations (Kornberg and Liberti, 1989). Recently, the β-lactoglobulin gene promoter linked to a reporter gene, chloramphenicol acetyl transferase (CAT) was cotransfected into Chinese hamster ovary (CHO) cells with a cDNA encoding the long form of the prolactin receptor (Leseuer *et al*, 1990). A 4.6 fold increase in CAT activity was observed in the presence of serum. In the absence of serum, CAT activity was increased 12 to 20 fold (Lesueur *et al*, 1990; Leseuer *et al*, 1991). A similar experiment using the short form of the receptor cotransfected with the β-lactoglobulin-reporter construct did not result in a prolactin-mediated increase in CAT activity (Leseuer *et al*, 1991). Both the long form and the truncated Nb2 form of the prolactin receptor were shown to mediate a prolactin-stimulated increase in CAT activity when cotransfected with a 2.3 kb of the 5' flanking region of rat β-casein-CAT construct (Ali *et al*, 1992). These experiments suggest that a "prolactin-responsive element" may be contained within the promoter region of the β-lactoglobulin gene and the 5' flanking region of the rat β-casein gene. Furthermore, the short form of the prolactin receptor is insufficient to mediate this response and serum may contain a factor which inhibits the response.

Prolactin regulated genes

The caseins are the most abundant proteins in the lactating mammary gland. They function to provide supersaturating concentrations of calcium, phosphates and amino acids in the milk. The casein genes belong to a multigene family with considerable divergence; however, the 5' flanking region is highly conserved. Synthesis and accumulation of casein mRNA in rat and mouse mammary gland explants requires insulin, hydrocortisone and prolactin (Rosen *et al*, 1986). All three hormones enhance casein transcription (Rosen *et al*, 1989). Prolactin causes a 2 to 4 fold increase in transcription while all three hormones together increase transcription 10 fold (Goodman and Rosen, 1990). Prolactin and hydrocortisone also stabilize the casein mRNA with prolactin causing a 125 fold increase in mRNA accumulation (Chomczynski *et al*, 1986). Insulin has no effect on stability. Chomczynski *et al* (1986) have also observed that the casein 5' flanking region is bidirectional and produces an antisense mRNA. This antisense mRNA is not hormonally regulated.

WAP (whey acidic protein) is the major whey protein in rodent milk. WAP is a member of the four disulfide core protein family (Hennighausen and Sippel, 1982) and its expression requires insulin, prolactin and glucocorticoids (Burdon *et al*, 1991). The 5' flanking sequences of WAP can confer tissue specific and hormone regulation on a heterologous gene (Andres *et al*, 1987).

α-lactalbumin is the major whey protein in the lactating mammary gland of most mammals except rodents (Brew and Hill, 1975). In the mouse, prolactin, hydrocortisone and insulin are required for α-lactalbumin expression, and epidermal growth factor, hydrocortisone and insulin are required for expression in the rat (see review: Vonderhaar and Ziska, 1989). Glucocorticoids are necessary for α-lactalbumin induction and accumulation; however, high concentrations inhibit accumulation (Ono and Oka, 1980) and secretion (Bhattacharjee and Vonderhaar, 1984). Progesterone inhibits α-lactalbumin production (Vonderhaar, 1977).

As stated previously, clinical studies have failed to establish a role for prolactin in human breast cancer. One problem associated with these studies is the lack of a definable end point of prolactin action. The prolactin-inducible protein (PIP/GCDFP-15) has now provided that end point in an easily manipulatable model system and may help to define the importance of prolactin in human breast cancer.

REGULATION OF TRANSCRIPTION

Eukaryotic gene expression is controlled by the binding of *trans*-acting sequence-specific transcription factors to *cis*-acting DNA elements (see review: Ptashne, 1988). These transcription factors can be either general transcription factors which form the preinitiation complex at the transcription start site or transcription factors which bind specific DNA regulatory elements and can activate or repress the transcription of a target gene. These DNA regulatory elements are usually in the 5' flanking region of genes, but have also been identified downstream of the transcription initiation site.

Regulatory transcription factors

Cell or tissue extracts contain *trans*-acting transcription factors which bind to *cis*-acting DNA elements and can be identified according to their sequence specificity in various *in vitro* DNA binding assays (see review: Mitchell and Tjian, 1989). The important domains in these *trans*-activating factors can be identified by deletion analyses which compare the binding properties and transcriptional activities of the deletion mutants to the wild type factor. The transcriptional activities of wild type and mutant factors can be assayed by transiently expressing them in tissue culture cells and measuring the level of transcription of a cotransfected reporter gene controlled by a promoter containing the factor-binding site. By using these types of assays, DNA-binding transcription factors were characterized as having separable DNA binding and transcriptional activation domains (see review: Ptashne, 1988).

DNA binding domains

Three different types of DNA binding domains have been identified. One of the DNA binding domains was first characterized in the RNA polymerase III transcription factor TFIIIA. TFIIIA binds to the internal control region of the 5S RNA gene through a "zinc finger" motif (Miller *et al*, 1985). The zinc finger consists of approximately 30 amino acids in which two cysteine and two histidine residues tetrahedrally coordinate a Zn^{+2} ion. Similar zinc fingers are found in the mammalian transcription factor Sp1 (Kadonaga *et*

al, 1987). A different type of zinc finger is found in the DNA binding domain of the nuclear hormone receptors in which two pairs of cysteines tetrahedrally coordinate the Zn^{+2} ion (Evans and Hollenberg, 1988; Freedman *et al*, 1988).

A second type of DNA binding domain is referred to as the homeodomain. This domain is approximately 60 amino acids long and was originally found in regulators of *Drosophila* embryogenesis (see review: Kessel and Gruss, 1990). The homeodomain has now been observed in vertebrate transcription factors, and is distantly related to the helix-turn-helix motif found in prokaryotic repressors. A subclass of homeodomain proteins are referred to as the POU domain proteins (Herr *et al*, 1988). This POU domain contains approximately 160 amino acids and contains a homeodomain as well as a conserved region called the POU box. POU is an acronym based on the first characterized proteins of this homeodomain subclass: the pituitary specific Pit-1, the octamer binding proteins Oct-1 and Oct-2 and the nematode developmental regulatory protein, *unc-86*.

The third type of DNA binding domain consists of a 30 amino acid region of basic residues followed by a region containing four leucine residues spaced 7 amino acids apart. The basic region is responsible for DNA binding and the leucine-rich region, referred to as a "leucine zipper", is responsible for dimerization of the protein (Landschulz *et al*, 1988; O'Shea *et al*, 1989; Sauer, 1990). This leucine zipper defines a number of transcription factors including C/EBP, *Jun*, *Fos*, *Myc* and CREB (Jones, 1990). Other transcription factors including *Myc*, MyoD or the *Drosophila* developmental gene, *achaete-scute*, contain a motif homologous to the leucine zipper structure consisting of a helix-loop-helix motif in which hydrophobic amino acids arranged on one side of paired helices form a dimerization domain (Dang *et al*, 1989; Barinaga, 1991).

Transcriptional activation functions (TAFs)

These DNA binding transcription factors contain one or more transcriptional activating

functions (TAFs) which are distinct from the DNA binding domain. Three types of common TAF motifs have been characterized (see review: Mitchell and Tjian, 1989). They do not appear to have conserved primary amino acid sequences but instead have regions of similar overall structure. The first defined activation regions were found in yeast. The transcription factors GAL4 and GCN4 contain regions of acidic amino acids which can form amphipathic α-helical structures (Brent and Ptashne, 1985; Hope and Struhl, 1986). Similarly one of the two transcriptional activation domains in the glucocorticoid receptor forms an acidic amphipathic α-helix (Hollenberg and Evans, 1988). These acidic activation domains are hypothesized to interact with a component of the preinitiation complex in a nonspecific fashion (see review: Struhl, 1991).

A second type of TAF was found in the transcription factor Sp1. In Sp1 the two most potent of the four regions which contribute to transcriptional activation were found to consist of 25% glutamine residues (Courey and Tjian, 1988). A number of other transcription factors in *Drosophila* and yeast, as well as the mammalian transcription factors Oct-1 and Oct-2, *Jun*, AP-2 and SRF have a glutamine rich region. The third type of transcriptional activation domain which characteristically contains 20 to 30% proline residues is found in the transcription factor CTF/NF-1 (Mermod *et al*, 1989). This proline rich region is also found in AP-2, *Jun*, Oct-2 and SRF (serum response factor). These TAFs have been hypothesized to interact with and potentially stabilize the binding of one of the general transcription factors which make up the preinitiation complex or with one of the subunits of RNA polymerase II.

The basal or general transcription complex

The specific transcription factors bind to regulatory elements and stimulate the assembly of the basal transcriptional apparatus at the cap site (see review: Ptashne and Gann, 1990). The basal transcriptional apparatus is made up of the core promoter elements, their associated binding factors and RNA polymerase II. The core elements of eukaryotic class II promoters are the TATA box and the Inr initiator element, which are targets of the regulatory proteins (Smale and Baltimore, 1989). Only the TATA box binding factor,

TFIID, and the Inr binding factor, TFII-I, have intrinsic promoter binding activity and are capable of nucleating the assembly of the preinitiation complex (PIC). A number of general transcription factors have been characterized, including TFIIA, IIB, IID, IIE, IIF, IIG/J, IIH and II-I (Buratowski *et al*, 1989). The chain of assembly of the preinitiation complex is binding of TFIID, followed by TFIIA, RNA polymerase II, TFIIF and TFIIE. Alternative preinitiation complexes containing either TFIID and TFIIA, or TFIID and TFII-I have been demonstrated, suggesting that TFII-I can substitute for TFIIA (Roeder, 1993). Although TFIID functions in binding the TATA box of promoters, those promoters which lack the TATA sequence still requires TFIID. The TFIID→TFIIA pathway binds and initiation formation of the PIC through the TATA box, whereas the TFIID→TFII-I pathway utilizes both the TATA and Inr sequences. A number of eukaryotic promoters which do not have TATA box sequences activate transcription using this pathway (Roeder, 1993).

TFIID is made up of the TATA binding protein (TBP) as well as TBP associated subunits (Lillie and Green, 1989). X-ray crystallographic studies have shown the TBP to form a "saddle" structure which sits on the DNA (Nikolov *et al*, 1992). The TBP has been shown to directly interact with TFIIA, TFIIB, TFII-I, transcription activation functions (TAFs), RNA polymerase II and DNA. TFII-I contains direct repeats with a 60 amino acid helix-loop-helix like DNA binding motif which could provide a mechanism of interaction with upstream regulatory factors. TFII-I also contains a lysine repeat motif which is involved in the binding of TFIIA.

Regulation of transcription

The core promoter elements and their associated binding factors have an intrinsic ability to initiate transcription *in vitro*; however, functional interactions are limited *in vivo* (Roeder, 1993). The binding affinity of the general transcription factors is weak and access to the binding sites is frequently blocked by chromatin structures. In addition, the general cofactor USA represses basal activator-independent transcription and potentiates activator-dependent transcription. High level induction of genes requires additional

cofactors which are nonessential for basal transcription (Roeder, 1993).

Such cofactors include the ligand-dependent nuclear hormone receptors. In the target cell, nuclear hormone receptors bind their appropriate ligand which causes dissociation of heat shock proteins from the receptor (O'Malley, 1993). This dissociation converts the receptor into an allosteric form capable of dimerizing and binding to its recognition sequence (hormone response element). The receptor can then recruit and stabilize the general transcription factors to target gene promoters and cause RNA polymerase II to initiate transcription (O'Malley, 1993).

Recent studies have shown that retinoic acid receptor (RAR) interacts with basal transcription factors to activate the transcription of genes. In embryonal carcinoma (EC) cells, RAR and the human TATA box-binding factor TFIID functionally interact to activate the RARβ2 promoter. This interaction requires both the TATA box and the retinoic acid response element (RARE) (Berkenstam *et al*, 1992). Cooperation is independent of both position and distance between the TATA box and the RARE. It does not specifically require the RARβ2 TATA box as the adenovirus major late promoter TATA box can substitute for the RARβ2 TATA box (Berkenstam *et al*, 1992). EC cells also contain an E1A-like activity and in COS cells, E1A cotransfection is necessary for TFIID and RAR cooperativity (Berkenstam *et al*, 1992). E1A has been shown to bind TFIID *in vitro* (Lee *et al*, 1991); therefore, E1A, or the EC cells' E1A-like activity, is hypothesized to mediate the RAR and TFIID interaction (Berkenstam *et al*, 1992).

Repression of transcription

Transcription factors can not only activate target genes but can also can repress their transcription. This repression occurs through three possible mechanisms (see review: Levine and Manley, 1989). The first mechanism is through direct blocking by the repressor protein on regulatory elements comprising the promoter. By this mechanism, the general transcription factors are blocked from interacting with the promoter or

upstream activator proteins are prevented from binding their DNA elements. The repression of estrogen activation by thyroid hormone occurs through this mechanism (Glass *et al*, 1988). The thyroid hormone receptor (TR) binds the consensus estrogen response element (ERE) *in vitro* and blocks estrogen receptor (ER) induced activation. TR binds as a dimer to its thyroid hormone response element (TRE). If the half sites of the element are adjacent (as in the TRE), then TR is an activator. If the half sites are separated (as in the ERE), then the TR is inactive (Glass *et al*, 1988).

The second mechanism of transcriptional repression is referred to as quenching (Han *et al*, 1989). The activator and repressor proteins bind to adjacent nonoverlapping DNA sequences and protein-protein interactions between the two proteins prevent the activator from making proper contact with the transcription complex. Glucocorticoid receptor (GR) has been shown to inhibit binding of the protooncogene *c-Jun* to the glucocorticoid response element (GRE) most likely by a protein-protein interaction. *c-Jun* is a component of the AP-1 transcription factor and *c-Jun* or AP-1 can block the binding of GR on a GR responsive promoter (Jonat *et al*, 1990; Schüle *et al*, 1990; Yang-Yen *et al*, 1990).

The final mechanism of transcriptional repression is referred to as squelching (Gill and Ptashne, 1988). In squelching, overexpression of a repressor results in the sequestration of other transcription factors which are essential for *trans*-activation by an activator. For example, in the rat prolactin gene, the 5' flanking region mediates repression in response to estrogen (Adler *et al*, 1988). This repression is not through direct contact of the ER DNA binding domain since the ERE can be mutated or the DNA binding domain can be destroyed without affecting the repression. Therefore, this effect may be through squelching. The DNA sequences required for ER repression correspond to the Pit-1 binding sites which are required for activation of the prolactin gene's transcription (Adler *et al*, 1988).

STEROID HORMONE RECEPTORS

The steroids, retinoids, thyroid hormones and vitamin D_3 are chemically unrelated hormones with diverse functions. The steroid hormones play a vital part in reproductive processes as well as the normal metabolic functions of the body. The retinoids are important in development and differentiation, thyroid hormone is necessary in normal metabolic function and vitamin D_3 is required for calcium regulation. Nevertheless, these hormones have a common link in that they exert their effects by interacting with a ligand-dependent transcription factor belonging to the nuclear hormone receptor superfamily.

The nuclear hormone receptor superfamily comprises a group of related genes including the receptors for the steroids (estrogen, glucocorticoid, progesterone, androgen and mineralicorticoid) as well as vitamin D_3, thyroid hormone and retinoic acid. Other members of this gene family are called orphan receptors as they do not yet have identified ligands (see review: Carson-Jurica *et al*, 1990).

Most of the members of the nuclear hormone receptor family are single proteins synthesized from single mRNAs. Exceptions to this generalization include: the progesterone receptor (PR) which has two forms, A and B, which are derived from alternate initiation of a single mRNA (Conneely *et al*, 1987; Gronemeyer *et al*, 1987; Misrahi *et al*, 1987); the thyroid hormone receptor (TR) of which two genes encode two receptors, α and β (Murray *et al*, 1988); and the retinoic acid receptor (RAR) and retinoid X receptor (RXR) in each of which three genes encode three receptor mRNAs, α, β and γ (see review: De Luca, 1991).

Receptor structure

During the past decade, the known members of the nuclear receptor gene superfamily have been cloned and characterized (see review: Carson-Jurica *et al*, 1990). These receptors have a similar modular structure of six domains (A-F): the most highly conserved in sequence being the C domain or DNA binding domain (DNA binding

domain). This DNA binding domain is composed of 68 amino acids and can be further subdivided into two DNA binding "zinc finger" motifs of 20 invariant amino acids, nine of which are cysteines (Freedman *et al*, 1988; Hollenberg and Evans, 1988). Each "zinc finger" motif consists of four cysteines which tetrahedrally coordinate a molecule of zinc. With some exceptions, the genes have eight exons with the first exon encoding the A/B domain and exons two and three encoding the first and second zinc DNA binding fingers, respectively. For the RAR, RXR and TR, the first several exons are alternately spliced to encode a variable domain A while the remaining exons encode identical domains B to F (see review: De Luca, 1991).

The three dimensional solution structure of the ER (Schwabe *et al*, 1990), GR (Härd *et al*, 1990) and RXR (Kliewer *et al*, 1993) DNA binding domains have been resolved by nuclear magnetic resonance (NMR) spectroscopy. These studies have revealed that the two zinc finger motifs of the receptor DNA binding domains fold to form a single structural domain consisting of two perpendicularly oriented α-helices similar in structure to the helix-turn-helix motif found in many DNA binding transcription factors. In addition, the RXR DNA binding domain contains a third α-helix directly downstream of the second zinc finger helix (Kliewer *et al*, 1993). This third helix is proposed to function as a dimerization interface mediating homodimerization and binding of the RXR to its response element.

Recently, the X-ray crystallographic structure of the glucocorticoid receptor DNA binding domain complexed with DNA was reported by Luisi *et al* (1991). They resolved the crystal structure of the GR DNA binding domain complexed to a DNA element comprised of two hexameric GRE half-sites separated by either three or four base pairs. The interaction of one subunit of the dimer with DNA leads to dimerization and cooperative DNA binding of the second subunit. The two subunits make several protein-protein contacts when bound to their respective half-sites. The recognition helices of the dimer subunits interact with successive major grooves of the DNA and do not contact the minor

groove. Luisi *et al* (1991) proposed a model for receptor binding in which one DNA binding domain binds specifically to DNA and the other binds nonspecifically. This study revealed that the DNA binding domain of GR, when dimerized, bound correctly to half-sites separated by three base pairs and not four revealing the importance of the half-site spacing in the hormone response elements. Furthermore, the study confirms the protein-DNA interactions predicted by the NMR spectroscopy studies and by receptor mutagenesis studies.

Hormone response element recognition

The DNA binding domain recognizes DNA elements refered to as hormone response elements. Three amino acids at the 3' base of the first zinc finger allow discrimination of binding sites or hormone response elements (HREs) among the different receptors (Danielson *et al*, 1989; Mader *et al*, 1989; Umesono and Evans, 1989) within a region of eight amino acids called the "P box". The P box of GR, PR, AR and MR contains the residues glycine, serine and valine and recognizes a HRE containing the consensus element TGTTCT (Danielsen *et al*, 1989). The ER P box contains the residues glutamine, glycine and alanine and recognizes the consensus element half-site, AGGTCA (Mader *et al*, 1989). If the specific residues of the P box of the ER are mutated to those of the GR, the mutant receptor recognizes a GRE and inversely, if the P box of the GR are mutated to those of the ER, the mutant receptor recognizes an ERE (Green *et al*, 1988). The RAR, TR and VDR P box contains the residues glutamine, glycine and glycine and the orphan receptors, COUP-TF and EAR-2 contain the residues glutamine, glycine and serine. The ER, RAR, TR, VDR, RXR, COUP-TF and EAR-2 all recognize the consensus half-site AGGTCA (Forman and Samuels, 1990). The "D box" at the 5' base of the second zinc finger recognizes spacing between the half-sites of the responsive element (Green and Chambon, 1989).

Hormone response elements

The nuclear hormone receptors have been classified into groups according to two criteria:

the recognition of a consensus HRE and the presence of certain amino acids responsible for that recognition. The group containing the receptors for the retinoids, thyroid hormones and vitamin D_3 contain the amino acids glutamine, glycine and glycine at the appropriate discriminatory positions and recognize the consensus half-site AGGTCA and its variations (Forman and Samuels, 1990). The thyroid hormone receptor (TR), the vitamin D_3 receptor (VDR) and the retinoic acid receptor (RAR) can discriminate between their HREs by recognizing a 3, 4 or 5 nucleotide space respectively, between the direct repeats of the consensus half-site sequence, AGGTCA (Näär *et al*, 1991; Umesono *et al*, 1991). Both TR and RAR can recognize and bind an inverted repeat of the consensus sequence (Umesono *et al*, 1988). Although these receptors have similar binding requirements, their ligands mediate very specific and different actions. This incongruity can be explained by the differential expression of these receptors in different cell types, by the interaction of these receptors with each other and with specific transcription factors and by the metabolism or alteration of ligands within specific cell types. As stated in the previous section, the GR, PR, AR and MR can recognize identical responsive elements containing the consensus half-site, TGTTCT (Strähle *et al*, 1987; Ham *et al*, 1988). Discrimination of the responsive elements between members of this group is controlled by the specific base pairs comprising the element and the context of the element in the hormone responsive promoter. The ER recognizes a palindrome of the consensus half-site, AGGTCA, separated by three base pairs (Klein-Hitpass *et al*, 1986; Martinez *et al*, 1987).

As mentioned above, the RXR heterodimerizes with the TR, VDR and RAR on DNA elements comprised of direct repeats of the core recognition half-site (AGGTCA) separated by three, four or five base pairs. Until recently, the polarity of these heterodimers on the hormone response element was unknown. The studies by Glass *et al* (1993) have revealed that the RXR is positioned over the upstream element. The C-terminal dimerization interface of RXR preferentially faces the 3' end of the core recognition half-site, while the dimerization interfaces of the RXR partners face in the

opposite direction. This preference determines polarity of the heterodimer on the DNA element.

The ligand binding domain / dimerization domain

The E domain or ligand binding domain (LBD) is complex, containing regions necessary for ligand binding as well as dimerization and also a region mediating hormone-relieved repression (Carlstedt-Duke *et al*, 1987; Godowski *et al*, 1987; Kumar and Chambon, 1988). The LBD has two regions of high sequence conservation between the members of the nuclear receptor family. The dimerization region encompasses nine hydrophobic heptad repeats similar to those seen in the leucine zipper and helix-loop-helix domains found in other transcription factors (Abel and Maniatis, 1989; Jones, 1990). These repeats, which consist of hydrophobic amino acids at positions one, five and eight of the heptad, form a hydrophobic interface on one side of an α helix which is thought to mediate dimerization between two receptors (Forman *et al*, 1989). The dimerization region is flanked on either side by ligand binding regions which are thought to interact directly with the ligand and indicate its specificity. A hormone-inactivation region, τ_i is observed between the N-terminal area of the C-terminal ligand binding domain and the dimerization region. This region is thought to confer transcriptional inactivation to the receptor in the absence of ligand binding. This inhibition is relieved by the binding of hormone. The TR and RAR have been observed to form heterodimers and this interaction allows the recognition and binding to specific TREs by the RAR (Glass *et al*, 1989). This cooperative interaction requires the DNA binding and ligand binding domains of both receptors. The dimerization domain in the LBD is not critical for DNA binding by the C domain but instead is hypothesized to be required to juxtapose the adjacent N- and C-terminal ligand binding regions of two receptors (Forman and Samuels, 1990). DNA binding domain negative mutants of TR can act in a dominant negative manner for both TR and RAR. These mutants are transcriptionally inactive and exert their effects by forming heterodimers with the wild type receptor (Glass *et al*, 1989; Sharif and Privalsky, 1991). These heterodimers are incapable of activating transcription (Herskowitz, 1989).

Other domains

The four largest receptors, GR, PR, AR and MR, contain an amino acid sequence highly similar to the SV40 nuclear translocation signal (NTS) (Kalderon *et al*, 1984; Landford *et al*, 1986). The estrogen receptor (ER), thyroid hormone receptor (TR), retinoic acid receptor (RAR) and the vitamin D receptor (VDR) have less similarity in this region (see review: Carson-Jurica *et al*, 1990). Most of these receptors have been observed to be nuclear in the presence or absence of their ligand; however, in the absence of ligand, the localization of GR and MR within the cell appears to be cytoplasmic (Wikstrom *et al*, 1987). All receptors are nuclear in the presence of their ligand.

In the absence of ligand, some of the inactive receptors are thought to be associated with other proteins and RNA in large complexes. One of the components of this complex is the 90 kDa heat shock protein (*hsp90*). *hsp90* has been associated with ER, GR, PR, AR and MR (Catelli *et al*, 1985; Redeuilh *et al*, 1987; Renoir *et al*, 1986; Sanchez *et al*, 1985). The receptors interact with *hsp90* through the C-terminal region (Denis *et al*, 1988). Two *hsp90* molecules are thought to interact with one molecule of receptor (Radanyi *et al*, 1989). The receptor is unable to bind DNA while complexed to *hsp90*. Upon binding the ligand, a process referred to as activation, *hsp90* is released and the activated receptor can dimerize and bind to specific DNA sequences referred to as hormone responsive elements (HREs). VDR, RAR and TR are not associated with *hsp90* (see review: Carson-Jurica *et al*, 1990; Yang *et al*, 1991).

Transactivation

The function of the nuclear receptors is to transcriptionally activate or repress genes in response to the binding of its ligand. The CAT or luciferase reporter system has been used as a model with which to identify the regions of the receptor protein responsible for this function. In the human GR, a hormone dependent activation domain has been identified in the LBD (Hollenberg and Evans, 1988). This 30 amino acid region has been called *tau2* to distinguish it from the activation domain in the N-terminus of the receptor,

called *tau1*. The transactivation domains of the human GR are acidic in nature, similar to the acidic activation domains (AAD) of the yeast transcription factors, GAL4 and GCN4 (Ma and Ptashne, 1987; Hope and Struhl, 1986). The human ER also has two regions which are responsible for the transactivating function of the receptor (Tora *et al*, 1989). These regions are called TAF1 and TAF2 (transcriptional activation function). The hormone-dependent TAF2 is located in the LBD and the hormone independent TAF1 is located in the N-terminus of the receptor. However, structurally these TAFs are different from the *tau1* and *tau2* of the GR in that they contain no acidic blobs or amphipathic helices common to many transcription factors. TAF1 is cell type specific and has been shown to have properties different from acidic activation domains (Tora *et al*, 1989). The TAFs and AADs interact with different factors to exert their transactivating effects (Tasset *et al*, 1990).

The nuclear receptors can activate or repress the expression of their target genes alone, through simple HREs or in combination with other transcription factors, including other nuclear receptors, through complex hormone regulatory regions in the promoters of target genes. Synergism between ER and PR or ER and GR has been observed in the chicken vitellogenin gene (Cato *et al*, 1988; Ankenbauer *et al*, 1988) and the GRE/PRE has been observed to mediate interactions with Sp1, OTF-1, NF-1 and the CACCC box binding protein (Strähle *et al*, 1988; Schüle *et al*, 1988). Also, the TR can heterodimerize with the RAR to enhance its binding to TREs (Glass *et al*, 1989) and RAR can form inactive heterodimers with RXR to inhibit activation through a RXRE (Mangelsdorf *et al*, 1991). However, RXR can heterodimerize with TR, VDR and RAR to enhance transactivation through TREs, VDREs and RAREs (Yu *et al*, 1991). Negative regulation of genes can be accomplished by the presence of a negative HRE. Several examples of negative GREs have been characterized (see review: Carson-Jurica *et al*, 1990). The TR can recognize and bind to an ERE and repress transactivation by the ER (Graupner *et al*, 1991). In the rat prolactin gene, ER can repress transactivation, an effect which is not mediated through the DNA binding domain or the LBD, but through an interaction with a pituitary specific

transcription factor, Pit-1 (Adler *et al*, 1988).

Many transcription factors are regulated by phosphorylation through kinases and phosphatases. Most of the members of the nuclear receptor family are phosphoproteins. The binding of their ligand is frequently associated with hyperphosphorylation in specific areas of the receptor. This observation suggests a function for phosphorylation in the function of the receptor; however, no conclusive evidence for this suggestion thus far exists (see review: Ortí *et al*, 1992).

Orphan receptors

Many of the receptors, classified as orphans due to a lack of an identified ligand, have sequence similarity to the RXRs and have been shown to heterodimerize with other nuclear receptors. Peroxisome proliferators activate an orphan receptor (PPAR) which forms heterodimers with RXRα to activate acyl CoA oxidase gene expression in response to clofibric acid or 9-*cis* RA. PPAR and RXRα act synergistically. The PPRE is AGGACAaAGGTCA (Osumi *et al*, 1991; Tugwood *et al*, 1992; Dreyer *et al*, 1992). RXRα and 9-*cis* RA can activate the PPRE. PPAR and clofibric acid can also activate the PPRE and the combination is synergistic. PPAR or RXRα does not bind to the PPRE efficiently in solution alone but strongly bind when mixed. PPAR-RXRα also binds DR+1 HREs like CRBPII but binds only weakly to the RARβ RARE and MLV-TRE and not at all to the VDRE in the osteopontin gene. Both receptors are highly expressed in liver and kidney: these tissues are principal sites of action of peroxisome proliferators. RXRα is the main protein in the liver which enhances PPAR binding to the PPRE (Kliewer *et al*, 1992c).

NGF1-B is a gene which is expressed early on in the response of PC12 pheochromocytoma cells to NGF and fibroblasts to serum (Milbrandt, 1988; Hazel *et al*, 1988). NGF1-B binds the element, AAAAGGTCA, and is a strong activator of transcription. The "A" box of 7 residues (344-350) at the C-terminus of the DNA binding

domain is necessary for recognition of the AT base pairs at the 5' end of the binding element and is required for 5' adjacent sequence recognition (Wilson *et al*, 1992). Deletion of the C-terminus, which is required for the dimerization of other receptors, does not reduce the affinity of NGF1-B to the NBRE (Wilson *et al*, 1992). However, deletion of the A box causes a loss in transactivating ability. A transactivating domain of 18 amino acids has been identified in the N-terminus and deletion of portions of the C-terminus cause a reduction in the ability to transactivate a reporter gene (Paulsen *et al*, 1992). Paulsen *et al* (1992) also characterized one C-terminus deleted mutant which is constitutively active in mammalian cell lines and is much less active in *Drosophila* S2 cells. They postulate that in insect cells lack a NGF1-B ligand or other necessary accessory factors.

ANDROGENS AND ANDROGEN RECEPTORS

Actions of androgen

In males, gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates release of luteinizing hormone (LH) from the pituitary. LH in turn stimulates secretion of androgens from the testes (for review see: Braunstein, 1986). The Leydig cells constitute the major endocrine component of the testes. The primary secretory product of the Leydig cells are the androgens: testosterone, dihydrotestosterone (DHT), dihydroepiandrosterone (DHEA) and androstenedione. Ninety five percent of total androgen is produced in the testes and the remainder is produced in the adrenal cortex. Of the total circulating testosterone in males, 60% is bound by sex hormone binding globulin, 38% is bound by albumin and 2% is free in the circulation. Adrenal androgens are primarily a source of precursors to testosterone which are peripherally converted to testosterone. They contribute minimally to total androgens in normal males but constitute a major source of androgens in females due to the low amount of ovarian androgen produced. In androgen target tissues, the microsomal enzyme, 5α-reductase converts testosterone to its more potent form, dihydrotestosterone (for review see: Braunstein, 1986).

Historically, the action of androgen on its target tissues has been classified as either anabolic or androgenic. Anabolic effects occur in tissues other than the reproductive tract, such as the kidney, liver, submaxillary gland and muscle. In the mouse kidney, androgens are responsible for hypertrophy of the tissue but do not stimulate DNA synthesis. In the mouse liver, androgens increase the rate of transcription of mRNA for major urinary proteins. In the submaxillary gland, testosterone increases DNA synthesis and stimulates cell proliferation. Androgenic effects encompass differentiation of the male phenotype and occur in reproductive tract tissues (Berger and Watson, 1989). In the fetal or neonatal male reproductive system, androgens prevent programmed cell death of the Wolffian ducts and induce cell death in the embryonic mammary epithelial rudiment. The morphogenesis and patterning of Wolffian duct structures, the prostate, the bulbourethral

glands and the seminal vesicle are all under the influence of androgens. Androgens stimulate growth of these target tissues and also regulate a variety of secretory proteins in the male reproductive tract (Cunha and Young, 1991).

Androgens are required for sexual differentiation in males from the establishment of gonadal sex to the development of male internal and external genitalia. They are also required for the maturation of the male reproductive system at puberty and are responsible for the development of male secondary sex characteristics (for review see: Braunstein, 1986). Testosterone and dihydrotestosterone exert their effects on target tissues by interacting with a nuclear androgen receptor. This androgen receptor is a member of the ligand-activated nuclear receptor superfamily of genes (Chang *et al*, 1988; Lubahn *et al*, 1988; Trapman *et al*, 1988; Tilley *et al*, 1989).

Androgen receptor gene structure

The human androgen receptor gene has a exon/intron structure similar to that of the other cloned steroid receptor genes and spans approximately 90 kilobases on chromosome Xq11-q12 (Brown *et al*, 1989). The first exon contains the 5' untranslated region of the mRNA and the region encoding the N-terminal portion of the receptor. Exons two and three encode the first and second "zinc fingers" respectively and exons four through eight encode the C-terminal portion of the receptor including the ligand binding domain (Kuiper *et al*, 1989). The mRNA, from the first in frame AUG, translates into a protein of 918 amino acids with a calculated molecular weight of 97 kDa (Liao *et al*, 1989). The region of the highest amino acid similarity between the androgen receptor and other members of the nuclear receptor family is in the DNA binding domain: the AR is 75 to 80 percent similar to GR, PR and MR and 40 to 56 percent similar to ER, TR, RAR and DR. The other domains have less sequence similarity: the AR is 50 to 54 percent similar to GR, PR and MR in the ligand binding domain. The N-terminal regions of the different steroid hormone receptors have the least similarity in terms of structure and length. The N-terminal of the AR protein is unusual in that it contains several oligo or polyamino acid

stretches (Liao *et al*, 1989), the function of which is unknown.

The AR promoter contains no CAAT and TATA consensus sequences typical of many gene promoters (Tilley *et al*, 1990). Instead, a GC rich region is present, including a Sp1 consensus sequence (Tilley *et al*, 1990; Faber *et al*, 1991). The promoter region also contains a 60 base pair homopurine sequence, containing GGGGA and GGGA motifs, the significance of which is unknown (Faber *et al*, 1991). This promoter has been shown to be utilized in human prostate, testes, genital skin fibroblasts, T-47D and MCF-7 human breast cancer cells and in LNCaP human prostatic carcinoma cells (Tilley *et al*, 1990).

Androgen receptor mRNA expression/cDNA cloning

In the mouse, the appearance of androgen receptor expression in the male reproductive tract has been followed in a spatial and temporal fashion (Cooke *et al*, 1991). Although all androgen responsive organs contain androgen receptor in the mesenchymal/stromal cells at all times measured, epithelial androgen receptor appears to be temporally expressed in a cranial to caudal fashion from the efferent ductules to the Wolffian derived organs and the urogenital sinus derived organs. Androgen receptor appears in the efferent ductule epithelium at day 16 of gestation. At day 19, androgen receptor appears in the epididymus and ductus deferens. At birth, the seminal vesicle and coagulating gland epithelium appears androgen receptor negative; however, at day 1 a weak signal is observed which increases strongly by day 2. In the prostatic epithelium androgen receptor is absent up to day 4 and increases thereafter. Androgen receptor appears in the bulbourethral gland epithelium at day 8 post partum (Cooke *et al*, 1991). The intraepithelial androgen receptor induces seminal vesicle secretory proteins, while the mesenchymal androgen receptor mediates epithelial morphogenesis and columnar cytodifferentiation and proliferation (Cunha and Young, 1991).

The hAR mRNA is a single 10.6 kb species consisting of a 1.1 kb 5' untranslated region, an open reading frame of 2.7 kb and a 6.8 kb 3' untranslated region (Faber *et al*, 1991).

Two sites of transcription initiation are present 13 base pairs apart and two functional polyadenylation signals exist 221 base pairs apart: the first signal (ATTAAA) induces polyadenylation to begin at three different sites downstream and the second signal (CATAAA) induces polyadenylation to begin at a single downstream site. Other polyadenylation signal-like sequences are present but have not been shown to be functional (Faber *et al*, 1991).

The cDNA of the hAR was cloned by a number of independent groups (Chang *et al*, 1988; Lubahn *et al*, 1988; Trapman *et al*, 1988; Tilley *et al*, 1989) and has been used to express the hAR protein or fragments of the protein in tissue culture cells (Quarmby *et al*, 1990b), bacteria (De Vos *et al*, 1991) and insect cells (Xie *et al*, 1992).

The levels of androgen receptor mRNA and protein are primarily regulated by androgen. The highest levels of androgen receptor mRNA has been detected in the seminal vesicle, ventral prostate and coagulating gland (Liao *et al*, 1989). In target organs, such as rat ventral prostate, coagulating gland, epididymus, seminal vesicle, kidney and brain (Quarmby *et al*, 1990a), androgen receptor is downregulated by dihydrotestosterone (Liao *et al*, 1989) as well as the synthetic androgen, R1881, and the antiandrogen, cyproterone acetate (Quarmby *et al*, 1990a). Androgens have also been observed to control androgen receptor mRNA stability (Rundlett *et al*, 1990) and increase the stability of the protein (Kemppainen *et al*, 1992). Androgen receptor mRNA is also autoregulated in the human prostate cancer cell line, LNCaP (Quarmby *et al*, 1990a).

The androgen receptor gene has also been shown to be regulated by the gonadotropins. Accordingly, the 5' flanking region of the mouse AR gene has been analyzed for the presence of a cAMP responsive element (CRE) (Linzey *et al*, 1992). The CRE-like sequence found within 1.5 kb of the gene promoter has been shown to be functional based on transfection studies and gel retardation studies. This effect of the CRE appears to be modulated by androgens, as increasing amounts of a transfected mouse AR expression

construct represses the forskolin induction of a CAT reporter construct containing the CRE. This effect, however, is not mediated through the putative ARE consensus sequence found in the same fragment of the 5' flanking region, as deletion of this ARE-like sequence did not affect the forskolin repression by mouse AR (Linzey *et al*, 1992).

Androgen responsive cell lines: LNCaP

LNCaP cells are a cell line derived from a supraclavicular lymph node metastasis of a malignant prostatic adenocarcinoma (Sonnenschein *et al*, 1989). They have been used extensively in the past as an androgen responsive human cell line. They contain both AR mRNA and protein (Tilley *et al*, 1990), but contain no detectable ER or PR. The LNCaP androgen receptor has a point mutation in the ligand binding domain at amino acid 877 causing a threonine to change to an alanine (Harris *et al*, 1991). The mutation in the androgen receptor in LNCaP cells has diminished its ability to discriminate between various ligands. This mutant receptor binds and is activated by androgen, estrogen, progesterone or antiandrogens (Newmark *et al*, 1992). In LNCaP cells hydroxyflutamide, estrogen and progesterone cause cell proliferation. Estrogen and progesterone binding is inhibited by androgens (Sonnenschein *et al*, 1989). The antiandrogen, hydroxyflutamide, progesterone and cyproterone acetate have been shown to increase transcription in cotransfection studies using a reporter construct and an AR identical to the LNCaP receptor (Harris *et al*, 1990; Veldscholte *et al*, 1990). Testosterone, the synthetic androgen, R1881, and cyproterone acetate causes down regulation of AR mRNA. Estrogen fails to down regulate AR mRNA both in LNCaP cells and in the normal rat prostate, suggesting that although estrogen has some agonist action for the LNCaP androgen receptor, it does not act as a true agonist (Quarmby *et al*, 1990a).

Dihydrotestosterone increases androgen receptor concentrations two fold in LNCaP cells after 24 hours of hormone treatment. Although androgens have been shown to downregulate the AR protein, they have also been shown to increase the half life of the protein (Kemppainen *et al*, 1992). Retinoic acid can inhibit this effect to control levels.

Additionally, the affinity of androgen receptor for the synthetic androgen R1881 is diminished two to three fold by retinoic acid. After 48 hours, the accumulation of the prostate-specific antigen (PSA), an androgen responsive gene, is diminished in the presence of retinoic acid. Therefore, the sensitivity of human prostatic cancer cells to androgens appears to be modulated by retinoic acid (Young *et al*, 1992).

Ligand binding

The ligand binding domain of the androgen receptor has a strong affinity for dihydrotestosterone (DHT) and the synthetic androgen, R1881. Transiently expressed recombinant androgen receptor has been shown to have the highest affinity for R1881, followed by DHT and testosterone (Kemppainen *et al*, 1992). DHT is ten fold more potent than testosterone due to its higher binding affinity. In tissues with low levels of androgen, testosterone requires conversion to DHT. In tissues, like the testes and Wolffian ducts, with high levels of androgen, testosterone does not require conversion to DHT (Deslypere *et al*, 1992).

The antiandrogen, flutamide binds androgen receptor very weakly: its metabolite, hydroxyflutamide is hypothesized to mediate its effects and is a much stronger antiandrogen (Simard *et al*, 1986). At high concentrations, the recombinant androgen receptor will bind estradiol, the synthetic antiprogestin/antiglucocorticoid, RU486, and the antiandrogens, cyproterone acetate and hydroxyflutamide (Wilson, 1992). Binding of R1881 to the androgen receptor can be inhibited between 50 to 80 percent by 100 fold molar excess of cyproterone acetate, estradiol, progesterone, hydroxyflutamide and RU486. Binding of R1881 to the recombinant androgen receptor is not inhibited by flutamide (Wilson, 1992). In transient transfection studies with an androgen receptor expression construct and a CAT reporter gene construct, R1881, DHT and testosterone can activate AR mediated transcriptional activation in a dose dependent manner (Wilson, 1992). Estradiol, progesterone, cyproterone acetate and RU486 at concentrations greater than 10^{-9} M stimulated CAT activity to 15 to 100% of the stimulation of 10^{-11} M R1881. A molar excess of flutamide and hydroxyflutamide did not stimulate CAT activity;

however, these compounds were able to inhibit the induction by R1881 as could cyproterone acetate (Wilson, 1992).

Nuclear translocation

The wild type androgen receptor is perinuclear in the absence of hormone and nuclear in the presence of hormone. The nuclear uptake of AR from the perinuclear region of cytoplasm is dependent on binding of the androgens, R1881, DHT or testosterone to the receptor. The 100 kDa hAR undergoes nuclear translocation in response to DHT treatment (Luke and Coffey, 1992). Hydroxyflutamide, cyproterone acetate, RU486, estradiol and progesterone can also stimulate nuclear uptake at high concentrations (5 x 10^{-8} M) but flutamide cannot (Kemppainen *et al*, 1992).

Deletion mutants have helped to define the amino acids involved in this hormone-dependent nuclear translocation. Deletion of 211 amino acids of N-terminus results in increased nuclear immunostaining and deletion of the C-terminus or ligand binding domain results in a constitutively nuclear mutant receptor with reduced transcriptional activity (Simental *et al*, 1991). A nuclear translocation signal (NTS) sequence similar to the NTS of the GR and homologous to the NLS of the SV40 large T antigen is required for nuclear translocation (Simental *et al*, 1991). This NTS is contained in the hinge region between amino acids 557 to 653 of the receptor. An additional hormone-dependent NTS is present in the DNA binding domain (Jenster *et al*, 1991). These NTS sequences in the hinge region and DNA binding domain contain basic amino acids that act as bipartite hormone dependent NTS (Wilson, 1992). Deletion of this NTS decreases nuclear translocation (increases perinuclear staining) in the presence of androgen but does not abolish nuclear staining; therefore, the NTS potentiates but is not wholly responsible for nuclear uptake. This deletion mutant is still transcriptionally active, which suggests that the minimal amounts of androgen receptor still entering the nucleus may be sufficient for activity. Deletion of both the N-terminus and this NTS produces a mutant receptor that is nuclear (Simental *et al*, 1991).

Androgen receptor phosphorylation

The androgen receptor appears as a doublet of 110 and 112 kDa. This size difference is due to phosphorylation (Kuiper *et al*, 1991). Both the hinge region and the N-terminus region contain phosphorylation sites. Two possible phosphorylation sites are present in the androgen receptor at amino acid 80 and 93. An apparent 1.8 fold increase in phosphorylation in response to androgen treatment is accompanied by tight nuclear binding (van Laar *et al*, 1991). A serine to alanine mutation at amino acid 93 decreases the phosphorylation in response to androgens, but this amino acid change does not affect the transcriptional activation function of the receptor (Wilson, 1992). When amino acids 51 to 211 are deleted, only one form of androgen receptor is observed. In all other deletions and mutations tested, the two forms of androgen receptor are observed. The fragment from amino acid 142 to 337 is also phosphorylated in response to R1881 in a manner similar to the wild type androgen receptor (Wilson, 1992).

AR phosphorylation appears to increase 2 to 4 fold after 3 hours of treatment with either R1881, DHT or testosterone, but not with estradiol, progesterone, cyproterone acetate, flutamide or hydroxyflutamide at a high concentration (100 nM). However, this apparent androgen stimulated increase in phosphorylation is due to an increase in the amount of AR protein because of a change in the degradation rate of the AR in response to androgen (Kemppainen *et al*, 1992). In the absence of androgen, the half life of the AR protein is one hour, while in the presence of androgen the half life is extended to six hours. Androgen does not seem to promote phosphorylation of the recombinant androgen receptor (Kemppainen *et al*, 1992). Inversely, RU486 (100 nM) does appear to increase the apparent phosphorylation of androgen receptor; however, this increase cannot be accounted for by an increase in the half life of the protein (Kemppainen *et al*, 1992).

Transactivation

The characterization of the transactivation function of the androgen receptor has been accomplished primarily through cotransfection of androgen receptor expression vectors

with a CAT reporter construct containing the MMTV GRE/PRE sequence (Simental *et al*, 1991) or a PRE consensus sequence (Jenster *et al*, 1991) into CV1 cells or HeLa cells, respectively. A deletion of 12 or 114 amino acids from the C-terminus is sufficient to inactivate the 918 amino acid wild type receptor (Jenster *et al*, 1991). A deletion of 250 amino acids of the C-terminus will produce a truncated AR which is constitutively active (Rundlett *et al*, 1990) at a level that is 10 to 40% of the wild type receptor (Simental *et al*, 1991; Jenster *et al*, 1991). A receptor encompassing amino acids 1 through 653 is constitutively active at the same level as the wild type receptor (Jenster *et al*, 1991). These ligand binding domain deletions suggest a transcriptional activation function in the LBD as well as a inhibitory function in absence of androgen (Simental *et al*, 1991). A 19 amino acid deletion in the ligand binding domain (amino acids 712 to 731) abolished hormone binding. This region has sequence similarity with other steroid receptors and may be a site of interactions with *hsp90* (Simental *et al*, 1991; Jenster *et al*, 1991). The DNA binding domain contains a low intrinsic transactivation function which is 1% that of the wild type receptor (Simental *et al*, 1991).

The main transactivation region is in the N-terminal region of the AR (Simental *et al*, 1991). The region of the androgen receptor between amino acids 141 to 338 has been identified as the transactivation domain. It is rich in acidic residues and has sequence similarity to the *tau1* acidic activation domain of glucocorticoid receptor GR and the activation domains of GCN4 and Gal4 (Jenster *et al*, 1991). A N-terminal peptide is itself inactive in transactivating the reporter gene, yet it can inhibit by 17% the transactivation activity of the wild type receptor at an equimolar concentration. This observation suggests that the N-terminal region competes for nuclear factors necessary for activity (Simental *et al*, 1991). This hypothesis is further supported by the observation that the activated estrogen receptor inhibits the androgen receptor mediated transactivation of MMTV due to a possible sequestration of necessary nuclear factors or possibly an ER-AR complex interaction (Leo and Kumar, 1992).

Further dissection of this transactivation region has defined three subregions. The first subregion from amino acid 141 to 199, when deleted, decreased the activity of the mutant receptor to 75% of the wild type. The adjacent subdomain from amino acid 198 to 240 contains an inhibitory sequence which when deleted enhances the activity of the mutant receptor one to two fold. The third subregion from amino acid 240 to 338 when deleted decreases activity of the mutant receptor to 60% of the wild type (Wilson, 1992). These observations have been confirmed by an independent group who have shown that deletion of amino acids 51 to 211, greatly reduces the activity of the mutant receptor and a deletion of amino acids 244 to 360 abolishes that ability of the mutant receptor to activate transcription (Jenster *et al*, 1991). The region between amino acids 51 to 211 contains three of the polyglutamine stretches (Jenster *et al*, 1991) which have been implicated in the androgen resistance disorder spinal and bulbar muscular atrophy (La Spada *et al*, 1991; Wilson, 1992). The significance of these repeats is not known.

Androgen responsive genes and androgen responsive elements

The effects of the androgen receptor are mediated through its interaction with *cis*-acting elements located near or within androgen regulated genes. The androgen response elements (ARE) which have been characterized to date are very similar in sequence to the response elements which mediate the effects of glucocorticoid, progesterone and mineralocorticoid and yet they interact specifically with the DNA binding domain of the androgen receptor [see Table 1, page 53]. A single base mutation in the ARE abolishes interaction of the AR DNA binding domain with the ARE and androgen responsiveness (De Vos *et al*, 1991). A number of models of androgen action have been studied in androgen responsive rodent tissues such as the steroid binding protein gene family in the rat prostate, the sex-limited protein (Slp) gene in mouse liver and a variety of mouse androgen responsive genes. The C3(1) gene, the mouse RP2 gene and the rat SVS (seminal vesicle secretory protein) IV and V genes are cellular genes that have been observed to be transcriptionally regulated by androgen. Androgens also negatively regulate genes, among them are TRPM-2 (T-repressed prostate message-2), TGFβ

(transforming growth factor β) and the androgen receptor gene itself (Riegman *et al*, 1991). The best described androgen responsive system is that of the mouse mammary tumor virus long terminal repeat (MMTV-LTR).

- Androgen regulated genes in breast cancer: MMTV-LTR

In addition to the PIP/GCDFP-15 gene discussed earlier, the mouse mammary tumor virus long terminal repeat (MMTV-LTR) is the best characterized androgen inducible promoter in breast cancer. The mouse mammary tumor virus induces the formation of mammary carcinomas in mice 4 to 9 months after infection. MMTV-LTR integrates into the genome of affected mice near critical regulatory genes which then are transcriptionally controlled by the steroid responsive promoter/enhancer of MMTV-LTR. The steroid responsive region has been localized to an area within the LTR, 200 base pairs upstream of the MMTV transcriptional start site (Buetti and Diggelmann, 1983). MMTV-LTR was initially found to be glucocorticoid inducible and to bind glucocorticoid receptor (Chandler *et al*, 1983). The same region was subsequently found also to bind progesterone receptor and mediate progesterone induction (von der Ahe *et al*, 1985). Two areas of interaction with the GR and PR were identified by a DNaseI footprinting assay, referred to as the distal and proximal footprints. GR and PR were found to bind to similar sequences in the distal footprint (base pair -192 to -162) but the proximal footprint pattern was found to be different for the two steroid hormone receptors. The distal footprint and proximal footprints contain one and three TGTTCT sequence motifs respectively [see Table 1, page 53]. These TGTTCT sequences and the sequences proximal to them define the 15 base pair steroid response elements of the MMTV-LTR (Ham *et al*, 1988). The GR and PR bind to the same MMTV steroid response element sequences (Ham *et al*, 1988) but they can be distinguished by DNaseI footprinting and methylation protection patterns (Chalepakis *et al*, 1988). These regions of DNA has been shown subsequently also to mediate androgen responsiveness as well as mineralocorticoid responsiveness (Ham *et al*, 1988). They can confer androgen responsiveness to the reporter gene, chloramphenicol acetyl transferase (CAT) by approximately 20 fold in CV1

Table 1 Androgen response elements

Published ARE sequences are indicated, bracketed by their position within the gene. Half-sites as indicated by the respective authors are indicated by capital letters.

Table 1→

GENE	SPECIES	RESPONSE ELEMENT	REFERENCE
tat (tyrosine aminotransferase)	mouse	$^{-2509}$TGTACAggaTGTTCT^{-2495}	Strähle *et al*, 1987
MMTV-LTR (mouse mammary tumor virus-long terminal repeat)	mouse	$^{-184}$GTTACAaacTGTTCT^{-170} $^{-128}$GTATCAaaaTGTTCT^{-114} $^{-107}$AGCTCTtagTGTTCT^{-93} $^{-92}$ATTTTCctaTGTTCT^{-78}	Ham *et al*, 1988
Slp (sex limited protein)	mouse	AGAACAggcTGTTTC	Adler *et al*, 1991
β-glucuronidase	mouse	^{7833}AGTACTtgtTGTTCT^{7847}	Lund *et al*, 1991
RP2	mouse	$^{-802}$GAAACAagcTGTTTC^{-788}	Rhee *et al*, 1991
PSA (prostate specific antigen)	human	$^{-170}$AGAACAgcaAGTGCT^{-156}	Riegman *et al*, 1991
ODC (ornithine decarboxylase)	mouse	$^{-921}$AGTCCCactTGTTCT^{-907}	Crozat *et al*, 1992
Probasin	rat	$^{-240}$atagcatcttGTTCTTagt^{-222} $^{-140}$gtaaagtactccAAGAACctattt^{-117}	McQueen *et al*, 1992
C3(1) of prostate (steroid-) binding protein	rat	^{1359}AGTACGtgaTGTTCT^{1373}	Tan *et al*, 1992
PIP (prolactin-inducible protein) [sequences containing ARE half-sites found in the PIP 5' flanking region]	human	$^{-1473}$TAATGAggcTGTTCT^{-1487} $^{-1183}$CACAAGcagTGTTCT^{-1169} $^{-1111}$AACTTCagcTGTTCT^{-1125} $^{-413}$AAGCAAagaTGTTCT^{-399} $^{-194}$GAGTCAgatTGTTCT^{-180}	Tsuyuki (unpublished) Myal *et al*, 1991

and HeLa cells (Rundlett *et al*, 1990). The AR has been shown to bind to the same sequences as GR and PR (Ham *et al*, 1988). The 15 base pair steroid response elements in MMTV-LTR have little sequence similarity among themselves, except for the common TGTTCT motif. They also differ in their ability to interact with the three different hormone receptors. The differing hormone responsiveness of the HREs are not reflections of the hormone receptor concentration in the assay systems (Ham *et al*, 1988). R5020, a synthetic progestin, can induce a CAT reporter gene 60 fold through the MMTV HRE region, while dexamethasome induces CAT activity 200 fold and DHT induces CAT activity 7 fold. The distal footprint HRE is more active as a PRE than a GRE and the relative spacing between the HREs also affects hormone inducibility (Ham *et al*, 1988). The region between the two footprints between base pairs -160 and -130 does not seem to contain any sequences required for hormone responsiveness as mutation of this area had no effect. However, a large insertion in this area diminished responsiveness to progesterone and abolished the effect of androgen which suggests that this region is more important in terms of orientation of the hormone receptor binding sites (Ham *et al*, 1988). Additionally, the hormonal response does not appear to involve other factors interacting with this area (Chalepakis *et al*, 1988). A strong, orientation-independent functional cooperation exists between the two hormone responsive areas. This observation leads to the conclusion that optimal hormonal induction requires the interaction between receptor molecules bound to the different binding sites. The interactions of the receptors with their binding sites and cooperation between the receptors is different for GR and PR (Ham *et al*, 1988). *In vitro* cooperation between the proximal and distal footprints is not found with GR but instead a cooperation between GR molecules on the proximal binding sites is seen (Perlmann, 1990).

In the MMTV-LTR, a binding site for nuclear factor-1 (NF-1) has been observed to be essential for glucocorticoid but not progesterone induction (Ham *et al*, 1988). NF-1 binding is hormone dependent with complexes forming on the MMTV hormone responsive region only in the presence of glucocorticoid. Therefore, binding of the

hormone receptor appears to facilitate NF-1 binding. In cotransfection studies in JEG-3 cells with a NF-1 cDNA, progesterone inducibility is enhanced 17 fold with the addition of NF-1 while glucocorticoid inducibility is enhanced 20 fold. However, GR binding has been observed to inhibit NF-1 binding and PR can compete with NF-1 for binding to DNA *in vitro*. The present hypothesis is that binding of the hormone receptors may alter the chromatin structure of MMTV such that NF-1 can bind to its sequence and initiate transcription (Brüggemeier *et al*, 1990). *In vitro*, NF-1 acts as a basal transcription factor. PR dependent transcription requires other mechanisms independent of NF-1. The MMTV HRE contains a distal octamer-binding transcription factor-1 (OTF-1) binding site which is important for hormone dependent transcription but does not influence basal transcription. The binding of OTF-1 is facilitated by PR or GR binding. Due to its lack of an activation domain, OTF-1 cannot activate a TATA box minimal promoter; however, bound OTF-1 may allow contact between the hormone receptor and TFIID (Brüggemeier *et al*, 1991).

At present, the MMTV-LTR promoter is thought to be basally silent due to a nucleosome positioned over the hormone regulatory region. Two independent mechanisms of transcriptional activation are hypothesized: 1) either the hormone receptors bind to their HRE and allow binding of NF-1 which forms a stable transcription complex or 2) the hormone receptors bind to their HRE and facilitate binding of OTF-1 which is necessary for formation of a stable transcription complex (Brüggemeier *et al*, 1991).

A negative modulation of glucocorticoid induction of MMTV has been observed in a rat hepatoma variant cell line (Tanaka *et al*, 1991). Nuclear extracts of this cell line contain a factor which binds preferentially to the coding strand of the MMTV promoter between base pairs -163 to -147. No overlap exists between this binding site and the GRE. This sequence has similarity with an AP-1 binding site but the factor has been shown not to be AP-1 or a serum response element binding factor. Furthermore, this factor is not dependent on new protein synthesis as its transcription is not affected by cycloheximide.

In addition to the MMTV-LTR promoter, the promoter of apolipoprotein D (ApoD) also exhibits androgen regulation. ApoD is a progesterone binding component of high density lipoprotein which has been originally observed as a major component of gross cystic disease of the breast. ApoD (GCDFP-24) concentration has been observed to be high in well differentiated carcinoma and lower in poorly differentiated, agressive carcinoma. The hormonal regulation of expression of ApoD appears to be inversely related to the effects of the hormones on cell proliferation. Estradiol represses expression of ApoD by 65% in these cells while dexamethasone increases ApoD expression by 16 fold and DHT increases ApoD expression by 22 fold. The combined effect of dexamethasone and DHT is an increase in ApoD expression by 28 fold. Estradiol appears to synergize positively with dexamethasone and DHT. Estradiol and dexamethasone increase ApoD mRNA by 50 fold, estradiol and DHT increase ApoD mRNA by 35 fold and all three hormones increase ApoD mRNA by 105 fold (Simard *et al*, 1992).

- Androgen regulated genes in the prostate:

The level of androgen in the prostate is mediated by the enzyme 5α-reductase, which converts testosterone to its more potent form, dihydrotestosterone. Prostate growth itself is dependent on DHT and not on testosterone (Imperato-McGinley *et al*, 1979). Castration decreases prostate size and DNA content and decreases intraprostatic testosterone and DHT (Wenderoth *et al*, 1983). Finasteride is an inhibitor of 5α-reductase which decreases conversion of testosterone to DHT by 95%. The intraprostatic level of testosterone is increased 10 fold by this drug and the prostate size decreases (Geller, 1990). Prostatic steroid binding protein (PSBP) gene induction and TRPM2 (T-repressed prostate message) repression is responsive to an increase in testosterone. TRPM2 has been used as a marker of programmed cell death (apoptosis) (Buttyan *et al*, 1989). Castration decreases, while finasteride has no effect on PSBP mRNA levels in the prostate; however, finasteride can enhance the negative effect of castration on PSBP mRNA. 5α-reductase inhibition induces cell loss through a mechanism independent of apoptosis (Rittmaster *et al*, 1991).

The rat prostatic steroid binding protein (PSBP) contains three peptides encoded by the C1, C2 and C3 genes. In the promoter or the first intron of each of these genes, high affinity androgen receptor binding sites have been identified (Rushmere *et al*, 1990). The C3(1) gene ARE has been characterized extensively and the bacterially expressed DNA binding domain of the rat androgen receptor has been shown in gel retardation experiments to interact with the MMTV-LTR or a gene fragment of the C3(1) first intron. This androgen receptor fragment can also protect two areas in the C3(1) first intron from digestion with DNase I (De Vos *et al*, 1991). The first intron of this gene contains two ARE-like sequences on opposite strands of the DNA fragment [see Table 1, page 53]. The second ARE has a higher affinity for the androgen receptor and mutations in this sequence reduce this affinity (Rushmere *et al*, 1990). Two oligonucleotide copies of the second ARE separated by 6 base pairs can synergize with each other in conferring 33 fold androgen inducibility to a CAT reporter construct. The reporter construct used is the chloramphenicol acetyl transferase (CAT) gene driven by the thymidine kinase (tk) promoter [-105→+51] of herpes simplex virus (Schüle *et al*, 1988; Eisenberg *et al*, 1985). The entire intron fragment of 0.5 kb can confer a 16 fold androgen inducibility to the reporter gene construct, tkCAT, while the second ARE alone confers a 7 fold induction. The first ARE is weak by itself but can enhance the activity of the second ARE. The activity of the 0.5 kb fragment cannot be entirely accounted for by interactions of the two AREs (Tan *et al*, 1992).

Prostatic specific antigen (PSA) is a 33 kDa serine protease (Watt *et al*, 1986) which is a member of the kallikrein gene family. PSA has an 82% nucleotide sequence identity with human glandular kallikrein (HGK) and is expressed in human prostatic epithelium (Chapdelaine *et al*, 1988). In LNCaP human prostatic carcinoma cells, a 1.6 kb PSA transcript is inducible by the androgens mibolerone and DHT, but not by dexamethasone or diethylstilbestrol (Young *et al*, 1991). The induction of PSA by androgens is reversed by treatment with hydroxyflutamide (Young *et al*, 1991). The PSA mRNA accumulates within 6 hours of treatment with R1881 and this accumulation has been determined by

nuclear run on assays to occur as a result of an increase in the level of transcription. The 5' flanking region of the PSA gene has been dissected to define regions conferring this androgen responsiveness. A 1.6 kb fragment upstream of and including the endogenous promoter can confer 110 fold enhancement of a reporter CAT gene with R1881 treatment (Riegman *et al*, 1991). LNCaP cell nuclear extracts contain protein factors that can protect four areas of the 5' flanking region (including base pairs -320 to +12) from digestion with DNase I. Three of these footprints have been identified to include the TATA box, the GC box and an ARE-like sequence, AGAACAgcaAGTGCT, from base pairs -170 to -156 [see Table 1, page 53]. The region from base pair -320 to -115 has been found to be essential for androgen inducibility, but the region from base pair -539 to -320 is also necessary for optimal androgen responsiveness (Riegman *et al*, 1991). TPA and phorbol esters have been shown to repress the androgen inducibility of PSA. This effect occurs through the protein kinase C pathway (Andrews *et al*, 1992).

The rat probasin gene, which is regulated by androgen *in vivo* (Dodd *et al*, 1983), encodes a nuclear and secreted protein of the rat dorsolateral prostate (Spence *et al*, 1989). Two areas of the 5' flanking region have been shown to bind recombinant AR peptides. These two areas, referred to as ARE-1 and ARE-2, are not effective as transcriptional enhancers individually, but mediate a 30 to 40 fold induction of the probasin promoter in combination. Unlike many previously characterized AREs, the sequence of ARE-2 is not similar to that of the consensus GRE (Rennie *et al*, 1993) [see Table 1, p 53].

- Androgen regulated genes in the mouse kidney and liver:

Androgen increases total RNA in mouse kidney proximal tubule epithelial cells by 40% per cell and causes Bowman's capsule cells to hypertrophy. These cells do not divide or synthesize DNA (Watson and Paigen, 1990) and the total number of AR molecules per kidney cell does not increase over time (Paigen and Peterson, 1981). The mouse kidney expresses a variety of androgen responsive genes. In the kidney, the androgen inducible mRNAs do not reach maximal levels until three to five days after androgen treatment.

The effect of androgens appears to be primarily at the level of transcription. These genes can be classified into two groups depending on the time needed for maximum induction and the presence of a lag phase after androgen treatment before measurable levels of mRNA appear (Watson and Paigen, 1990). The group one genes reach maximal induction in two to three days and the group two genes reach maximal induction after eight days. The group two genes also have a lag phase of several hours before the mRNA is detectable. These genes also tend to have lower basal rates of synthesis. Since other steroid hormones seem to act at a very early time after hormone treatment to increase transcription of genes, Watson and Paigen (1990) have proposed two models to explain the considerably longer times involved in androgen-induced transcriptional activation. In model one, they hypothesize the existence of an androgen induced intermediate factor which must accumulate to a minimum concentration before genes can be induced (Watson and Paigen, 1990). In model two, they suggest that each gene must have many androgen receptor binding sites and the number of binding sites that interact with androgen determines the level of transcriptional activity (Almagor and Paigen, 1988).

RP2 is a 43 kDa protein of unknown function. It is expressed in all tissues but is induced by testosterone in the kidney only. The increase in RP2 mRNA has been shown to be both transcriptional and posttranscriptional (Berger and Watson, 1989). RP2 mRNA transcription increases three to five fold and accumulation increases 10 to 12 fold in response to androgen. In some mouse species, the androgen-induced increase in RP2 mRNA is minimal and this minor increase is not a transcriptional effect. This phenomenon is due to the presence of at least two promoters in the RP2 gene, the major promoter being sensitive to testosterone. The reduced androgen induction of RP2 mRNA in these species of mouse is due to alterations in function of the major promoter (Rheaume *et al*, 1989). In *Mus domesticus*, RP2 is androgen responsive: in *M. caroli* the androgen inducibility of RP2 is very low. *M. caroli* has a functional androgen receptor as the ADH (alcohol dehydrogenase) and ODC (ornithine decarboxylase) genes are inducible by androgen. Nuclear extracts of control and androgen treated kidneys from *M. domesticus* contain a factor which binds the RP2 promoter from base pairs -247 to -269 in the 5' flanking region of the gene. This factor, named RPBF-1 (RP binding factor) is

present in the control kidney nuclear extracts of *M. caroli* but is superseded in androgen treated kidney nuclear extracts by RPBF-2 which recognizes a distinct but overlapping region of the RP2 gene from base pairs -265 to -290. The RP2 gene contains an ARE-like sequence, GAAACAagcTGTTTC, at position -802 to -788 which can bind the DNA binding domain of the androgen receptor (Rhee *et al*, 1991) [see Table 1, page 53].

Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthesis pathway. In female mice the level of ODC enzyme is increased 300 to 500 fold by testosterone. The level of mRNA increases 10 to 20 fold caused primarily at a transcriptional level, although androgens also stabilize the ODC protein (Berger and Watson, 1989). An ARE-like sequence 900 base pairs upstream of the transcription start site [see Table 1, page 53] has been shown to bind androgen receptor weakly and the ODC gene 5' flanking region can confer androgen responsiveness to a CAT reporter gene construct (Crozat *et al*, 1992).

The mouse sex-limited protein (Slp) is androgen inducible in the liver and kidney. The androgen responsive region was first identified as a DNase I hypersensitive site in liver chromatin (Adler *et al*, 1991). The GRE consensus sequence in this region by itself is necessary but not sufficient for androgen induction. Specificity for the androgen receptor is determined by interaction with specific accessory factors and not differences in the hormone responsive element receptor binding sites (Robins *et al*, 1992). The full length Slp gene including 10 kb of 5' flanking region is inducible by androgens approximately twelve fold (Loreni *et al*, 1988). A 162 base pair fragment can confer androgen inducibility on a CAT reporter gene to a similar level (Adler *et al*, 1991). This fragment contains three sequences with similarity to the GRE consensus in the 3' region. The third hormone response element AGAACAggcTGTTTC binds androgen receptor weakly and is necessary for a strong hormonal response but is not sufficient alone [see Table 1, page 53]. HRE3, when multimerized, can synergize with itself in conferring androgen responsiveness to a CAT reporter gene (Adler *et al*, 1991). Sequences upstream can

greatly enhance the androgen responsiveness of HRE3. Two areas of this 162 base pair fragment are protected from DNase I digestion by proteins from several different cell types. A 17 base pair repeat (containing one mismatch) occurs in the 5' region of the fragment and the first footprint covers the second of these repeats. The second footprint overlaps the first HRE and the two footprints are separated by an area unresponsive to androgen (Adler *et al*, 1991). Slightly different patterns of gel retardation are observed between nuclear extracts from T-47D cells and CV1 cells using the 5' region or an oligonucleotide to the first footprint. The 5' region containing the 17 base pair repeats alone is unresponsive to androgen, but placed next to the third HRE confers androgen inducibility to a CAT reporter gene to the same level as the wild type gene. The nonresponsive first and second HREs can be involved in androgen inducibility as the combination of the 5' region and all three HREs can induce CAT activity more than three fold over the 5' region and HRE3 alone (Adler *et al*, 1991).

- Androgen regulated genes in the rodent submaxillary gland:

The submaxillary gland in rodents is an androgen responsive tissue and expresses a variety of androgen regulated proteins including renin, EGF, NGF and a TRH-like protein isolated from the rat submandibular gland, called SMR-1. Another rat submandibular gland mRNA, SMR-2, has recently been cloned and is related to the glutamine rich proteins (Rosinski-Chupin and Rougeon, 1990). EGF and four trypsin-like proteinases F,D,A and P are expressed in the submandibular gland in hypophysectomized mice. EGF is negatively regulated by hypophysectomy. Proteinase A has been identified as a βNGF endopeptidase and proteinase D is an EGF binding protein. Hypophysectomized male mice express only proteinase F which is negatively regulated by T_3. Hypophysectomy decreases the levels of proteinase F in female mice an effect which is reversed by DHT treatment. Proteinases D, A and P are induced by DHT, T_3 and dexamethasone from the highest to lowest level, respectively. T_3 and DHT have additive effects on these proteinases and T_3 and dexamethasone act synergistically. T_3 synergized with both dexamethasone and DHT (9 and 6 fold, respectively) in the induction of EGF in the submandibular gland (Hosoi *et al*, 1992).

Androgen resistance

The study of the structure of the androgen receptor has been aided by the existence of a number of models in which naturally occuring specific genetic defects in the androgen receptor gene are responsible for a phenotypic change. The disorder which has proved most useful in the dissection of androgen receptor structure is that of androgen insensitivity syndrome (AIS), an X-chromosome linked defect which affects males. Patients with AIS are affected with phenotypic gradations from complete testicular feminization to undervirilization (McPhaul *et al*, 1991). Androgen insensitivity syndrome is due to mutations or abnormal expression of the androgen receptor (see review: McPhaul *et al*, 1991) or of the enzyme 5α-reductase (Imperato-McGinley *et al*, 1974) which is responsible for the conversion of testosterone to the more potent androgen, dihydrotestosterone. In the androgen receptor gene, these mutations seem to encompass predominantly point mutations, rather than gross structural changes (Liao *et al*, 1989; see review: McPhaul *et al*, 1991). The study of these mutations has led to the elucidation of receptor structure-function correlations as well as the effect of receptor structure on phenotype (Lubahn *et al*, 1989).

Four different catagories of receptor defects have been established for AIS: receptor binding negative, receptor binding reduced, receptor binding abnormal and receptor binding positive (McPhaul *et al*, 1991). Receptor binding is defined by an androgen binding assay in genital skin fibroblast culture (Zoppi *et al*, 1992). In the receptor binding negative group, a small percentage of patients have been described with major alterations in the 3' terminus of their androgen receptor corresponding to the ligand binding domain. These alterations can be either the result of deletions or insertions in the AR gene or single base substitutions which cause aberrant splicing of introns, frameshift mutations or premature termination codons. Additionally, a number of single amino acid substitutions have been described which appear to affect the structure of the androgen receptor such that it is incompetent to bind androgen. Finally, the level of androgen receptor mRNA or protein can be decreased by decreased synthesis or stability (McPhaul

et al, 1991).

An animal model for receptor negative binding is the testicular feminized (*Tfm*) mouse (Lyon and Hawkes, 1970). Mouse AR has 97% sequence similarity with rat AR and 83% with human AR. The DNA binding domain and ligand binding domains are identical (Chang *et al*, 1988). The *Tfm* mouse model is a result of a single base deletion in the region of the mRNA coding for the N-terminus which results in a frameshift mutation (Charest *et al*, 1991). The *Tfm* male mouse has female genitalia, has no Wolffian duct structures and does not respond to testosterone (Lyon *et al*, 1973). *Tfm* mice have approximately 10% of the level of AR mRNA found in normal mice because the mutated androgen receptor mRNA is unstable (Charest *et al*, 1991). A deletion at base pair 1107 to 1112 causes 41 missense amino acids and a premature termination codon at base pair 1235 to 1237. The *Tfm* receptor is 66 kDa in size (Gaspar *et al*, 1991).

In qualitatively abnormal receptor binding, no major alterations in gene structure appear to be present and normal levels of androgen binding are observed in the fibroblast culture assay. However, mutations have been identified in the ligand binding domain which causes the receptor protein to have a decreased affinity for dihydrotestosterone or an increased ligand dissociation. Also, an increased thermal instability of the receptor protein has been described (McPhaul *et al*, 1991).

In quantitative abnormal receptor binding, the androgen receptors appear to be normal but the level of ligand binding is reduced (McPhaul *et al*, 1991). This defect is illustrated by the testicular feminized (*Tfm*) rat model of androgen insensitivity. In the *Tfm* rat, a single amino acid mutation of the ligand binding domain results in defective processing of the receptor to a form incompetent to bind ligand (Yarbrough *et al*, 1990). In addition, androgens do not down regulate AR mRNA (Quarmby *et al*, 1990a).

In the receptor binding positive but androgen resistant group, the AR binds androgen, but

due to a defect in DNA binding domain, the receptor does not bind DNA and does not transcriptionally activate target genes. In these patients, exon three coding for the DNA binding domain has been spliced out or a single amino acid substitution has disrupted the zinc finger structure (McPhaul *et al*, 1991). From studies of Zoppi *et al* (1992), one incomplete testicular feminization patient has mutations in base pairs 595 and 615 of the AR mRNA (exon 3) in the region of the DNA binding domain. A complete testicular feminization patient has been observed to have one of the same mutations at base pair 615. This difference suggests that amino acid 595 can partially restore AR DNA binding.

The androgen receptor is similar in structure to other members of the steroid receptor family, but is unique in that its N-terminal region contains a number of polyamino acid sequences, notably a long tract of 13 to 30 glutamine residues. The function of these sequences in AR is unknown. X-linked spinal and bulbar muscular atrophy (SMBA/Kennedy's disease) is a disorder associated with androgen insensitivity. SBMA is a adult onset motoneuronal disease caused by a mutation in the N-terminus of the AR. Thirty nine to sixty CAG repeats encoding glutamines are observed in patients instead of the 13 to 30 repeats found in control subjects in the polymorphic region coding for the polyglutamine sequence in the N-terminus of the AR (La Spada, 1991; Wilson, 1992).

RETINOIDS AND RETINOIC ACID RECEPTORS

Actions of retinoic acid

All-*trans* retinol, commonly known as vitamin A, is specifically required for vision. Its derivative, all-*trans* retinoic acid, can fulfil all other functions of retinoids (Morriss-Kay, 1992). The maintenance of epithelial integrity in the superficial linings of the body is dependent on retinoic acid as is the expression of the keratin genes in these tissues (De Luca, 1991). In the chicken embryo, both the vascular system (Thompson *et al*, 1969) and the correct pattern formation of the developing limb (reviewed in Tabin, 1991) require retinoids and their receptors and binding proteins.

Retinoic acid stimulates the differentiation of the F9 mouse embryonal carcinoma cell line into primitive endoderm which progresses to either visceral endoderm or, in the presence of both retinoic acid and cAMP, parietal endoderm. Various protein products are characteristic of these stages. In visceral endoderm, an increase in α-fetoprotein (Hogan *et al*, 1981) and apolipoprotein E (Basheeruddin *et al*, 1987) levels are observed and in parietal endoderm, an increase in laminin, type IV collagen and entactin (Carlin *et al*, 1983; Marotti *et al*, 1985) levels are observed. Increases in the levels of the keratin genes, K8 and K18, the histocompatibility antigen, H2, and β2 macroglobulin (Croce *et al*, 1981) are common to both differentiation pathways.

In mammalian pregnancy, retinoic acid acts as a powerful teratogen (Lammer *et al*, 1985) and embryos develop abnormally under conditions of vitamin A excess (Cohlan, 1953). Under conditions of vitamin A deficiency, the columnar epithelia of the body undergoes squamous metaplasia and keratinization (Wolbach and Howe, 1925). The malformations resulting from either an excess or a deficiency of retinoic acid constitute distinct and different patterns (Morriss-Kay *et al*, 1992).

Retinoic acid induces differentiation of epithelial cells (Ruberte *et al*, 1990) and

teratocarcinoma stem cells (Strickland *et al*, 1990). Retinoic acid directs cells to undergo normal differentiation and thus may prevent epithelial tumorigenesis (Sporn *et al*, 1976). In rodents, tumorigenesis of the skin and the respiratory, buccal, stomach and mammary gland epithelium is inhibited by retinoids (De Luca, 1978; Moon *et al*, 1983). Mammary cancer in animals can be prevented by retinoids in combination with other hormones (Lippman *et al*, 1987; Sporn *et al*, 1976; Moon and Mehta, 1990) and proliferation of human breast cancer (HBC) cells is inhibited by retinoic acid alone or in combination with antiestrogens (Fontana, 1987; Fontana *et al*, 1990; Fontana *et al*, 1988; Marth *et al*, 1985; Wetherall and Taylor, 1986; Koga and Sutherland, 1991).

Retinoic acid receptor cloning and structure

The first human retinoic acid receptor (RARα) was cloned in 1987 (Petkovich *et al*, 1987; Giguere *et al*, 1987). The second retinoic acid receptor was cloned when the integration site of hepatitis B virus in human liver DNA was found to have sequence similarities to the oncogene, *v-erbA* (Dejean *et al*, 1986). In 1987, the cDNA for this integration site was cloned (de Thé *et al*, 1987) and called *hap*: it was found to be a second retinoic acid receptor and was designated RARβ (Brand *et al*, 1988). A third retinoic acid receptor (RARγ) was cloned in mouse (Zelent *et al*, 1989), while attempting to clone mouse RARα and β (de Thé *et al*, 1987; Petkovich *et al*, 1987; Giguere *et al*, 1987).

The three RAR genes are similar to each other. The retinoic acid receptor (RAR) has a domain structure similar to that of the other members of the steroid receptor gene family. The C domains (DNA binding domain) of the RARs have 93 to 95% amino acid identity with each other. The E domains (ligand binding domain) have 85 to 90% amino acid identity, the B domains have 75 to 86% identity and the D domains have 61 to 74% identity. The A and F domains are variable (De Luca, 1991). A *trans*-activating function (TAF) has been discovered in the RAR E region but no TAF has been found thus far in the A/B region (Nagpal *et al*, 1992b). The multiple protein species seen when RARγ is expressed in COS1 cells are due to ligand independent phosphorylation in the A/B regions

and possibly in the D region (Rochette-Egly *et al*, 1991). The amino acid sequence of the RARs has been conserved in evolution from mouse to human (Zelent *et al*, 1989). The three receptor subtypes have different affinities for different retinoids. The RARα has the least retinoic acid affinity, RARβ has a 10 fold higher affinity and RARγ has the highest affinity for retinoic acid (Lehmann *et al*, 1991).

RAR α, β and γ are found on mouse chromosomes 17, 3 and 12 and human chromosomes 11, 14 and 15, respectively (Ishikawa *et al*, 1990; Mattei *et al*, 1991). RARα and β are homologous to TRα and β (Petkovich *et al*, 1987; Mattei *et al*, 1988). TRα and β are located on mouse chromosomes 17 and 3, respectively (Thompson *et al*, 1987; Gareau *et al*, 1988).

RAR isoforms

The three RAR genes generate several different mRNA isoforms resulting from the use of two promoters and differential splicing of the exons comprising the A region of the receptor (Rochette-Egly *et al*, 1991). The isoforms have identical DNA and ligand binding domains (Lehmann *et al*, 1991). These different isoforms have a spatial and temporal pattern of distribution in both the adult mouse and in embryogenesis (Rochette-Egly *et al*, 1991).

The mouse RARα has seven isoforms which have identical B to F domains. The individual A domains are derived from alternate splicing of the first eight exons of the RARα gene (Leroy *et al*, 1991a). These isoforms are differentially expressed in various tissues and cell lines and are differently regulated by retinoic acid. Two of the RARα isoforms are predominantly expressed: mouse RARα1 is ubiquitous and independent of retinoic acid and RARα2 is tissue specifically expressed and is inducible by retinoic acid (Leroy *et al*, 1991b). The human and mouse RARα1 promoters resemble those of housekeeping genes (Leroy *et al*, 1991a; Brand *et al*, 1990).

The RARα2 and the RARβs are inducible by retinoic acid (de Thé *et al*, 1989; Zelent *et al*, 1989) and a retinoic acid response element (RARE) has been found in the promoters of the RARα2 and RARβ2 genes (de Thé *et al*, 1989; Sucov *et al*, 1990; Leroy *et al*, 1991b). The RARα2 RARE consists of two direct repeats of the consensus motif, AGTTCA, with a spacing of 5 base pairs. All three RARs bind to the RARE of RARα2. Mutation of this RARE diminishes the retinoic acid inducibility (Leroy *et al*, 1991b). Mouse RARα2 transcription is mediated by an alternative promoter which contains no consensus TATA box, but instead has a degenerate A+T rich sequence which is also found in the human RARα2 and the mouse RARβ2 promoters.

The mouse RARβ has three different isoforms which are highly conserved between mouse and human. These isoforms have a differential pattern of expression in mouse tissues: RARβ2 is predominantly expressed in retinoic acid-treated embryonal carcinoma cells and embryonic stem cells while β1 and β3 are predominantly expressed in fetal and adult brain (Zelent *et al*, 1991) suggesting the involvement of β1 and β3 in the development of the central nervous system and β2 in the early stages of fetal development (Zelent *et al*, 1991). RARβ4 uses the same retinoic acid responsive downstream promoter as RARβ2 and initiates at a non-AUG initiation codon CUG (Nagpal *et al*, 1992a).

The mouse RARγ has seven isoforms which differ in the A region and are derived from alternate splicing of the first seven exons (Kastner *et al*, 1990). The RARγ isoforms are conserved in evolution from mouse to human. Mouse RARγ2 is not retinoic acid inducible.

Retinol binding proteins

After intestinal absorption, vitamin A (all-*trans* retinol) binds to cellular retinol binding protein II (CRBPII). While in this complex, the retinol is enzymatically converted to retinyl esters and is incorporated into chylomicrons for transport to the liver (Ong, 1987; MacDonald and Ong, 1988). The main retinol carrier in the blood is the 21 kDa retinol

binding protein (Goodman, 1984) which is synthesized in the liver and secreted into the bloodstream. The retinol binding protein then associates reversibly with a 55 kDa protein (*trans*-thyretin) which prevents glomerular filtration by the kidney (Blomhoff *et al*, 1990). In addition to the retinol binding protein (RBP), three forms of cellular retinol binding protein (CRBP), two forms of cellular retinoic acid binding protein (CRABP), three forms of retinoic acid receptor (RAR) and three forms of the newly isolated retinoid X receptor (RXR) have been characterized (De Luca, 1991).

RAR and binding protein expression

The distribution patterns of RAR and binding proteins determines the quantitative control of RAR activation and the qualitative differences in the effects of activated RARs on gene expression in different cell types (Morriss-Kay, 1992). All mammalian cell lines have endogenous RAR (Sucov *et al*, 1990). Every organ of the mouse fetus expresses RARα (Dollé *et al*, 1990). Although RARα is ubiquitous, RARβ and γ have more restricted patterns. RARα and β is expressed in adult brain specifically in the hippocampus and hypothalamus (Giguere *et al*, 1987; Benbrook *et al*, 1988; Zelent *et al*, 1989). Endodermal tissues such as trachiobronchial, intestinal and genital tract epithelium express RARβ. RARγ is expressed in the skin (Elder *et al*, 1991). In the late gestational stages, RARβ and γ are mutually exclusive (Dollé *et al*, 1990).

In different organs, CRBP and RARβ expression regions are found to either overlap or reciprocate (Dollé *et al*, 1990). CRBPI, CRABPI and CRABPII are all present in embryos (Crow and Ong, 1985). Areas of high CRBP and CRABP expression are distinct (Dollé *et al*, 1990). CRBPI and II are highly specific for retinol binding and CRABPI and II are highly specific for retinoic acid binding (Chytil and Ong, 1987). CRABP may accentuate the formation of steep retinoic acid gradients (Dollé *et al*, 1990). In areas where retinoic acid will be required at high concentrations, CRBP functions to concentrate retinol; whereas in areas not requiring high concentrations of retinoic acid, CRBPII is not present in embryos and is involved in the intestinal absorption of vitamin A instead.

Positive retinoic acid response elements

In 1986, Sap and colleagues observed that TR and RAR recognized similar hormone response elements. RARα was subsequently found to bind a synthetic TRE (Umesono *et al*, 1988). In 1991, Umesono and colleagues made a proposal that delineated the response elements for a number of the members of the nuclear receptor superfamily. This proposal has become known as the 3-4-5 rule. The estrogen response element half-site, AGGTCA, is defined as a sequence motif which is recognized by a number of different receptors. A direct repeat of this motif separated by three base pairs defines a vitamin D response element (DRE), a direct repeat separated by four base pairs defines a thyroid hormone receptor response element (TRE) and a direct repeat separated by five base pairs defines a retinoic acid response element (RARE). A direct repeat without intervening base pairs constitutes a negative TRE. The palindrome of this motif separated by three base pairs (AGGTCAnnnTGACCT) has previously been shown to be an estrogen response element (ERE), and an inverted palindrome (AGGTCATGACCT) constitutes a TRE (Umesono *et al*, 1991) and can recognize both the RAR and TR (Näär *et al*, 1991). The thyroid hormone receptor has been shown to be able to bind with high affinity to the ERE but is transcriptionally inactive (Glass *et al*, 1988) [see Table 2, page 73].

Thyroid hormone and retinoic acid increase growth hormone levels in the rat pituitary GH_1 and GH_3 cell lines, both individually and additively (Glass *et al*, 1988; Bedo *et al*, 1989). In GH_1 cells, thyroid hormone and retinoic acid synergistically activates rat GH-CAT constructs (Bedo *et al*, 1989). The palindromic TRE of the rat growth hormone gene mediates both T_3 and retinoic acid induction and is activated by RARα (Umesono *et al*, 1988). The RARα alone has a low affinity for this element, but high affinity is achieved with the addition of F9 teratocarcinoma whole cell extract. The activity in the F9 cell extract is the thyroid hormone receptor which enhances RAR binding to the TRE by heterodimerizing with it and increasing its binding affinity to the TRE (Glass *et al*, 1989).

The heterodimerization of the RAR and TR allows a cooperative increase in binding of RARα to some thyroid hormone response elements (TREs) (Glass *et al*, 1989). Mutations in the DNA binding domain or the C-terminus of the receptor abolishes this interaction. The heterodimer is positive for the palindromic TRE but is negative for the TRE of α-myosin heavy chain gene, suggesting that the heterodimer has the ability to recognize a distinct subset of DNA response elements (Glass *et al*, 1989). A region in the ligand binding domain is similar in sequence between the RAR and TR. This region contains eight hydrophobic heptad repeats similar to the leucine zipper dimerization domain found in other transcription factors. This region has been shown to mediate interaction between chick *c-erbA* and rat TR (Forman *et al*, 1989). The heptad repeat region by itself can act as a dominant negative for chick *c-erbA*, rat TR and RAR. TR, like RAR, has α and β subtypes derived from alternative splicing. Truncated RAR expression vectors transfected into F9 embryonal carcinoma cells have a dominant negative effect (Duester *et al*, 1991).

In F9 cells, after 24 to 28 hours of exposure to retinoic acid, transcription of the mouse laminin B1 (LB1) gene increases (Wang *et al*, 1985). This effect is cycloheximide sensitive (Wang and Gudas, 1988). Retinoic acid induction of the LB1 gene is mediated by the binding of RAR to the promoter. The LB1 RARE has been characterized and consists of three TGACC-like repeats from base pairs -471 to -432 of the promoter (Vasios *et al*, 1989) [see Table 2, page 73]. All three RARs can bind to the LB1 RARE. The slow kinetics of induction of the retinoic acid response of the mouse LB1 gene suggests that an additional retinoic acid inducible LB2 promoter specific activator is required or a negative regulatory mechanism must be relieved (Vasios *et al*, 1991).

Table 2 Retinoic acid response elements

Published RARE sequences are indicated, bracketed by their position within the gene. Half-sites as indicated by the respective authors are indicated by capital letters.

Table 2→

GENE	SPECIES	RESPONSE ELEMENT	REFERENCE
palindromic TRE and RARE	synthetic	AGGTCATGACCT	Umesono *et al*, 1988
Growth hormone TRE and RARE	rat	$^{-182}$GATCAgggacgTGACCgca^{-164}	Umesono *et al*, 1988
Laminin B1	mouse	$^{-468}$tTGACCctttttctaaggcctTAACCtagcTCACCtc^{-434}	Vasios *et al*, 1989
RARβ (retinoic acid receptor β)	human mouse	$^{-53}$GGTTCAccgaaAGTTCA^{-37}	de Thé *et al*, 1900 Sucov *et al*, 1990
CP-H (complement factor H)	mouse	$^{-143}$AGGTCActgacAGGCA^{-132}	Munos-Canores *et al*, 1990
Osteocalcin Ap-1 binding site (VDRE)	human	$^{-500}$GGTCAcTCACC^{-510}	Schüle *et al*, 1990
ADH-3 (alcohol dehydrogenase-3)	human	GGGTCAttcagAGTTCA	Duester *et al*, 1991
RARα2 (retinoic acid receptor α2)	mouse	$^{-55}$AGTTCAgcaagAGTTCA^{-39}	Leroy *et al*, 1991b
PEPCK (phospho*enol* pyruvate carboxykinase)	rat	$^{-451}$TGACCTTTGGCCGTGGGA^{-434}	Lucas *et al*, 1991
Oxytocin	human	$^{-162}$TGACCTTGACCCcggcccaggccctgctaatgaagaggaaagcccgtacgcactcggccTGACCCacggcgaccctctgTGACCA^{-78}	Richard and Zingg, 1991
ApoA1 (apolipoprotein A1)	human	$^{-192}$AGGGCAgGGGTCAagGGTTCA^{-212}	Rottman *et al*, 1991
CRBP-I (cellular retinol binding protein I)	mouse	$^{-1111}$AGGTCAaaAGGTCA^{-998}	Smith *et al*, 1991
DR+5 (direct repeat + 5)	synthetic	AGGTCAccaggAGGTCA	Umesono *et al*, 1991
CRABPII (cellular retinoic acid binding protein II)	mouse	$^{-1168}$AGTTCAccAGGTCA^{-1145} $^{-654}$TGACCTcTGCCCT^{-642}	Durand *et al*, 1992
MIEP (major IE promoter of cytomegalovirus)	viral	$^{-299}$TGGCATtaTGCCCAgtacaTGACCT^{-275}	Ghazal *et al*, 1992
Oxytocin (negative RARE)	rat	$^{-170}$CCTGAggcggTGACCTGACCcca^{-148}	Lipkin *et al*, 1992

Negative retinoic acid response elements

All three RARs can repress AP-1 mediated gene expression. The transcription factor *Jun* can block osteocalcin gene induction by retinoic acid (Schüle *et al*, 1991). Both the DNA binding domain and LBD of the receptor are required. RARα inhibits binding of AP-1 to the AP-1 site but does not bind the AP-1 binding site itself. The GR DNA binding domain can substitute for the RAR DNA binding domain; therefore, the effect is not likely due to a retinoic acid mediated induction of another factor. The mechanism by which RARα inhibits AP-1 binding to DNA is either through a protein-protein interaction or through the blocking or destabilizing of the interaction between *Jun* and another factor which facilitates DNA binding by RARα (Schüle *et al*, 1991).

A high affinity binding site for specifically negative regulation by RAR has been observed in the oxytocin gene (Lipkin *et al*, 1992). A strong constitutive transactivation domain from the VP16 protein of HSV (herpes simplex virus), fused to the N-terminus of RAR acts as a transactivator of TRE/RAREs; however, this construct represses the oxytocin negative RARE [see Table 2, page 73]. This effect suggests a model in which different protein/protein and protein/DNA interactions impose differences in the conformation of the RAR on the palindromic TRE or the oxytocin negative RARE (Lipkin *et al*, 1992).

Promyelocytic leukaemia

Patients with acute promyelocytic leukaemia (APL) can experience morphologically complete remissions through treatment with all *trans*-retinoic acid, which induces differentiation of the malignant clone (Huang *et al*, 1988; Castaigne *et al*, 1990; Warrell *et al*, 1991). Retinoic acid causes differentiation of cultures of primary bone marrow cells from APL patients into mature granulocytes (Breitman *et al*, 1981). The t(15;17) (q22:q12-21) translocation associated with APL has been detected in 90% of patients (Kakizuka *et al*, 1991) and recently, with improved molecular methods, 100% of patients (Biondi *et al*, 1991). Two chimeric genes are formed by this translocation: *PML-RAR* and *RAR-PML* (Alcalay *et al*, 1992). The *PML* (promyelocytic leukaemia) gene is

expressed in APL cells as a fusion of *PML* and RARα. This unique mRNA encodes a fusion protein between RARα and *PML* which is found in the leukaemic cells from acute promyelocytic leukaemia patients. Myeloid differentiation is blocked by *PML-RAR* and this inhibition is relieved by RA treatment (Kakizuka *et al*, 1991). The RARα gene localization close to the t(15;17) breakpoint and the sensitivity of APL cells to retinoic acid led to the molecular cloning of the APL translocation. The translocation rearrangement occurs in the second exon of the RARα gene on chromosome 17. A single locus on chromosome 15 contains a cluster of breakpoints. A cysteine rich region, which is present in a family of DNA binding proteins, suggests that the *PML* protein may be a transcription factor (Kakizuka *et al*, 1991).

The *PML* gene has been characterized (Kakizuka *et al*, 1991; de Thé *et al*, 1991) and includes a proline rich region which is presumed to be a DNA binding domain with atypical zinc fingers, an α-helical region with a leucine zipper motif and a N-terminal serine rich region with presumed serine/threonine phosphorylation sites. *PML* is expressed as a family of transcripts derived from alternative splicing of the 3' coding exon (Kakizuka *et al*, 1991; de Thé *et al*, 1991). *PML/RARα* fusions from the 15q+ derivative contains the N-terminal *PML* DNA binding domain and leucine zipper region fused to the DNA binding domain and LBD of RARα (de Thé and Dejean, 1992). The chromosome 17 breakpoint is located in intron two of the RARα gene and the chromosome 15 breakpoint clusters occur in two introns of the *PML* gene. Therefore fusion proteins contain variable lengths of *PML* in the α-helical region (de Thé and Dejean, 1992).

PML/RARα mRNA is less abundant than RARα mRNA; however, the fusion protein is in excess to the normal receptor (Kastner *et al*, 1992). Also observed is a C-terminal truncated *PML* gene product and a 17p+ derived transcript containing the N-terminus of RARα fused to the C-terminal domain of *PML*. This product is presumed nonfunctional as it contains neither DNA binding domain nor other functional regions (de Thé and Dejean, 1992). *PML/RARα* is not a functionally normal RAR; however, deletion of the

PML part of the fusion protein will restore the *trans*-activating function of the receptor (Kakizuka *et al*, 1991; de Thé *et al*, 1991; Kastner *et al*, 1992). *PML* and *PML/RARα* form dimers through a probable coiled/coil interaction (de Thé and Dejean, 1992) and the expression of *PML/RARα* in HL60 cells impairs or blocks retinoic acid induced differentiation (de Thé and Dejean, 1992). Therefore, *PML/RAR* may represent a new class of dominant negative oncogene (Kakizuka *et al*, 1991) which may antagonize the retinoic acid induced gene expression which in turn leads to differentiation. Alternatively, if *PML* is a key regulatory gene in promyelocytic differentiation or growth control, then the fusion protein may render the *PML* function retinoic acid dependent. However, retinoic acid administration does not seem to restore a normal *PML* profile (de Thé and Dejean, 1992).

Coregulators

Certain proteins have been observed to associate with nuclear hormone receptors and stabilize their binding to the appropriate HRE. This observation has led to the recognition that proteins from nuclear extracts from a variety of tissues and cell lines can interact with these nuclear hormone receptors, stabilize their DNA binding and enhance their transcriptional activity (Rosen *et al*, 1991). For TR, a variety of thyroid hormone receptor accessory proteins (TRAPs) have been suggested, based on the presence of heat-labile, trypsin-sensitive activities in the nuclear extracts of many cell lines and tissues (Murray and Towle, 1989; Darling *et al*, 1991). These proteinaceous activities enhance the formation of TR-TRE complexes and require the DNA binding and ligand binding domains of the TR. These proteins also have TRE-specific sequence requirements: both half-sites of the rat growth hormone promoter TRE are necessary for TRAP binding to occur (Beebe *et al*, 1991). The VDR has also been associated with a nuclear accessory factor (NAF) which is necessary for its interaction with the osteocalcin VDRE (Liao *et al*, 1990).

The RARβ response element mediates responsiveness to retinoic acid differently in

different cell types (Sucov *et al*, 1990). The RAR has been associated with several "coregulators" from a number of cell lines which differ in molecular size (Glass *et al*, 1990). Multiple cross linking products form distinct cell type specific heterodimers (Glass *et al*, 1990). The HeLa cell line contains two proteins of molecular weight, 65 and 55 kDa, which have been seen to interact with RAR. As well, a 45 kDa protein has been isolated in HL60 cells (Glass *et al*, 1990) and a 42 kDa protein has been observed in liver cells (Lazar *et al*, 1991). A variety of cell lines, including Schneider cells from *Drosophila*, COS7, F9 (Yang *et al*, 1991), GC and CV1 cells (Glass *et al*, 1990), have been observed to contain RAR binding factors (RBFs) of varying sizes. The fact that insect cells possess RBFs similar to those of mammalian cells suggests an evolutionary importance in their function.

Retinoid X receptors

The retinoid X receptor (RXR) was originally cloned as an orphan receptor based on its sequence similarity with the estrogen receptor (Mangelsdorf *et al*, 1990). The RXRs have since been found to comprise a family of separate genes which code for receptors for a stereoisomer of all-*trans* retinoic acid, 9-*cis* retinoic acid (Heyman *et al*, 1992; Levin *et al*, 1992). RXRα is incapable of high affinity binding of retinoic acid and binds only at high concentrations (10^{-6}M) (Mangelsdorf *et al*, 1990; Mangelsdorf *et al*, 1991).

Three types of RXRs have been cloned, α, β and γ, each of which produces a number of isoforms by alternative splicing. The RXRs bind an HRE with a spacing preference of one nucleotide between the direct repeats of the consensus sequence (Mangelsdorf *et al*, 1991). Recently RXRα was found to bind the same HRE as the orphan receptors, COUP-TF (chicken ovalbumin upsteram promoter transcription factor) and EAR-2. *In vivo*, COUP-TF can interact with RXR and interfere with its transactivation function by forming inactive heterodimers (Kliewer *et al*, 1992b). Ligand-dependent transactivation functions which are promoter context-dependent have been shown in the ligand binding domain of the RARs and RXRs (Nagpal *et al*, 1992b). The N-terminal A/B regions of

the RARs and RXRs have a modulating function, also promoter context-dependent, which have been shown to cooperate with the C-terminal transactivation functions (Nagpal *et al*, 1992b).

RXR structure and expression

Cloning of RXRα revealed 75% amino acid identity with the RARs in the ligand binding domain and 61% in the DNA binding domain. The DNA binding domain of RXRα has 53% amino acid identity with TRβ and less than 27% in the ligand binding domain (Mangelsdorf *et al*, 1990). The 4.8 kb rat RXRα mRNA is abundant in the liver, muscle, lung, kidney and heart (Mangelsdorf *et al*, 1990). HeLa cells contain mRNA transcripts for RXRα and RXRγ. Retinoid X receptor has a less specific pattern of expression than RAR (Mangelsdorf and Evans, 1992). RARs have been detected in vertebrates only; however, RXRs are expressed in invertebrates as well (Oro *et al*, 1990). *Xenopus* eggs contain RARα and RARγ and RXRα and RXRγ (Blumberg *et al*, 1992). *Xenopus* RAR and RXRs are differentially expressed during development (Blumberg *et al*, 1992).

Human RXRβ was cloned as the HeLa cell factor which interacts with RAR to promote its binding to RAREs (Yu *et al*, 1991; Leid *et al*, 1992). RXRβ was also subsequently found to be the HeLa cell factor, TRAP, which interacts with TR to enhance its binding to TREs (Leid *et al*, 1992). RXRβ heterodimerizes with RAR, VDR and TR and enhances binding of the receptors to their cognate response elements. The affinity of binding is such that heterodimerization between RXR and the other receptors is much preferred over homodimerization of any of the receptors or heterodimerization of TR and RAR. RXRβ can be defined as a coregulator because it forms heterodimers with the specific receptor and increases DNA binding and transcriptional activation. Moreover, it has intrinsic DNA binding activity (Yu *et al*, 1991). HeLa cell nuclear extract contains two RBFs (RAR binding factors): the major protein which has now been found to be RXRβ and the minor 55 kDa protein which is hypothesized to be the human counterpart of mouse RXRα or γ. The smaller RBF proteins found in HL60 cells and in liver are

probably too small to be RXRs although they could be truncated, alternately spliced forms (Leid *et al*, 1992) such as are found in the RAR gene family. The RXR genes and their isoforms are well conserved between species but are not as well conserved between RXR types.

The RXR dimerization domain

The regions of TRα necessary for dimerization and interaction with nuclear auxiliary proteins are part of the DNA binding domain and a region in the C-terminus (Zhang *et al*, 1991). A DNA binding domain-negative mutant of chick *c-erbA* (a protooncogene encoding TR) can act in a dominant negative fashion with rat TR and RAR. The mutant *c-erbA* receptor has been shown to abolish the effects of TR and RAR by forming nonfunctional heterodimers with them. These heterodimers cannot bind DNA (Forman *et al*, 1989). Higher levels of TR can repress RAR (Graupner *et al*, 1989; Hudson *et al*, 1990).

The dimerization domain mediating this interaction is localized to the C-terminus of the receptor (the ligand-binding domain). These C-terminus sequences have been shown to act as dimerization interfaces in the ER and TR (Glass *et al*, 1989; Forman *et al*, 1989; Fawell *et al*, 1990). This region extends further toward the DNA binding domain in the RAR than in the ER. This domain contains nine heptad repeats consisting of hydrophobic amino acids at positions one and eight and charged amino acids with hydrophobic side chains at position five (Forman *et al*, 1989). These repeats are reminiscent of the leucine zipper dimerization motif in some transcription factors. This domain is hypothesized to form an α helix such that a hydrophobic dimerization interface forms on one side of the helix. Deletions or mutations in the ninth heptad have been shown to abolish RXR/RAR heterodimer formation (Forman and Samuels, 1990). Similarly, the addition of four amino acids to position 422 of the TR resulted in a breaking of the helix and loss of TR/RAR heterodimerization (Glass *et al*, 1989). N-terminal deletions do not affect RXR interactions with TRα; however, deletions of 60 or 75 amino acids from the C-terminus

abolishes heterodimerization. The dimerization domain of the RXR, RAR, TR and VDR which contain the heptad repeats seems to be necessary and sufficient to mediate heterodimerization between RXR and the other receptors. The dimerization function found in the C domain of other receptors does not seem to be required for dimerization in RXR but may have a role in regulating complex protein-protein interactions (Forman and Samuels, 1990). Recognition of the retinoid X response element is mediated through the DNA binding domain of the RXR. If the RXR DNA binding domain first zinc finger is mutated, no binding occurs.

Retinoid X response elements

The RXRα and CRBPII genes are coexpressed in the small intestine. The CRBPII element consists of five AGGTCA motifs with spacing of 1 base pair between the motifs from base pairs -639 to -605 in the CRBPII promoter [see Table 2, page 73]. The CRBPII response element acts as a RXRE and not a RARE. Both RXRβ homodimers and RAR/RXR heterodimers bind the CRBPII hormone response element while RAR homodimers alone do not. RAR can inhibit RXR transactivation of the CRBPII element (Mangelsdorf *et al*, 1991). This repression by RAR depends on the C-terminus of the receptor (Kliewer *et al*, 1992). The RXR/RAR heterodimer can bind both the direct repeat element with a spacing of five base pairs (DR+5) to cause transactivation and also the DR+1 to inhibit transactivation (Yu *et al*, 1991).

Ligand for retinoid X receptor

The ligand for the three RXRs has been shown to be 9-*cis* retinoic acid. 9-*cis* RA is 40 fold more potent than all-*trans* RA in activating RXRα and also activates RARα (Heyman *et al*, 1992). 9-*cis* RA is generated from all-*trans* RA *in vivo* as a result of intracellular conversion. Other examples of intracellular conversion of ligands for nuclear receptors include the aromatization of testosterone to estrogen, the hydroxylation of corticosterone to aldosterone and the dehydrogenation and isomerization of pregnenolone to progesterone. 9-*cis* RA can induce the formation of RXR homodimers in the absence of

DNA and high concentrations of all-*trans* RA can cause DNA binding by RXR homodimers (Zhang *et al*, 1992).

The CRBPII RARE is activated by the binding of RXR homodimers (Mangelsdorf *et al*, 1991) and is repressed by RXR/RAR heterodimers. The CRBPI RARE does not bind RXR in the presence of 9-*cis* RA, but is activated by RAR/RXR heterodimers in the presence of 9-*cis* RA (Mangelsdorf *et al*, 1991). The ApoA1 RARE binds RXR homodimer strongly in the presence of 9-*cis* RA and binds the homodimer weakly in the presence of all-*trans* RA. However, maximal induction of the ApoA1 element requires both the RXR and RAR (Zhang *et al*, 1992). The RARβ RARE binds the homodimer weakly and the RAR/RXR heterodimer strongly (Mangelsdorf *et al*, 1991).

The specific function of 9-*cis* RA appears to be to promote the formation of RXR homodimers and to enhance their binding to RXREs. Recently, the osteopontin VDRE, which has a direct repeat with a three base pair spacer (DR+3) motif, has been shown to be activated synergistically by vitamin D and 9-*cis* RA in the presence of VDR and RAR. The DR+6 VDRE has been shown be activated by vitamin D specifically in the presence of VDR alone (Carlberg *et al*, 1993). These studies have led to the concept that 9-*cis* RA is a general permissive factor which synergizes with other nuclear receptor ligands to amplify hormonal signals (Green, 1993).

RESEARCH HYPOTHESIS AND OBJECTIVES

Breast cancer is frequently a hormone responsive disease. Although the main focus of research in breast cancer is the role of estrogen in the development of the disease, other hormones, such as androgens and retinoids, have been implicated in breast cancer pathology and have been used in various therapies.

PIP has been proposed to be a marker of apocrine differentiation and has been associated with breast cancer. Additionally, PIP is regulated by a variety of hormones in a manner inversely correlated with the effect on cell growth. Hormones which inhibit cell growth or cause differentiation induce PIP gene expression and hormones which cause cell proliferation inhibit PIP gene expression. Therefore, PIP may be useful as a marker to study the state of differentiation in breast tissue potentially leading to cancer and is an ideal gene to study the molecular mechanisms of hormone regulation of gene expression.

The *cis*-acting DNA elements which interact with *trans*-acting factors in gene regulation are often found in the 5' flanking region of genes. The PIP gene is regulated by many different hormones. In order to study the molecular mechanisms of hormone regulation of the PIP gene, I hypothesized that some of the *cis*-acting hormone response elements responsible for this complex regulation may be found in the 5' flanking region of the PIP gene. Therefore, the objectives of this study were to:

1) Characterize the effect of retinoic acid, estrogen and androgen on PIP gene expression.
2) Characterize the 5' flanking region of the PIP gene for DNA elements necessary for conferring responsiveness to androgen, estrogen and retinoic acid.
3) Examine the molecular mechanisms of action of the hormones regulating the PIP gene and their interactions.

MATERIALS

All restriction endonucleases and DNA modifying enzymes were purchased from Amersham, Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs or USBC. The androgen receptor antibody used in the gel mobility shift assays was purchased from Monosan (Netherlands). The retinoic acid receptor antibodies used in the gel mobility shift assays were a gift of Dr. P. Chambon (Strasbourg, France). They are mouse ascites antibodies raised to the F domain of the three human RAR isoforms. The androgen receptor peptide (GST-AR2) used in the DNase I footprinting and gel mobility shift assays was provided by Dr. P. Rennie (Rennie *et al*, 1993). This GST-AR2 was made as an IPTG-induced glutathione-S-transferase fusion protein in *E. coli*. The estrogen receptor used in the DNase I footprinting and gel mobility shift assays was transcribed and translated from a ER expression vector (pT7βhER) using the TnT T7 coupled reticulocyte lysate system (Promega). The pT7βhER was a gift of Dr. S. Tsai (Houston, USA). 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. pTKCAT is a pUC19 based vector containing the thymidine kinase promoter adjacent to the *E. coli* chloramphenicol acetyl transferase gene. It was kindly provided by Dr. P. Cattini (Dept. of Physiology, University of Manitoba) (Cattini and Eberhardt, 1987). Other vectors used in these studies are M13mp18 and 19 (purchased from BRL), pVZ1 (a gift of Dr. M.L. Duckworth, Dept. of Physiology, University of Manitoba) and MMTV-TK-CAT (a gift of Dr. L. Murphy, Dept. of Biochemistry, University of Manitoba).

METHODS

1. SUBCLONING OF DNA FRAGMENTS INTO PLASMID VECTORS:

The necessity for specific DNA clones for experimental manipulations of the PIP cDNA and for gene sequencing required subcloning of the DNA subfragments from recombinant plasmids and phage. Usually the DNA fragment of interest was cleaved from the parent clone by digesting 50 to 100 μg of plasmid with the appropriate restriction endonuclease at a concentration of one to three units/μg for two to four hours at the temperature recommended by the manufacturer. The DNA subfragment was then recovered by gel electrophoresis either by using a low melting point agarose TBE (89mM Tris/89mM boric acid/2mM EDTA) gel or by eluting the DNA fragment into a well containing 5X TBE buffer cut in a 1% agarose TBE gel with ethidium bromide at a concentration of 0.5 μg/mL for visualization (both methods are found in Maniatis *et al*, 1982). The DNA subfragment was then purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and concentrated by ethanol precipitation. The purified DNA subfragment was then quantitated. A fraction of the purified DNA was electrophoresed through an agarose TBE gel adjacent to a known quantity of λ*HindIII*/ϕX174*HaeIII* digest marker DNA and the quantity of ethidium bromide staining was compared. A two to ten fold molar excess of purified DNA insert was then mixed with the specific vector which was linearized by digestion with the appropriate restriction endonuclease to produce ends compatible with that of the insert. A total reaction volume of 20 μL was achieved with the addition of 2 μL of 10X ligase buffer (0.5M Tris, pH7.4/0.1M $MgCl_2$/0.1M DTT/10mM spermidine/10mM ATP/1 mg/mL BSA) and 4 to 8 units of T_4 DNA ligase and water. The reaction was allowed to proceed at room temperature for four to twelve hours and a portion was subsequently used to transform the appropriate competent bacterial host.

In the case of DNA fragments subcloned into M13RF phage, 5 to 10 μL of the ligation mixture was mixed with 200 μL of competent *E. coli* strain JM101 cells and incubated on ice for 30 minutes. A heat shock of 90 seconds was then applied and 200μL of warm

YT broth (8 g/L bactotryptone/5 g/L bactoyeast extract/5 g/L NaCl) was added. The transformed cells were then plated by mixing gently with 3.5 mL of warm YT top agarose containing 0.3mM IPTG (isopropyl thiogalactoside) and 0.017% X-gal and poured onto YT agarose plates containing 50 μg/mL ampicillin. The plates were incubated overnight at 37°C to allow plaques to develop and were inspected for blue color development the following morning. Due to the disruption by the insertion of the subcloned DNA fragment of a mutant form of the *lacZ* gene which is inducible by IPTG, the recombinant plaques appeared white. The color of nonrecombinant plaques was due to the conversion of the color indicator X-gal to a blue chromophor by the β-galactosidase enzyme.

2. RESTRICTION ENDONUCLEASE DIGESTION AND AGAROSE GEL ELECTROPHORESIS:

The analysis of large fragments of DNA is facilitated by restriction endonuclease mapping. A more detailed map of the PIP gene than that published in Myal *et al* (1991) was required for further experimental manipulation. Accordingly, the PIP cDNA (prior to sequencing) and gene were digested by restriction endonucleases to formulate a detailed restriction map. Briefly, the isolated DNA subfragments (1 to 3 μg) to be analyzed were cleaved with specific restriction endonucleases (1 to 3 units/μg) for two to four hours at the temperature recommended by the manufacture. The reaction was then loaded onto a 0.8 to 1.5% agarose TBE gel and electrophoresed in 1X TBE buffer at 80 to 120 volts. Visualization of DNA bands is accomplished by the addition of ethidium bromide at 0.5 μg/mL. Ethidium bromide interchelates with DNA and fluoresces under ultraviolet light. By running marker DNA of known size, the pattern generated by a particular restriction endonclease can be analyzed and the positions of restriction endonuclease recognition sequences can be deduced. By using combinations of restriction endonucleases, a map of recognition sequences can be generated. Restriction endonucleases which cleave a given fragment of DNA into one to five fragments is ideal for such analysis. Restriction endonucleases which cleave a given fragment of DNA frequently are not particularly helpful because they generate a complex pattern of fragments which are difficult to interpret.

3. SOUTHERN TRANSFER AND HYBRIDIZATION:

After the DNA samples were fractionated on an agarose gel, the gel was soaked in an excess of 1.5M NaCl/0.5 NaOH for one hour at room temperature with gentle agitation. After this denaturing step the gel was transferred to a neutralizing solution of 1.5M NaCl/1M Tris (pH8.0) for one hour at room temperature. The gel was then transferred to nitrocellulose paper in 20X SSC (3M NaCl/0.3M sodium citrate, pH7.0) as outlined in Maniatis *et al* (1982). After 12 hours, the nitrocellulose was rinsed in 6X SSC, air dried and baked for two hours at 80°C. The nitrocellulose filter was then prehybridized in a plastic bag for at least two hours at 42°C and a cDNA probe which had been radioactively labelled by nick translation or random priming and denatured by boiling was added to the prehybridization solution (6X SSC/0.5% SDS/5X Denhardt's solution/100μg/mL denatured salmon sperm DNA/10mM EDTA). Hybridization was continued at 42°C for at least 6 to 8 hours and the blot was washed in 2X SSC/0.5% SDS for 15 minutes at room temperature and then in 0.1X SSC/0.1% SDS at 65°C. The nitrocellulose filters were then autoradiographed for 6 to 24 hours at -70°C with an intensifying screen.

4. DNA LABELLING:

Nick translation: DNA fragments were labelled with [^{32}P]-α-dCTP for the purposes of hybridization using nick translation. *E. coli* DNA polymerase I adds nucleotides to 3' -OH ends to nicked double stranded DNA. The 5' to 3' exonuclease activity of the enzyme also can remove nucleotides from the 5' side of the nick. In nick translation, these activities are exploited to replace existing nucleotides with radioactive nucleotides. The materials used for nick translation were obtained in a kit from Amersham.

Random priming: DNA can also be labelled with [^{32}P]-α-dCTP by random priming. Double stranded DNA is denatured by boiling and annealed to random oligonucleotide primers. These primers are extended by reverse transcriptase in the presence of one radioactive nucleotide and other unlabelled nucleotides. The materials used for random priming were obtained either in a kit from Amersham (using *E. coli* DNA polymerase I) or Pharmacia (using T_7 polymerase).

End labelling: DNA was end labelled with Klenow fragment for the purposes of DNase I footprinting and gel mobility shift assays. The DNA fragment with a protruding 5' end was incubated at 37°C in the presence of Klenow fragment and the appropriate [^{32}P]-α labelled nucleotides and cold nucleotides. The unincorporated nucleotides were then separated by use of Ultrafree MC filter columns (Millipore).

5. SINGLE STRANDED PHAGE PURIFICATION:

Single stranded DNA was then prepared for sequencing. Usually 4 to 12 single white plaques were isolated by picking individual agarose plugs with a small bore Pasteur pipettes and adding them to 1 mL cultures of JM101 bacteria. The bacterial cultures were 1:100 dilutions of an overnight culture of bacteria with YT broth and agitating gently at 37°C for 90 minutes. The infected cultures were allowed to incubate with gentle agitation for four to five hours and the phage were harvested from the medium by removal of the bacteria by centrifugation followed by precipitation of the phage with 0.5M NaCl/4% PEG at room temperature. The supernatant was then removed by centrifugation and the remaining phage pellet was resuspended in 10mM Tris (pH7.4)/0.1mM EDTA. The phage protein was then extracted by an equal volume of water saturated phenol, followed by a second extraction with chloroform:isoamyl alcohol (24:1) and the DNA was precipitated at -70°C with 0.2M sodium acetate (pH5.5) and 2.2 volumes of ethanol. The phage DNA pellet was recovered by centrifugation and resuspended in 10mM Tris (pH7.4)/0.1mM EDTA and stored at -20°C.

6. TRANSFORMATION:

In the case of DNA subcloned into plasmids, 5 to 10 µL of the ligation mixture was added to 200 µL of a competent *E. coli* bacterial host and incubated on ice for 30 minutes. A heat shock of 90 seconds was then applied and 200 µL of LB broth was added and the mixture was allowed to incubate at 37°C for 30 minutes. The transformed cells were then spread onto LB agar plates containing 50 µg/mL ampicillin and 0.017% X-gal if the plasmid contained the lacZ color selection system. The plates were then incubated at 37°C overnight and were inspected for colony formation the following

morning. Colonies chosen for analysis were picked with a flame sterilized wire loop into 5 mL LB broth containing 50 μL/mL ampicillin. The cultures were allowed to incubate with agitation for 10 to 16 hours and the recombinant plasmids were harvested either by the alkaline lysis method of Maniatis (Maniatis *et al*, 1982), the method of Serghini *et al* (1989) or the preferred method of Chowdhury (1991). Briefly, equal volumes of bacterial culture and phenol:chloroform:isoamyl alcohol are mixed and vortexed vigorously. This mixture is centrifuged and the aqueous layer containing the plasmid is retained. The plasmid is then precipitated from the aqueous layer by isopropanol and pelleted by centrifugation.

7. PLASMID DNA AMPLIFICATION AND PURIFICATION:

Large scale preparations of plasmid were accomplished by a modification of the alkaline lysis method of Maniatis *et al* (1982). A 5 mL confluent culture of transformed bacteria was added to 500 mL of LB broth in a large culture flask and allowed to reach an A_{260} of 0.8 before 0.17 mg/mL chloramphenicol was added to amplify the recombinant plasmid DNA selectively. The large flask was then allowed to incubate with gentle agitation for 16 hours. The bacteria were recovered by centrifugation for 10 minutes at 11,000 x g (8000 rpm) in a JA10 rotor. The pellet was then resuspended in 9 mL lysis buffer (50mM glucose/25mM Tris, pH8.0/10mM EDTA) and transferred to 40 mL Oak ridge tubes where 1 mL of egg white lysozyme (20 mg/mL) was added. This mixture was allowed to incubate with gentle agitation for 30 minutes on ice and then 10 mL of 0.2N NaOH/1% SDS solution was added for another 30 minutes incubation. Finally, 10 mL of potassium acetate solution (3M with respect to potassium/5M with respect to acetate) was added for a final 30 minute incubation. The plasmid DNA was collected in the supernatant by centrifuging the lysed cell slurry in a fixed-angle JA20 rotor at 40,000 x g (18000 rpm) for 30 minutes. The supernatant was extracted by an equal volume of phenol:chloroform:isoamyl alcohol and precipitated with 0.6 volumes of isopropanol for 10 minutes at room temperature in 30 mL Corex tubes. The DNA was recovered by centrifugation in a JA20 rotor at 27,000 x g (15000 rpm) for 10 minutes and redissolved in 6 mL 10mM Tris /1mM EDTA. Cesium chloride (6.6g) was added and dissolved by

vigorous vortexing. This mixture was then placed in a Ti75 polyallomer heat sealed tube and 0.5 mL of 1 mg/mL ethidium bromide was added. The excess tube space was filled with mineral oil and the tubes were heat sealed. The plasmid DNA was separated from the bacterial and nicked DNA by centrifugation in a fixed-angle Ti75 rotor for 16 to 18 hours at 200,000 x g (55000 rpm) followed by 45 minutes at 130,000 x g (45000 rpm). The plasmid DNA was visualized using a long wave ultraviolet light. The lower band was specifically removed using a 3 mL syringe with a 16 gauge needle after a vent hole was created in the tube air space by a 16 gauge needle. The ethidium bromide-containing plasmid solution was brought to a total volume of 4 mL with water and extracted with excess volumes of isoamyl alcohol three to four times to remove the ethidium bromide. The plasmid was then precipitated with 0.2M sodium acetate and 2.2 volumes of ethanol at -20°C for two or more hours. The purified plasmid was recovered by centrifuging in a JA20 rotor for 30 minutes at 27,000 x g (15000 rpm) and redissolved in water and the quantity was calculated from the absorbance at a wavelength of 260 nm.

8. SINGLE STRANDED DNA SEQUENCING:

Single stranded DNA was sequenced using the Sanger *et al* (1977) dideoxy method of sequencing. Briefly, 1/4 of a minipreparation of single stranded DNA as outlined above was used in a sequencing reaction. Single stranded DNA (7 μL) was allowed to anneal to 1 pmol of primer with 1.5 μL 100mM Tris (pH8)/50mM $MgCl_2$ by incubating the mixture in a total volume of 10 μL for 5 minutes at 65°C then allowing it to cool slowly to room temperature. Four aliquots of [^{35}S]-α-dATP were lyophilized and resuspended in 2 μL of each dideoxynucleotide (0.5 mM). To each tube, 2 μL of annealed DNA was added and 1 unit of the Klenow fragment of DNA polymerase I. Nineteen minutes later, 2 μL of cold deoxynucleotide (0.125 mM dNTPs) was added and further incubated for 15 minutes. Finally 5 μL of formamide dye mix was added and the tubes were boiled for 3 minutes and immediately placed on ice. The samples were loaded onto a 6% polyacrylamide gel containing 7M urea and was run in 1X Winter's TBE buffer (133mM Tris/45mM boric acid/3mM EDTA) for 2 to 4 hours at 1600 V and 30 mA.

9. DOUBLE STRANDED DNA SEQUENCING:

The sequencing of double stranded DNA requires an additional step to denature the plasmid prior to starting the sequencing reaction. Double stranded DNA (1 μg/10 μL) was mixed with 10 μL of 0.4M sodium acetate and incubated at room temperature for 5 minutes. The mixture was then neutralized with 4 μL of 2M ammonium acetate (pH4.7) and precipitated with three volumes of ethanol. After recovery by centrifugation, the DNA was resuspended in 7 μL water and the sequencing procedure was similar to the single stranded DNA procedure outlined above. The earlier single-stranded sequencing employed Klenow, while the later double-stranded sequencing used Sequenase. The materials used in sequencing using Sequenase were obtained in a kit from USBC.

10. EXONUCLEASE III (*ExoIII*) GENERATED DELETIONS:

ExoIII deletions were made using the method of Henikoff (1984). *ExoIII* enzyme 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. Therefore to prepare plasmid for *ExoIII* digestion, the plasmid DNA was digested with a restriction endonuclease producing a 3' protruding end which was unavailable to digestion and a restriction endonuclease producing a 5' end which was accessible to digestion. The *ExoIII* digestion was carried out at 35°C in 80 μL of digestion buffer (66mM Tris, pH8.0/0.66mM $MgCl_2$) and 4 units/μL of enzyme. By varying the times of digestion, unidirectional nested deletions can be easily obtained. Under these conditions, 200 base pairs are removed every 30 seconds. Aliquots of 7.5 μL were removed every 30 seconds and the reaction was stopped and the remaining single stranded DNA was digested by addition of S1 nuclease buffer (40mM potassium acetate/300mM NaCl/1.3mM $ZnSO_4$/7% glycerol/0.25 units/μL S1 nuclease). The linear DNA molecules were then blunt ended by addition of Klenow buffer (0.66mM Tris/3.3mM $MgCl_2$/100 units/mL Klenow) and chased with unlabelled 4μM dNTP mix. The aliquots were then circularized by addition of 20mM Tris/4mM $MgCl_2$/0.4mM ATP/0.4mM DTT/2% PEG/0.2 units T_4 DNA ligase. After 30 minutes at room temperature, the ligation mixture was used to transform the appropriate competent

bacterial host.

11. CELL CULTURE:

The human breast cancer cell lines T-47D and ZR-75-1 were obtained from sources described by Shiu (1979). The T-47D cell line was derived from the pleural effusion of a patient with a ductal breast carcinoma. This epithelial cell line was characterized by Horwitz *et al* (1978) to contain high levels of PR, moderate levels of AR and low levels of GR. It has also been shown by Freake *et al* (1981) to contain ER and VDR. The ZR-75-1 cell line was obtained from the ascitic effusion of a patient with an infiltrating ductal carcinoma. These cells were characterized to contain receptors for androgen, progesterone, glucocorticoid and estrogen (Engel *et al*, 1978). 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 mg/ml, respectively), bovine insulin (10 mg/ml), and 5 or 10% (v/v) fetal bovine serum. This medium is referred to as complete medium. 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.

5α-dihydrotestosterone (DHT), retinoic acid (RA), 17β-estradiol (E), 6α-methyl-17α-hydroxy-progesterone acetate (MPA) and dexamethasone (DEX) were obtained from Sigma Chemical Co. Stock solutions of 1.0 X 10^{-2}M in ethanol were prepared and stored in the dark at -20°C. Human growth hormone and human prolactin were a gift of Drs. H.G. Friesen and I. Worsley (Dept. of Physiology, University of Manitoba). Stock solutions of prolactin (prepared in water) and growth hormone (prepared in 0.05M ammonium bicarbonate) were prepared at a concentration of 1 mg/mL and frozen in aliquots at -20°C.

For cells grown for RNA isolation, stock cells were plated at 2 X 10^3 cells/cm^2 and left to attach to the plates for 48 hours in insulin free medium containing 5% fetal bovine

serum. Cells were then changed to hormone depleted media containing 5% charcoal stripped fetal bovine serum and maintained for 48 hours. Various hormones were then added for varying times dependent on the experiment. Control cells for steroid or retinoic acid treatment were treated with media containing ethanol to the same concentration as the hormone treatment. After the appropriate time of hormone treatment, cells were harvested for RNA isolation.

12. RNA ISOLATION:

RNA was isolated by the guanidium isothiocyanate/cesium chloride method (Chirgwin *et al*, 1979). Briefly, medium was removed from the tissue culture plates and 7 mL of guanidium isothiocyanate solution (4M guanidium isothiocyanate/0.5% sarkosyl/0.1M β mercaptoethanol/5mM sodium citrate, pH7.0) was added. The cells were scraped with a rubber policeman and the cell suspension was transferred to a polypropylene tube and drawn through an 18 gauge needle several times. This solution was then carefully overlaid on a 5.7M cesium chloride/0.1M EDTA, pH7.5 cushion in a polyallomer Ti75 heat seal tube. This tube was then heat sealed and centrifuged in the Ti75 rotor for 20 hours at 80,000 x g (35000 rpm). After centrifugation, the RNA pellet was recovered from the tube and dissolved in water. The quantity of RNA was calculated from the absorbance at a wavelength of 260 nm.

13. NORTHERN ANALYSIS AND HYBRIDIZATION:

Northern analysis of formamide and formaldehyde denatured RNA was performed as in Maniatis *et al* (1982). Samples containing 10 to 30 μg of total RNA were prepared by adding 2.7 μL of gel running buffer (GRB = 40mM MOPS, pH7.0/10mM sodium acetate/1mM EDTA, pH8.0), 10 μL formamide and 4.7 μL formaldehyde to a total volume of 30 μL. The samples were incubated at 65°C for 3 minutes and 3 μL of loading buffer (LB = 50% glycerol/1mM EDTA/0.4% bromophenol blue and xylene cyanol) was added. The samples were loaded onto a denaturing formaldehyde agarose gel (1% agarose/1X GRB/2.2M formaldehyde/0.5 μg/mL ethidium bromide) and electrophoresed in 1X GRB. After the RNA had been fractionated on the gel, the RNA

was transferred to nitrocellulose as described in Maniatis *et al* (1982). After overnight transfer in 20X SSC, the nitrocellulose filter was rinsed in 2X SSC, air dried and baked for two hours at 80°C. The hybridization conditions for Northern analysis are identical to those for Southern analysis outlined on page 82. The nitrocellulose filters were washed in 2X SSC/1% SDS at room temperature for 15 minutes and in 0.2X SSC/0.1% SDS at 65°C. The nitrocellulose filters were then autoradiographed for 24 to 36 hours at -70°C with an intensifying screen. Quantitation of relative amounts of specific mRNA transcripts was performed by densitometric scanning of the autoradiographs using the computer software, SCANPLOT version 4.01 (Cunningham Engineering).

14. TRANSIENT TRANSFECTION AND CAT ASSAYS:

Cells used for transient transfection were primarily ZR-75-1 human breast cancer cells; however, in some experiments T-47D human breast cancer cells were used. These cells were plated at a density of $1.0X10^6$ cells per 10 cm dish, in insulin-free medium containing fetal bovine serum. To study the effect of steroid hormones on the transfected constructs, it was necessary to deplete the endogenous levels of steroids in both the cells and the medium. This depletion was done by culturing the cells for 48 hours in phenol red free medium containing 5% steroid-depleted (charcoal treated) fetal bovine serum for 48 h after plating. The cells were then transfected with the appropriate construct (15 μg) and in some instances co-transfected with a steroid hormone receptor expression vector (2.5-5 μg) using the calcium phosphate precipitation method (Howley *et al*, 1983). In this method, 0.5 mL of 2X calcium chloride-DNA solution (0.25M $CaCl_2$/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 minutes to allow a fine $CaPO_4$-DNA precipitate to form. Equal amounts of this precipitated solution (450 μL) were then added to two identical dishes of hormone depleted cells containing 7 ml of fresh medium. Four hours later, DNA uptake by the cells was enhanced by removing the medium from the dishes and treating the cells with a 25% glycerol DMEM solution for 8-10 min. Following this treatment the cells were washed with DMEM, 10 mL of fresh medium was added and one

of the matched plates was treated with the appropriate hormone (final concentration 10^{-8}M). The cells were then incubated for 36 to 42 hours in order to express the CAT gene. After this incubation cells were isolated using trypsin/EDTA and resuspended in 200 μL of 100mM Tris/0.1% TritonX-100. This solution lysed the cells after 15 minutes incubation on ice. The cellular debris was pelleted by microcentrifugation for 5 minutes. To remove any endogenous acetylases that may degrade the acetylated products, the supernatant was heated at 65°C for 10 minutes 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 (50 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, pH 7.5 and adding 62 μL of CAT buffer (40 μL water/20 μL Tris, pH7.0/2 μL [^{14}C]-chloramphenicol) and 10 μL of acetyl coenzyme A (70 μg/ml) which is the source of the acetyl group. Acetylation of the labelled chloramphenicol was allowed to proceed for 4 to 6 hours at 37°C. After the incubation, acetylated products were extracted with ethyl acetate (600 μL) and lyophilized. 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 to 96 hours later. Acetylated chloramphenicol product was quantitated by scraping the isolated 3-mono-acetylated [^{14}C]-chloramphenicol product from the TLC plate into a scintillation cocktail and then scintillation counts were measured. Significant differences in CAT expression between hormone treated and untreated transfected cells were confirmed by statistical analysis of multiple experiments.

15. β-GALACTOSIDASE ASSAY:

An alternate method of standardizing the amounts of cell extract used in the CAT assays was to cotransfect the expression vectors with a β-galactosidase expression vector (CH110; Pharmacia). This vector contains a functional *lacZ'* gene driven by the SV40 early promoter. When transiently expressed in tissue culture cells, CH110 expresses the

β-galactosidase enzyme. The levels of β-galactosidase can be determined by assaying the extracts of transfected cells for enzyme activity (Maniatis *et al*, 1982). Briefly, 30 μL of the cell extract is mixed with o-nitrophenyl-β-D-galactopyranoside (at a concentration of 4.5 mg/mL) in the presence of 1.0 mM $MgCl_2$ and 45 mM β-mercaptoethanol. This reaction is in a total volume of 300 μL in 0.1 M sodium phosphate buffer. The reaction is incubated for 30 minutes at 37°C until a yellow color has developed. The reaction is then stopped by adding 500 μL of 1 M Na_2CO_3. The optical density of the reactions is determined at a wavelength of 420 nM and is compared to a standard curve of β-galactosidase enzyme. Unfortunately, the transient expression of the β-galactosidase enzyme was affected by the treatment of the tissue culture cells with retinoic acid. Therefore, this method of standardization of the cell extracts was abandoned.

16. STATISTICAL ANALYSIS:

The statistical significance of the results from the transient expression studies was determined primarily by the student's t-test. Unfortunately, due to the lack of a transfection efficiency control, the individual PIPCAT constructs were not comparable with each other. Therefore, to determine the significance of response to hormone treatment, each construct was tested individually for its increase in mean induction over the base line provided by control cells (not treated with hormone). The statistical method used was the one-tailed student's t-test with a p value of 0.05 [see Table 4]. For the combination hormone treatment, a two-tailed t-test was applied using SigmaStat (Jandel Scientific) and the difference in the mean value of two hormone treatments was compared and the p values were calculated [see Table 5].

17. PREPARATION OF NUCLEAR EXTRACTS:

Nuclear extracts were made by the procedure of Dignam *et al* (1983). Briefly, cells were harvested with trypsin and counted. The cells were resuspended and swelled with buffer A (10mM HEPES, pH7.9/1.5mM $MgCl_2$/10mMKCl/0.5mM DTT). Then the cells were broken open with a Dounce homogenizer and the nuclei were recovered by centrifugation at 600 x g (2000 rpm) for 10 minutes at 4°C in a JS-13 swinging bucket rotor. The

nuclei were broken open with a Dounce homogenizer in buffer C (20mM HEPES, pH7.9/25% glycerol/0.42M NaCl/1.5mM MgCl2/0.2mM EDTA/1.0mM PMSF/1.0mM DTT) and the nuclear membranes were removed by centrifugation at 25,000 x g (12,500 rpm) for 20 minutes at 4°C in a JS-13 swinging bucket rotor. The nuclear extract was dialyzed in buffer D (20mM HEPES, pH7.9/20% glycerol/0.1M KCl/0.2mM EDTA/1mM PMSF/1.0mM DTT) and centrifuged at 200,000 x g (55000 rpm) in the Ti75 rotor to remove all insoluble matter. Finally, the nuclear extract was aliquoted and frozen at -70°C.

18. DNASE I FOOTPRINTING ASSAY:

The DNase I footprinting assay (Galas and Schmitz, 1978) is useful for identifying proteins that bind to a specific fragment of DNA. In brief, the DNA fragment was end labelled specifically at one end only, usually using T_4 polynucleotide kinase to phosphorylate or Klenow fragment to add a radioactive nucleotide to a 5' protruding end generated by appropriate restriction digests. The footprinting reaction included the labelled DNA fragment (40,000 cpm), a specific nuclear extract or the AR peptide (GST-AR2: Rennie *et al*, 1993) in buffer D in a total volume of 12.5 µL (as outlined above), 0.5 µg of a nonspecific competitor DNA, poly (dI-dC) and magnesium (20 mM) and calcium (3.3 mM) ions. This mixture was incubated on ice for binding to occur and then subjected to limited DNase I digestion. The concentration of DNase I was such that a single random nick is produced per molecule of labelled DNA and was determined by performing the DNase I digestion on the labelled DNA fragment with various concentrations of the enzyme, in the absence of any nuclear extract or other proteins. The reaction was then stopped with a solution (120mM Tris, pH7.5/15mM EDTA/180mM NaCl/1% SDS) containing 100 µg proteinase K and 10 µg yeast transfer RNA. After 25 minutes at 37°C the mixture was extracted with phenol:chloroform:isoamyl alcohol and precipitated with sodium acetate and ethanol. The DNA was recovered by centrifuging, run on a 7% sequencing polyacrylamide urea gel and autoradiographed. The protein binding sites were identified by comparing the pattern or ladder of DNA bands produced in the presence of proteins with the pattern of bands produced in the absence of proteins.

Proteins which protected an area of the DNA fragment by binding to it were seen as bands missing from the ladder. If the protein altered the conformation of DNA such that DNase I could preferentially nick a specific site in the fragment, then the resulting hypersensitive site was seen as a darker band.

19. GEL MOBILITY SHIFT ASSAY:

The gel mobility shift assay reveals binding interactions between proteins and a specific fragment of DNA. The method is described briefly as in Garner and Rezvin (1981). Nuclear extracts from cells were used to specifically retard [^{32}P] labelled DNA fragments which contain binding sites for proteins in the extract. Nuclear extract (5 µg protein) or the AR peptide (GST-AR2: Rennie *et al*, 1993) and 4 µg of the nonspecific DNA competitor, poly (dI-dC) were incubated on ice for 15 minutes in buffer (20mM Tris, pH7.5/100mM KCl/1mM $MgCl_2$/0.1mM EDTA/0.5mM DTT/10% glycerol). A 5' end [^{32}P] labelled DNA fragment (0.2 ng) was added and incubated on ice for a further 15 minutes. In specific samples, 1 µL of antibodies to AR or RAR was added and incubated for an additional 15 minutes on ice. The RAR antibodies have been shown to specifically retard RAR/RARE complexes in gel mobility shift assays (Rochette-Egly *et al*, 1991). A 5% polyacrylamide gel was loaded with the samples and run in 0.5X TBE (45mM Tris/45mM boric acid/2mM EDTA). After electrophoresis, the gel was autoradiographed.

20. CONSTRUCTS:

Sequencing of the PIP cDNA: The PIP cDNA was subcloned into the *EcoRI* site of pBR322 by Dr. L. Murphy (Murphy *et al*, 1987). For sequencing purposes, the cDNA was subcloned into the *EcoRI* site in the multiple cloning region of M13mp18 and mp19 and transformed into competent *E. coli* strain JM101. As well, the fragments generated by digestion of the cDNA with *PstI* (2 sites) and *HpaII* (1 site) were subcloned into the appropriate sites in the multiple cloning region (MCR) of M13 to ensure that the full sequence could be obtained by overlapping subclones.

Restriction analysis of the PIP gene: The 14 kilobase insert of the PIP gene was cloned by Dr. Y. Myal (Myal *et a*l, 1991). It was released from the λ phage vector EMBL3A and subcloned into pUC9 and transformed into competent *E. coli* strain DH5α. For sequencing analysis of the 5' flanking region of the PIP gene, a 2 kb fragment from the *SalI* site of the 5' arm of the λ phage to the *PstI* site located within the first exon of the mRNA was cloned into the *SalI* to *PstI* site of pUC9 and transformed into the competent *E. coli* strain DH5α. This plasmid (referred to as 2.0gPIP) was cleaved with *SphI* and *HincII* to generate ends appropriate for exonuclease III digestion and subclones of varying lengths were generated and sequenced.

The *NheI* to *SphI* 1.5 kilobase fragment of 2.0gPIP was subcloned into the *XbaI* to *SphI* site of the MCR of pVZ1 and transformed into competent *E. coli* strain TG1λ. This plasmid is referred to as 1.5gPIP.

Transient transfections: The *NarI* to *SphI* 1.5 kilobase fragment of 1.5gPIP was subcloned into the *NarI* to *SphI* site of pSV40CAT and transformed into the competent *E. coli* strain RR1. This construct is referred to as 1.5gPIPsvCAT.

The *SphI* to *BglII* fragment of 1.5gPIPsvCAT containing the SV40 early promoter was removed by cleaving with those endonucleases and circularizing the remaining DNA, generating the 1.5SB-PIP-CAT construct.

Exonuclease III digestion utilizing the *SacI* to *SmaI* site of 1.5SB-PIP-CAT was used to create deletions from the 5' end of 1.5SB-PIP-CAT. These plasmids were designated the SB-PIP-CAT constructs and range in size from 1.5 kilobases to 140 base pairs. The smallest construct contains the region from -100 to +40 of the PIP promoter and is referred to as SB18S-PIP-CAT.

DNase I footprinting and gel mobility shift assays: To create constructs with inserts of

convenient size for DNase I footprinting and gel mobility shift assays, the 1.5 kilobase insert of 1.5gPIP was digested with *FokI* and *AvaI* which generated DNA fragments ranging in size from 87 to 326 base pairs. These fragments were blunt ended with Klenow fragment and subcloned into the *SmaI* site of pVZ1. These constructs are referred to as PIP probes A, C, D, E and F depending on their localization in the PIP 5' flanking region in a 5' to 3' direction.

PIP probe C' was created by cleaving SB1-PIP-CAT (containing -1144 to +43) with *FokI* and *NsiI* to generate a fragment from -1144 to -1030. This fragment was blunt ended with Klenow fragments and subcloned into the *SmaI* site of pVZ1.

The *FokI/AvaI* fragments of the 1.5 kb insert of 1.5gPIP were cleaved from the pVZ1 vector with *HindIII* and *NsiI* and were subcloned into the *HindIII* to *NsiI* site of pSV40CAT. These resulting subclones referred to as APIP-SV-CAT through GPIP-SV-CAT were cleaved with *NcoI* to *NotI*, thus removing the SV40 early promoter, and the corresponding fragment of SB18S-PIP-CAT containing the PIP promoter was subcloned into that site. These subclones are referred to as APIP-CAT through FPIP-CAT (GPIP-CAT was not created as it would only contain two copies of the PIP promoter).

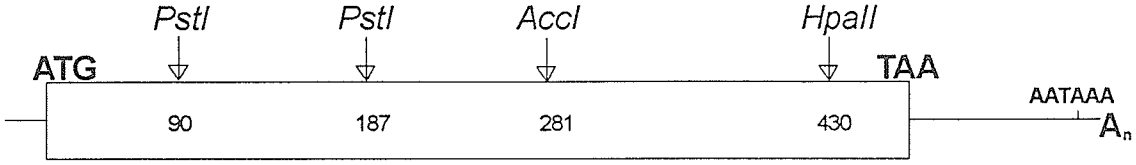
RESULTS

Cloning and characterization of the PIP cDNA and gene

The PIP cDNA was cloned from a λgt11 phage library made with mRNA derived from the T-47D human breast cancer cell line (Murphy *et al*, 1987a). I sequenced the 565 base pair PIP cDNA, identifying an open reading frame encoding a protein of 146 amino acids. Relevant features of this cDNA are an ATG initiation codon at base pair 26 fulfilling the requirements outlined by Kozak (1983), a termination codon located at base pair 464 and a polyadenylation signal at base pair 545 followed by a poly A tail after base pair 565 [Fig. 1]. The PIP cDNA was found to have sequence similarity to a mouse protein isolated from the submaxillary gland, an androgen dependent tissue (Windass *et al*, 1984). The amino acid sequences, when compared to human PIP, exhibit 51% sequence identity and 67% similarity when conserved amino acid changes are allowed. The major difference between PIP and the mouse submaxillary gland protein (mSMG) was that the derived amino acid sequence of the mSMG protein did not appear to contain a signal peptide. Subsequently, this submaxillary gland protein was resequenced in our laboratory and a sequencing difference was identified in the 5' sequence of the cDNA which caused a shift in the reading frame (Myal, pers. comm.). The overall effect of this shift was to increase the amino acid similarity in the N-terminus of the protein, indicating a potential signal peptide [Fig. 2].

Figure 1 The PIP cDNA.
A) Partial restriction map of the PIP cDNA depicting the restriction endonuclease sites used in generating PIP clones for sequencing. B) Sequence of the PIP cDNA. The initiation codon (position 26) and the termination codon (position 464) are double underlined. The polyadenylation signal (position 545) is singly underlined.

Fig. 1→



CACATTGCCT TTTGTTTTCT CCAGCATGCG CTTGCTCCAG CTCCTGTTCA
GGGCCAGCCC TGCCACCCTG CTCCTGGTTC TCTGCCTGCA GTTGGGGGCC 100
AACAAAGCTC AGGACAACAC TCGGAAGATC ATAATAAAGA ATTTTGACAT
TCCCAAGTCA GTACGTCCAA ATGACGAAGT CACTGCAGTG CTTGCAGTTC 200
AAACAGAATT GAAAGAATGC ATGGTGGTTA AAACTTACCT CATTAGCAGC
ATCCCTCTAC AAGGTGCATT TAACTATAAG TATACTGCCT GCCTATGTGA 300
CGACAATCCA AAAACCTTCT ACTGGGACTT TTACACCAAC AGAACTGTGC
AAATTGCAGC CGTCGTTGAT GTTATTCGGG AATTAGGCAT CTGCCCTGAT 400
GATGCTGCTG TAATCCCCAT CAAAAACAAC CGGTTTTATA CTATTGAAAT
CCTAAAGGTA GAATAATGGA AGCCCTGTCT GTTTGCCACA CCCAGGTGAT 500
TTCCTCTAAA GAAACTTGGC TGGAATTTCT GCTGTGGTCT ATAA*AATAAA*
CTTCTTAACA TGCTTA$_n$

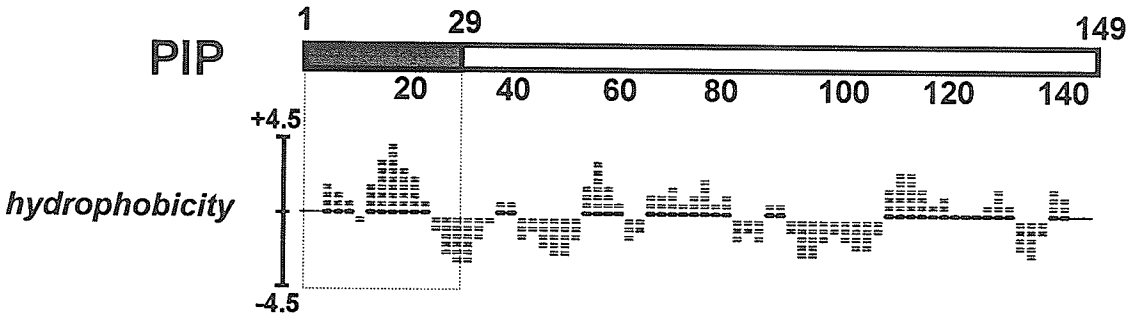
The PIP protein derived from the cDNA sequence has a calculated molecular weight of 16.5 kDa. The mature PIP is glycosylated (Shiu and Iwasiow, 1985) and a glycosylation consensus site (Asn-X-Thr) is located at amino acids 105 to 107. PIP is a secreted protein and the N-terminal sequence of the protein is rich in hydrophobic residues suggesting the presence of a signal peptide [Fig. 2]. The hydropathy plot of the amino acid sequence of PIP was derived using the Pustell Sequence Analysis Program (International Biotechnologies, Inc.). The N-terminal of PIP is blocked and therefore, based on the hydropathy plot and the consensus criteria for signal peptide sequences, the glycine at amino acid 24 was proposed to be the N-terminal residue of PIP. Recently, PIP has been reported to be isolated from human seminal plasma and the peptide sequence has been determined (Schaller *et al*, 1991). It has been called secretory actin-binding protein (SABP) and has been characterized with regard to its protein sequence. The SABP (PIP) is a 118 amino acid peptide with a calculated molecular weight of 13.5 kDa. The N-terminal residue is a pyroglutamic acid (amino acid 29) in contrast to the glycine suggested from the hydrophobicity plot [Fig. 2]. The aspartic acid residue at amino acid 105 has been confirmed to be the site of a N-glycosidic carbohydrate moiety and carbohydrate accounts for 22% of the molecular weight of PIP. Two disulfide bonds exist between cysteines 37 and 63 and cysteines 61 and 95.

Figure 2 Derived amino acid sequence of PIP and comparison with a protein expressed in mouse submaxillary gland.

A) Amino acid identity between the two sequences is double underlined and conservative changes are singly underlined. B) Hydropathy plot of derived amino acid sequence of PIP indicating the proposed hydrophobic signal peptide sequence. Residues indicated above the center line are hydrophobic and residues indicated below the center line are hydrophilic. The confirmed 29 amino acid signal peptide is shaded in the PIP amino acid line diagram and boxed in the hydrophobicity plot. The hydrophobicity plot was derived using the Pustell sequence analysis program (International Biotechnologies, Inc.).

Fig. 2→

```
Met Arg Leu Leu Gln Leu Leu Phe Arg Ala Ser Pro Ala Thr Leu 15
--- --- Met Gly Gly Leu Ser Phe Thr Phe Ser Ala Val Thr Leu 13

Leu Leu Val Leu Cys Leu Gln Leu Gly --- --- Ala Asn Lys Ala 28
Phe Leu Val Leu Cys Leu Gln Leu Gly Ile Ile Glu Ser Gln Asp 28

Gln Asp Asn Thr Arg Lys Ile Ile Ile Lys Asn Phe Asp Ile Pro 43
Asp Glu Asn Val Arg Lys Pro Leu Leu Ile Glu Ile Asp Val Pro 43

Lys Ser Val Arg Pro Asn Asp Glu Val Thr Ala Val Leu Ala Val 58
Ser Thr Ala Gln Glu Asn Gln Glu Ile Thr Val Gln Val Thr Val 58

Gln Thr Glu Leu Lys Glu Cys Met Val Val Lys Thr Tyr Leu Ile 73
Glu Thr Gln Thr Arg Glu Cys Met Val Ile Lys Ala Tyr Leu Val 73

Ser Ser Ile Pro Leu Gln Gly Ala Phe Asn Tyr Lys Tyr Thr Ala 88
Ser Asn Glu Pro Met Glu Gly Ala Phe Asn Tyr Val Gln Thr Arg 88

Cys Leu Cys Asp Asp Asn Pro Lys Thr Phe Tyr Trp Asp Phe Tyr 103
Cys Leu Cys Asn Asp His Pro Ile Arg Phe Phe Trp Asp Ile Ile 103

Thr Asn Arg Thr Val Gln Ile Ala Ala Val Val Asp Val Ile Arg 118
Ile Thr Arg Thr Val Thr Phe Ala Thr Val Ile Asp Ile Val Arg 118

Glu Leu Gly Ile Cys Pro Asp Asp Ala Ala Val Ile Pro Ile Lys 133
Glu Lys Asp Ile Cys Pro Asn Asp Met Ala Val Val Pro Ile Thr 133

Asn Asn Arg Phe Tyr Thr Ile Glu Ile Leu Lys Val Glu 146 human PIP
Ala Ser Arg Tyr Tyr Thr Tyr Asn Thr Val Arg Met Asn 146 mouse PIP
```



In the process of characterizing the 5' flanking region of the PIP gene, a detailed restriction map of the gene was required. Accordingly, I performed single and double digestions of the entire 14 kb genomic PIP clone (cloned by Dr. Y. Myal) and determined the number and location of restriction endonuclease sites [Fig. 3]. In this work, the sequences and partial restriction map determined by Myal *et al* (1991) proved very useful. Putative CAAT and TATA box sequences are located at base pairs -32 and -25, respectively in the 5' flanking region of the PIP gene (Myal *et al*, 1991). The 5' flanking region encompassed by the λ phage clone was approximately 2.0 kb. I cloned and sequenced most of this fragment. Relevant features of the 5' flanking region include a 250 base pair *Alu* repeat sequence approximately 1.6 to 1.8 kb from the transcription initiation site. A perfect consensus ERE is located at base pair -1198 and six ARE half-sites are located at base pairs -1487, -1183, -1125, -413, -375 and -194 [Fig. 4].

Figure 3 Restriction map of the genomic PIP clone.
A) List of restriction endonucleases used to cleave the genomic PIP clone and the number of cleavage sites. B) Representation of the PIP gene indicating the restriction endonuclease cleavage sites. One kilobase is represented by 1.5 cm. The exons are represented as filled boxes.

Fig. 3→

Restriction endonucleases which **do not** cleave the PIP gene	*AatI*, *BssHI* (50°C), *ClaI*, *NaeI*, *NarI*, *NotI*, *PvuI*, *SacII*, *SalI*, *SfiI* (50°C), *XhoI*, *XmaIII*
Restriction endonucleases which cleave the PIP gene **once**	*BamHI*, *BglII*, *KpnI*, *NheI*, *SmaI* (25°C)
RE which cleave the PIP gene **twice**	*BglI*, *EcoRV*, *HindIII*, *NcoI*
RE which cleave the PIP gene **three** times	*EcoRI*, *NdeI*, *Tth111I* (65°C), *XbaI*
RE which cleave the PIP gene **four** times	*AvaII*, *HpaII*, *NsiI*
RE which cleave the PIP gene **five** times	*AccI*, *NciI*
RE which cleave the PIP gene **six** times	*HincII*, *PstI*, *PvuII*
RE which cleave the PIP gene **seven** times	*SphI*

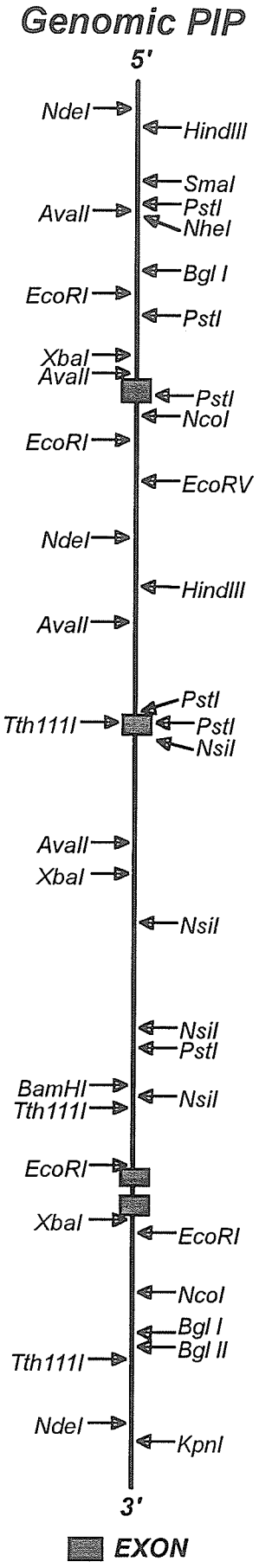


Figure 4 Sequence of the 5' flanking region of the PIP gene.
Sequences within the 5' flanking region of the PIP gene which share sequence similarity with the GRE/PRE/ARE consensus sequence [GGTACAnnnTGTTCT] are indicated by italics. Within these italicized sequences, the core GRE half-site TGTTCT is double underlined. The consensus ERE sequence is shaded and double underlined. The CAAT and TATA box sequences are indicated by single underlines and single underlined italics, respectively. The first 46 basepairs of the first exon, including the 5' untranslated region up to the initiation codon are indicated by italics.

Fig. 4→

GTAACCATCA CAGCAGTCAT GGCCTGAATA AGACTTGTCC TAGGCCAGGC -1815
ACAGTGGCTC ACACCTGTAA TCCCAGCAAT TTGGGAGGCC GAGGCAGGTG
GATCACTTGA GGTCAGGAAT GTAAGATCAG CTTGGCCAAC ATGGTGAAAC -1715
CCCATCTCTA CCAAAAATAC AAAAATTAGC CGGGTATGGT GGCACCTGCC
TATAATCCCC ACTACTCGGG TGGCTGAGGC ATGAGAATTT CCTGAACCCG -1615
GGAGTGGAGG CTGCAGTGAG CTGAGATCAT GCCACTGCAC TCCAGCCTGG
ATGAGTGAGA TTCTGTCTCA AAAAAAAAAG ATTGTCCTGT ACATTGTAGG -1515
TATTGCAGTT TCCTGCTAGC TCACAGA*AGA ACAGCCTCAT TA*CCTTCAAT
AGATGCTCTT AACCCATCGT CATCTGCCTT CCTCTGGACA CTGCCTTTTG -1415
GGAGTGCTGG AGTTGCATCC TCAAGGACAG GGCTATGCTT CAAAGAGCCA
AGCAAAGAAC TTTTTCCTGC ATTTTCCCAC ACAGACACTT CCTAGATTCT -1315
TCTGCCTTAT GCCTGCCTTG GTCAGTGTAG CTAGGATGTG CTTCAAATGG
AAATTATGCA AGTGAGAAGG ACCAGGTGTA AAGACAATTT AATTGTGGGA -1215
AAAGGCTTAC AAAGGA*AGGT CATTGTGACC TCACAAGCAG TGTTCT*TGAG
TGGATAGGAC ATAAACAGGG AAGAAAGCAT GTGATCATG*A GAACAGCTGA* -1115
*AGTT*CTGAGT GAGCAATGAC ATGAATGGTG TCCAATTCAA AGAAAAGAGA
AGGTAGATCC AGGAGCTGAA TAAACAGACA GAGCAATTTT GAGAGATACA -1015
TCCAGCAAGC AGAAGCACAC ACTTACACTT TAGCTCTGAA CTCTGATTTT
CACTCTGTTG CCAGAGAGGC TCTTCCTGGG CAGCTGTGTG CCATGCAAAT -915
GATAGAAACA AATCCCATAG TATTGTAAGT CATAGGTTAA GGAGCAGATT
TAATTTCATA AGGAGAAATA ACACAATGCC AGTGTCAGCA AGAGCAAGTC -815
TCCCCATCAT CCCAAGGAGA GGAATTCTGA CTGACCCATG TCAATAAGAA
AATAAGCCAG CTCTTTGGTG CCAAGAAGTC TGATTTGGGG GTGCCAGCTA -715
TTTATAGGAA TTGTCTAGAA TTAAAATATC ACCCTGCCTT GGGGATTGTG
GGGCAGCTGG GGATCTTGGA CATCTGCAGT ATATTGCAAC CACACCTGAA -615
ATTAAGAAGG ACTCAATGAC AATAAACATC TTTACATCAC CCAGTGTCTT
GAACTCACTT CCCCATCATG TAGAGCAGCT CAGGTCTTCT CTAGCTCTTC -515
CACCTCATGA TGGGTAGTGA CTGCTAGGGA AGTCCTGATC ATCCTAGCCC
TCCAAGAAAT GTTTGAATAG CATTCGATCT TGAACTCACT ATAATTTGAT -415
C*AAGCAAAGA TGTTCT*ATGT CTCTGGTCTG AACCTGCCA*C CACAGCTGTG*
TTCTCTGCTT TCAGAGACCA GGGGAGCAGT GACTGAAGCT AAGACTACAC -315
ATAGCACTTC AGACAGGCAA TAGAGGAAAA GAAAACTGAA TCTGTAAACT
CGGGAAAAAC TGGTGTATCT TTTGAGGTCC CAGCCATTTT GAGAAACTCT -215
GATGACTGAA AAAATAAGGA *AGAACAATCT GACTC*CTCTC TCAAACGCTG
ACATTCTAGA CTGAGTGTTG ATTTTTTCTT AGAAAAACAA ACTTTGGGTC -115
AACAAGGAAA GATCACGAGT GGGGAGGGTG AATGGGTGAT TCACCTCATG
GATCTCCCTG GCAGCTCTTG CACTTCCTTG AT*CAATCTG T ATATAA*GAAA -15
TGCTGGGCAC CTGG*GACACC ACTTCTCTGG GACACATTGC CTTCTGTTTT* +36
CTCCAGCATG

Hormonal regulation of the PIP mRNA

The most potent steroid in inducing the PIP gene is the androgen, dihydrotestosterone. DHT [10^{-8}M] causes the mRNA level of PIP to increase gradually from approximately 24 hours to a maximal level at 5 days [shown up to 2 days in Fig. 5]. This increase has been shown to occur at the level of transcription (Murphy *et al*, 1987a). Since the expression of PIP in ZR-75-1 cells is extremely low and at the limit of detection by Northern analysis, I have used T-47D human breast cancer cells to investigate the effects of different hormone treatments on PIP gene expression. The T-47D cell line was derived from the pleural effusion of a patient with a ductal breast carcinoma. This epithelial cell line was characterized by Horwitz *et al* (1978) and shown to contain high levels of PR, moderate levels of AR and low levels of GR. Furthermore, T-47D cells have also been shown by Freake *et al* (1981) to contain ER and VDR. The resulting autoradiographs were quantitated by densitometric scanning and the calculated intensities for the different hormone treatments were standardized to the levels of either the 28S ribosomal RNA or G3PD. Either probe is acceptable as a standardization marker as neither is affected by the hormone treatments in T-47D cells. Quantitation of relative amounts of specific mRNA transcripts was performed by densitometric scanning of the autoradiographs using the computer software, SCANPLOT version 4.01 (Cunningham Engineering).

I have shown that in T-47D HBC cells, PIP mRNA levels increase gradually within 1 hour after retinoic acid [10^{-8}M] treatment, maximally at three to four hours (9 to 10 fold) and thereafter decrease to control levels [Fig. 6]. DHT and retinoic acid treatment in combination does not cause any increase in PIP mRNA accumulation over that of DHT alone after 24 hours of hormone treatment [Fig. 7]. I have also studied the combination of retinoic acid, dihydrotestosterone and estradiol. Estradiol has been reported to decrease the level of PIP/GCDFP-15 synthesis in ZR-75-1 HBC cells and inhibit the induction of PIP by androgens (Simard *et al*, 1989). In T-47D cells, estradiol [10^{-8}M] inhibits the induction of the PIP gene by both DHT and retinoic acid after 24 hours of hormone treatment [Fig. 8].

Figure 5 Induction of the PIP gene by androgen.
30 μg of total RNA from T-47D human breast cancer cells treated with [10^{-8}M] dihydrotestosterone for the times indicated were electrophoresed on a denaturing agarose gel and subjected to Northern analysis. The probe used was the nick translated PIP cDNA.

Fig. 5→

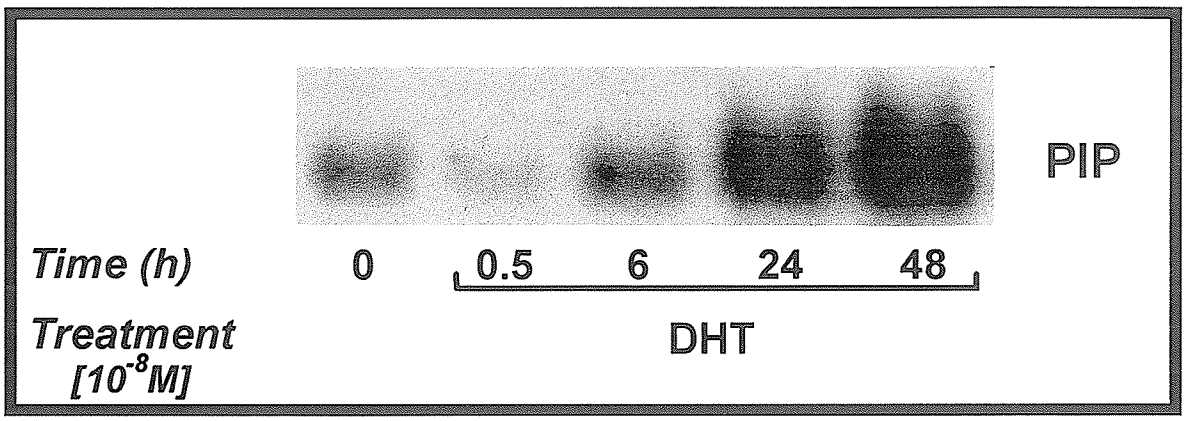

PIP
Time (h)
0
0.5
6
24
48
Treatment
[10-8M]
DHT

Figure 6 Induction of the PIP gene by retinoic acid.
30 μg of total RNA from T-47D HBC cells (treated with [10^{-8}M] retinoic acid for the times indicated) were electrophoresed on a denaturing agarose gel and subjected to Northern analysis. The probe used was the nick translated PIP cDNA. The nitrocellulose was subsequently probed with nick translated mouse glyceraldehyde-3-phosphate dehydrogenase (G3PD). Quantitation of relative amounts of specific mRNA transcripts was performed by densitometric scanning of the autoradiographs using the computer software, SCANPLOT version 4.01 (Cunningham Engineering). Fold inductions with respect to control T-47D total RNA standardized to G3PD are indicated below.

Fig. 6→

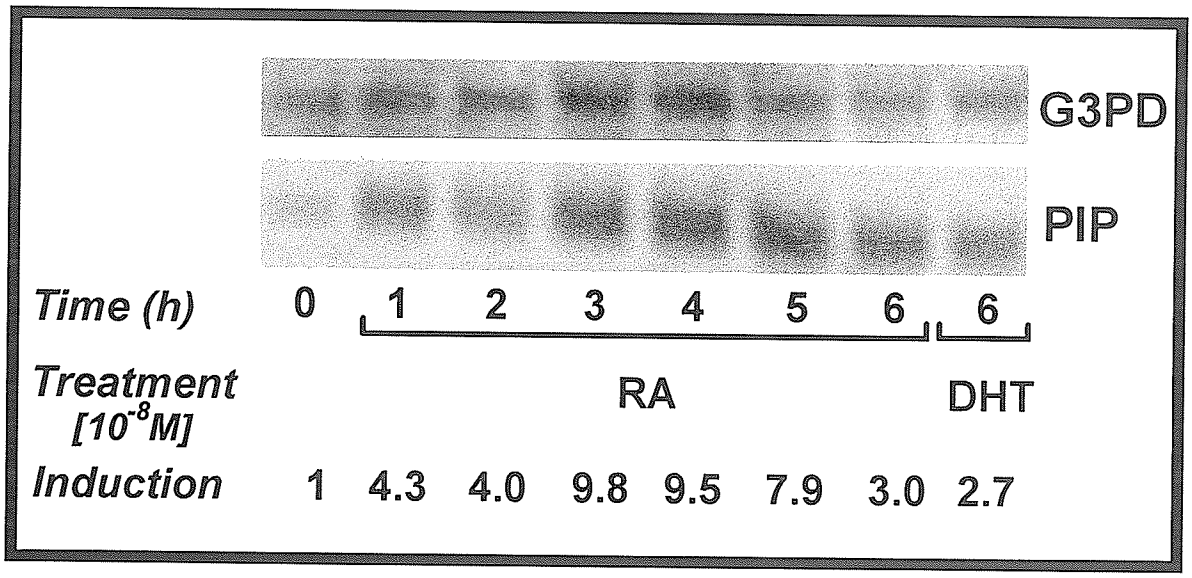
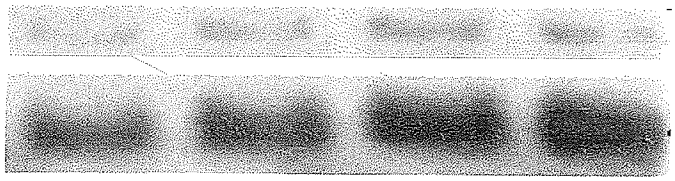

G3PD
PIP
Time (h)
0
1
2
3
4
5
6
6
Treatment [10-8M]
RA
DHT
Induction
1
4.3
4.0
9.8
9.5
7.9
3.0
2.7

Figure 7 Induction of the PIP gene by retinoic acid, dihydrotestosterone and both hormones combined.

30 μg of total RNA from T-47D HBC cells (treated for 24 hours with [10^{-8}M] retinoic acid, [10^{-8}M] dihydrotestosterone or both hormones combined) were electrophoresed on a denaturing agarose gel and subjected to Northern analysis. The probe used was the nick translated PIP cDNA. The nitrocellulose was subsequently probed with nick translated mouse G3PD. Fold inductions with respect to control T-47D total RNA standardized to G3PD are indicated below.

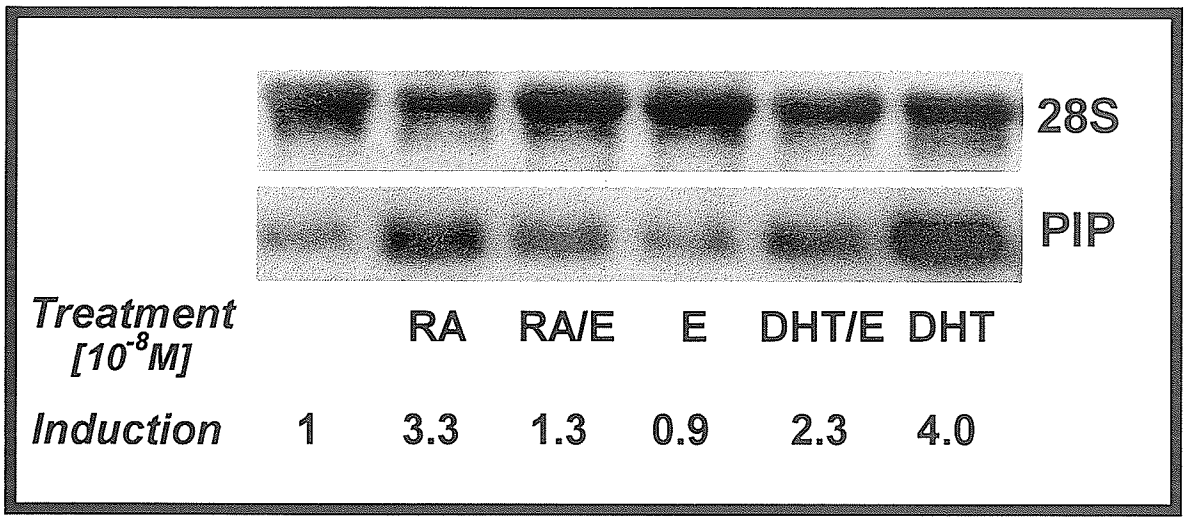
Fig. 7→



Treatment [10^{-8}M]	-	RA	DHT	RA/DHT
Induction	1	1.6	2.6	2.2

Figure 8 Induction of the PIP gene by retinoic acid and dihydrotestosterone and inhibition of induction by estrogen. 30 μg of total RNA from T-47D HBC cells (treated for 24 hours with [10^{-8}M] hormones as indicated) were electrophoresed on a denaturing agarose gel and subjected to Northern analysis. The probe used was the random primed PIP cDNA. The nitrocellulose was subsequently probed with a random primed 28S probe. Fold inductions with respect to control T-47D total RNA standardized to 28S are indicated below.

Fig. 8→


28S
PIP
Treatment [10-8M]
RA
RA/E
E
DHT/E
DHT
Induction
1
3.3
1.3
0.9
2.3
4.0

Hormonal regulation of the 5' flanking region of the PIP gene

To address the question of the hormonal inducibility of the PIP gene, the 5' flanking region was ligated to the reporter gene, chloramphenicol acetyl transferase (CAT). The 5' flanking region used in these experiments spanned an area from -1499 to +43 encompassing the six ARE half-sites and the ERE as well as a portion of the first exon including the entire untranslated region. The *Alu* repeat region was avoided in these constructs.

Initially, the base vector used in these studies was SVCAT, a pBR322 based vector containing the CAT gene driven by the SV40 early promoter lacking the enhancer sequences. A fragment of the 5' flanking region of the PIP gene including the promoter was ligated in front of the SV40 promoter to create PIPSVCAT [-790 $\rightarrow$ +43] [Fig. 9]. The MMTV (mouse mammary tumor virus long terminal repeat) is a well characterized promoter which has been shown to bind and mediate the hormonal response to glucocorticoid, progesterone and androgen through their respective receptors (Chalepakis *et al*, 1988; Ham *et al*, 1988). MMTVTKCAT was used as a positive control for androgen induction and these constructs (PIPSVCAT or MMTVTKCAT) were transiently expressed in ZR-75-1 or T-47D human breast cancer cells which endogenously express PIP at low and high levels, respectively. After treatment of the cells with the androgen, dihydrotestosterone [10^{-8}M], the cell extracts were assayed for the presence of the CAT enzyme. Although the MMTVTKCAT control showed inducibility in the presence of androgen, the PIPSVCAT construct was uninducible [Fig. 10]. Thus, I prepared new PIP constructs using the thymidine kinase (tk) promoter.

Using TKCAT which is a pUC based vector containing the CAT gene driven by the thymidine kinase promoter, varying lengths of the 5' flanking region of the PIP gene including the promoter were ligated in front of the TK promoter to create the PIPTKCAT constructs [Fig. 9]. These constructs were transiently expressed in T-47D HBC cells. Unfortunately, the basal construct TKCAT, containing no PIP sequences, was inducible by DHT in these cells and any induction observed in the PIPTKCAT constructs was

therefore suspect [Fig. 11].

Figure 9 Constructs used in the transient expression studies.
The PIP gene is indicated at the top and includes 2.0 kilobases of the 5' flanking region and the first exon. The first construct is PIPSVCAT and contains [-790 → +43] of the PIP gene driven by the SV40 early promoter. The reporter gene is chloramphenicol acetyl transferase (CAT). The following four constructs are PIPTKCAT constructs and contain varying lengths of the PIP gene as indicated, driven by the thymidine kinase promoter. The reporter gene is CAT. The remainder of the constructs are PIPCAT constructs and contain varying lengths of the PIP gene as indicated, driven by the homologous PIP promoter. The reporter gene is CAT. Dashed lines indicate relative positioning of the androgen responsive element (ARE) half-sites.

Fig. 9→

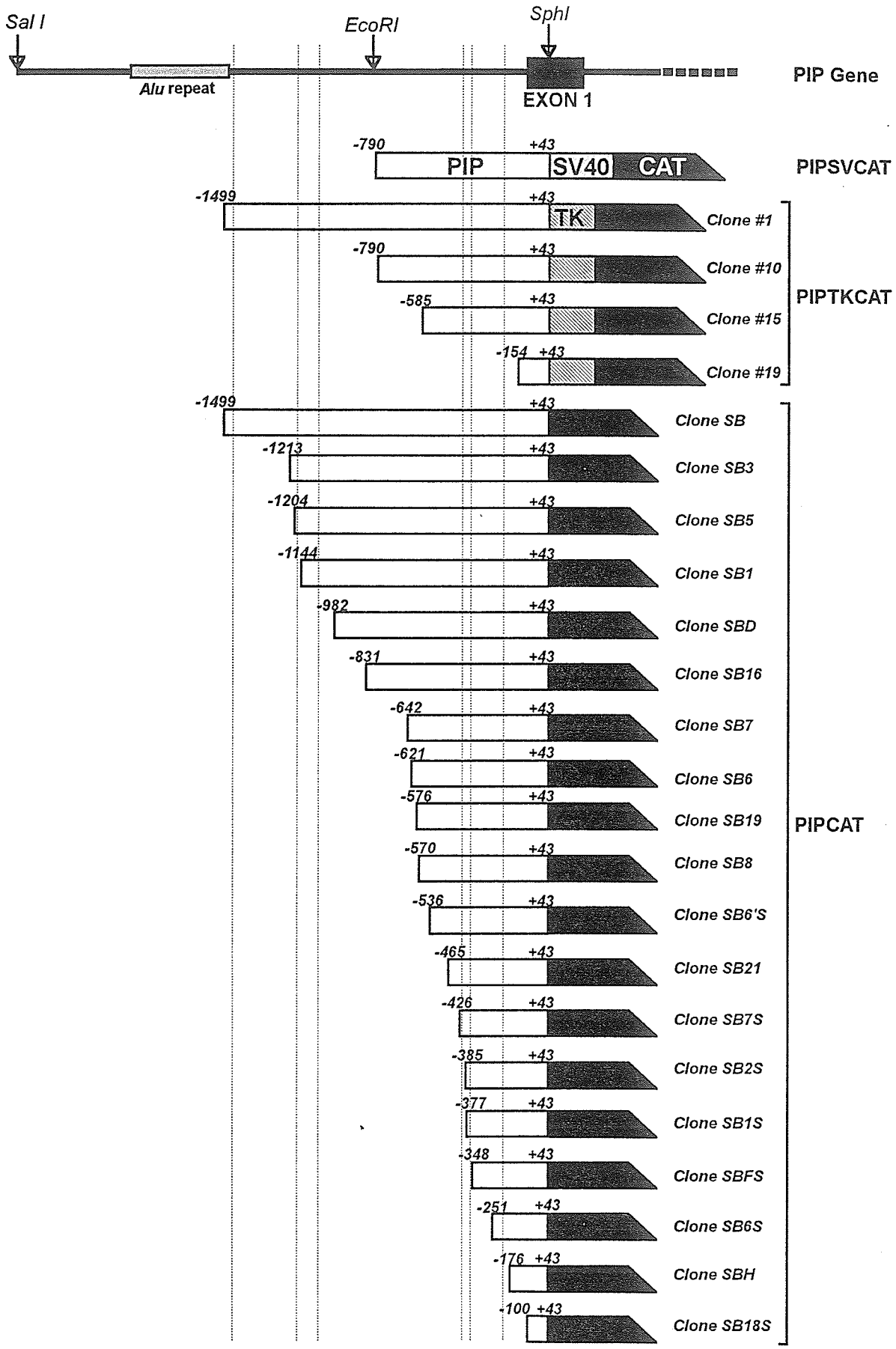

Sal I
EcoRI
SphI
Alu repeat
EXON 1
PIP Gene
-790
+43
PIP
SV40
CAT
PIPSVCAT
-1499
+43
TK
Clone #1
-790
+43
Clone #10
-585
+43
Clone #15
-154
+43
Clone #19
PIPTKCAT
-1499
+43
Clone SB
-1213
+43
Clone SB3
-1204
+43
Clone SB5
-1144
+43
Clone SB1
-982
+43
Clone SBD
-831
+43
Clone SB16
-642
+43
Clone SB7
-621
+43
Clone SB6
-576
+43
Clone SB19
-570
+43
Clone SB8
-536
+43
Clone SB6'S
-465
+43
Clone SB21
-426
+43
Clone SB7S
-385
+43
Clone SB2S
-377
+43
Clone SB1S
-348
+43
Clone SBFS
-251
+43
Clone SB6S
-176
+43
Clone SBH
-100
+43
Clone SB18S
PIPCAT

Figure 10 PIPSVCAT is not androgen inducible in T-47D and ZR-75-1 human breast cancer cells.

Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] dihydrotestosterone. Control cells were treated with media containing ethanol to the same concentration as the hormone treatment. The SVCAT construct is the base construct into which the PIP[-790 $\rightarrow$ +43] was inserted. MMTVTKCAT is a positive control for androgen induction.

Fig. 10→

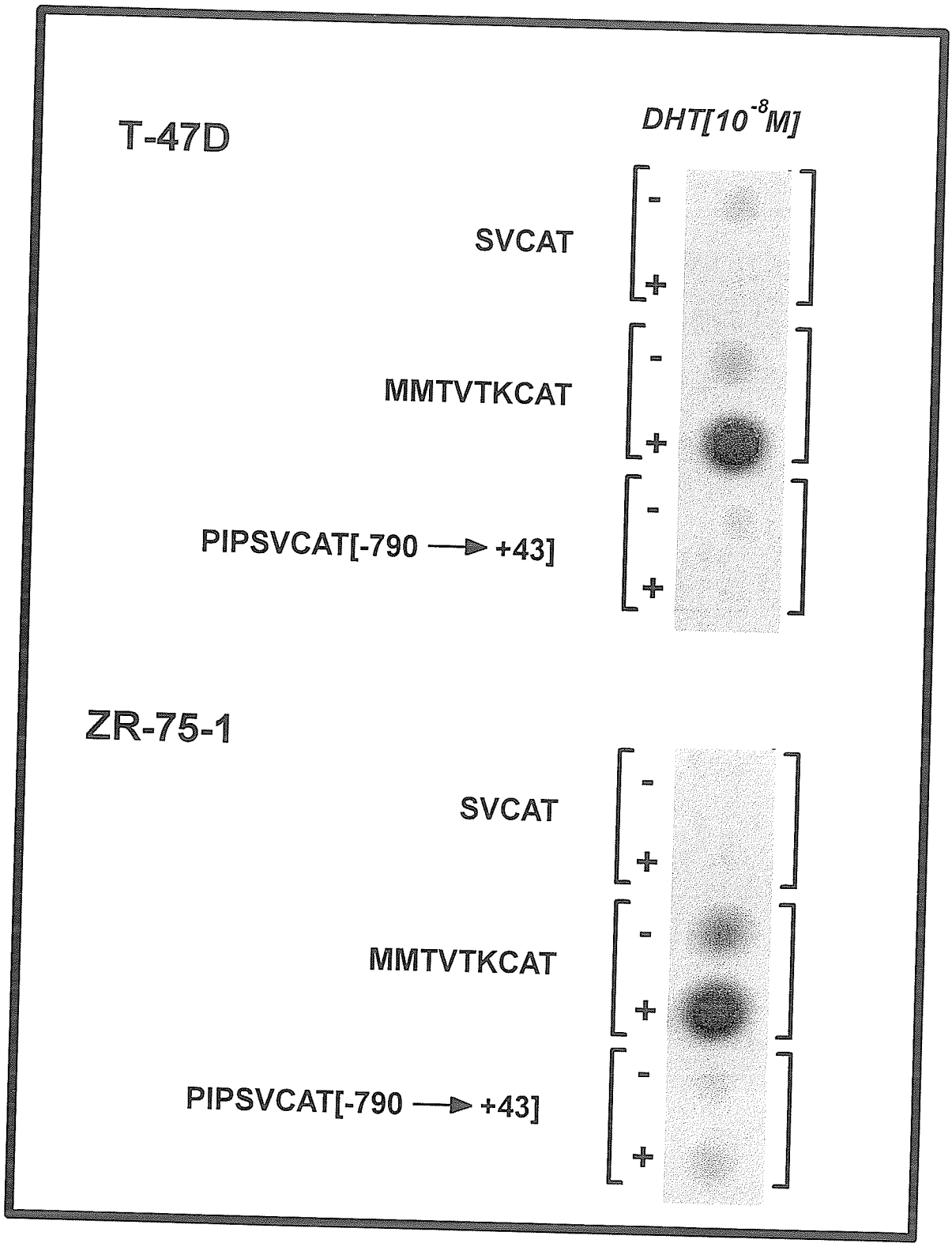
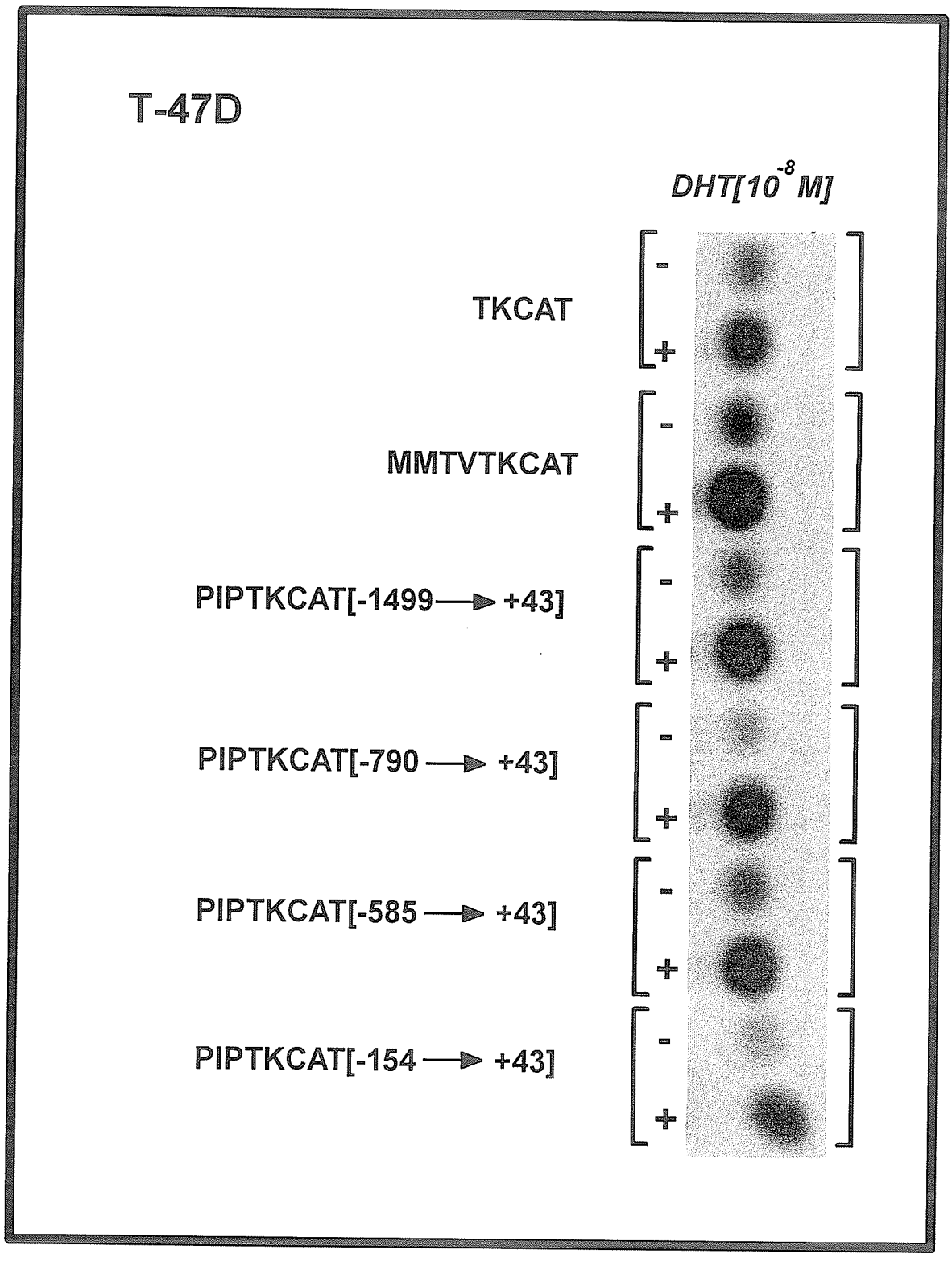

T-47D
DHT[10-8M]
SVCAT
-
+
MMTVTKCAT
-
+
PIPSVCAT[-790 → +43]
-
+
ZR-75-1
SVCAT
-
+
MMTVTKCAT
-
+
PIPSVCAT[-790 → +43]
-
+

Fig. 11→

Figure 11 TKCAT is androgen inducible in T-47D HBC cells.
Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] dihydrotestosterone or ethanol. The TKCAT construct is the base construct into which the varying lengths of PIP, as indicated, were inserted. MMTVTKCAT is a positive control for androgen induction.


T-47D
DHT[10-8 M]
TKCAT
MMTVTKCAT
PIPTKCAT[-1499 → +43]
PIPTKCAT[-790 → +43]
PIPTKCAT[-585 → +43]
PIPTKCAT[-154 → +43]
-
+

Finally, PIPCAT constructs containing only the PIP promoter and no heterologous promoter were created [Fig. 9]. These constructs were transiently expressed in ZR-75-1 cells which were then treated with dihydrotestosterone [10^{-8}M] or prolactin/growth hormone [1 μg/mL] [Fig. 12]. These hormones have the greatest effect on the levels of PIP mRNA in human breast cancer cells. Prolactin and growth hormone are equipotent in induction of PIP mRNA and act through the prolactin receptor (Murphy *et al*, 1987a); therefore, in most experiments, growth hormone was used due to its availability. The PIPCAT[-1499→+43] construct was observed to be inducible by DHT but not by prolactin or growth hormone.

Further dissection of the 5' flanking region of the PIP gene was accomplished by the production of nested deletions of the PIP sequence from 5' to 3' [Fig. 9]. All constructs were sequenced to ensure the correct PIP sequences in the CAT vector. All constructs tested from PIPCAT[-1499 → +43] to [-251 → +43] were inducible by androgen with the exception of PIPCAT[-1144 → +43] which was not inducible although its background CAT activity was high. PIPCAT[-176 → +43] (not shown) and [-100 → +43] were not inducible by androgen [Fig. 13]. The effect of other steroids on PIPCAT induction was tested. Retinoid acid [10^{-8}M] was found to induce PIPCAT expression in constructs between and including PIPCAT[-1499 → +43] and PIPCAT[-1144 → +43]. Smaller constructs, or those including PIPCAT[-982 → +43], were not inducible with retinoic acid [Fig. 14].

Combinations of different hormones were used to test CAT induction in PIPCAT [-1204 → +43]. Retinoic acid [10^{-8}M] and DHT [10^{-8}M] act synergistically in inducing PIPCAT expression. T_3 [10^{-8}M] minimally induces PIPCAT expression and can diminish the inducibility of PIPCAT by retinoic acid [Fig. 15]. PIPCAT constructs are minimally inducible by dexamethasone [10^{-8}M] [Fig. 16]. Estrogen [10^{-8}M] alone induces PIPCAT expression minimally and the effect is not dependent on concentration. Retinoic acid induces PIPCAT expression in a concentration dependent manner from [10^{-7}] to [10^{-11}M]. When both hormones are used, estrogen synergizes with retinoic acid and this effect is

not concentration-dependent between estradiol concentrations from [10^{-7}] to [10^{-10}M] are used [Fig. 17].

Figure 12 PIPCAT[-1499 → +43] is inducible by androgen but not by prolactin in ZR-75-1 HBC cells.

Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] dihydrotestosterone or ethanol. PIPCAT[-1499 → +43] contains the homologous PIP promoter (no heterologous promoter).

Fig. 12→

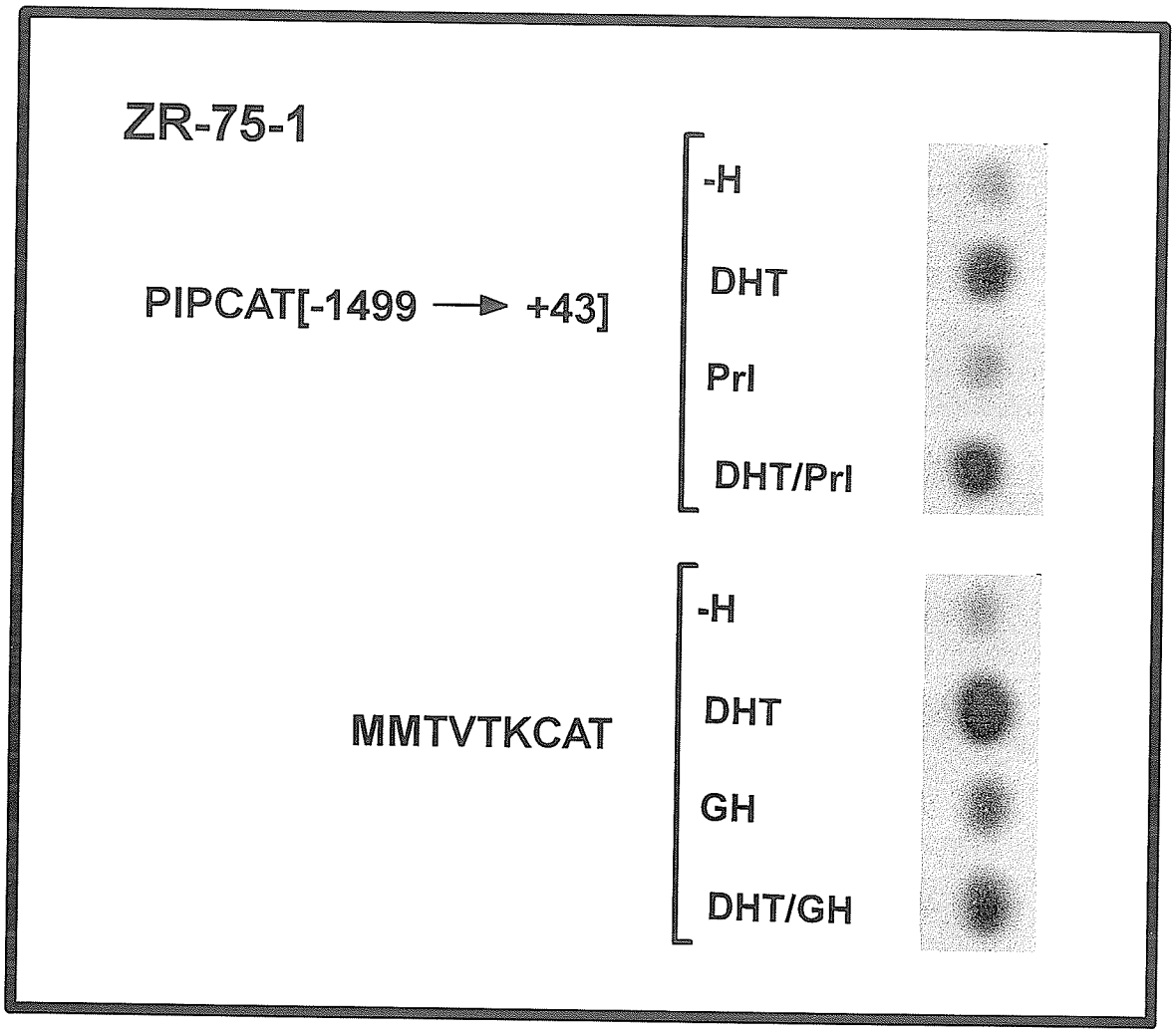

ZR-75-1
PIPCAT[-1499 → +43]
-H
DHT
Prl
DHT/Prl
MMTVTKCAT
-H
DHT
GH
DHT/GH

Figure 13 Androgen inducibility of PIPCAT constructs transfected into ZR-75-1 HBC cells.

Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] dihydrotestosterone or ethanol. The autoradiograph was used to identify radioactive spots which were scraped and scintillation counted to calculate fold increases with androgen treatment.

Fig. 13→

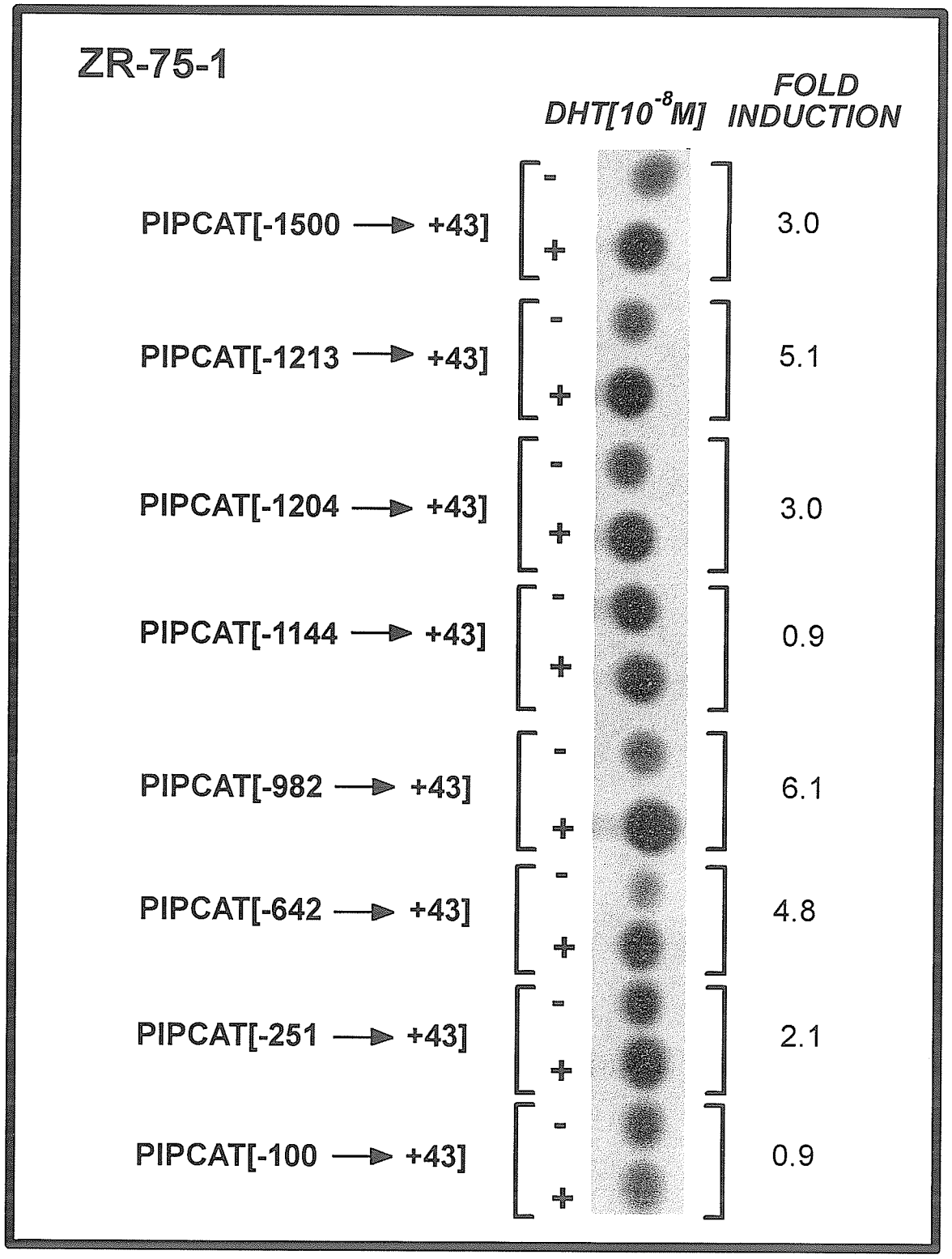

ZR-75-1
DHT[10-8M]
FOLD INDUCTION
PIPCAT[-1500 → +43]
-
+
3.0
PIPCAT[-1213 → +43]
-
+
5.1
PIPCAT[-1204 → +43]
-
+
3.0
PIPCAT[-1144 → +43]
-
+
0.9
PIPCAT[-982 → +43]
-
+
6.1
PIPCAT[-642 → +43]
-
+
4.8
PIPCAT[-251 → +43]
-
+
2.1
PIPCAT[-100 → +43]
-
+
0.9

Figure 14 Retinoic acid inducibility of PIPCAT constructs transfected into ZR-75-1 HBC cells.

Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] retinoic acid or ethanol. The autoradiograph was used to identify radioactive spots which were scraped and scintillation counted to calculate fold increases with retinoic acid treatment.

Fig. 14→

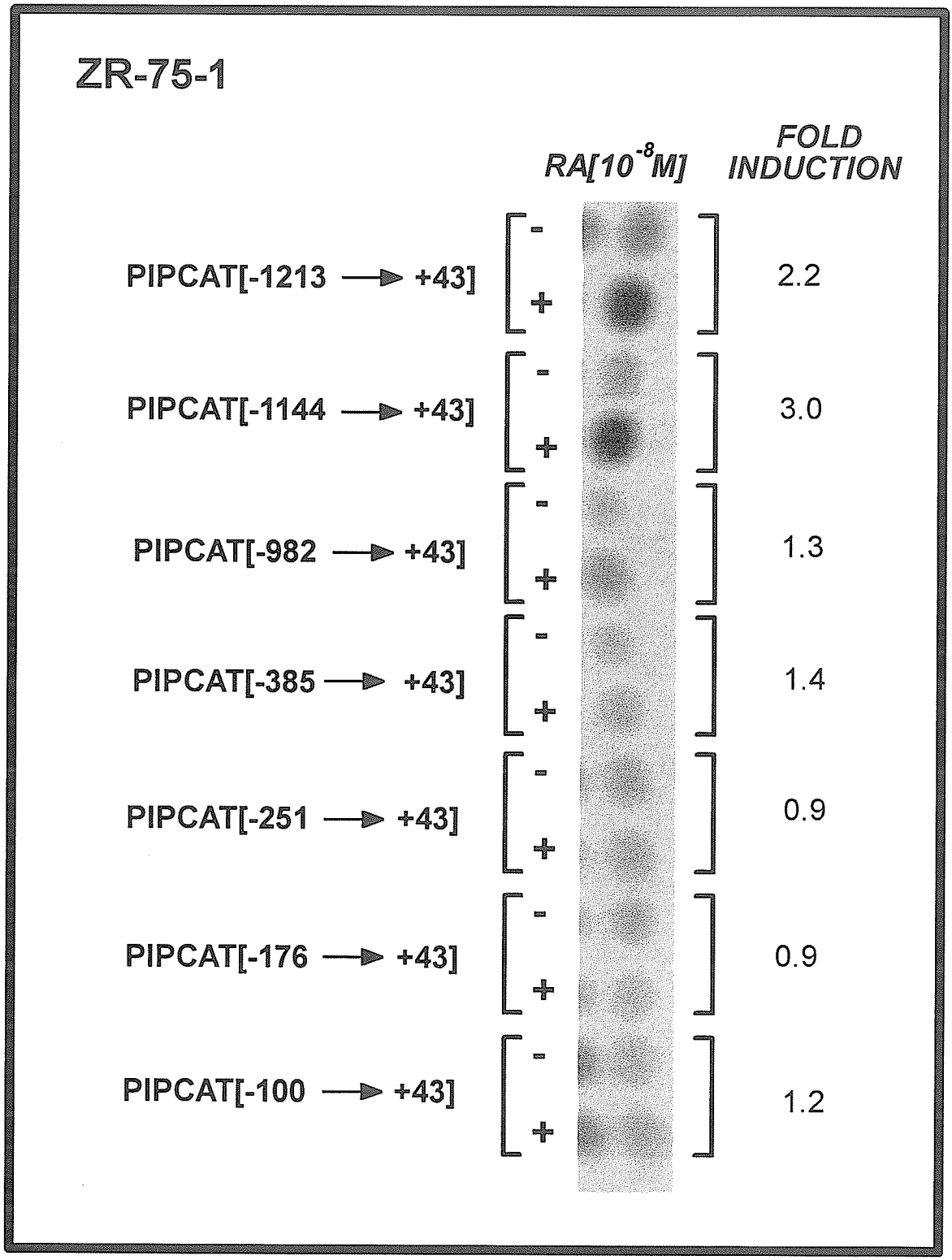

ZR-75-1
RA[10^-8 M]
FOLD INDUCTION
PIPCAT[-1213 → +43]
- +
2.2
PIPCAT[-1144 → +43]
- +
3.0
PIPCAT[-982 → +43]
- +
1.3
PIPCAT[-385 → +43]
- +
1.4
PIPCAT[-251 → +43]
- +
0.9
PIPCAT[-176 → +43]
- +
0.9
PIPCAT[-100 → +43]
- +
1.2

Figure 15 Dihydrotestosterone and thyroid hormone affect retinoic acid induction of PIPCAT[-1204 → +43] transfected into ZR-75-1 HBC cells.
Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] hormones as indicated or ethanol. The autoradiograph was used to identify radioactive spots which were scraped and scintillation counted to calculate fold increases with hormone treatment.

Fig. 15→

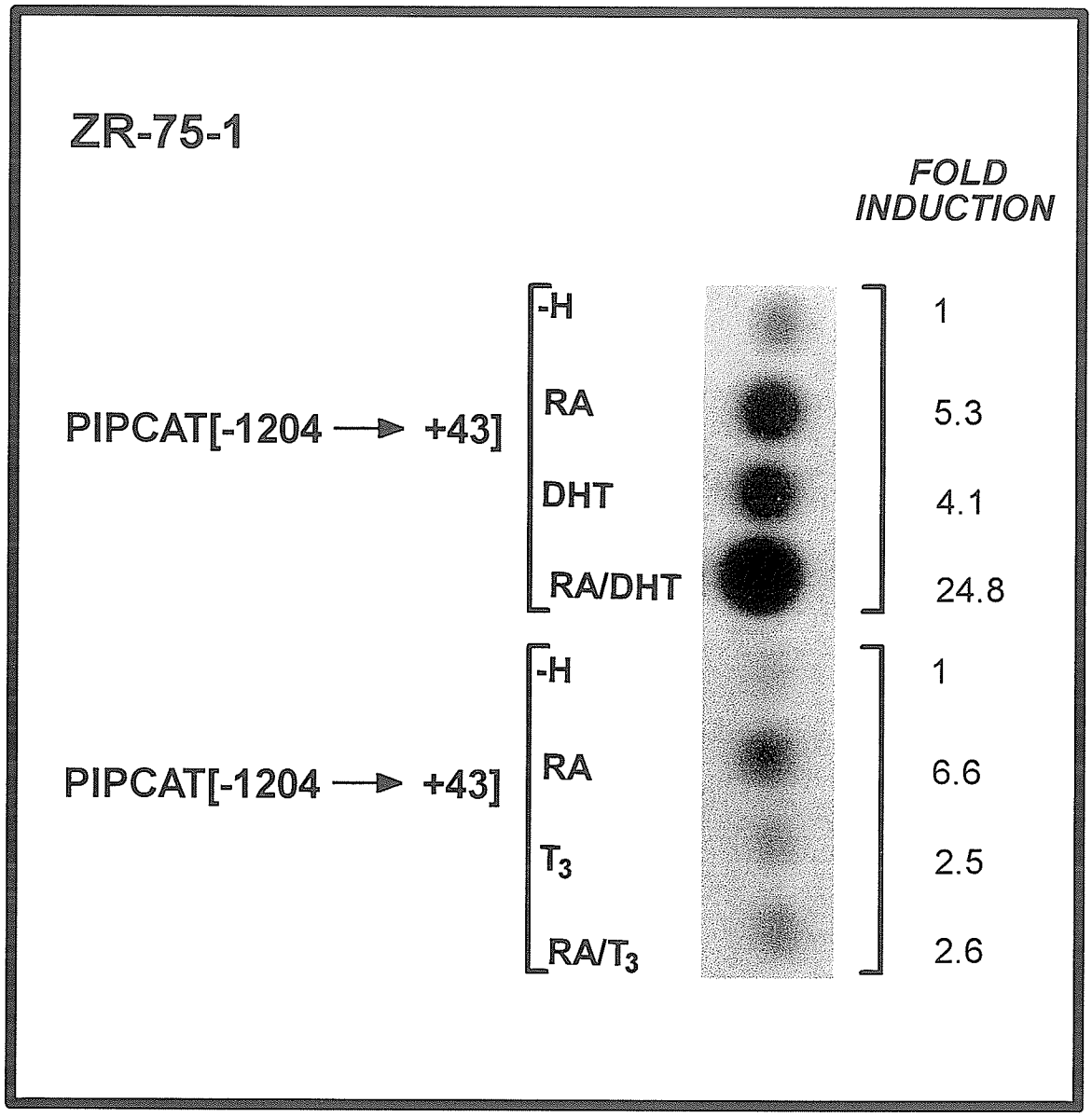

ZR-75-1
FOLD INDUCTION
PIPCAT[-1204 → +43]
-H
RA
DHT
RA/DHT
1
5.3
4.1
24.8
PIPCAT[-1204 → +43]
-H
RA
T3
RA/T3
1
6.6
2.5
2.6

Figure 16 Dihydrotestosterone strongly and estrogen or dexamethasone weakly induce PIPCAT expression in ZR-75-1 HBC cells.
Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] hormones as indicated or ethanol. The autoradiograph was used to identify radioactive spots which were scraped and scintillation counted to calculate fold increases with hormone treatment.

Fig. 16→

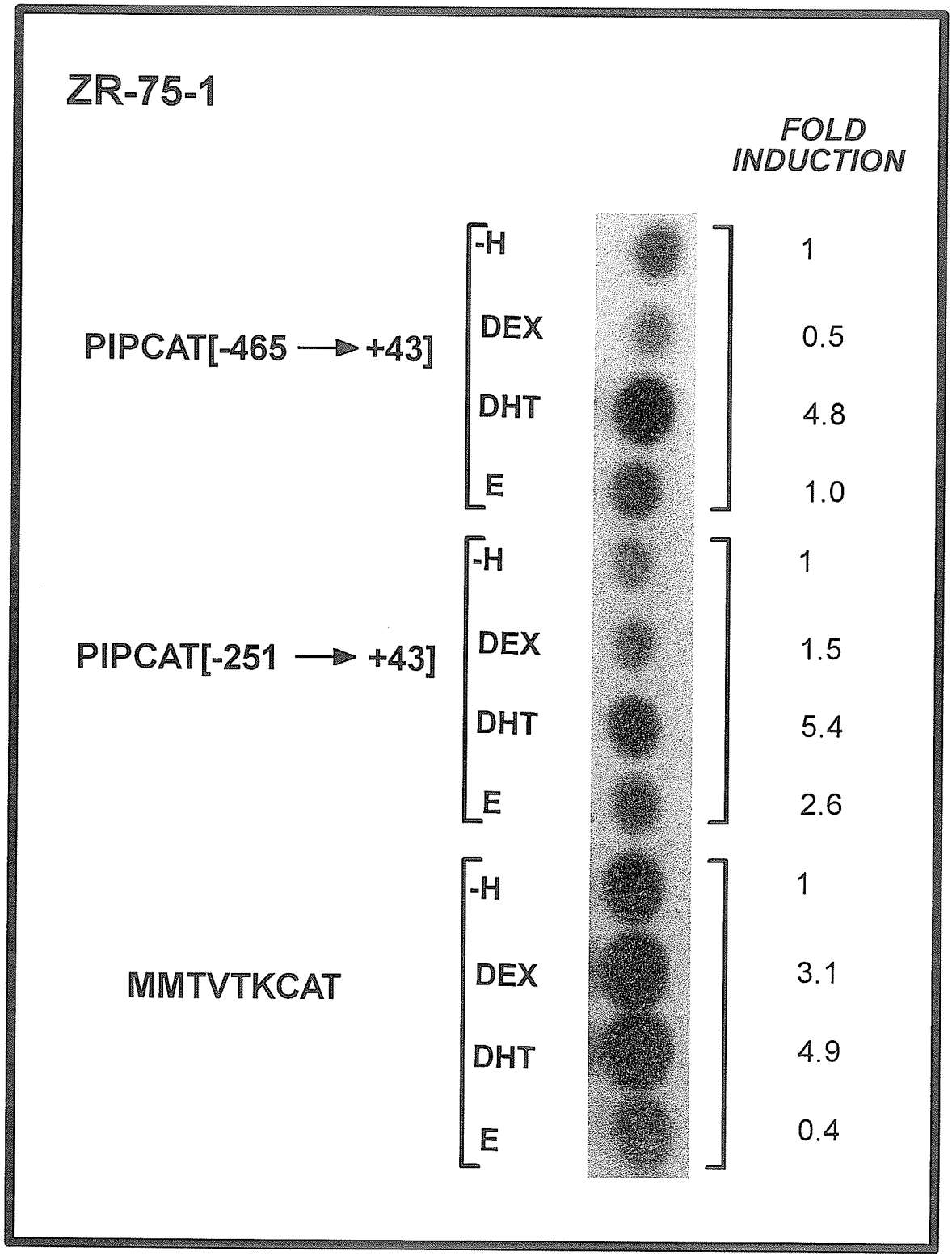
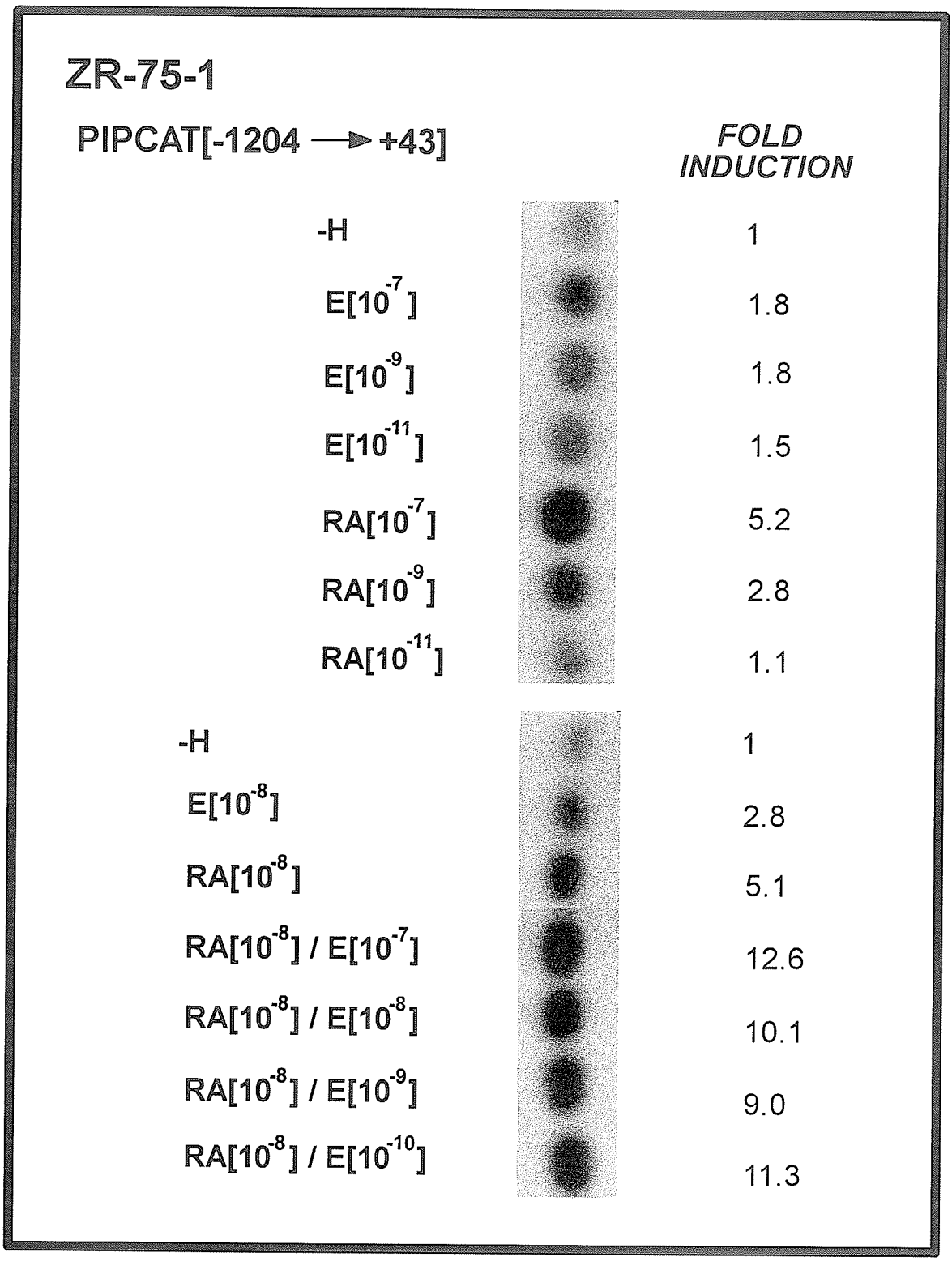

ZR-75-1
FOLD INDUCTION
PIPCAT[-465 → +43]
-H
DEX
DHT
E
1
0.5
4.8
1.0
PIPCAT[-251 → +43]
-H
DEX
DHT
E
1
1.5
5.4
2.6
MMTVTKCAT
-H
DEX
DHT
E
1
3.1
4.9
0.4

Figure 17 Estrogen and retinoic acid synergize in inducing PIPCAT[-1204 → +43] in ZR-75-1 HBC cells.

Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] hormones as indicated or ethanol. The autoradiograph was used to identify radioactive spots which were scraped and scintillation counted to calculate fold increases with hormone treatment.

Fig. 17→


ZR-75-1
PIPCAT[-1204 ⟶ +43]
FOLD INDUCTION
-H
1
E[10-7]
1.8
E[10-9]
1.8
E[10-11]
1.5
RA[10-7]
5.2
RA[10-9]
2.8
RA[10-11]
1.1
-H
1
E[10-8]
2.8
RA[10-8]
5.1
RA[10-8] / E[10-7]
12.6
RA[10-8] / E[10-8]
10.1
RA[10-8] / E[10-9]
9.0
RA[10-8] / E[10-10]
11.3

Other cell lines were used in transient expression studies of the PIPCAT constructs. Only the human breast cancer cell lines, T-47D and ZR-75-1, expressed the CAT enzyme after hormone treatment. The HBC cell lines BT-474 and MCF-7 did not transiently express PIPCAT. Additionally, the "normal" breast epithelial cell line, HBL-100 [Fig. 18], the cervical carcinoma cell line, HeLa, and the prostatic carcinoma cell line, LNCaP (data not shown), did not transiently express PIPCAT. Of these cell lines, only the T-47D HBC cells express high levels of the PIP gene and ZR-75-1 and BT-474 HBC cells express low levels.

As a control for transfection efficiency between constructs, the expression vector, CH110, which expresses the β-galactosidase enzyme, was cotransfected with the PIPCAT constructs. β-galactosidase enzyme can be easily assayed in the extracts of transfected cells. Unfortunately, the expression of β-galactosidase was severely depressed with retinoic acid treatment and hormone combinations containing retinoic acid (to approximately 30% of non-treated cell extracts). Therefore, the β-galactosidase control was abandoned and cell extracts were standardized with respect to total proteins in the extracts [see Table 3].

Figure 18 PIPCAT[-1204 → +43] is inducible in T-47D and ZR-75-1 HBC cells but not in HBL-100, BT-474, MCF-7, HeLa and LNCaP cells.
Transient transfections and CAT assays were carried out as outlined in **Methods**. Transfected cells were treated with [10^{-8}M] dihydrotestosterone as indicated or ethanol. MMTVTKCAT is a positive control for androgen induction.

Fig. 18→

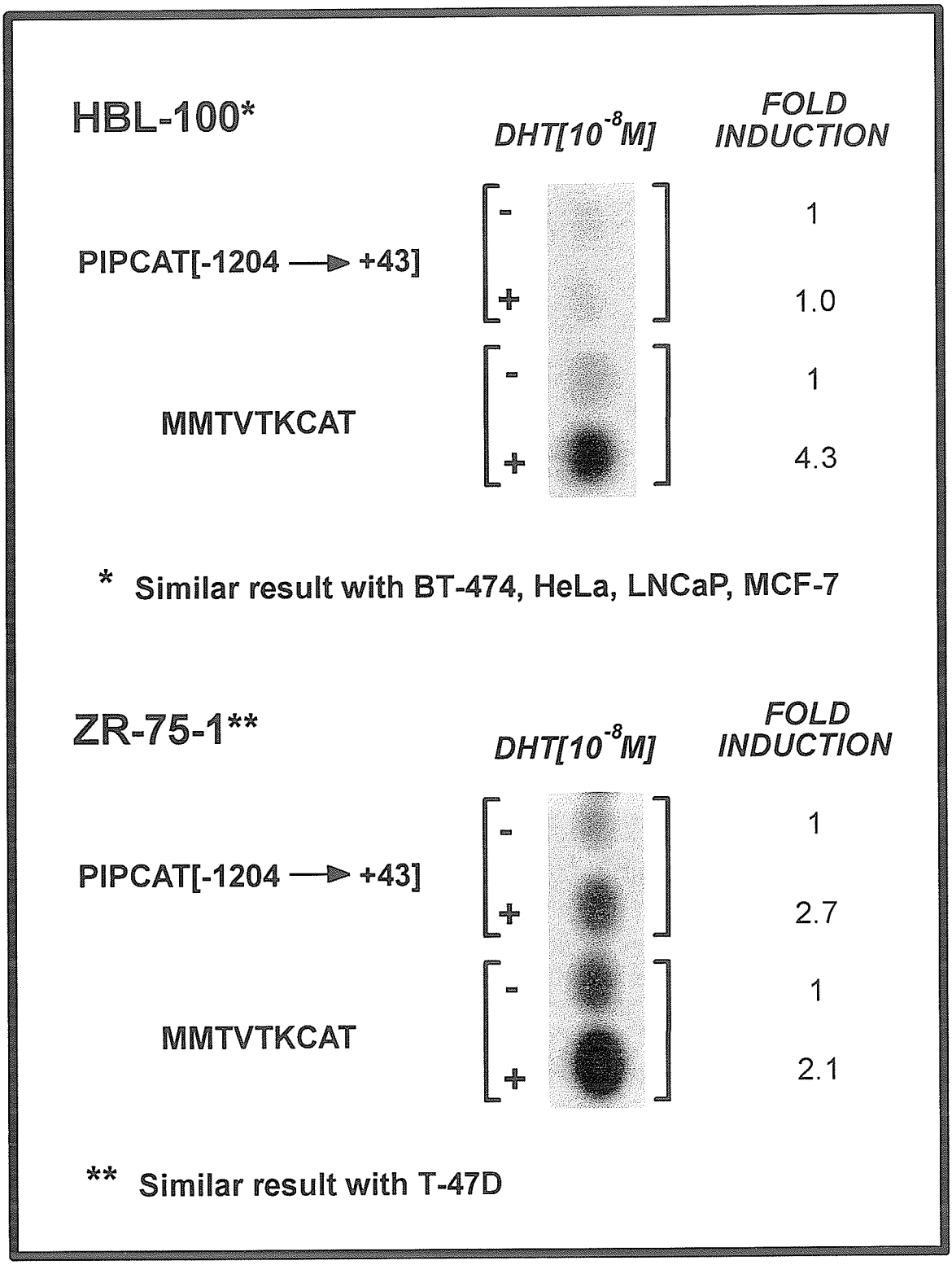

HBL-100*
DHT[10-8M]
FOLD INDUCTION
PIPCAT[-1204 → +43]
-
+
1
1.0
MMTVTKCAT
-
+
1
4.3
* Similar result with BT-474, HeLa, LNCaP, MCF-7
ZR-75-1**
DHT[10-8M]
FOLD INDUCTION
PIPCAT[-1204 → +43]
-
+
1
2.7
MMTVTKCAT
-
+
1
2.1
** Similar result with T-47D

Table 3 Retinoic acid suppresses expression of β-galactosidase in ZR-75-1 HBC cells. Transient transfections were carried out as outlined in **Methods**. A) ZR-75-1 HBC cells were transfected with the β-galactosidase expression vector, CH110, treated with varying concentrations of retinoic acid and cell extracts were assayed for β-galactosidase activity. B) ZR-75-1 HBC cells were transfected with the β-galactosidase expression vector, CH110, treated with hormones as indicated and cell extracts were assayed for β-galactosidase activity. Percent activity was calculated with respect to transfected cells not treated with hormones.

Table 3→

A.

RA [10^{-11}]	+		
RA [10^{-9}]		+	
RA [10^{-7}]			+
	93%	73%	47%

B.

DHT [10^{-8}]	+			+	
E_2 [10^{-8}]		+			+
RA [10^{-8}]			+	+	+
	91% (±12%)	120%	35% (±12%)	34% (±13%)	31%

To summarize these results, the PIP gene is inducible by dihydrotestosterone and retinoic acid with different time courses of induction and these inductions are inhibited by estradiol. The PIP gene 5' flanking region contains potential response elements for both androgen and estrogen receptors but not for retinoic acid receptor. Areas of the PIP gene 5' flanking region including the PIP promoter have been shown to confer androgen and retinoic acid responsiveness to the reporter CAT gene. The androgen responsive region extends between -1499 and -251 of the 5' flanking region. One construct which spans -1144 to +43 is not androgen inducible. The retinoic acid responsive region extends between -1499 and -982 of the 5' flanking region and more 3' sequences do not confer retinoic acid inducibility on the reporter gene, CAT [see Table 4]. The fold inductions for constructs indicated by an asterisk are statistically significant ($p \leq 0.0025$) as determined by a one-tailed student's t-test.

PIPCAT [-1204→+43] was treated with hormones, individually and in combination, to compare their effects on PIPCAT induction. These results are summarized in Table 5. The induction by dexamethasone by itself is not statistically significant, nor does dexamethasone significantly affect the inductions of the other hormones. The inductions of DHT, estradiol and retinoic acid are all individually statistically significant, however, and only DHT significantly affects the induction of PIPCAT [-1204→+43] by retinoic acid ($p = 0.009$) or estradiol ($p = 0.011$) as determined by the Student's t-test.

The effects of treatment of various hormones, individually and in combination, on PIP mRNA and PIPCAT [-1204→+43] expression is compared and summarized in Table 6. Dexamethasone induces PIP mRNA expression minimally and PIPCAT expression is increased 1 to 2 fold above background. This increase, however, is not statistically significant. DHT (6 to 10 fold) and RA (10 fold) both induce PIP mRNA levels significantly and increase PIPCAT expression 4 and 5 fold, respectively (statistically significant; $p \leq 0.05$). Estradiol has no effect on PIP mRNA levels, yet increases PIPCAT expression by 2 to 3 fold (statistically significant; $p \leq 0.05$). Prolactin and growth hormone are equipotent with DHT (5 fold) in inducing the PIP mRNA, yet they have no

effect in increasing PIPCAT expression. The treatment of T-47D cells with simultaneous addition of DHT and estradiol leads to an estrogen inhibition of DHT-induced PIP mRNA expression. DHT and estradiol treatment does not affect PIPCAT expression significantly differently from DHT treatment alone. DHT and retinoic acid do not affect each other in inducing PIP mRNA expression; however, DHT and retinoic acid treatment increases PIPCAT expression additively a significant difference from DHT treatment alone ($p = 0.009$). Finally, estradiol inhibits retinoic acid induction of PIP mRNA levels, yet simultaneous addition of both hormones increases PIPCAT expression significantly over the expression of estradiol alone ($p = 0.011$).

Table 4 Summary of PIPCAT induction by dihydrotestosterone and retinoic acid. The induction of the PIPCAT[X $\rightarrow$ +43] constructs by either dihydrotestosterone or retinoic acid were averaged and standard deviations were calculated. A one-tailed student's t-test was applied to the means and statistical significance at $p \leq 0.0025$ was determined. Statistically significant values are indicated with an asterisk. For constructs assayed less than three times, no standard deviations are provided. For representative assays, see figures 13 and 14.

Table 4→

PIPCAT constructs [x→+43]	DHT [10^{-8}]	s.d.	n		RA [10^{-8}]	s.d.	n
-1500	4.0*	1.0	4				
-1213	5.4*	2.3	4		4.0	-	1
-1204	3.7*	1.7	11		5.2*	1.2	5
-1144	1.1	0.5	5		3.2	-	1
-982	4.1*	1.5	3		1.6	0.7	4
-831	4.5*	1.5	3				
-642	3.7*	1.1	4		0.9	-	1
-621	2.7	-	2				
-576	2.8	-	2				
-570	4.3*	1.5	3				
-536	3.1	-	1				
-465	3.1*	1.6	5				
-426	4.4	-	2				
-385	1.1	-	2		1.3	-	2
-377	3.2	-	2				
-348	3.5	-	1				
-251	1.6*	0.4	6		1.2	-	2
-176	1.0	0.6	4		1.8	-	2
-100	0.9	0.2	7		1.5	-	2

Table 5 Summary of PIPCAT[-1204 → +43] induction by hormones and hormone combinations.

The mean induction of PIPCAT[-1204 → +43] by hormones and hormone combinations were determined and a two-tailed student's t-test was applied to compare between groups. The DHT treatment mean is statistically different from the DHT/RA treatment mean and is indicated with an asterisk ($p = 0.009$). The estradiol treatment mean is statistically different from the estradiol/RA treatment mean and is indicated with two asterisks ($p = 0.011$). The other treatment groups are not significantly different from each other.

Table 5→

PIPCAT [-1204 → +43]	DEX	DHT	Estradiol	RA
DEX	1.9 ± 0.6 n = 3			
DHT	2.7 n = 2	3.7 ± 1.7* n = 11		
Estradiol	2.8 ± 2.6 n = 3	4.4 ± 2.3 n = 3	2.5 ± 0.6** n = 3	
RA	3.7 ± 1.5 n = 3	7.1 ± 2.6* n = 4	7.5 ± 1.7** n = 4	5.2 ± 1.2 n = 5

* p = 0.009; ** p = 0.011

Table 6 Summary of effects of different hormones on induction of the PIP mRNA in T-47D HBC cells and PIPCAT[-1204 → +43] in ZR-75-1 HBC cells.

The results from figures 5 through 8 and 12 through 17 are summarized. The first column summarizes the PIPCAT[-1204→+43] CAT assay results when transiently transfected cells are treated with different hormones and hormone concentrations. Statistically significant inductions are indicated for individual hormone treatments by an asterisk ($p \leq 0.025$). For hormone combinations, the treatment groups are compared to the appropriate individual treatment groups. For example, the DHT/E_2 treatment group is not significantly different from DHT or E_2 alone.

Table 6→

	CAT ASSAY PIPCAT[-1204→+43]	PIP mRNA
DEX [10^{-8}]	↑ 1 to 2 fold	↑
DHT [10^{-8}]	↑ 4 fold*	↑↑↑ (6 to 10 fold)
E_2 [10^{-8}]	↑ 2 to 3 fold*	no effect
GH/PRL	no effect	↑↑↑ (5 fold)
RA [10^{-8}]	↑ 5 fold*	↑↑↑ (10 fold)
DHT + E_2 [10^{-8}]	E_2 does not affect DHT induction (4 fold)	E_2 inhibits DHT induction
DHT + RA [10^{-8}]	additive (7 fold)*	no effect
E_2 + RA [10^{-8}]	additive/synergistic (7 fold)*	E_2 inhibits RA induction

* statistically significant (see Tables 4 and 5)

Analysis of the 5' flanking region of the PIP gene for hormone responsive elements

As a result of the previous transient expression studies, we were interested in examining the 5' flanking region of the PIP gene for possible binding sites for the receptors for androgen, estrogen and retinoic acid. I performed gel mobility shift studies, initially with probe C, a fragment from -1268 to -1030 encompassing the consensus ERE and two downstream ARE half-sites [Fig. 19]. Nuclear extracts from ZR-75-1 HBC cells were observed to contain proteins that complexed with probe C in a concentration dependent manner. Increasing amounts of nuclear extract resulted in a correspondingly larger number of shifted protein-DNA complexes [Fig. 20]. Nuclear extracts from GH_4C_1 rat pituitary cells, HeLa cervical carcinoma cells and MCF-7 and ZR-75-1 human breast cancer cells contained proteins that bound to probe C [-1268 → -1030], probe D [-1030 → -821], probe E [-821 → -495] and probe F [-495 →-272], although considerably different pattern of shifted protein-DNA complexes were observed in different cell types [Fig. 21]. Probes C and F contain ARE half-site sequences, while probes D and E do not contain consensus binding sites for any of the characterized mammalian transcription factors [Fig. 19]. Probe E was slightly contaminated with higher molecular weight DNAs as seen in the first lane in the lower autoradiograph. It is interesting to note that the pattern of shifted complexes with HeLa and ZR-75-1 nuclear extracts is similar. A purified peptide, GST-AR2, containing the DNA-binding domain of the androgen receptor was shown to bind strongly and cause a specific shift in the mobility of probe C at a concentration of 0.1 μg [Fig. 22]. This androgen receptor peptide was produced as a glutathione-S-transferase fusion protein in *E. coli* by Rennie *et al* (1993) and the GST protein alone was used as a control for these experiments. The androgen receptor peptide, at these concentrations, did not bind to and cause a shift in the mobility of probes A, C', D, E or F. Probe C' [-1144 → -1030] consists of a fragment of probe C containing only the second ARE half-site and does not contain the consensus ERE or the ARE half-site adjacent to the ERE. Again, probe E is contaminated with higher molecular weight DNAs but does not show a specific pattern of mobility shift in the presence of the AR peptide. Increasing amounts of the androgen receptor peptide (0.1 to 0.8 μg) caused an increasing number of higher shifted protein-probe C complexes [Fig. 23]. The androgen

receptor peptide also bound to probe D [-1030→-821] and probe F [-495→-272] [Fig. 24] at the higher concentrations (≥ 0.2 μg). Probe F contains two ARE half-sites at base pairs -413 and -375. Probe D does not contain any potential AREs [Fig. 19] by comparison of the sequence with known AREs [Table 1]. The androgen receptor also bound to a *c-myc* [-63 → +268] probe and a vitellogenin A2 [-331 → -87] probe but at higher concentrations (≥ 0.2 μg) of androgen receptor peptide [Fig. 25]. The specific binding of the AR peptide to probe C is much more pronounced than to the other probes. The vitellogenin A2 probe is slightly contaminated with higher molecular weight DNAs.

Figure 19 Constructs used in DNase I footprinting and gel mobility shift assays. The 5' flanking region of the PIP gene is indicated by a solid line, the potential AREs are indicated by solid filled boxes and the consensus ERE is indicated by a grey filled box. The unfilled boxes indicate the fragments of the 5' flanking region of the PIP gene used in DNase I footprinting and gel mobility shift assays.

To create these constructs, the 1.5 kilobase insert of 1.5gPIP was digested with *FokI* (sites at -1413, -1268, -1030, -821 and -495) and *AvaI* (site at -272) which generated DNA fragments ranging in size from 87 to 326 base pairs. These fragments were blunt ended with Klenow fragment and subcloned into the *SmaI* site of pVZ1. These constructs are referred to as PIP probes A, C, D, E and F depending on their localization in the PIP 5' flanking region in a 5' to 3' direction. PIP probe C' was created by cleaving SB1-PIP-CAT (containing -1144 to +43) with *FokI* and *NsiI* to generate a fragment from -1144 to -1030. This fragment was blunt ended with Klenow fragment and subcloned into the *SmaI* site of pVZ1.

Fig. 19→

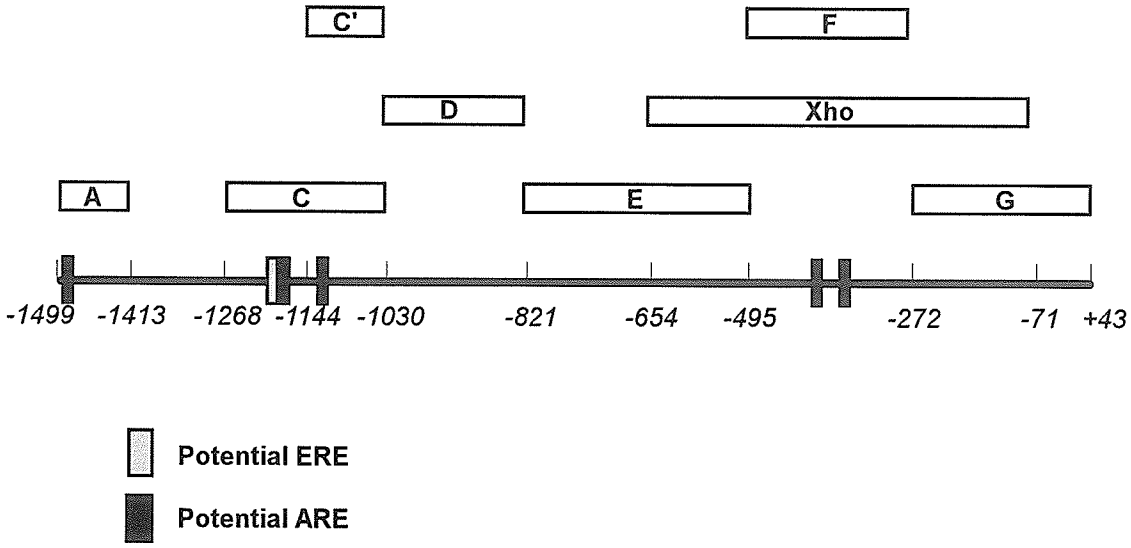
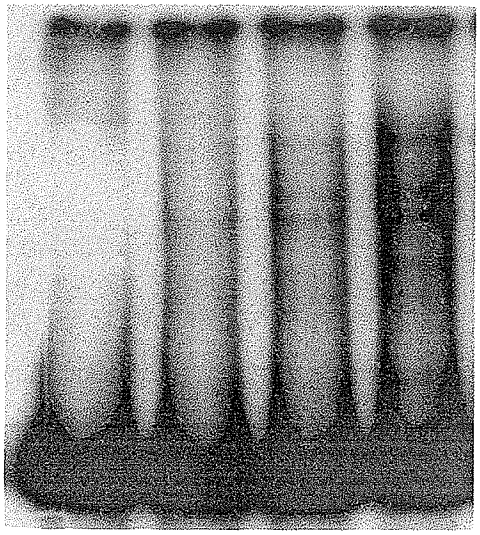

C'
F
D
Xho
A
C
E
G
-1499
-1413
-1268
-1144
-1030
-821
-654
-495
-272
-71
+43
Potential ERE
Potential ARE

Figure 20 Nuclear proteins in ZR-75-1 nuclear extracts bind PIP probe C. Increasing amounts of ZR-75-1 nuclear extracts were incubated with 20000 cpm of end-labelled probe C and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel.

Fig. 20→

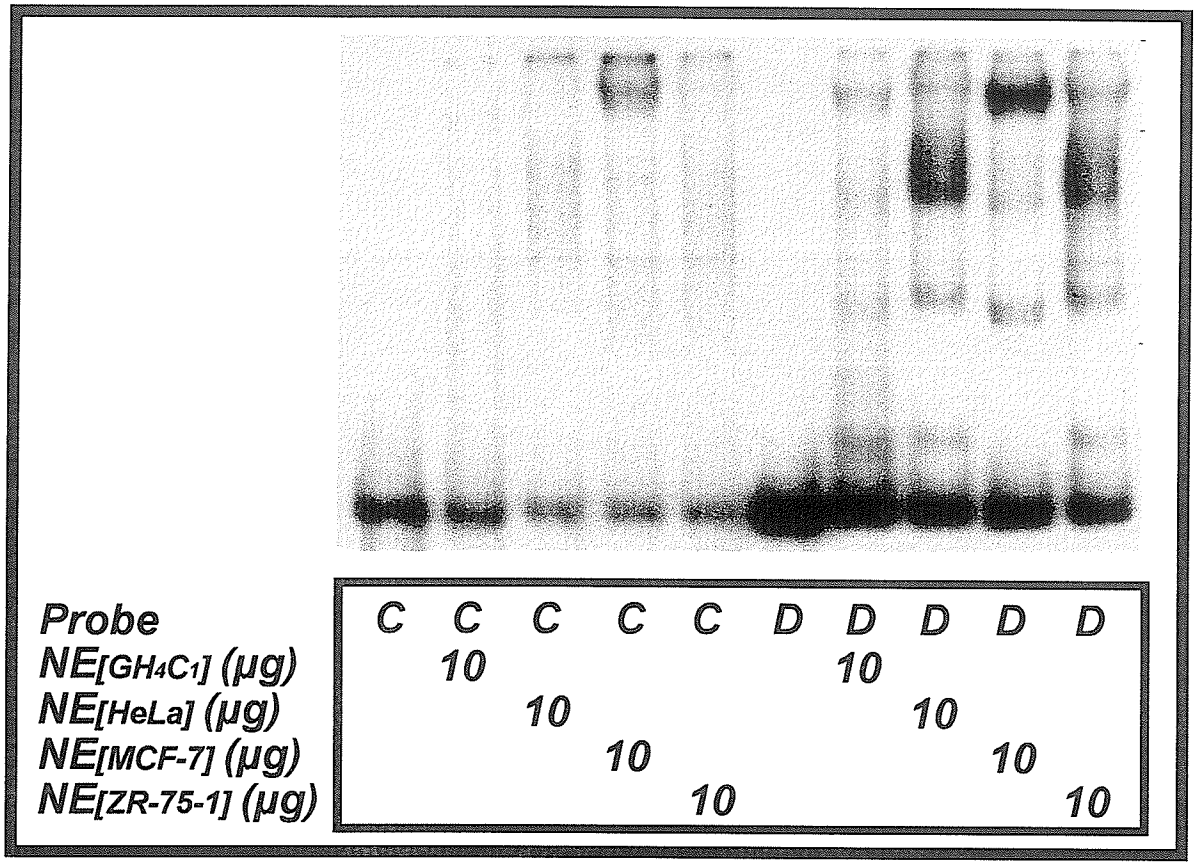


$NE_{[ZR\text{-}75\text{-}1]}$ (μg)	0	2.5	5.0	7.5

Figure 21 Nuclear extracts from different cell types bind PIP probes C, D, E and F.

Nuclear extracts from GH_4C_1 rat pituitary cells, HeLa human cervical carcinoma cells, MCF-7 human breast cancer cells and ZR-75-1 human breast cancer cells were incubated with 20000 cpm of end-labelled probes C, D, E and F (see page 97 and figure 19) and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel.

Fig. 21→


Probe
NE[GH4C1] (µg)
NE[HeLa] (µg)
NE[MCF-7] (µg)
NE[ZR-75-1] (µg)
C C C C C D D D D D
10 10
10 10
10 10
10 10

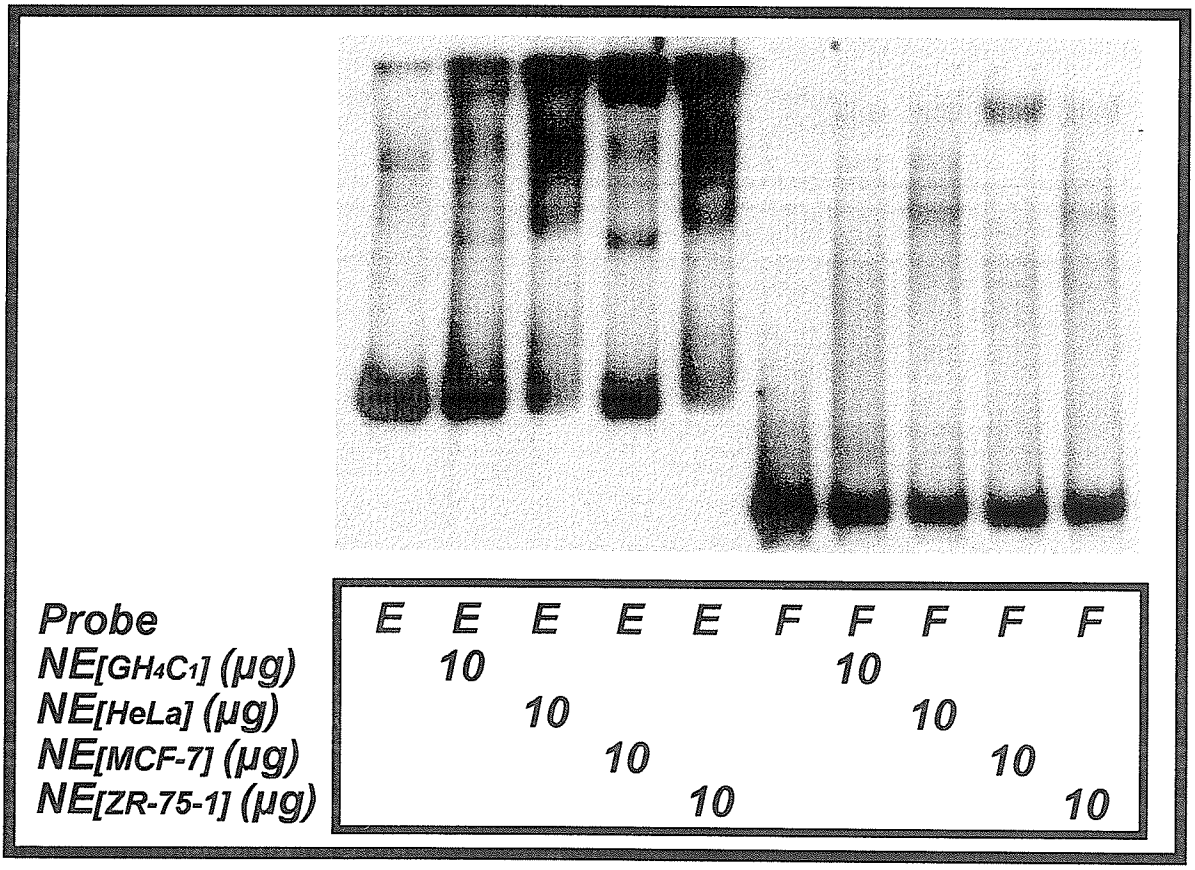
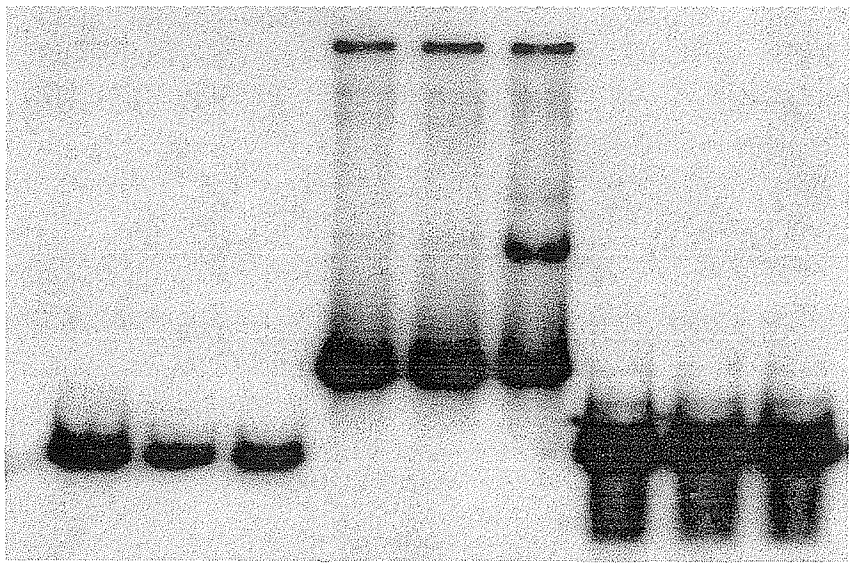

Probe
NE[GH4C1] (µg)
NE[HeLa] (µg)
NE[MCF-7] (µg)
NE[ZR-75-1] (µg)
E E E E E F F F F F
10 10
10 10
10 10
10 10

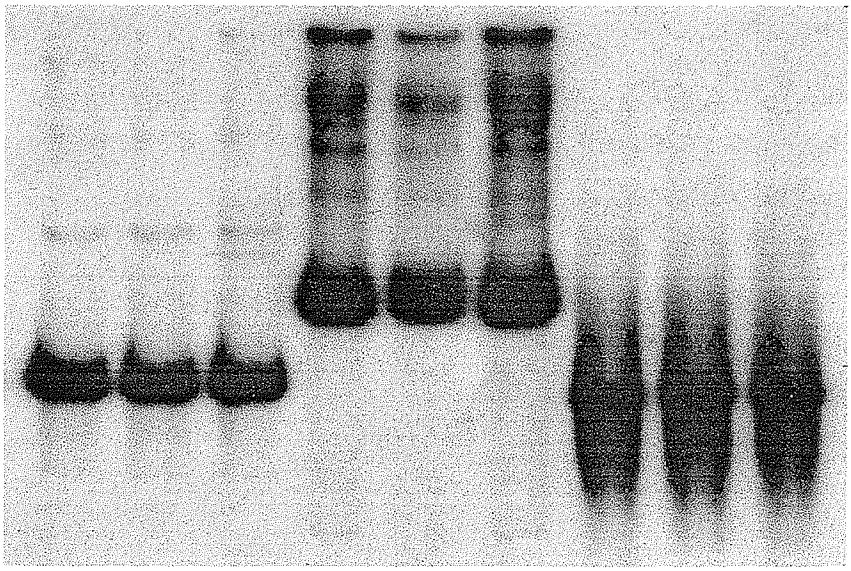
Figure 22 Androgen receptor binds PIP probe C, but not probes A, C', D, E and F.

Purified androgen receptor (0.1 μg) was incubated with 20000 cpm of end-labelled probes A, C, C', D, E and F and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel. The control for androgen receptor is the purified GST fusion protein alone.

Fig. 22→



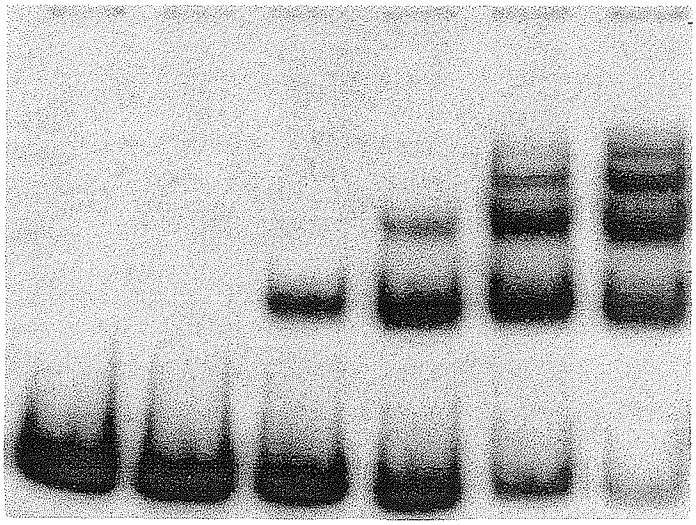
Probe	A	A	A	C	C	C	C'	C'	C'
Control (µg)		0.1			0.1			0.1	
hAR (µg)			0.1			0.1			0.1



Probe	D	D	D	E	E	E	F	F	F
Control (µg)		0.1			0.1			0.1	
hAR (µg)			0.1			0.1			0.1

Figure 23 Androgen receptor binds PIP probe C.
Increasing amounts of purified androgen receptor were incubated with 20000 cpm of end-labelled probe C and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel. The control for androgen receptor is the purified GST fusion protein alone.

Fig. 23→

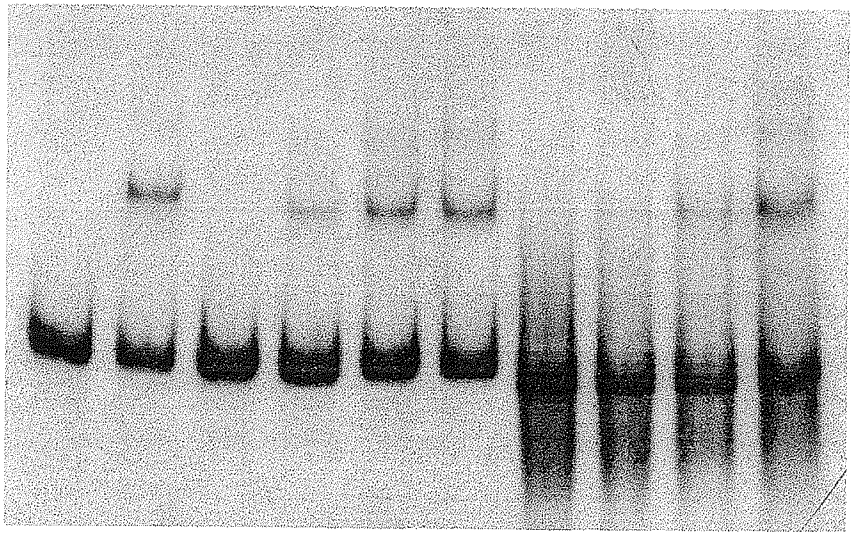


Control (µg)	4				
hAR (µg)		0.1	0.2	0.4	0.8

Figure 24 Androgen receptor binds PIP probe C, but also PIP probes D and F at higher concentrations (≥0.2 μg).

Purified androgen receptor was incubated with 20000 cpm of end-labelled probes C, D and F and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel.

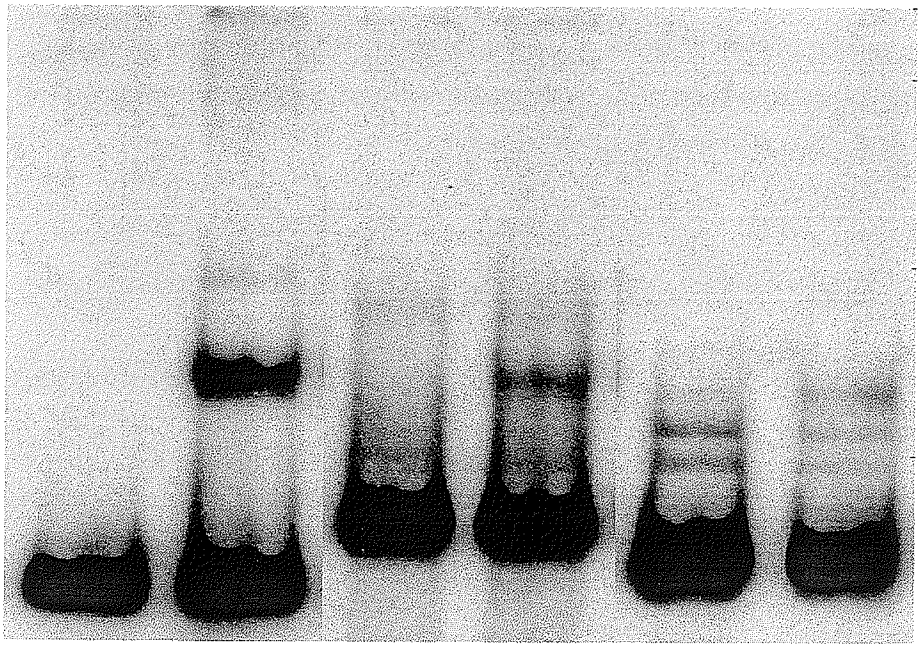
Fig. 24→



Probe	C	C	D	D	D	D	F	F	F	F
Control (µg)	0.2		0.2				0.1			
hAR (µg)		0.2		0.2	0.4	0.8		0.2	0.4	0.8

Figure 25 Androgen receptor binds PIP probe C but also the *c-myc* promoter and the vitellogenin A2 promoter at higher concentrations (≥ 0.2 µg).
Purified androgen receptor was incubated with 20000 cpm of end-labelled probe C, the *c-myc* promoter and the vitellogenin A2 promoter and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel.

Fig. 25 →



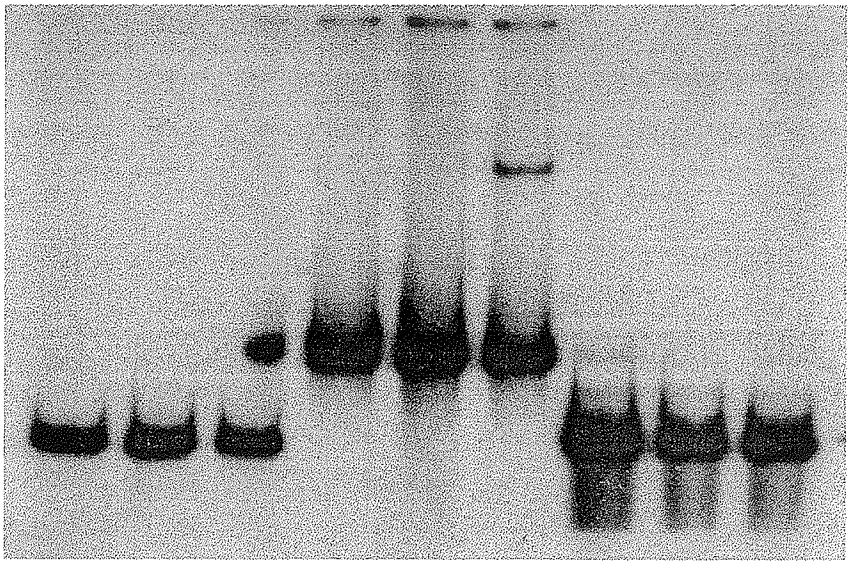
Probe	C	c-myc	Vit A2
hAR (0.2 µg)	+	+	+

Estrogen receptor was transcribed and translated from a T7 expression construct (pT7βhER: Tsai, Houston, Texas) using a rabbit reticulocyte lysate transcription/translation protocol (Promega). The estrogen receptor containing lysate was then used in mobility shift assays to examine the interaction of estrogen receptor with probe C. The control for these experiments was a rabbit reticulocyte lysate which had been programmed by the plasmid vector pVZ alone. Probe C contains the only sequence similar to a consensus ERE in the 5' flanking region of the PIP gene. Estrogen receptor specifically caused a shift in probe C as a single band but did not shift probes A, C', D, E or F [Fig. 26]. Since the consensus ERE and the closest downstream potential ARE are adjacent to each other, the effect of estrogen receptor on androgen receptor binding was examined. With increasing amounts of estrogen receptor added simultaneously to androgen receptor, the protein-DNA complex representing the androgen receptor decreased and was replaced by that of the estrogen receptor [Fig. 27]. In figure 27, comparing the second and fifth lanes, the band representing probe C shifted by the AR peptide suggests that the ER may enhance the binding of the AR peptide to probe C. However, upon many repetitions of this assay, this enhancement is an artifact of this particular assay and is not observed in other similar assays.

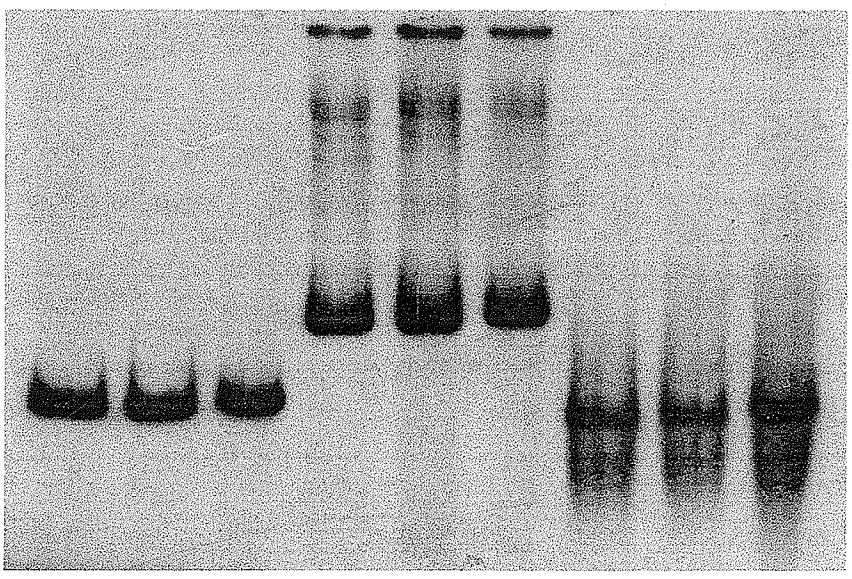
Figure 26 Estrogen receptor binds PIP probe C but not PIP probes A, C', D, E and F.

Translated estrogen receptor was incubated with 20000 cpm of end-labelled probes A, C, C', D, E and F and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel. The control for translated estrogen receptor is rabbit reticulocyte lysate programmed with the plasmid vector pVZ1 alone.

Fig. 26→



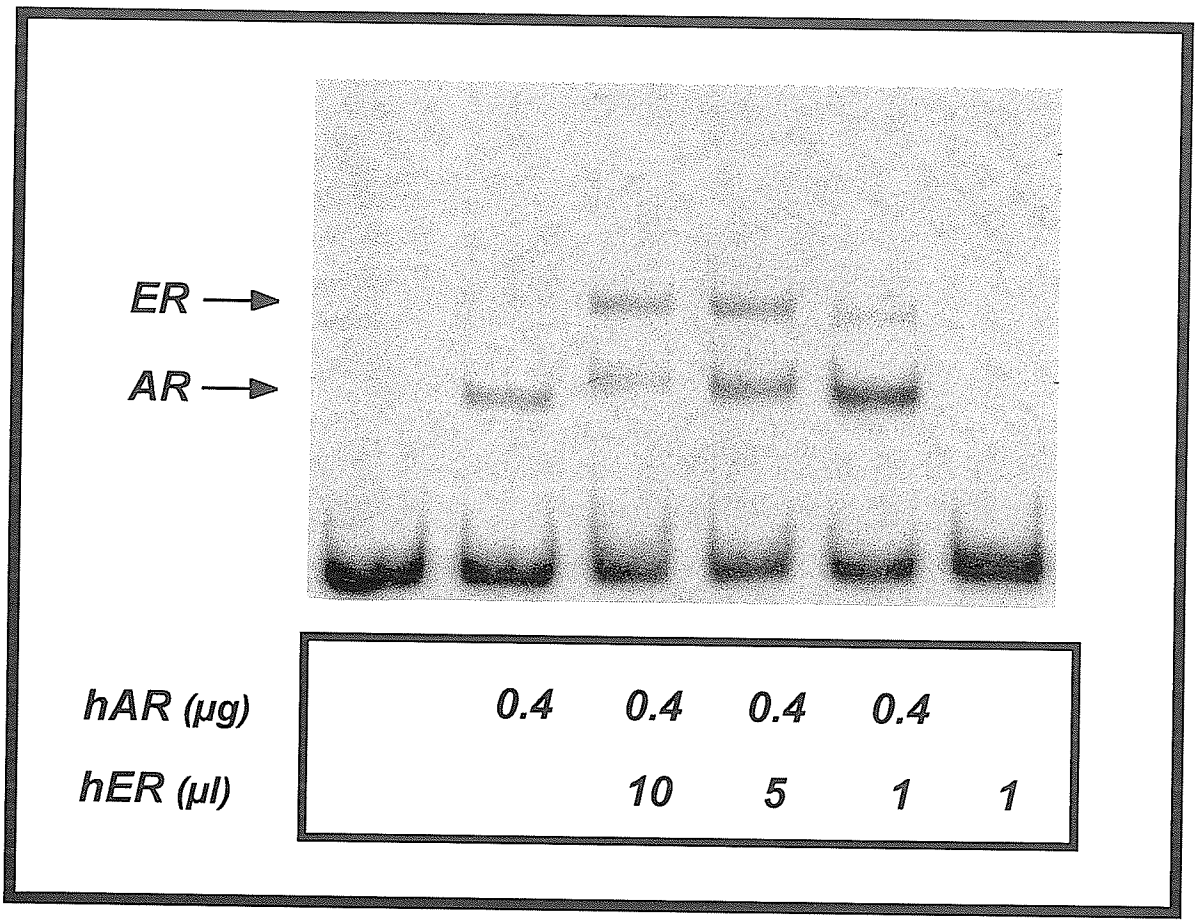
Probe	A	A	A	C	C	C	C'	C'	C'
Control (µl)		2			2			2	
hER (µl)			2			2			2



Probe	D	D	D	E	E	E	F	F	F
Control (µl)		2			2			2	
hER (µl)			2			2			2

Figure 27 Increasing amounts of translated estrogen receptor causes decreased androgen receptor binding to PIP probe C.
Increasing amounts of translated estrogen receptor was incubated with standard amounts of androgen receptor and 20000 cpm of end-labelled probe C and the protein-DNA complexes were resolved on a 5% 0.5X TBE-polyacrylamide gel.

Fig. 27→


ER
AR
hAR (µg)
hER (µl)
0.4
0.4
0.4
0.4
10
5
1
1

Although no retinoic acid response element-like sequence has been found in the PIP gene 5' flanking region, retinoic acid induces PIP expression both at the level of mRNA and in CAT assays. Retinoic acid receptor has been observed to compete for an ERE and thereby exert an antiestrogenic effect (De Verneuil and Metzger, 1990; Näär *et al*, 1991). We obtained mouse ascites antibodies raised to the F domain of the three isoforms of human RAR and thus far have been unable to show an interaction between RAR antibodies and proteins in ZR-75-1 nuclear extract. I am currently continuing these experiments.

In order to further characterize the actual sequences comprising the binding sites for the nuclear hormone receptors, I performed DNase I footprinting assays. DNase I footprinting analysis of probe C shows that the purified androgen receptor peptide binds to a specific site in the DNA around the area of the first ARE half-site (base pairs -1198 to -1178). Estrogen receptor was shown to bind to a slightly larger area on probe C, base pairs -1205 to -1169, including sequences bound by the androgen receptor [Fig. 28]. Additionally, nuclear extracts from ZR-75-1 HBC cells can protect an area on probe C, base pairs -1150 to -1131, which is not protected by nuclear extracts of GH_4C_1 and HeLa cells (not shown). This protected area is not bound by the androgen receptor peptide (GST-AR2) nor by the translated estrogen receptor [Fig. 29].

Figure 28 Androgen receptor and estrogen receptor protect overlapping areas on PIP probe C from DNase I digestion.

DNase I footprinting assays were performed as outlined in **Methods**. The control for AR was the glutathione-S-transferase protein alone and the control for ER was rabbit reticulocyte lysate programmed by the plasmid vector, pVZ.

Fig. 28→

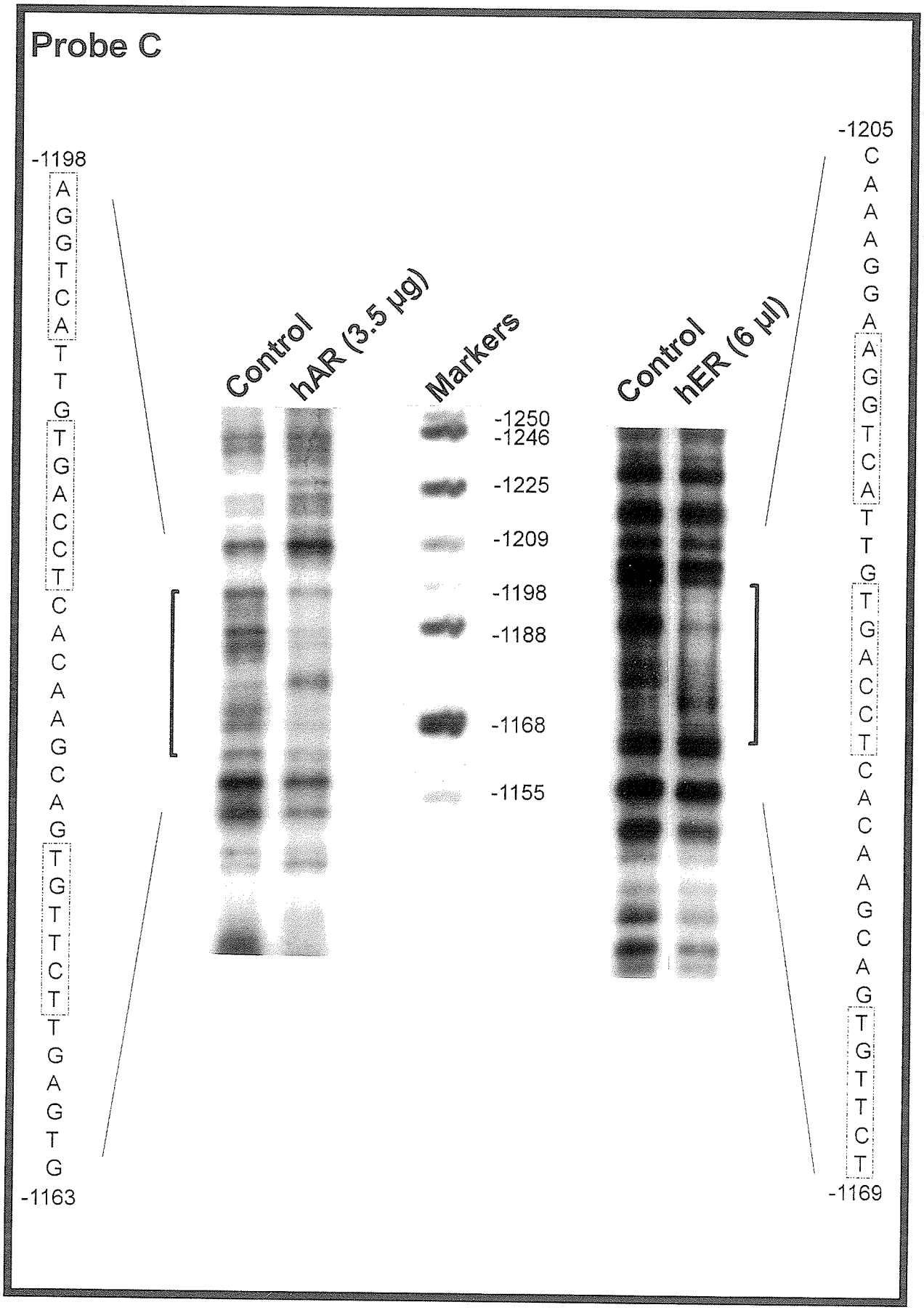
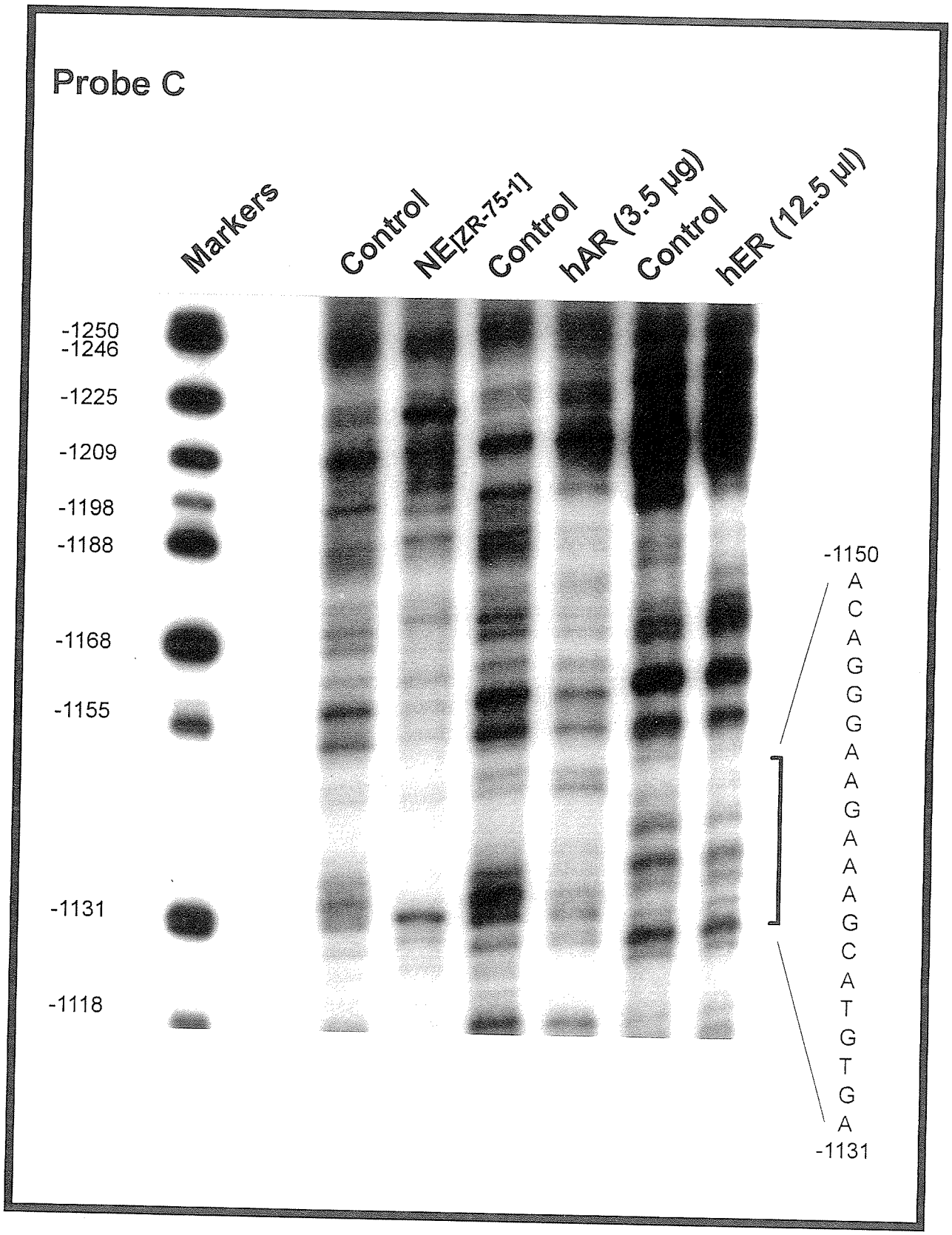

Probe C
-1198
AGGTCA
TTG
TGACCT
CACAAGCAG
TGTTCT
TGAGTG
-1163
Control
hAR (3.5 µg)
Markers
-1250
-1246
-1225
-1209
-1198
-1188
-1168
-1155
Control
hER (6 µl)
-1205
CAAAGGAAGGTCA
TTG
TGACCT
CACAAGCAG
TGTTCT
-1169

Figure 29 A nuclear protein from ZR-75-1 nuclear extracts protects an area [-1150 → -1131] on PIP probe C which is not protected by androgen receptor or estrogen receptor.

DNase I footprinting assays were performed as outlined in **Methods**. The control for AR was the glutathione-S-transferase protein alone and the control for ER was rabbit reticulocyte lysate programmed by the plasmid vector, pVZ.

Fig. 29→


Probe C
Markers
Control
NE[ZR-75-1]
Control
hAR (3.5 µg)
Control
hER (12.5 µl)
-1250
-1246
-1225
-1209
-1198
-1188
-1168
-1155
-1131
-1118
-1150
A
C
A
G
G
G
A
A
G
A
A
A
A
G
C
A
T
G
T
G
A
-1131

To summarize these results, the 5' flanking region of the PIP gene appears to contain a binding site for androgen receptor, which corresponds to an ARE half-site. The 5' flanking region also contains a consensus ERE [AGGTCAttgTGACCT] found at base pair -1198 which binds ER. These binding sites correspond to overlapping sequences on probe C as shown by DNase I footprinting assays. The translated ER and the purified AR DNA binding domain compete for binding to their overlapping binding sites as shown by mobility shift assays. Additionally, a potentially tissue specific factor appears to protect the area between -1150 to -1131 from DNase I digestion.

Figure 30 Sequence of the 5' flanking region of the PIP gene.
The sequence which is protected by the translated estrogen receptor [-1205→-1169] is singly underlined. The consensus ERE is shaded and double underlined. The sequence which is protected by the androgen receptor DNA binding domain [-1198→-1178] is double underlined. The sequence protected by a nuclear factor from ZR-75-1 HBC cells [-1150→-1131] is indicated by double underlined italics. The CAAT and TATA box sequences are indicated by single underlines and single underlined italics, respectively. The first 46 basepairs of the first exon, including the 5' untranslated region up to the initiation codon are indicated by italics.

Fig. 30→

GTAACCATCA CAGCAGTCAT GGCCTGAATA AGACTTGTCC TAGGCCAGGC -1815
ACAGTGGCTC ACACCTGTAA TCCCAGCAAT TTGGGAGGCC GAGGCAGGTG
GATCACTTGA GGTCAGGAAT GTAAGATCAG CTTGGCCAAC ATGGTGAAAC -1715
CCCATCTCTA CCAAAAATAC AAAAATTAGC CGGGTATGGT GGCACCTGCC
TATAATCCCC ACTACTCGGG TGGCTGAGGC ATGAGAATTT CCTGAACCCG -1615
GGAGTGGAGG CTGCAGTGAG CTGAGATCAT GCCACTGCAC TCCAGCCTGG
ATGAGTGAGA TTCTGTCTCA AAAAAAAAAG ATTGTCCTGT ACATTGTAGG -1515
TATTGCAGTT TCCTGCTAGC TCACAGAAGA ACAGCCTCAT TACCTTCAAT
AGATGCTCTT AACCCATCGT CATCTGCCTT CCTCTGGACA CTGCCTTTTG -1415
GGAGTGCTGG AGTTGCATCC TCAAGGACAG GGCTATGCTT CAAAGAGCCA
AGCAAAGAAC TTTTTCCTGC ATTTTCCCAC ACAGACACTT CCTAGATTCT -1315
TCTGCCTTAT GCCTGCCTTG GTCAGTGTAG CTAGGATGTG CTTCAAATGG
AAATTATGCA AGTGAGAAGG ACCAGGTGTA AAGACAATTT AATTGTGGGA -1215
AAAGGCTTAC AAAGGAAGGT CATTGTGACC TCACAAGCAG TGTTCTTGAG
TGGATAGGAC ATAA*ACAGGG AAGAAAGCAT GTGA*TCATGA GAACAGCTGA -1115
AGTTCTGAGT GAGCAATGAC ATGAATGGTG TCCAATTCAA AGAAAAGAGA
AGGTAGATCC AGGAGCTGAA TAAACAGACA GAGCAATTTT GAGAGATACA -1015
TCCAGCAAGC AGAAGCACAC ACTTACACTT TAGCTCTGAA CTCTGATTTT
CACTCTGTTG CCAGAGAGGC TCTTCCTGGG CAGCTGTGTG CCATGCAAAT -915
GATAGAAACA AATCCCATAG TATTGTAAGT CATAGGTTAA GGAGCAGATT
TAATTTCATA AGGAGAAATA ACACAATGCC AGTGTCAGCA AGAGCAAGTC -815
TCCCCATCAT CCCAAGGAGA GGAATTCTGA CTGACCCATG TCAATAAGAA
AATAAGCCAG CTCTTTGGTG CCAAGAAGTC TGATTTGGGG GTGCCAGCTA -715
TTTATAGGAA TTGTCTAGAA TTAAAATATC ACCCTGCCTT GGGGATTGTG
GGGCAGCTGG GGATCTTGGA CATCTGCAGT ATATTGCAAC CACACCTGAA -615
ATTAAGAAGG ACTCAATGAC AATAAACATC TTTACATCAC CCAGTGTCTT
GAACTCACTT CCCCATCATG TAGAGCAGCT CAGGTCTTCT CTAGCTCTTC -515
CACCTCATGA TGGGTAGTGA CTGCTAGGGA AGTCCTGATC ATCCTAGCCC
TCCAAGAAAT GTTTGAATAG CATTCGATCT TGAACTCACT ATAATTTGAT -415
CAAGCAAAGA TGTTCTATGT CTCTGGTCTG AACCTGCCAC CACAGCTGTG
TTCTCTGCTT TCAGAGACCA GGGGAGCAGT GACTGAAGCT AAGACTACAC -315
ATAGCACTTC AGACAGGCAA TAGAGGAAAA GAAAACTGAA TCTGTAAACT
CGGGAAAAAC TGGTGTATCT TTTGAGGTCC CAGCCATTTT GAGAAACTCT -215
GATGACTGAA AAAATAAGGA AGAACAATCT GACTCCTCTC TCAAACGCTG
ACATTCTAGA CTGAGTGTTG ATTTTTTCTT AGAAAAACAA ACTTTGGGTC -115
AACAAGGAAA GATCACGAGT GGGGAGGGTG AATGGGTGAT TCACCTCATG
GATCTCCCTG GCAGCTCTTG CACTTCCTTG ATCAATCTG*T ATATAA*GAAA -15
TGCTGGGCAC CTGG*GACACC ACTTCTCTGG GACACATTGC CTTCTGTTTT* +36
CTCCAGCATG

DISCUSSION

Cloning and characterization of the PIP cDNA and gene

I have sequenced the PIP cDNA and this information has allowed the determination of the amino acid sequence of PIP as well as some structural features of the protein, such as a potential glycosylation site and the presence of a signal peptide (Murphy *et al*, 1987a). Unfortunately, the sequence alone has not been helpful in suggesting a function for PIP; however, in searching a database for similar sequences, we (Murphy, Tsuyuki and Shiu) have identified three sequences of interest: (1) mSMG, (2) GCDFP-15 and (3) SABP. The mouse submaxillary gland protein (mSMG) appears to be the murine equivalent of PIP (Windass *et al*, 1984) and is a useful tool in developing a mouse model for the investigation of PIP's function. Additionally, the identification of this protein in an androgen-dependent tissue led to the investigation of androgen regulation of the PIP gene and to the identification of PIP in human saliva (Murphy *et al*, 1987a). The discovery that PIP is identical to Gross Cystic Disease Fluid Protein (GCDFP-15) and the extensive studies of hormonal regulation of GCDFP-15 in diseases of the breast and breast cancer cell lines (see Introduction: p. 10 to 15) have confirmed many of the observations made by Murphy *et al* (1987a) and have suggested further studies of hormonal regulation. PIP has also been discovered to be identical to SABP and the characterization of SABP through protein analysis (Schaller *et al*, 1991) has confirmed the primary structure of PIP, the glycosylation signal proposed by Murphy *et al* (1987a) and has identified the actual N-terminal residue of the mature PIP as glutamic acid. Further, this study has also provided some insight into the possible functions of PIP. Since SABP was isolated from seminal plasma by actin affinity chromatography, Akiyama and Kimura (1990) suggest that PIP/SABP may have a role in the gelation of semen. However, no evidence to support this hypothesis is provided. Furthermore, the lack in PIP/SABP of amino acid motifs common to other actin binding proteins (Tellam *et al*, 1989) and PIP/SABP being a secreted protein with a tissue distribution different from other actin-binding proteins casts uncertainty as to the relevance of its actin-binding ability. PIP/GCDFP-15 has been

observed to bind fibrinogen with high affinity. However, it does not interfere with or induce clotting and does not block plasmin's digestion of fibrinogen; therefore, the biological significance of this finding is undetermined (Haagensen *et al*, 1990). Perhaps the actin- and fibrinogen-binding properties of PIP may not be specific to actin and fibrinogen, but may reflect an affinity of PIP for other proteins. The questionable significance of PIP's binding affinities and the observation that the addition of PIP to human breast cancer cells in culture does not have any obvious effect (Shiu *et al*, 1987) have left the biological function of PIP unknown.

Hormonal regulation of the PIP mRNA

The PIP gene is regulated positively by prolactin, androgen and retinoic acid and negatively by estrogen. In order to study the effects of these hormones, I have performed transient expression studies in the human breast cancer cell lines, T-47D and ZR-75-1. As described previously, T-47D cells contain high levels of PR, moderate levels of AR, ER and VDR and low levels of GR while ZR-75-1 cells have been shown to contain AR, PR, GR and ER (Engel *et al*, 1978). Both cell lines contain prolactin receptors (Shiu, 1979).

- Androgen

The effects of androgen have been shown to take place at a transcriptional level (Murphy *et al*, 1987a) as have the effects of prolactin (Myal *et al*, 1991). The time course of induction of PIP mRNA after human breast cancer cells are treated with androgen is typical of many androgen regulated genes, in that unlike many other steroids working through their receptors at a transcriptional level, androgen frequently increases the transcription of target genes slowly (Watson and Paigen, 1990). PIP mRNA levels are detectable 12 to 24 hours after androgen treatment of human breast cancer cells and continue to rise to maximal levels at 5 days (Murphy *et al*, 1987a; also Fig. 5).

- Retinoic acid

The time course of induction of retinoic acid is considerably different from that of androgen. PIP mRNA levels begin to increase within one hour after cells are treated with retinoic acid and reach maximal levels (10 fold) at 3 hours [Fig. 6]. Thereafter, PIP mRNA levels slowly decrease to control levels. At 24 hours, therefore, PIP mRNA levels have begun to increase as a result of androgen treatment and are considerably decreased as a result of retinoic acid treatment. Androgen and retinoic acid do not appear to affect each other's time course of PIP mRNA induction. A time course of induction by both hormones follows a biphasic pattern which corresponds to the induction by retinoic acid during the first 24 hours, followed by the induction by DHT up to 5 days.

- Estrogen

Estrogen has been reported to inhibit the androgen induced synthesis of PIP in ZR-75-1 cells (Simard *et al*, 1989). In studying the effects of the estrogen inhibition of androgen and retinoic acid on PIP gene expression in T-47D HBC cells, I chose 24 hours as a time where androgen has induced detectable levels of PIP mRNA and induction by retinoic acid is still detectable. Estrogen appears to inhibit PIP induction by either androgen or retinoic acid at 24 hours of simultaneous estrogen/DHT or estrogen/RA treatment. Estrogen by itself has no effect or is slightly inhibitory on PIP mRNA levels in ZR-75-1 HBC cells [Fig. 8].

Haagensen and coworkers (Haagensen *et al*, 1990) have proposed that PIP/GCDFP-15 is a marker of apocrine differentiation and that hormones which cause cell proliferation will inhibit PIP expression and hormones which cause differentiation will activate PIP expression. The role of retinoic acid as an agent of differentiation is well documented, as is the role of estrogen in cellular proliferation. Androgen has a role in the sexual differentiation in males and frequently has antiestrogenic effects in estrogen target tissues. Therefore, the results I obtain from the hormone regulation studies are in accordance with this apocrine differentiation hypothesis.

Hormonal regulation of the 5' flanking region of the PIP gene

Androgen, estrogen and retinoic acid have been shown to affect gene transcription through interaction with specific nuclear receptors which recognize specific *cis*-acting DNA elements within the regulated gene. These hormone response elements are often found in the 5' flanking region of the gene; therefore, I cloned and sequenced the 5' flanking region of the PIP gene and analyzed this region for AREs, EREs and RAREs.

The sequencing of the 5' flanking region of the PIP gene revealed a perfect consensus ERE [AGGTCAn_3TGACCT] 1.2 kb from the transcription start site as well as five sequences with some sequence similarity to AREs [Fig. 4] These five sequences all contain the ARE half-site TGTTCT. Interestingly, in a small region from -1198 to -1110 the consensus ERE and two ARE half-sites are located in proximity to each other. The consensus ERE and the more 5' ARE half-site are adjacent to each other and are separated from the more 3' ARE half-site by 49 base pairs. This area was of considerable interest as a region of interaction with nuclear hormone receptors.

The PIPSVCAT[-790 $\rightarrow$ +43] construct initially used in these studies is uninducible by androgen in T-47D and ZR-75-1 HBC cells [Fig. 9 and 10]. This construct contains two promoters, the PIP promoter and the SV40 early promoter. The double promoter was suggested to possibly interfere with the proper translation of the CAT protein (Cattini, pers. comm.) and this suggestion has been supported by the androgen inducibility of PIPCAT [-831 $\rightarrow$ +43] which contains the identical PIP 5' flanking sequence plus 41 base pairs but lacks the SV40 early promoter.

The PIPTKCAT constructs used in these studies were found to be inducible by androgen in T-47D and ZR-75-1 HBC cells; however, the base vector TKCAT was also observed to be inducible in these cells [Fig. 11]. In view of the results with the double promoter-containing PIPSVCAT construct, we assumed that any androgen induction I observed with the PIPTKCAT constructs was due to the TK promoter and that the PIP sequences 5' to the promoter were not functional. Studies using this vector were not pursued; however,

in a study by Schüle *et al* (1988), the pUC plasmid used to create their tkCAT expression vector was found to contain a functional transcription factor binding site which could confer glucocorticoid responsiveness onto the thymidine kinase promoter. In order to study the cooperativity of the GR and another transcription factor, the CACCC-box binding factor, Schüle *et al* (1988) deleted a fragment of the pUC vector from their tkCAT expression vector. On examination of the tkCAT expression vector I used to make the PIPTKCAT constructs, I discovered that this binding site is present. This binding site may be responsible for the androgen induction seen with PIPTKCAT constructs.

During the transient expression studies discussed below, I attempted to monitor transfection effeciencies by cotransfecting the HBC cells with a β-galactosidase expression vector and measuring β-galactosidase levels in the cell protein extracts. Unfortunately, retinoic acid treatment inhibits β-galactosidase expression in these cells [see Table 3]; therefore, I have not compared relative CAT inductions between constructs. I have used the CAT assay results only to identify hormone response within constructs.

The PIPCAT constructs containing only the PIP promoter were transiently expressed in ZR-75-1 HBC cells [Fig. 12 to 18]. I chose to use ZR-75-1 cells in all subsequent transient transfections because they gave a much more consistent PIPCAT and MMTVtkCAT expression. These transient expression studies determined that a small area [-1144 to -982] of the PIP 5' flanking region conferred responsiveness on the reporter gene to retinoic acid while a much larger area [-1500 to -251] conferred responsiveness to androgen. The retinoic acid responsive region was defined with two constructs: PIPCAT[-1144 → +43] and larger constructs were responsive to retinoic acid and PIPCAT[-982 → +43] and smaller constructs were not. The androgen responsive region spanned a much larger area and the constructs spanning this area were not uniform in their androgen inducibility. One construct, PIPCAT[-1144 → +43], did not exhibit any androgen inducibility although it was positively regulated by retinoic acid. Constructs smaller than PIPCAT[-1144 → +43] were positively regulated by androgen suggesting that the lack of androgen responsiveness of PIPCAT[-1144 → +43] might be of interest.

PIPCAT[-1144 → +43] contains only the 3' ARE half-site of the potentially hormone responsive region mentioned above. I hypothesized that perhaps both of the ARE half-sites were required for binding of AR and subsequent transactivation.

In most PIPCAT constructs, the data points for the retinoic acid induction are insufficient to perform statistical analysis. This problem is due to the unfortunate use of the β-galactosidase expression vector to standardize for transfection efficiency. The CAT assay values determined from extracts of cells transiently cotransfected with CH110 and retinoic acid treated were discarded because the amount of cell extract added to the acetylation reactions was not equal between the control cells and the retinoic acid treated cells.

The effect of dihydrotestosterone and retinoic acid is synergistic in induction of PIPCAT expression in ZR-75-1 cells [Fig. 15]. In T-47D cells, the time course of induction of the PIP mRNA is very different for the two hormones and no synergism is observed. This discrepancy may be the result of the difference between the hormonal induction of the endogenous gene versus the expression of the CAT protein driven by a hormone response element-containing fragment of DNA. In transient expression studies, the time course of induction is dependent on the transcription and stability of the CAT protein.

The increase in PIP gene expression observed after dexamethasone treatment has been reported to be through the glucocorticoid receptor, as the inductions by androgens and dexamethasone are additive (Simard *et al*, 1989). In the transient expression studies I performed with PIPCAT[-1204 → +43], I observed minimal inducibility of PIPCAT expression by dexamethasone alone (no statistical significance) and no additive effect with dihydrotestosterone, retinoic acid or estrogen. This minimal induction (1 to 2 fold) by dexamethasone is potentially due to a limiting concentration of glucocorticoid receptor relative to the concentration of androgen receptor. These results cannot determine the presence or absence of the glucocorticoid response element in the area of the PIP 5' flanking region [-1204 to +43] encompassed in my studies, which is responsible for the induction of PIP mRNA by dexamethasone observed by Simard and colleagues. This

question can be easily addressed by cotransfecting the glucocorticoid receptor with PIPCAT in the transient expression assays.

Thyroid hormone (T_3) alone, minimally (2 fold) induces PIPCAT expression and reduces the induction by retinoic acid [Fig. 15]. Presumably this phenomenon is a result of interaction between the thyroid hormone receptor and the retinoic acid receptor as has been shown for the TRE of the α-myosin heavy chain gene, in which the homodimer of TR is active and the heterodimer of TR and RAR is inactive (Glass *et al*, 1989).

The effect of estrogen and retinoic acid on the induction of PIPCAT[-1204 → +43] is unexpected. Estrogen, when added to transiently transfected ZR-75-1 cells, induces PIPCAT expression minimally. This effect does not appear to be concentration dependent, yet a consistent 2 fold induction is seen between estradiol concentrations of 10^{-7} to 10^{-11}M [Fig. 17]. In combination with retinoic acid, however, a synergistic increase of 7 fold induction is observed. These results are statistically significant. This induction is again not dependent on the concentration of estrogen used. This concentration independence suggests that the lowest concentration of estrogen used [10^{-11}M] is sufficient to modulate estrogen's effect on the stimulation of the PIP gene by retinoic acid. This concentration is low relative to physiological levels of steroid hormones. It will be necessary to repeat these experiments using estrogen concentrations less than 10^{-11}M. In T-47D HBC cells, estrogen inhibits the induction of the PIP mRNA by retinoic acid and has little effect by itself. Therefore the *in vivo* mRNA result directly contrasts the transient expression assay results. Additionally, the presence of a perfect consensus ERE would suggest transcriptional activation by ER instead of the inhibition I observe. Thus far, all consensus EREs described in the literature are stimulatory. To my knowledge, the PIP ERE sequence is potentially the first inhibitory consensus ERE. I attempted to explain these results by examining the specific binding of the ER and AR to the 5' flanking region of the PIP gene with gel mobility shift assays and DNase I footprinting assays.

Analysis of the 5' flanking region of the PIP gene for hormone responsive elements

In order to analyze the areas of the 5' flanking region of the PIP gene, I divided the 1500 base pairs of the region of interest into 7 restriction fragments of approximately equal length [see Fig. 19]. These fragments included probe A which contained the first ARE half-site, probe C which contained the consensus ERE and the second and third ARE half-sites and probe F which contained the fourth and fifth ARE half-sites. The probes D and E contained sequences between probes C and F which did not contain any consensus sequences to any nuclear hormone receptors or transcription factors. Probe G contained the PIP promoter sequences. Additionally, a fragment of probe C, containing the second ARE half-site but not the consensus ERE and the first ARE half-site, was created and designated probe C'.

- Androgen

Initially, probe C was the main focus of study. Using gel mobility shift assays, I determined that probe C was specifically bound by nuclear proteins from ZR-75-1 nuclear extracts [Fig. 20]. Using a purified AR peptide corresponding to the DNA binding domain made in *E. coli* (GST-AR2), I was able to show that AR bound specifically to probe C [Fig. 23] and at higher concentrations to probe F and interestingly to probe D which lacks potential ARE sequences [Fig. 24]. Equally surprising was the observation that the purified AR when used at this high concentration (with respect to the DNA probe) the AR peptide bound other DNAs also [Fig. 25] (*c-myc* promoter and vitellogenin A2 promoter). This result suggests the purified AR peptide at high concentration can nonspecifically interact with DNA. Nonetheless, at the 0.1 μg concentration used in these studies, the AR bound specifically to probe C and not to other PIP sequences or nonspecific sequences. Of the two ARE half-sites found in probe C, only the 3' half-site is included in probe C'. Since probe C' does not bind the purified AR protein, the 5' half-site is most likely the DNA element responsible for binding the AR.

In order to better define the sequence of the ARE in probe C, DNase I footprinting assays were performed. The androgen receptor DNA binding domain was found to specifically

protect a region between [-1198 to -1178] which includes part of the potential ARE defined by the 5' ARE half-site. Androgen receptor binds a site in probe C at low concentrations and sites within probe D and F at higher concentrations. The androgen induction of PIP mRNA may be a result of binding of the AR to a number of sites with varying affinities along the 5' flanking region of the PIP gene. Although the ARE in fragment C [-1198 to -1178] binds AR at low concentrations, the CAT assays would suggest that this ARE is not critical, as its deletion does not result in total loss of CAT activity. This suggestion can be confirmed by using the DNase I footprinting assay to test whether the AR DNA binding domain can bind to and protect areas of PIP probes A and F (which contain ARE half-sites) from DNase I digestion. Probes D and E which do not contain ARE half-site sequences should not be protected by the AR peptide.

The difference between the synergism observed between retinoic acid and androgen in the CAT assays and the induction of PIP mRNA in HBC cells may be a result of the difference between a transient expression system and the *in vivo* expression of genes. In CAT assays, steroid/retinoid hormones bind their nuclear receptors which in turn bind their cognate hormone response elements and activate transcription of a reporter gene. The reporter gene is translated into a stable protein which is assayed after 36 to 48 hours to allow time for the protein to accumulate. In tissue culture, we assay for the presence of a mRNA which responds to different hormones with different time courses of induction and different rates of degradation. Another factor which may explain the difference between the mRNA and CAT results may be the fact that the context of the hormone response elements is different between the transient expression system and the *in vivo* expressed gene. In CAT assays, the HRE is contained in a defined linear fragment of DNA while *in vivo* in HBC cells, the HREs are part of a large regulatory region existing in a natural chromatin organization. This structural difference between the *in vitro* and *in vivo* HRE may account for the spatial and temporal variations between the expression of PIP mRNA and PIP regulated CAT.

- Estrogen

The 5' flanking region of the PIP gene contains a perfect consensus ERE base pairs -1198 to -1184. Using an ER translated in a rabbit reticulocyte lysate, I was able to show that ER bound specifically to probe C and not to probe A, C', D, E or F. It is important to note that probe C' is identical to the 3' portion of probe C but lacks the consensus ERE and the 5' ARE half-site. Therefore, it would appear that the consensus ERE is the DNA element which binds the ER. To confirm this conclusion, DNase I footprinting assays were performed. Estrogen receptor footprints from [-1205 to -1169] encompassing the perfect consensus ERE. The binding of ER to this consensus ERE suggests that this element may be responsible for the inhibition of androgen-induced PIP expression. A unique characteristic of the PIP consensus ERE that distinguishes it from consensus EREs of other genes is that the PIP ERE might confer transcriptional inhibition and not transcriptional activation. To the best of our knowledge the PIP ERE may be the first example of an inhibitory ERE and the observation that the footprint spans 37 basepairs (much longer then the expected 15 basepairs) suggests that sequences bordering the consensus ERE may be important in defining its inhibitory function. It is necessary to determine the role of the PIP consensus ERE in estrogen inhibition of the PIP gene. By mutating the sequences of the consensus ERE and the adjacent ARE (within the PIPCAT constructs) and transiently expressing the constructs in ZR-75-1 cells, we can determine the function of these sequences in hormone activation and repression of PIP gene expression. The synergism of estradiol with retinoic acid in CAT assays is observed using the construct PIPCAT [-1204 $\rightarrow$ +43] which lacks one base pair of the footprint protected by the translated ER. If the lack of this base pair disrupts correct binding of the ER to its element, estradiol may transactivate instead of inhibit CAT expression. Indeed, I observe a slight induction by estradiol in PIPCAT[-1204 $\rightarrow$ +43].

- Androgen and estrogen

To determine if the androgen receptor and estrogen receptor binding sites could be identified, DNase I footprinting assays were performed. ZR-75-1 nuclear extract as well as the translated ER and purified AR DNA binding domain were used to protect probe

C from digestion with DNase I. The translated ER bound to and protected a fragment from -1205 to -1169 including the consensus ERE. The purified AR bound to and protected a slightly smaller fragment from -1198 to -1178 including the consensus ERE and part of the adjacent ARE half-site. These binding sites overlap. I have shown that estrogen can inhibit the action of androgen in increasing PIP gene expression in human breast cancer cells. I propose that estrogen inhibits PIP gene expression by androgen through an interference with the binding of the androgen receptor to the androgen response element in the 5' flanking region of the gene. To support this hypothesis, gel mobility shift assays were performed on probe C in which differing amounts of translated ER were added to a standard amount of purified AR. These experiments showed that as more ER is present, less AR binding is seen. I suggest that the estrogen receptor binds very strongly to the consensus ERE and can compete with binding of the androgen receptor to the adjacent overlapping ARE.

- Retinoic acid

In the regulation of transcription of the PIP gene by hormones, a complex interaction between the different hormone receptors seems to be responsible for the pattern of induction observed in human breast cancer cells. I suggest, based on the CAT assay results, that the retinoic acid induction of PIP mRNA is a result of RAR binding to a single site or sites within the region [-1144 to -982].

To examine the role of retinoic acid in induction of PIP gene expression, I have obtained mouse ascites antibodies raised to the F domains of the three isoforms of human RAR. These antibodies have been used by other investigators in gel mobility shift assays where they have observed the antibodies to supershift specific protein-DNA complexes (Rochette-Egly *et al*, 1991). I have used these antibodies in gel mobility shift experiments where the antibodies were used to supershift specific protein-DNA complexes containing RAR. According to a recent study of human breast cancer cell lines (Roman *et al*, 1992), RARα is present at high levels predominantly in ER+ cell lines, RARβ is present in predominantly ER- cell lines and RARγ is present at lower levels in all cell

lines tested. Unfortunately, ZR-75-1 HBC cells were not included in the panel of cells tested. However, since ZR-75-1 HBC cells are ER+ and PIP has been correlated with ER+ HBC cell lines, I would expect that the RAR isoform which is responsible for the responsiveness of PIP to retinoic acid would be either RARα or possibly RARγ. Recent studies have shown that retinoic acid receptor can negatively regulate the induction of genes by competing with the estrogen receptor for binding sites (Demirpence *et al*, 1992). The possibility that in the PIP gene the RAR binds to and transactivates through the ERE is unlikely, as the PIPCAT construct in which the ERE is deleted is still responsive to retinoic acid.

The PIPCAT construct [-1144 → +43] is not inducible by androgens although it is inducible by retinoic acid. This construct does not contain the consensus ERE and the adjacent 5' ARE half-site of probe C although it did contain the 3' ARE half-site [-1125 to -1111] as well as the two ARE half-sites closer to the PIP promoter. When the 5' end of this construct is deleted to create PIPCAT[-982 → +43], the androgen inducibility returns and the retinoic acid inducibility is lost. These results suggest that the region [-1144 to -982] contains a RARE and a sequence which results in inhibition of androgen inducibility. When ZR-75-1 nuclear extracts were used to protect probe C (which contained the 5' boundary of this construct) a protected region was seen between -1150 to -1131 which did not appear to correspond with the pattern of bands observed with the purified AR or the translated ER. This protected area was not seen with GH_4C_1 or HeLa nuclear extracts. If this factor is necessary for AR to bind to the 3' ARE half-site in probe C, then the disruption of this site may result in loss of inducibility. Since PIPCAT[-1144 → +43] is inducible by retinoic acid, this putative factor binding interaction may be AR specific. I suggest that a nuclear protein from ZR-75-1 HBC cells, which is neither AR nor ER, binds to this site which is adjacent to the downstream 5' ARE half-site [-1125 → -1111] and can potentially interact with the androgen receptor binding to this site. This hypothesis could be tested by combining purified androgen receptor and ZR-75-1 nuclear extract together in a gel mobility shift assay to test whether androgen receptor binding is affected. This potential interaction may be important in view of the

complete loss of androgen inducibility in this construct.

To summarize, androgen receptor binds to an ARE sequence at base pairs [-1198 → -1178] in the 5' flanking region of the gene. Estrogen receptor binds to an overlapping site at base pairs [-1205 → -1169] and may prevent the AR from binding to its response element. To my knowledge, this observation may be the first example of estrogen inhibition of androgen stimulation of a gene by direct competition of their receptors. Retinoic acid receptor activates PIP expression through a region between [-1144 → -982]; the specific sequence has yet to be identified. I propose that the estrogen receptor may bind to the region of the consensus ERE and prevent both androgen receptor and retinoic acid receptor from binding to their cognate response elements.

FUTURE STUDIES

The PIP gene is clearly retinoic acid responsive in HBC cells; however, I have been thus far unsuccessful at defining the mechanism by which this responsiveness occurs. I have concluded from the transient expression studies that a region encompassing bp -1144 to -982 can confer retinoic acid responsiveness to the reporter gene CAT. This region however, contains no consensus sequences corresponding to RAREs or to any other characterized DNA response elements. I will use the RAR antibodies to preabsorb RAR from ZR-75-1 nuclear extracts and use the DNase I footprinting assay to determine if specific sequences are no longer protected in the absence of RAR. I will also translate the three RARs using the same expression vector which was used to make translated ER. This protein will be used to perform gel mobility shift assays to determine if the RAR protein can bind to and retard any of the PIP probes, especially probe C' which contains the retinoic acid responsive sequence defined by the transient expression studies. If the RAR binds to and transactivates the PIP gene in a conventional manner, these experiments should identify a DNA element which confers retinoic acid responsiveness. I suggest that this sequence will be unconventional, as sequence analysis does not reveal any similarities to previously published RARE consensus sequences within the 5' flanking region of the PIP gene. If I cannot define a RARE, other mechanisms of retinoic acid transactivation must be considered. Could RAR be interacting with an existing nuclear factor? Gel mobility shift assays with a number of different nuclear extracts suggest that the 5' flanking PIP probes bind a number of different nuclear proteins. RAR could be either stabilizing an accessory factor or binding to an inhibitory factor, preventing it from inhibiting PIP transactivation.

An androgen receptor binding site in probe C has been defined using the gel mobility shift and DNase I footprinting assays; however, other AREs must exist in the 5' flanking region of the PIP gene as the removal of the probe C ARE half-site does not abolish androgen responsiveness in the smaller PIPCAT constructs. Since the AR DNA binding domain binds to the other PIP probes at high concentrations, I can use the DNase I

footprinting assay to characterize other potential AREs which perhaps have a weaker affinity for the AR. The DNase I footprinting assay will also be used to better characterize the binding of ER and AR to both strands of probe C.

Although I have demonstrated the binding of the AR DNA binding domain peptide and the translated ER to the 5' flanking region of the PIP gene, I have yet to show that these elements are functional and act as hormone responsive elements. To clearly define the role of the AR and ER binding sites I have identified in the 5' flanking region of the PIP gene, I will perform site-directed mutagenesis within the consensus ERE and the adjacent ARE. These studies will determine if these sequences are functional hormone response elements which are responsible for the androgen induction and the estrogen inhibition of the PIP mRNA. The method I will use to produce mutated PIPCAT constructs is described by Higuchi *et al* (1988) and Vallette *et al* (1989). Using as a template the PIPCAT[-1204→+43] which contains the consensus ERE sequence and five ARE half-sites and is inducible by DHT and retinoic acid, I will prepare mutant primers to the consensus ERE sequence or the adjacent ARE half-site and use polymerase chain reaction (PCR) to generate mutated sequences within the ERE/ARE region. Primers made to both strands of the ERE or ARE half-site sequences will contain specific mismatches disrupting the wild type sequence. These primers and primers located on either side of the PIP 5' flanking region insert of PIPCAT[-1204→+43] will be used to incorporate the mutated primer sequence into PIPCAT[-1204→+43] by PCR. This mutant construct can be assayed for a change in hormone responsive function by transiently expressing it in ZR-75-1 cells and treating the cells with DHT, estradiol or retinoic acid.

In summary, I have defined a multi-steroid regulated region of the PIP gene which contains a binding site for AR and ER and potentially, RAR. If I can demonstrate that these DNA elements are functional, this study will be the first example of estrogen inhibition of androgen stimulation of a gene by direct competition for their receptor binding sites. The human PIP/GCDFP-15 gene provides an excellent model with which to study the interaction of nuclear hormone receptors on transcriptional regulation.

LITERATURE CITED

Abel, T. and Maniatis, T. (1989). Gene regulation: action of leucine zippers. *Nature* **341**:24-25.

Adler, S., Waterman, M.L., He, X. and Rosenfeld, M.G. (1988). Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain. *Cell* **52**:685-695.

Adler, A.J., Scheller, A., Hoffman, Y. and Robins, D.M. (1991). Multiple components of a complex androgen-dependent enhancer. *Mol. Endocrinol.* **5**:1587-1596.

Akiyama, K. and Kimura, H. (1990). Isolation of a new actin-binding protein from human seminal plasma. *Biochem. Biophys. Acta* **1040**:206-210.

Alcalay, M., Zangrilli, D., Fagioli, M., Pandolfi, P.P., Mencarelli, A., Lo Coco, F., Biondi, A., Grignani, F. and Pelicci, P.G. (1992). Expression pattern of the *RARα-PML* fusion gene in acute promyelocytic leukaemia. *Proc. Natl. Acad. Sci. USA* **89**:4840-4844.

Alexander, I.E., Clarke, C.L., Shine, J. and Sutherland, R.L. (1989). Progestin inhibition of progesterone receptor gene expression in human breast cancer cells. *Mol. Endocrinol.* **3**:1377-1386.

Ali, S., Pellegrini, I. and Kelly, P.A. (1991). A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. *J. Biol. Chem.* **266**:20110-20117.

Ali, S., Edery, M., Pellegrini, I., Lesueur, L., Paly, J., Djiane, J. and Kelly, P.A. (1992). The Nb2 form of prolactin receptor is able to activate a milk protein gene promoter. *Mol. Endocrinol.* **6**:1242-1248.

Allan, G.F., Leng, X., Tsai, S.Y., Weigel, N.L., Edwards, D.P., Tsai, M-J. and O'Malley, B.W. (1992). Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. *J. Biol. Chem.* **267**:19513-19520.

Allegra, J.C., Lippman, M.E., Thompson, E.B., Simon, R., Barlock, A., Green, L., Huff, K.K., Do, H.M.T. and Aitken, S.C. (1979). Distribution, frequency and quantitative analysis of estrogen, progesterone, androgen and glucocorticoid receptors in human breast cancer. *Cancer Res.* **39**:1447-1454.

Andres, A.C., Schonenberger, C.A., Groner, B., Hennighausen, L., LeMeur, M. and Gerlinger, P. (1987). Ha-*ras* oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation and tumor induction in transgenic mice. *Proc. Natl. Acad. Sci. USA* **84**:1299-1303.

Andrews, P.E., Young, C.Y-F., Montgomery, B.T. and Tindall, D.J. (1992). Tumor-promoting phorbol ester down-regulates the androgen induction of prostate-specific antigen in a human prostatic adenocarcinoma cell line. *Cancer Res.* **52**:1525-1529.

Ankenbauer, W., Strähle, U. and Schutz, G. (1988). Synergistic action of glucocorticoid and estradiol responsive elements. *Proc. Natl. Acad. Sci. USA* **85**:7526-7530.

Apfel, C., Bauer, F., Crettaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W. and Klaus, M. (1992). A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. *Proc. Natl. Acad. Sci. USA* **89**:7129-7133.

Armstrong, O.T., Knudsen, K.A. and Miller, L.S. (1970). Effects of prolactin upon cholesterol metabolism and progesterone biosynthesis in corpora lutea of rats hypophysectomized during pseudopregnancy. *Endocrinology* **86**:634-641.

Asselin, J., Melançon, R., Moachon, G. and Bélanger, A. (1980). Characteristics of binding to estrogen, androgen, progestin and glucocorticoid receptors in 7,12-dimethybenz(a)anthracene-induced mammary tumors and their hormonal control. *Cancer Res.* **40**:1612-1622.

Bagchi, M.K., Tsai, M-J., O'Malley, B.W. and Tsai, S.Y. (1992). Analysis of the mechanism of steroid hormone receptor-dependent gene activation in cell-free systems. *Endocrine Rev.* **13**:525-535.

Balkan, W., Colbert, M., Bock, C. and Linney, E. (1992). Transgenic indicator mice for studying activated retinoic acid receptors during development. *Proc. Natl. Acad. Sci. USA* **89**:3347-3351.

Barinaga, M. (1991). Dimers direct development. *Science* **251**:1176-1177.

Basheerudin, K., Stein, P., Strickland, S. and Williams, D.L. (1987). Expression of the murine apolipoprotein E gene is coupled to the differentiated state of F9 embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA* **84**:898-907.

Baxter, R.C., Zaltsman, Z. and Turtle, J.R. (1984). Rat growth hormone (GH) but not prolactin (PRL) induces both GH and PRL receptors in female rat liver. *Endocrinology* **114**:1893-1901.

Bazan, J.F. (1990). Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* **87**:6934-6938.

Beatson, G.T. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* **2**:104-107, 162-167.

Bedo, G., Santisteban, P. and Aranda, A. (1989). Retinoic acid regulates growth hormone gene expression. *Nature* **339**:231-233.

Beebe, J.S., Darling, D.S. and Chin, W.W. (1991). 3,5,3'-triiodothyronine receptor auxiliary protein (TRAP) enhances receptor binding by interactions within the thyroid hormone response element. *Mol. Endocrinol.* **5**:85-93.

Benbrook, D., Lernhardt, E. and Pfahl, M. (1988). A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature* **333**:669-672.

Ben-Jonathan, N., Arbogast, L.A. and Hyde, J.F. (1989). Neuroendocrine regulation of prolactin release. *Prog. Neurobiol.* **33**:399-447.

Berger, F.G. and Watson, G. (1989). Androgen-regulated gene expression. *Annu. Rev. Physiol.* **51**:51-65.

Berkenstam, A., del Mar Vivanco Ruiz, M., Barettino, D., Horikoshi, M. and Stunnenberg, H.G. (1992). Cooperativity in transactivation between retinoic acid receptor and TFIID requires an activity analogous to E1A. *Cell* **69**:401-412.

Berrodin, T.J., Marks, M.S., Ozato, K., Linney, E. and Lazar, M.A. (1992). Heterodimerization among thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, chicken ovalbumin upstream promoter transcription factor and an endogenous liver protein. *Mol. Endocrinol.* **6**:1468-1478.

Berry, M., Metzger, D. and Chambon, P. (1990). Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonist activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO J.* **9**:2811-2818.

Bhattacharjee, M. and Vonderhaar, B.K. (1984). Thyroid hormones enhance the synthesis and secretion of α-lactalbumin by mouse mammary tissue *in vitro*. *Endocrinology* **155**:1070-1077.

Biondi, A., Rambaldi, A., Alcalay, M., Pandolfi, P.P., LoCoco, F., Diverio, D., Rossi, V., Mencarelli, A., Longo, L., Zangrilli, D., Masera, G., Barbui, T., Mandelli, F., Grignani, F. and Pelicci, P.G. (1991). RARα gene rearrangements as a genetic marker for diagnosis and monitoring in acute promyelocytic leukemia. *Blood* **77**:1418-1422.

Blomhoff, R., Green, M.H., Berg, T. and Norum, K.R. (1990). Transport and storage of vitamin A. *Science* **250**:399-404.

Blumberg, B., Mangelsdorf, D.J., Dyck, J.A., Bittner, D.A., Evans, R.M. and De Roberts, E.M. (1992). Multiple retinoid-responsive receptors in a single cell: families of retinoid X receptors and retinoic acid receptors in the *Xenopus* egg. *Proc. Natl. Acad. Sci. USA* **89**:2321-2325.

Blomhoff, R., Green, M.H., Berg, T. and Norum, K.R. (1990). Transport and storage of vitamin A. *Science* **250**:399-404.

Bonadonna, G. (1992). Evolving concepts in the systemic adjuvant treatment of breast cancer. *Cancer Res.* **52**:2127-2137.

Boncinelli, E., Simeone, A., Acampora, D. and Mavilio, F. (1991). *Hox* gene activation by retinoic acid. *Trends Genet.* 7:329-334.

Boutin, J-M., Jolicoeur, C., Okamura, H., Gagnon, J., Edery, M., Shirota, M., Banville, D., Dusanter-Fourt, I., Djiane, J. and Kelly, P.A. (1988). Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. *Cell* **53**:69-77.

Boutin, J-M., Edery, M., Shirota, M., Jolicoeur, C., Lesueur, L., Ali, S., Gould, D., Djiane, J. and Kelly, P.A. (1989). Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. *Mol. Endocrinol.* **3**:1455-1461.

Boylan, J.F. and Gudas, L.J. (1991). Overexpression of the cellular retinoic acid binding protein-I (CRABP-I) results in a reduction in differentiation-specific gene expression in F9 teratocarcinoma cells. *J. Cell Biol.* **112**:965-979.

Boylan, J.F. and Gudas, L.J. (1992). The level of CRABP-I expression influences the amounts and types of all-*trans*-retinoic acid metabolites in F9 teratocarcinoma stem cells. *J. Biol. Ch*em. **267**:21486-21491.

Brand, N., Petkovich, M., Krust, A., Chambon, P., De Thé, H., Marchio, A., Tiollais, P. and Dejean, A. (1988). Identification of a second human retinoic acid receptor. *Nature* **332**:850-853.

Brand, N., Petkovich, M. and Chambon, P. (1990). Characterization of a functional promoter for the human retinoic acid receptor α (hRAR-α). *Nucleic Acids Res.* **18**:6799-6806.

Braunstein, G.D. (1986). The testes. In: *Basic and clinical endocrinology, 2nd ed.* (Greenspan, F.S. and Forsham, P.H., eds.), pp. 353-355, Appleton-Century-Crofts, East Norwalk, Connecticut.

Breitman, T.R., Collins, S.J. and Keene, B.R. (1981). Terminal differentiation of promyelocytic leukaemic cells in response to retinoic acid. *Blood* **57**:1000-1004.

Brent, R. and Ptashne, M. (1985). A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* **43**:729-736.

Brew, K. and Hill, R.L. (1975). Lactose biosynthesis. *Rev. Physiol. Biochem. Pharmacol.* **72**:105-157.

Brown, C.J., Goss, S.J., Lubahn, D.B., Joseph, D.R., Wilson, E.M., French, F.S. and Willard, H.F. (1989). Androgen receptor locus on the human X-chromosome: regional localization to Xq11-12 and description of a DNA polymorphism. *Am. J. Hum. Genet.* **44**:264-269.

Brüggemeier, U., Rogge, L., Winnacker, E-L. and Beato, M. (1990). Nuclear factor I acts as a transcription factor of the MMTV promoter but competes with steroid hormone receptors for DNA binding. *EMBO J.* **9**:2233-2239.

Brüggemeier, U., Kalff, M., Franke, S., Scheidereit, C. and Beato, M. (1991). Ubiquitous transcription factor OTF-1 mediates induction of the MMTV promoter through synergistic interaction with hormone receptors. *Cell* **64**:565-572.

Buckley, A.R., Montgomery, D.W., Kipler, R., Putnam, C.W., Zukoski, C.F., Gout, P.W., Beer, C.T. and Russell, D.M. (1986). Prolactin stimulation of ornithine decarboxylase and mitogenesis in Nb2 node lymphoma cells: the role of protein kinase C and calcium mobilization. *Immunopharmacology* **12**:37-51.

Buckley, A.R., Crowe, P.D. and Russell, D.M. (1988). Rapid activation of protein kinase C in isolated rat liver nuclei by prolactin, a known mitogen. *Proc. Natl. Acad. Sci. USA* **85**:8649-8653.

Buetti, E. and Diggelmann, H. (1983). Glucocorticoid regulation of mouse mammary tumor virus: identification of a short essential DNA region. *EMBO J.* **2**:1423-1429.

Buratowski, S., Hahn, S., Guarente, L. and Sharp, P.A. (1989). Five intermediate complexes in transcription initation by RNA polymerase II. *Cell* **56**:549-561.

Burdon, T., Sankaran, L., Wall, R.J., Spencer H, and Hennighausen, L. (1991). Expression of a whey acidic protein transgene during mammary development. *J. Biol. Chem.* **266**:6909-6914.

Burridge, K., Nuckolls, G., Otey, C., Pavalko, F., Simon, K. and Turner, C. (1990). Actin-membrane interaction in focal adhesions. *Cell Differ. Dev.* **32**:337-342.

Buttyan, R., Olsson, C.A., Pintar, J., Chang, C., Bandyk, M., Ng, P. and Sawczuk, I. (1989). Induction of the TRPM-2 gene in cells undergoing programmed death. *Mol. Cell Biol.* **9**:3473-3481.

Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L.J., Grippo, J.F. and Hunziker, W. (1993). Two nuclear signalling pathways for vitamin D. *Nature* **361**:657-660.

Carlin, B.E., Durkin, M.E., Bender, B., Jaffe, R. and Chung, A.E. (1983). Synthesis of laminin and entactin by F9 cells induced with retinoic acid and dibutyryl cyclic AMP. *J. Biol. Chem.* **258**:7729-7737.

Carlstedt-Duke, J., Strömstedt, P-E., Wrange, O., Bergman, T., Gustafsson, J-A. and Jornvall, H. (1987). Domain structure of the glucocorticoid receptor protein. *Proc. Natl. Acad. Sci. USA* **84**:4437-4440.

Carr, D. and Friesen, H.G. (1976). Growth hormone and insulin binding to human liver. *J. Clin. Endocrinol. Metab.* **42**:484-493.

Carson-Jurica, M.A., Schrader, W.T. and O'Malley, B.W. (1990). Steroid receptor family: structure and functions. *Endo. Rev.* **11**:201-220.

Castaigne, S., Chomienne, C., Daniel, M.T., Ballerini, P., Berger, R., Fenaux, P. and Degos, L. (1990). All *trans*-retinoic acid as a differentiation therapy for acute promyelocytic leukaemia: I. Clinical results. *Blood* **76**:1704-1709.

Catelli, M-G., Binart, N., Jung-Testas, I., Renoir, J.M., Baulieu, E-E., Feramisco, J.R. and Welch, W.J. (1985). The common 90 kDa protein component of non-transformed 8S steroid receptors is a heat shock protein. *EMBO J.* **4**:3131-3135.

Cato, A.C., Heitlinger, E., Ponta, H., Klein-Hitpass, L., Ryffel, G.U., Bailly, A., Rauch, C. and Milgrom, E. (1988). Estrogen and progesterone receptor-binding sites on the chicken vitellogenin II gene: synergism of steroid hormone action. *Mol. Cell Biol.* **8**:5323-5330.

Chalbos, D., Haagensen, D.E., Parish, T. and Rochefort, H. (1987). Identification and androgen regulation of two proteins released by T-47D human breast cancer cells. *Cancer Res.* **47**:2787-2792.

Chalepakis, G., Arnemann, J., Slater, E., Brüller, H-J., Gross, B. and Beato, M. (1988). Differential gene activation by glucocorticoids and progestins through the hormone regulatory element of mouse mammary tumor virus. *Cell* **53**:371-382.

Chandler, V.L., Maler, B.A. and Yamamoto, K.R. (1983). DNA sequences bound specifically by glucocorticoid receptor *in vitro* render a heterologous promoter hormone responsive *in vivo*. *Cell* **33**:489-499.

Chang, C., Kokontis, J. and Liao, S. (1988). Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science* **240**:324-326.

Chapdelaine, D., Paradis, G., Tremblay, R.R. and Dube, J.Y. (1988). High level of expression in the prostate of a human expression in the prostate of a human glandular kallikrein mRNA related to prostate-specific antigen. *FEBS Lett.* **236**:205-208.

Charest, N.J., Zhou, Z-X., Lubahn, D.B., Olsen, K.L., Wilson, E.M. and French, F.S. (1991). A frameshift mutation destabilizes androgen receptor messenger RNA in the *Tfm* mouse. *Mol. Endocrinol.* **5**:573-581.

Chirgwin, J.M.,Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.

Chomczynski, P., Qasba, P. and Topper, Y.J. (1986). Transcriptional and post-transcriptional roles of glucocorticoids in the expression of the rat 25,000 molecular weight casein gene. *Biochem. Biophys. Res. Commun.* **134**:812-818.

Chowdhury, K. (1991). One step "miniprep" method for the isolation of plasmid DNA. *Nucleic Acids Res.* **19**:2792.

Chytil, F. and Ong, D.E. (1987). Intracellular vitamin A-binding proteins. *Annu. Rev. Nutrition* 7:321-325.

Clarke, C.L., Roman, S.D., Graham, J., Koga, M. and Sutherland, R.L. (1990). Progesterone receptor regulation by retinoic acid in the human breast cancer cell line T-47D. *J. Biol. Chem.* **265**:12694-12700.

Clarke, C.L., Graham, J., Roman, S.D. and Sutherland, R.L. (1991). Direct transcriptional regulation of the progesterone receptor by retinoic acid diminishes progestin responsiveness in the breast cancer cell line T-47D. *J. Biol. Chem.* **266**:18969-18975.

Cohlan, S.Q. (1953). Excessive intake of vitamin A as a cause of congenital anomalies in the rat. *Science* **117**:535-536.

Cole, M.P., Jones, C.T.A. and Todd, I.D.H. (1971). A new antioestrogenic agent in late breast cancer: an early clinical appraisal of ICI 46,474. *Br. J. Cancer* **25**:270-275.

Collette, J., Hendrick, J-C., Jaspar, J-M. and Franchimont, P. (1986). Presence of α-lactalbumin, epidermal growth factor, epithelial membrane antigen and groww cystic disease fluid protein (15,000 daltons) in breast cyst fluid. *Cancer Res.* **46**:3728-3733.

Conneely, O.M., Dobson, A.D.W., Tsai, M-J., Beattie, W.G., Toft, D.O., Huckaby, C.S., Zarucki, T., Schrader, W.T. and O'Malley, B.W. (1987). Sequence and expression of a functional chicken progesterone receptor. *Mol. Endocrinol.* **1**:517-525.

Cooke, N.E., Szpirer, C. and Levan, G. (1986). The related genes encoding growth hormone and prolactin have been dispersed to chromosome 10 and 17 in the rat. *Endocrinology* **119**:2451-2454.

Cooke, P.S., Young, P. and Cunha, G.R. (1991). Androgen receptor expression in developing male reproductive organs. *Endocrinology* **128**:2867-2873.

Cosman, D., Lyman, S.D., Idzerda, R.L., Beckmann, M.P., Park, L.S., Goodwin, R.G. and March, C.J. (1990). A new cytokine receptor superfamily. *Trends Biosci.* **15**:265-270.

Courey, A. and Tjian, R. (1988). Analysis of Sp1 *in vitro* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* **55**:887-898.

Croce, C.M., Linnenbach, A., Huebner, K., Parnes, J.R., Margulies, D.H., Appella, E. and Seidman, J.G. (1981). Control of expression of histocompatibility antigens (H-2) and beta 2-microglobulin in F9 teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**:5754-5758.

Crow, J.A. and Ong, D.E. (1985). Cell-specific immunohistochemical localization of a cellular retinol-binding protein (type two) in the small intestine of the rat. *Proc. Natl. Acad. Sci. USA* **82**:4707-4747.

Crozat, A., Palvimo, J.J., Julkunen, M. and Jänne, O.A. (1992). Comparison of androgen regulation of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase gene expression in rodent kidney and accessory sex organs. *Endocrinology* **130**:1131-1144.

Cunha, G.R. and Young, P. (1991). Inability of *Tfm* (testicular feminization) epithelial cells to express androgen-dependent seminal vesicle secretory proteins in chimeric tissue recombinants. *Endocrinology* **128**:3293-3298.

Cunningham, B.C., Bass, S., Fuh, G. and Wells, J.A. (1990). Zinc mediation of the binding of human growth hormone to the human prolactin receptor. *Science* **250**:1709-1712.

Danielson, M., Hinck, L. and Ringold, G.M. (1989). Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* **57**:1131-1138.

Dang, C.V., McGuire, M., Buckmire, M. and Lee, W.M.F. (1989). Involvement of the "leucine zipper" region in oligomerization and transforming activity of human *c-myc* protein. *Nature* **337**:644-666.

Dao,T.L. and Huggins, C. (1955). Bilateral adrenalectomy in the treatment of cancer of the breast. *A. M. A. Arch. Surg.* **71**:645-657.

Darling, D.S., Beebe, J.S., Burnside, J., Winslow, E.R. and Chin, W.W. (1991). 3,5,3'-triiodothyronine (T_3) receptor-auxiliary protein (TRAP) binds DNA and forms heterodimers with the T_3 receptor. *Mol. Endocrinol.* **5**:73-84.

Dauvois, S., Li, S., Martel, C. and Labrie, F. (1989). Inhibitory effect of androgens on the growth of dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma in the rat. *Breast Cancer Res. Treat.* **14**:299-306.

Dauvois, S., Simard, J., Dumont, M., Haagensen, D.E. and Labrie, F. (1990). Opposite effects of estrogen and the progestin R5020 on cell proliferation and GCDFP-15 expression in ZR-75-1 human breast cancer cells. *Mol. Cell. Endocrinol.* **73**:171-178.

Dauvois, S., Geng, C-S., Lévesque, C., Mérand, Y. and Labrie, F. (1991). Additive inhibitory effects of an androgen and the antiestrogen EM-170 on estradiol-stimulated growth of human ZR-75-71 breast tumors in athymic mice. *Cancer Res.* **51**:3131-3135.

Davis, J.H. and Linzer, D.I.H. (1989). Expression of multiple forms of prolactin receptor in mouse liver. *Mol. Endocrinol.* **3**:674-680.

Day, R.N., Koike, S., Sakai, M., Muramatsu, M. and Maurer, R. (1990). Both Pit-1 and the estrogen receptor are required for the estrogen responsiveness of the rat prolactin gene. *Mol. Endocrinol.* **4**:1964-1971.

Dejean, A., Bougueleret, L., Grzeschik, K.H. and Tiollais, P. (1986). Hepatitis B virus DNA integration in a sequence homologous to *v-erb-A* and steroid receptor genes in a hepatocellular carcinoma. *Nature* **322**:70-72.

de Launoit, Y., Dauvois, S., Dufour, M., Simard, J. and Labrie, F. (1991). Inhibition of cell cycle kinetics and proliferation by the androgen 5α-dihydrotestosterone and antiestrogen N,n-butyl-N-methyl-11-[16'α-chloro-3',17β-dihydroxy-estra-1',3',5'-(10')triene-7'α-yl] undecanamide in human breast cancer ZR-75-1. *Cancer Res.* **51**:2797-2802.

de Luca, L.M. (1978). Vitamin A. In: *Fat soluble vitamins.* (De Luca, H.F., ed.), pp. 1-67, Plenum, New York.

de Luca, L.M. (1991). Retinoids and their receptors in differentiation, embryogenesis and neoplasia. *FASEB J.* **5**:2924-2933.

Demirpence, E., Pons, M., Balaguer, P. and Gagne, D. (1992). Study of an antiestrogenic effect of retinoic acid in MCF-7 cells. *Biochem. Biophys. Res. Comm.* **183**:100-106.

Denis, M., Gustafsson, J-A. and Wikstrom, A.C. (1988). Interaction of the Mr = 90,000 heat shock protein with the steroid-binding domain of the glucocorticoid receptor. *J. Biol. Chem.* **263**:18520-18523.

Deslypere, J.P., Young, M., Wilson, J.D. and McPhaul, M.C. (1992). Testosterone and 5α-dihydrotestosterone interact differently with the androgen receptor to enhance transcription of the MMTV-CAT reporter gene. In: The 74th annual meeting of the Endocrine Society, #300, San Antonio, Texas.

de Thé, H., Marchio, A., Tiollais, P. and Dejean, A. (1987). A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature* **330**:667-670.

de Thé, H., Marchio, A., Tiollais, P. and Dejean, A. (1989). Differential expression and ligand regulation of the retinoic acid receptor α and β genes. *EMBO J.* **8**:429-433.

de Thé, H., del Mar Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H. and Dejean, A. (1990). Identification of a retinoic acid response element in the retinoic acid receptor β gene. *Nature* **343**:177-180.

de Thé, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. and Dejean, A. (1991). The *PML-RARα* fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukaemia encodes a functionally altered RAR. *Cell* **66**:675-684.

de Thé, H. and Dejean, A. (1992). Retinoic acid receptor α in acute promyelocytic leukaemia. In: *Growth regulation by nuclear hormone receptors (Vol. 14).* (Parker, M.G., ed.), pp. 195-203, Cold Spring Harbor laboratory press, New York.

de Verneuil, H. and Metzger, D. (1990). The lack of transcriptional activation of the *v-erbA* oncogene is in part due to a mutation present in the DNA binding domain of the protein. *Nucleic Acids Res.* **18**:4489-4497.

de Vos, P., Claessens, F., Winderickx, J., Van Dijck, P., Celis, L., Peeters, B., Rombauts, W., Heyns, W. and Verhoeven, G. (1991). Interaction of androgen response elements with the DNA-binding domain of the rat androgen receptor expressed in *Escherichia coli*. *J. Biol. Chem.* **266**:3439-3443.

Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluable extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475-1489.

Dilley, W.G., Leight, G.S., Silva, J.S., Ammirata, S., Haagensen, D.E. and Wells, S.A. (1983). Androgen stimulation of gross cystic disease fluid protein and carcinoembryonic antigen in patients with metastatic breast carcinoma. *J. Natl. Cancer Inst.* **70**:69-74.

Dilley, W.G., Haagensen, D.E., Leight, G.S., Ammirata, S., Davis, S.R., Silva, J.S., Zamcheck, N., Lokich, J.J. and Wells, S.A. (1986). Fluoxymesterone stimulation of tumor marker secretion in patients with breast carcinoma. *Breast Cancer Res. Treat.* **8**:205-215.

DiMattia, G.E., Gellersen, B., Bohnet, H.G. and Friesen, H.G. (1988). A human B-lymphoblastoid cell line produces prolactin. *Endocrinology* **122**:2508-2517.

DiMattia, G.E., Gellersen, B., Duckworth, M.L. and Friesen, H.G. (1990). Human prolactin gene expression: the use of an alternative non-coding exon in decidua and the IM-9-P3 lymphoblast cell line. *J. Biol. Chem.* **265**:16412-16421.

Djiane, J., Durand, P. and Kelly, P.A. (1977). Evolution of prolactin receptors in rabbit mammary gland during pregnancy and lactation. *Endocrinology* **100**:1348-1356.

Dodd, J.C., Sheppard, P.C. and Matusik, R.J. (1983). Characterization and cloning of highly abundant mRNAs of the rat dorsal prostate. *J. Biol. Chem.* **258**:10731-10737.

Dollé, P., Ruberte, E., Leroy, P., Morriss-Kay, G. and Chambon, P. (1990). Retinoic acid receptors and cellular retinoid binding proteins I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110**:1133-1151.

Dreyer, C.L., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992). Control of the peroxisomal beta oxidation pathway by a novel family of nuclear hormone receptors. *Cell* **68**:879-887.

Duester, G., Shean, M.L., McBride, M.S. and Stewart, M.J. (1991). Retinoic acid response element in the human alcohol dehydrogenase gene *ADH3*: implications for regulation of retinoic acid synthesis. *Mol. Cell. Biol.* **11**:1638-1646.

Dumont, M., Dauvois, S., Simard, J., Garcia, T., Schachter, B. and Labrie, F. (1989). Antagonism between estrogen and androgens on GCDFP-15 gene expression in ZR-75-1 cells and correlation between GCDFP-15 and estrogen as well as progesterone receptor expression in human breast cancer. *J. Steroid Biochem.* **34**:397-402.

Durand, B., Saunders, M., Leroy, P., Leid, M. and Chambon, P. (1992). All-*trans* and 9-*cis* retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. *Cell* **71**:73-85.

Dusanter-Fourt, I., Gaye, P., Belair, L., Petridou, B., Kelly, P.A. and Djiane, J. (1991). Prolactin receptor gene expression in the rabbit: identification, characterization and tissue distribution of several PRL receptor mRNA encoding a unique precursor. *Mol. Cell. Endocrinol.* **77**:181-192.

Edery, M., Jolicoeur, C., Levi-Meyrueis, C., Dusanter-Fourt, I., Pétridou, B., Boutin, J-M., Lesueur, L., Kelly, P.A. and Djiane, J. (1989). Identification and sequence analysis of a second form of prolactin receptor by molecular cloning of complementary DNA from rabbit mammary gland. *Proc. Natl. Acad. Sci. USA* **86**:2112-2116.

Eisenberg, S.P., Coen, D.M. and McKnight, S.L. (1985). Promoter domains required for expression of plasmid-borne copies of the herpes simplex virus thymidine kinase gene in virus-infected mouse fibroblasts and microinjected frog oocytes. *Mol. Cell Biol.* **5**:1940-1947.

Elder, J.T., Fisher, G.J., Zhang, Q-Y., Eisen, D., Krust, A., Chambon, P. and Voorhees, J.J. (1991). Retinoic acid receptor gene expression in human skin. *J. Invest. Dermatol.* **96**:425-433.

Engel, L.W., Young, N.A., Tralka, T.S., Lippman, M.E., O'Brien, S.J. and Joyce, M.J. (1978). Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Res.* **38**:3352-3364.

Evans, R.M. and Hollenberg, S.M. (1988). Zinc fingers: gilt by association. *Cell* **51**:1-3.

Faber, P.W., van Rooij, H.C.J., van der Korput, H.A.G.M., Baarends, W.M., Brinkmann, A.O., Grootegoed, J.A. and Trapman, J. (1991). Characterization of the human androgen receptor transcription unit. *J. Biol. Chem.* **266**:10743-10749.

Fawell, S.E., Lees, J.A., White, R. and Parker, M.G. (1990). Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell* **60**:953-962.

Fisher, E.R. and Fisher, B. (1963). Experimental studies of factors influencing hepatic metastasis. XIV. Effect of prolactin. *Cancer Res.* **23**:1532-1538.

Fisher, B. (1992). The evolution of paradigms for the management of breast cancer: a personal perspective. *Cancer Res.* **52**:2371-2383.

Fontana, J.A., Hobbs, P.D. and Dawson, M.I. (1988). Inhibition of mammary carcinoma growth by retinoidal benzoic acid derivatives. *Exp. Cell Biol.* **56**:254-263.

Fontana, J.A., Mezu, A.B., Cooper, B.N. and Miranda, D. (1990). Retinoid modulation of estradiol-stimulated growth and of protein synthesis and secretion in human breast carcinoma cells. *Cancer Res.* **50**:1997-2002.

Fontana, J.A., Nervi, C., Shao, Z-M. and Jetten, A.M. (1992). Retinoid antagonism of estrogen-responsive transforming growth factor α and pS2 gene expression in breast carcinoma cells. *Cancer Res.* **52**:3938-3945.

Forman, B.M., Yang, C-R., Au, M., Casanova, J., Ghysdael, J. and Samuels, H. (1989). A domain containing leucine zipper like motifs mediate novel *in vivo* interactions between the thyroid hormone and retinoic acid receptors. *Mol. Endocrinol.* **3**:1610-1626.

Forman, B.M. and Samuels, H.H. (1990). Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol. Endocrinol.* **4**:1293-1301.

Forsyth, I.A. (1988). The roles of placental lactogen and prolactin in mammogenesis: an overview of *in vivo* and *in vitro* studies. In: *Prolactin gene family and its receptors.* (Hoshino, K., ed.), pp.137, Elsevier, Amsterdam.

Francis, M.J.O. and Hill, D.J. (1975). Prolactin-stimulated production of somatomedin by rat liver. *Nature* **255**:167-168.

Frawley, L.S. (1989). Mammosomatotropes: current status and possible functions. *Trends Endocrinol. Metab.* **1**:31.

Freake, H.C., Marcocci, C., Iwasaki, J. and MacIntyre, I. (1981). 1,25-dihydroxyvitamin D_3 specifically binds to a human breast cancer cell line (T-47D) and stimulates growth. *Biochem. Biophys. Res. Commun.* **101**:131-1138.

Freedman, L.P., Luisi, B.F., Korszun, Z.R., Basavappa, R., Sigler, P.B. and Yamamoto, K.R. (1988). The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* **334**:543-546.

Freemark, M. and Comer, M. (1989). Purification of a distinct placental lactogen receptor, a new member of the growth hormone/prolactin receptor family. *J. Clin. Invest.* **83**:883-889.

Galas, D. and Schmitz, A. (1978). DNase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5**:3157-3170.

Gareau, J.L., Houle, B., Leduc, F., Bradley, W.E.C. and Dobrovic, A. (1988). A frequent HindDIII RFLP on chromosome 3p21-25 detected by a genomic *erbA* beta sequence. *Nucleic Acids Res.* **16**:1223.

Garner, M.M. and Rezvin, A. (1981). A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose regulatory system. *Nucleic Acids Res.* **9**:3047-3060.

Gaspar, M-L., Meo, R., Bourgarel, P., Guenet, J-L. and Tosi, M. (1991). A single base deletion in the *Tfm* androgen receptor gene creates a short-lived messenger RNA that directs internal translation initiation. *Proc. Natl. Acad. Sci. USA* **88**:8606-8610.

Gebert, J.F., Moghal, N., Frangioni, J.V., Sugarbaker, D.J. and Neel, B.G. (1991). High frequency of retinoic acid receptor beta abnormalities in human lung cancer. *Oncogene* **6**:1859-1868.

Geller, J. (1990). Effect of finasteride, a 5α-reductase inhibitor on prostate tissue androgens and a prostate-specific antigen. *J. Clin. Endocrinol. Metab.* **71**:1552-1555.

Gellersen, B., DiMattia, G.E., Friesen, H.G. and Bohnet, H.G. (1989). Prolactin (PRL) mRNA from human decidua differs from pituitary PRL mRNA but resembles the IM-9-P3 lymphoblast PRL transcript. *Mol. Cell. Endocrinol.* **64**:127-130.

Ghazal, P., DiMattei, C., Giulietti, E., Kliewer, S., Umesono, K. and Evans, R.M. (1992). Retinoic acid receptors initiate induction of the cytomegalovirus enhancer in embryonal cells. *Proc. Natl. Acad. Sci. USA* **89**:7630-7634.

Giguere, V., Ong, E.S., Segui, P. and Evans, R.M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* **330**:624-629.

Giguere, V., Lyn, S., Yip, P., Siu, C-H. and Amin, S. (1990). Molecular cloning of cDNA encoding a second retinoic acid-binding protein. *Proc. Natl. Acad. Sci. USA* **87**:6233-6237.

Gill, G. and Ptashne, M. (1988). Negative effect of the transcriptional activator Gal4. *Nature* **334**:721-724.

Glass, C.K., Holloway, J.M., Devary, O.V. and Rosenfeld, M.G. (1988). The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell* **54**:313-323.

Glass, C.K., Lipkin, S.M., Devary, O.V. and Rosenfeld, M.G. (1989). Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. *Cell* **59**:697-708.

Glass, C.K., Devary, O.V. and Rosenfeld, M.G. (1990). Multiple cell type-specific proteins differentially regulate target sequence recognition by the α retinoic acid receptor. *Cell* **63**:729-738.

Glass, C.K., Kurokawa, R., Rosenfeld, M., Yu, V.C., Näär, A., Silverman, S., Kyakumoto, S. and Han, Z. (1993). Differential orientations of the C-terminal dimerization interface determine binding site selection by nuclear receptor heterodimers. In: Mechanism of action of retinoids, vitamin D, and steroid hormones, March 15-20, Banff, Canada.

Glick, A.B., Flanders, K.C., Danielpour, D., Yuspa, S.H. and Sporn, M.B. (1989). Retinoic acid induces transforming growth factor β2 in cultured keratinocytes and mouse epidermis. *Cell Regul.* **1**:87-89.

Godowski, P.J., Rusconi, S., Miesfeld, R. and Yamamoto, K.R. (1987). Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* **325**:365-368.

Golander, A., Hurley, T., Barrett, J. and Handwerger, S. (1979). Synthesis of prolactin by human decidua *in vitro*. *J. Endocrinol.* **82**:263-267.

Goodman, D.S. (1984). Plasma retinol binding protein. In: *The retinoids*. (Sporn, M.B., Roberts, A.B. and Goodman, D.S., eds.), pp.41-88, Academic press, Orlando.

Goodman, H.S. and Rosen, J.M. (1990). Transcriptional analysis of the mouse β-casein gene. *Mol. Endocrinol.* **4**:1661-1670.

Grattarola, R. (1976). Ovariectomy alone or in combination with dexamethasone in patients with advanced breast cancer and high levels of testosterone excretion. *J. Natl. Cancer Inst.* **56**:11-19.

Graupner, G., Wills, K.N., Tzukerman, M., Zhang, X.K. and Pfahl, M. (1989). Dual regulatory role for thyroid-hormone receptors allows control of retinoic-acid receptor activity. *Nature* **340**:653-656.

Graupner, G., Zhang, X-K., Tzukerman, M., Wills, K., Hermann, T. and Pfahl, M. (1991). Thyroid hormone receptors repress estrogen receptor activation of a TRE. *Mol. Endocrinol.* **5**:365-372.

Green, S. and Chambon, P. (1988). Nuclear receptors enhance our understanding of transcriptional regulation. *Trends Genet.* **4**:309-314.

Green, S., Kumar, V., Theulaz, I., Wahli, W. and Chambon, P. (1988). The N-terminal DNA-binding "zinc-finger" of the oestrogen and glucocorticoid receptors determine target gene specificity. *EMBO J.* 7:3037-3044.

Green, S. and Chambon, P. (1989). Chimeric receptors used to probe the DNA-binding domain of the estrogen and glucocorticoid receptors. *Cancer Res.* **49**:2282S-2285S.

Green, S. (1993). Promiscuous liaisons. *Nature* **361**:590-591.

Groham, S., Marshall, J., Mettlin, C., Rzepka, T., Nemoto, T. and Byers, T. (1982). Diet in the epidemiology of breast cancer. *Am. J. Epidemiol.* **116**:68-75.

Gronemeyer, H., Turcotte, B., Quirin-Stricker, C., Bocquel, M.T., Meyer, M.E., Krozowski, Z., Jeltsch, J.M., Lerouge, T., Garnier, J.M. and Chambon, P. (1987). The chicken progesterone receptor: sequence, expression and functional analysis. *EMBO J.* **6**:3985-3994.

Gruber, J.R., Ohno, S. and Niles, R.M. (1992). Increased expression of protein kinase C_{α} plays a key role in retinoic acid-induced melanoma differentiation. *J. Biol. Chem.* **267**:13356-13360.

Gudas, L.J. (1992). Retinoids, retinoid-responsive genes, cell differentiation and cancer. *Cell Growth Diff.* **3**:655-662.

Haagensen, D.E., Mazoujian, G., Dilley, W.G., Pederson, C.E., Kister, S.J. and Wells, S.A. (1979). Breast gross cystic disease fluid analysis: I. Isolation and radioimmunoassay for a major component protein. *J. Natl. Cancer Inst.* **62**:239-247.

Haagensen, D.E., Silva, J.S., Leight, G.S., Dilley, W.G. and Wells, S.A. (1981). Fluoxymesterone stimulates plasma concentrations of gross cystic disease fluid protein in patients with metastatic breast carcinoma. *Surg. Forum* **32**:413-414.

Haagensen, D.E. and Mazoujian, G. (1986). Biochemistry and immunohistochemistry of fluid proteins of the breast in gross cystic disease. In: Diseases of the Breast, 3rd ed. (Haagensen, C.D., ed.), pp. 474-500, W.B. Saunders Co., Philedelphia.

Haagensen, D.E., Dilley, W.G., Mazoujian, G. and Wells, S.A. (1990). Review of GCDFP-15: An apocrine marker protein. *Ann. N.Y. Acad. Sci.* **586**:161-173.

Hallenbeck, P.L., Marks, M.S., Lippoldt, R.E., Ozato, K. and Nikodem, V.M. (1992). Heterodimerization of thyroid hormone (TH) receptor with H-2RIIBP (RXRβ) enhances DNA binding and TH-dependent transcriptional activation. *Proc. Natl. Acad. Sci. USA* **89**:5572-5576.

Ham, J., Thomson, A., Needham, M., Webb, P. and Parker, M. (1988). Characterization of response elements of androgen, glucocorticoids and progestins in mouse mammary tumour virus. *Nucleic Acids Res.* **16**:5263-5277.

Han, K., Levine, M.S. and Manley, J.L. (1989). Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* **56**:573-583.

Härd, T., Kellenbach, E., Boelens, R., Maler, B.A., Dahlman, K., Freedman, J.P., Carlstedt-Duke, J., Yamamoto, K.R., Gustafsson, J-A. and Kaptein, R. (1990). Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* **249**:157-160.

Harigaya, T., Smith, W.C. and Talamantes, F. (1988). Hepatic placental lactogen receptors during pregnancy in the mouse. *Endocrinology* **122**:1366-1372.

Härkönen, P.L., Mäkelä, S.I., Valve, E.M., Karhukorpi, E-K. and Väänänen, H.K. (1991). Differential regulation of carbonic anhydrase II by androgen and estrogen in dorsal and lateral prostate of the rat. *Endocrinology* **128**:3219-3227.

Harris, S.E., Rong, Z., Harris, M.A. and Lubahn, D.B. (1990). Androgen receptor in human prostate carcinoma LNCaP/ADEP cells contains a mutation which alters the specificity of the steroid-dependent transcriptional activation region. In: The 72nd Annual Meeting of the Endocrine Society, #275, Atlanta, Georgia.

Harris, S.E., Harris, M.A., Rong, Z., Hall, J., Judge, S., French, F.S., Joseph, D.R., Lubahn, D.B., Simental, J.A. and Wilson, E.M. (1991). In *Molecular and cellular biology of prostate cancer* (Karr, J.P., Coffey, D.S., Smith, R.G. and Tindall, D.J., eds.), pp. 315-330, Plenum Press, New York.

Harvey, C., Jackson, S.M., Siddiqui, S.K. and Gutierrez-Hartmann, A. (1991). Structure-function analysis of the rat prolactin promoter: phasing requirements of proximal cell-specific elements. *Mol. Endocrinol.* **5**:836-843.

Hazel, T.G., Nathans, D. and Lau, L.F. (1988). A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* **85**:8444-8448.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**:351-359.

Hennighausen, L.G. and Sippel, A.E. (1982). Mouse whey acidic protein is a novel member of the famiy of "four-disulfide core" proteins. *Nucleic Acids. Res.* **10**:2677-2684.

Henttu, P., Liao, S. and Vihko, P. (1992). Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. *Endocrinology* **130**:766-772.

Herr, W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A., Rosenfeld, M.G., Finney, M., Ruvkin, G. and Horvitz, H.R. (1988). The POU domain: a large conserved region in the mamalian *Pit-1*, *Oct-1*, *Oct-2*, and *Caenorhabditis elegans unc-86* gene products. *Genes Dev.* **3**:1513-1516.

Herskowitz, I. (1987). Functional inactivation of genes by dominant negative mutations. *Nature* **329**:219-222.

Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M. and Thaller, C. (1992). 9-*cis* retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**:397-406.

Higuchi, R., Krummel, B. and Saiki, R. (1988). A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**:7351-7367.

Ho, K.C., Tan, J-A., Marschke, K.B. and French, F.S. (1992). Complex response elements of the rat ventral prostate 20 kD protein gene enhance androgen but not glucocorticoid stimulation of transcription. In: The 74th annual meeting of the Endocrine Society, #569, San Antonio, Texas.

Hoeffler, J.P. and Frawley, L.S. (1987). Liver tissue produces a patent lactogenic factor that partially mimics the actions of prolactin. *Endocrinology* **120**:1679-1681.

Hogan, B.L., Taylor, A. and Adamson, E. (1981). Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* **291**:235-237.

Hollenberg, S.M. and Evans, R.M. (1988). Multiple and cooperative *trans*-activation domains of the human glucocorticoid receptor. *Cell* **55**:899-906.

Hope, I.A. and Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* **46**:885-894.

Horwitz, K.B., Zava, D.T., Thilagar, A.K., Jensen, E.M. and McGuire, W.L. (1978). Steroid receptor analyses of nine human breast cancer cell lines. *Cancer Res.* **38**:2434-2437.

Hosoi, K., Maruyama, S., Ueha, T., Sato, S. and Gresik, E.W. (1992). Additive and/or synergistic effects of 5α-dihydrotestosterone, dexamethasone and triiodo-L-thyronine on induction of proteinases and epidermal growth factor in the submandibular gland of hypophysectomized mice. *Endocrinology* **130**:1044-1055.

Howley, P., Sarver, N. and Law, M-F. (1983). Eukaryotic cloning vectors derived from bovine papillomavirus DNA. *Methods in Enzymol.* **101**:387-402.

Hsieh, J-T., Zhau, H.E., Wang, X-H., Liew, C-C. and Chung, L.W.K. (1992). Regulation of basal and luminal cell-specific cytokeratin expression in rat accessory sex organs. *J. Biol. Chem.* **267**:2303-2310.

Hu, L., Crowe, D.L., Rheinwald, J.G., Chambon, P. and Gudas, L.J. (1991). Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.* **51**:3972-3981.

Huang, M.E., Ye, Y-C., Chen, S-R., Zhao, J-C., Gu, L-J., Cai, J-R., Zhao, L., Xie, J-X., Shen, Z-X. and Wang, Z-Y. (1988). Use of all *trans*-retinoic acid in the treatment of acute promyelocytic leukaemia. *Blood* **72**:567-572.

Hwang, P., Murray, J.B., Jacobs, J.W., Niall, H.D. and Friesen, H.G. (1974). Human amniotic fluid prolactin. Purification by affinity chromatography and amino-terminal sequence. *Biochemistry* **13**:2354-2358.

Imperato-McGinley, J., Guerrero, L., Gautier, T. and Peterson, R.E. (1974). Steroid 5α-reductase deficiency in Man: an inherited form of pseudohermaphroditism. *Science* **186**:1213-1215.

Imperato-McGinley, J., Peterson, R.E., Gautier, T. and Sturla, E. (1979). Male pseudo-hermaphrodism secondary to 5α-reductase deficiency - a model for the role of androgens in both the development of the male phenotype and the evolution of a male gender identity. *J. Steroid Biochem.* **11**:637-645.

Ing, N.H., Beekman, J.M., Tsai, S.Y., Tsai, M-J. and O'Malley, B.W. (1992). Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). *J. Biol. Chem.* **267**:17617-17623.

Ishikawa, T., Umesono, K., Mangelsdorf, D.J., Aburatani, H., Stanger, B.Z., Shibasaki, Y., Imawari, M., Evans, R.M. and Takaku, F. (1990). A functional retinoic acid receptor encoded by the gene on human chromosome 12. *Mol. Endocrinol.* **4**:837-844.

Jahn, G.A., Biolez-Bodja, F., Belair, L., Kerdelhue, B., Kelly, P.A., Djiane, J. and Edery, M. (1991a). Effect of DMBA on the expression of prolactin receptors and IGFI genes in rat mammary gland. *Biomed. Pharmacother.* **45**:15-22.

Jahn, G.A., Edery, M., Belair, L., Kelly, P.A. and Djiane, J. (1991b). Prolactin receptor gene expression in rat mammary gland and liver during pregnancy and lactation. *Endocrinology* **128**:2976-2984.

Jenster, G., van der Korput, H.A.G.M., van Vroonhoven, C., van der Kwast, T.H., Trapman, J. and Brinkmann, A.O. (1991). Domains of the human androgen receptor involved in steroid binding, transcriptional activation and subcellular localization. *Mol. Endocrinol.* **5**:1396-1404.

Jonat, C., Rahmsdorf, H.J., Park, K-K., Cato, A.C.B., Gebel, S., Ponta, H. and Herrlich, P. (1990). Antitumor promotion and antiinflammation down modulation of AP-1 (*Fos/Jun*) activity of glucocorticoid hormone. *Cell* **62**:1189-1204.

Jones, N. (1990). Transcriptional regulation by dimerization: two sides to an incestuous relationship. *Cell* **61**:9-11.

Jordan, V.C. (1974). Antitumor activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J. Steroid Biochem.* **5**:354.

Kadonaga, J.T., Carner, K.R., Masiarz, F.R. and Tjian, R. (1987). Isolation of a cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**:1079-1090.

Kakizuka, A., Miller, W.H., Umesono, K., Warrell, R.P., Frankel, S.R., Murty, V.V.V.S., Dmitrovsky, E. and Evans, R.M. (1991). Chromosomal translocation t(15;17) in human acute promyelocytic leukaemia fuses RARα with a novel putative transcription factor, *PML*. *Cell* **66**:663-674.

Kalderon, D., Roberts, B.L., Richardson, W.D. and Smith, A.E. (1984). A short amino acid sequence able to specify nuclear location. *Cell* **39**:499-509.

Kastner, P., Krust, A., Mendelsohn, C., Garnier, J.M., Zelent, A., Leroy, P., Staub, A. and Chambon, P. (1990). Murine isoforms of retinoic acid receptor γ with specific patterns of expression. *Proc. Natl. Acad. Sci. USA* **87**:2700-2704.

Kastner, P., Brand, N., Krust, A., Leroy, P., Mendelsohn, C., Petkovich, M., Zelent, A. and Chambon, P. (1991). Retinoic acid nuclear receptors. In: *Developmental patterning of the vertebrate limb*. (Hinchligge, J.R., Hurle, J. and Summerbell, D., eds.), Plenum, New York.

Kastner, P., Perez, A., Lutz, Y., Rochette Egly, C., Gaub, M.P., Durand, B., Lanotte, M., Berger, R. and Chambon, P. (1992). Structure, localization and transcriptional properties of two classes of retinoic acid receptor α fusion proteins in acute promyelocytic leukaemia: structural similarities with a new family of oncoproteins. *EMBO J.* **11**:629-642.

Keller Wood, M. and Bland, K.I. (1991). Breast physiology in normal, lactating and diseased states. In: *The Breast: comprehensive management of benign and malignant diseases.* (Bland, K.I. and Copeland, E.M., eds.), pp. 36-45, Saunders, Philadelphia.

Kelly, P.A., Djiane, J., Postel-Vinay, M-C. and Edery, M. (1991). The prolactin/growth hormone receptor family. *Endocrine Rev.* **12**:235-251.

Kemppainen, J.A., Lane, M.V., Sar, M. and Wilson, E.M. (1992). Androgen receptor phosphorylation, turnover, nuclear transport and transcriptional activation. *J. Biol. Chem.* **267**:968-974.

Kessel, M. and Gruss, P. (1990). Murine developmental control genes. *Science* **249**:374-379.

Kledzik, G.S., Marshall, S., Campbell, A.A., Gelato, M. and Meites, J. (1976). Effects of castration, testosterone, estradiol and prolactin on specific prolactin-binding activity in ventral prostate of male rats. *Endocrinology* **98**:373-379.

Klein-Hitpass, L., Schorpp, M., Wagner, U. and Ryffel, G. (1986). An estrogen responsive element derived from the 5' flanking region of the *Xenopus* vitellogenin A2 gene functions in transfected human cells. *Cell* **46**:1053-1061.

Kliewer, S.A., Umesono, K., Mangelsdorf, D.J. and Evans, R.M. (1992a). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. *Nature* **355**:446-449.

Kliewer, S.A., Umesono, K., Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A. and Evans, R.M. (1992b). Retinoid X receptor - COUP-TF interactions modulate retinoic acid signaling. *Proc. Natl. Acad. Sci. USA* **89**:1448-1452.

Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A. and Evans, R.M. (1992c). Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* **358**:771-774.

Kliewer, S., Lee, M., Wright, P. and Evans, R. (1993). Solution structure of the retinoid X receptor α DNA-binding domain: identification of a novel helix required for cooperative, homodimeric DNA binding. In: Mechanism of action of retinoids, vitamin D, and steroid hormones, March 15-20, Banff, Canada.

Koga, M. and Sutherland, R.L. (1991). Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D_3 or antiestrogen to inhibit human breast cancer cell proliferation. *J. Steroid Biochem. Mol. Biol.* **39**:455-460.

Kopan, R. and Fuchs, E. (1989). The use of retinoic acid to probe the relation between hyperproliferation-associated keratins and cell proliferation in normal and malignant epidermal cells. *J. Cell Biol.* **109**:295-307.

Kornberg, L.J. and Liberti, J.P. (1989). Nb2 cell mitogenesis: effect of lactogens on cAMP and protein phosphorylation. *Biochim. Biophys. Acta* **1011**:205-211.

Kozak, M. (1983). Comparison of initiation of protein synthesis in procaryotes, eucaryotes and organelles. *Microbiol. Rev.* **47**:1-45.

Krust, A., Kastner, P., Petkovich, M., Zelent, A. and Chambon, P. (1989). A third human retinoic acid receptor, hRAR-γ. *Proc. Natl. Acad. Sci. USA* **86**:5310-5314.

Kuiper, G.G.J.M., Faber, P.W., van Rooij, H.C.J., van der Korput, J.A.G.M., Ris-Stalpers, C., Klaassen, P., Trapman, J. and Brinkmann, A.O. (1989). Structural organization of the human androgen receptor gene. *J. Molec. Endoc.* **2**:1-4.

Kuiper, G.G.J.M., de Ruiter, P.E., Grootegoed, J.A. and Brinkmann, A.O. (1991). Synthesis and post-translational modification of the androgen receptor in LNCaP cells. *Mol. Cell. Endocrinol.* **80**:65-73.

Kumar, V. and Chambon, P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55**:145-156.

Labrie, F., Simard, J., Poulin, R., Hatton, A-C., Labrie, C., Dauvois, S., Zhao, H., Petitclerc, L., Couet, J., Dumont, M. and Haagensen, D.E. (1990). Potent antagonism between estrogens and androgens on GCDFP-15 expression and cell growth in the ZR-75-1 human breast cancer cells. *Ann. N.Y. Acad. Sci.* **586**:174-187.

Ladias, L.A.A. and Karathanasis, S.K. (1991). Regulation of the apolipoprotein AI gene by ARP1, a novel member of the steroid receptor superfamily. *Science* **251**:561-565.

Lammer, E.J., Chen, D.T., Hoar, R.M., Agnish, N.D., Benke, P.J., Braun, J.T., Curry, C.J., Fernhoff, P.M., Grix, A.W., Lott, I.T., Richard, J.M. and Sun, S.C. (1985). Retinoic acid embryopathy. *N. Engl. J. Med.* **313**:837-841.

Landford, R.E., Kanda, P. and Kennedy, R.C. (1986). Induction of nuclear transport with a synthetic peptide homologous to the SV40 antigen transport signal. *Cell* **46**:575-582.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**:1759-1764.

Larsen, J.L. and Dufau, M.L. (1988). Modulation of prolactin-stimulated Nb2 lymphoma cell mitogenesis by cholera toxin and pertussis toxin. *Endocrinology* **123**:438-444.

la Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* **352**:77-79.

Lazar, M.A., Berrodin, J.J. and Harding, H.P. (1991). Differential DNA binding by monomeric, homodimeric and potentially heterodimeric forms of the thyroid hormone receptor. *Mol. Cell. Biol.* **11**:5005-5015.

Lea, O.A., Kvinnsland, S. and Thorsen, T. (1989). Improved measurement of androgen receptors in human breast cancer. *Cancer Res.* **49**:7162-7167.

le Doussal, V., Zangerle, P.F., Collette, J., Spyratos, F., Hacene, K., Briere, M., Franchimont, P. and Gest, J. (1985). Immunohistochemistry of a component protein of the breast cystic disease fluid with mol. wt. 15,000. *Eur. J. Cancer Clin. Oncol.* **21**:715-725.

Lee, W.S., Cheng Kao, C., Bryant, G.O., Lui, X. and Berk, A.J. (1991). Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. *Cell* **67**:365-376.

Lehmann, J.M., Dawson, M.I., Hobbs, P.D., Husmann, M. and Pfahl, M. (1991). Identification of retinoids with nuclear receptor subtype-selective activities. *Cancer Res.* **51**:4804-4809.

Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J-Y., Staub, A., Garnier, J-M., Mader, S. and Chambon, P. (1992). Purification, cloning and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68**:377-395.

Leis, H.P. (1991). Prognostic parameters for breast carcinoma. In: *The Breast: comprehensive management of benign and malignant diseases.* (Bland, K.I. and Copeland, E.M., eds.), pp. 331-350, Saunders, Philadelphia.

Leo, M. and Kumar, M.V. (1992). Modulation of androgen induced gene transcription by estrogen receptor cDNA. In: The 74th annual meeting of the Endocrine Society, #209, San Antonio, Texas.

Lerner, L.J. and Jordan, V.C. (1990). Development of antiestrogens and their use in breast cancer: Eighth Cain Memorial Award lecture. *Cancer Res.* **50**:4177-4189.

Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J.M., Kastner, P., Dierich, A. and Chambon, P. (1991a). Multiple isoforms of the mouse retinoic acid receptor alpha are generated by alternative splicing and differential induction by retinoic acid. *EMBO J.* **10**:59-69.

Leroy, P., Nakshatri, H. and Chambon, P. (1991b). Mouse retinoic acid receptor α2 isoform is transcribed from a promoter that contains a retinoic acid response element. *Proc. Natl. Acad. Sci. USA* **88**:10138-10142.

Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J.M., Kastner, P., Dierich, A. and Chambon, P. (1992). In: *Retinoids in normal development and teratogenesis.* (Morriss-Kay, G., ed.), Oxford Univ. Press, Oxford.

Lesueur, L., Edery, M., Paly, J., Clark, J., Kelly, P.A. and Djiane, J. (1990). Prolactin stimulates milk protein promoter in CHO cells cotransfected with prolactin receptor cDNA. *Mol. Cell. Endocrinol.* **71**:R7-R12.

Lesueur, L., Edery, M., Ali, S., Paly, J., Kelly, P.A. and Djiane, J. (1991). Comparison of long and short forms of the prolactin receptor on prolactin-induced milk protein gene transcription. *Proc. Natl. Acad. Sci. USA* **88**:824-828.

Leung, D.W., Spencer, C.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J. and Wood, W.I. (1987). Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature* **330**:537-543.

Levin, A.A., Sturzenbecker, L.J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J.F. (1992). 9-*cis* retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. *Nature* **355**:359-361.

Levine, M. and Manley, J.L. (1989). Transcriptional repression of eukaryotic promoters. *Cell* **59**:405-408.

Lewis, J. and Martin, P. (1989). Vertebrate development. Limbs: a pattern emerges. *Nature* **342**:734-735.

Liao, S., Kokontis, J., Tetsujun, S. and Hiipakka, R.A. (1989). Androgen receptors: structures, mutations, antibodies and cellular dynamics. *J. Steroid Biochem.* **34**:41-51.

Liao, J., Ozono, K., Sone, T., McDonnell, D.P. and Pike, J.W. (1990). Vitamin D receptor interaction with specific DNA requires a nuclear protein and 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA* **87**:9751-9755.

Lillie, J.W. and Green, M.R. (1989). Activator's target in sight. *Nature* **341**:279-280.

Lindzey, J., Grossman, M. and Tindall, D. (1992). cAMP and androgen regulation of the 5' flanking region of the mouse androgen receptor gene. In: The 74th annual meeting of the Endocrine Society, #1490, San Antonio, Texas.

Linzer, D.I.H. and Talamantes, F. (1985). Nucleotide sequence of mouse prolactin and growth hormone mRNAs and expression of these mRNAs during pregnancy. *J. Biol. Chem.* **260**:9574-9579.

Lipkin, S.M., Nelson, C.A., Glass, C.K. and Rosenfeld, M.G. (1992). A negative retinoic acid response element in the rat oxytocin promoter restricts transcriptional stimulation by heterologous transactivation domains. *Proc. Natl. Acad. Sci. USA* **89**:1209-1213.

Lippman, S.M., Kessler, J.F. and Meyskens, F.L. (1987). Retinoids as preventative and therapeutic anticancer agents (part 1). *Cancer Treat. Rep.* **71**:391-405.

Loreni, F., Stavenhagen, J., Kalff, M. and Robins, D.M. (1988). A complex androgen-responsive enhancer resides 2 kilobases upstream of the mouse Slp gene. *Mol. Cell Biol.* **8**:2350-2360.

Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S. and Wilson, E.M. (1988). Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* **240**:327-330.

Lucas, P.C., O'Brien, R.M., Mitchell, J.A., Davis, C.M., Imai, E., Forman, B.M., Samuels, H.H. and Granner, D.K. (1991). A retinoic acid response element is part of a pleiotropic domain in the phospho*enol*pyruvate carboxykinase gene. *Proc. Natl. Acad. Sci. USA* **88**:2184-2188.

Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R. and Sigler, P.B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352**:497-505.

Luke, M.C. and Coffey, D.S. (1992). The expression of human androgen receptor cDNA via recombinant baculovirus infected Sf9 insect cells. In: The 74th annual meeting of the Endocrine Society, #1006, San Antonio, Texas.

Lund, D.S., Gallagher, P.M., Wang, B., Porter, S.C. and Ganschow, R.E. (1991). Androgen responsiveness of the murine β-glucuronidase gene is associated with nuclease hypersensitivity, protein binding and haplotype-specific sequence diversity within intron 9. *Mol. Cell. Biol.* **11**:5426-5434.

Lyon, M.F. and Hawkes, S.G. (1970). X-linked gene for testicular feminization in the mouse. *Nature* **227**:1217-1291.

Lyon, M.F., Hendry, I. and Short, R.V. (1973). The submaxillary glands as test organs for response to androgen in mice with testicular feminization. *J. Endocrinol.* **58**:357-362.

Ma, J. and Ptashne, M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* **48**:847-853.

MacDonald, P.N. and Ong, D.E. (1988). Evidence for a lecithin-retinol acyltransferase activity in the rat small intestine. *J. Biol. Chem.* **263**:12478-12482.

Maden, M., Ong, D.E., Summerbell, D. and Chytil, F. (1988). Spatial distribution of cellular protein binding to retinoic acid in the chick limb bud. *Nature* **335**:733-735.

Mader, S., Kumar, V., de Verneuil, H. and Chambon, P. (1989). Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* **338**:271-274.

Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. (1990). A nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**:224-229.

Mangelsdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E.S. and Evans, R.M. (1991). A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* **66**:555-561.

Mangelsdorf, D.J. and Evans, R.M. (1992). Vitamin A receptors: new insights on retinoid control of transcription. In: *Retinoids in normal development and teratogenesis.* (Morriss-Kay, G.M., ed.), pp. 27-50, Oxford Univ. Press, Oxford.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Mant, D. and Vessey, M.P. (1991). Epidemiology of breast cancer. In: *The Breast: comprehensive management of benign and malignant diseases.* (Bland, K.I. and Copeland, E.M., eds.), pp. 235-246, Saunders, Philadelphia, PA.

Marks, M.S., Levi, B-Z., Segars, J.H., Driggers, P.H., Hirschfeld, S., Nagata, T., Appella, E. and Ozato, K. (1992). H-2RIIBP expressed from a baculovirus vector binds to multiple hormone response elements. *Mol. Endocrinol.* **6**:219-230.

Marotti, K.R., Brown, G.D. and Strickland, S. (1985). Two-stage hormonal control of type IV collagen mRNA levels during differentiation of F9 teratocarcinoma cells. *Dev. Biol.* **108**:26-31.

Marth, C., Bock, G. and Daxenbichler, G. (1985). Effect of 4-hydroxyphenylretinamide and retinoic acid on proliferation and cell cycle of cultured human breast cancer cells. *J. Natl. Cancer Inst.* **75**:871-875.

Marth, C., Daxenbichler, G. and Dapunt, O. (1986). Synergistic antiproliferative effect of human recombinant interferons and retinoic acid in culture breast cancer cells. *J. Natl. Cancer Inst.* 77:1197-1202.

Martinez, E., Givel, F. and Wahli, W. (1987). The estrogen responsive element as an inducible enhancer: DNA sequence requirements and conversion to a glucocorticoid responsive element. *EMBO J.* **6**:3719-3727.

Mattei, M.G., Petkovich, M., Mattei, J.F., Brand, N. and Chambon, P. (1988). Mapping of the human retinoic acid receptor to the q21 band of chromosome 17. *Hum. Genet.* **80**:186-188.

Mattei, M.G., Rivière, M., Krust, S., Ingvarsson, S., Vennstrom, B., Islam, M.Q., Kastner, P., Zelent, A. and Chambon, P. (1991). Chromosomal assignment of retinoic acid receptor (RAR) genes in the human, mouse and rat genomes. *Genomics* **10**:1061-1069.

McDonnell, D.P., Vegeto, E. and O'Malley, B.W. (1992). Identification of a negative regulatory function for steroid receptors. *Proc. Natl. Acad. Sci. USA* **89**:10563-10567.

McGuire, W.L., Tandon, A.K., Allred, D.C., Chamness, G.C. and Clark, G.M. (1990). How to use prognostic factors in axillary node-negative breast cancer patients. *J. Natl. Cancer Inst.* **82**:1006-1015.

McPhaul, M.J., Marcelli, M., Tilley, W.D., Griffin, J.E. and Wilson, J.D. (1991). Androgen resistance caused by mutations in the androgen receptor gene. *FASEB J.* **5**:2910-2915.

McQueen, S.A., Sheppard, P.C., Hamel, A., Cheng, H., Snoek, R., Bruchovsky, N., Rennie, P.S. and Matusik, R.J. (1992). Characterization of two *cis*-acting DNA elements responsible for androgen regulation of the prostatic probasin gene. In: The 74th annual meeting of the Endocrine Society, #1508, San Antonio, Texas.

Mendel, D.B., Holbrook, N.J. and Bodwell, J.E. (1985). Degradation without apparent change in size of molybdate-stabilized nonactivated glucocorticoid-receptor complexes in rat thymus cytosol. *J. Biol. Chem.* **260**:8736-8740.

Mermod, N., O'Neill, E.A., Kelly, T.J. and Tjian, R. (1989). The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. *Cell* **58**:741-753.

Mick, C.C.W. and Nicoll, C.S. (1985). Prolactin directly stimulates the liver *in vivo* to secrete a factor (synlactin) which acts synergistically with the hormone. *Endocrinology* **116**:2049-2053.

Milbrandt, J. (1988). Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* **1**:183-188.

Miller, J., McLachlan, A.D. and Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from *Zenopus* oocytes. *EMBO J.* **4**:1609-1614.

Miller, W.R., Telford, J., Dixon, J.M. and Hawkins, R.A. (1985). Androgen receptor activity in human breast cancer and its relationship with oestrogen and progestogen receptor activity. *Eur. J. Cancer Clin. Oncol.* **21**:539-542.

Miller, W.R., Shivas, A.A., Franchimont, P. and Haagensen, D. (1988). Breast gross cystic disease protein 15 in human breast cancer in culture. *Eur. J. Cancer Clin. Oncol.* **24**:223-228.

Misrahi, M., Loosfelt, H., Atger, M., Meriel, C., Zerah, V., Dessen, P. and Milgrom, E. (1988). Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. *Biochem. Biophys. Res. Commun.* **143**:740-748.

Mitchell, P.J. and Tjian, R. (1989). Transcription regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**:371-378.

Miyajima, N., Kadowaki, Y., Fukushige, S-I., Shimuizu, S-I., Semba, K., Yamanashi, Y., Matsubara, K-I., Toyoshima, K. and Yamamoto, T. (1988). Identification of two novel members of *erbA* superfamily by molecular cloning: the gene products of the two are highly related to each other. *Nucleic Acids Res.* **16**:11057-11074.

Moon, R.C., Thompson, H.J., Becci, P.J., Grubbs, C.J., Gander, R.J., Newton, D.L., Smith, J.M., Phillips, S.L., Henderson, W.R., Mullen, L.T. and Brown, C.C. (1979). N-(4-hydroxyphenyl)retinamide, a new retinoid for prevention of breast cancer in the rat. *Cancer Res.* **39**:1339-1346.

Moon, R.C., McCormick, D.L. and Mehta, R.G. (1983). Inhibition of carcinogenesis by retinoids. *Cancer Res.* **43**:24696-24755.

Moon, R.C. and Mehta, R.G. (1990). Chemoprevention of mammary cancer by retinoids. *Basic Life Sci.* **52**:213-224.

Morishige, W.K. and Rothchild, I. (1974). Temporal aspects of the regulation of corpus luteum function by luteinizing hormone, prolactin and placental luteotrophin during the first half of pregnancy in the rat. *Endocrinology* **95**:260-274.

Morris, P.L. and Saxena, B.B. (1980). Dose- and age-dependent effects of prolactin (PRL) on luteinizing hormone- and PRL-binding sites in rat Leydig cell homogenates. *Endocrinology* **107**:1639-1645.

Morriss-Kay, G. (1992). Retinoic acid receptors in normal growth and development. In: *Growth regulation by nuclear hormone receptors (Vol. 14).* (Parker, M.G., ed.), pp. 181-193, Cold Spring Harbor Laboratory Press, New York.

Munos-Canores, P., Vik, D.P. and Tack, B.F. (1990). Mapping of a retinoic acid responsive element in the promoter region of the complement factor H gene. *J. Biol. Chem.* **265**:20065-20068.

Murphy, L.C., Tsuyuki, D., Myal, Y. and Shiu, R.P.C. (1987a). Isolation and sequencing of a cDNA clone for a prolactin-inducible protein (PIP). *J. Biol. Chem.* **262**:15236-15241.

Murphy, L.C., Lee-Wing, M., Goldenberg, G.J. and Shiu, R.P.C. (1987b). Expression of the gene encoding a prolactin-inducible protein by human breast cancer *in vivo*: correlation with steroid receptor status. *Cancer Res.* **47**:4160-4164.

Murphy, L.J., Tachibana, K. and Friesen, H.G. (1988). Stimulation of hepatic insulin-like growth factor-I gene expression by ovine prolactin: evidence for intrinsic somatogenic activity in the rat. *Endocrinology* **122**:2027-2033.

Murray, M.B., Zilz, N.D., McCreary, N.L., MacDonald, M.J. and Towle, H.C. (1988). Isolation and characterization of rat cDNA clones for two distinct thyroid hormone receptors. *J. Biol. Chem.* **263**:12770-12777.

Murray, M.B. and Towle, H.C. (1989). Identification of nuclear factors that enhance binding of thyroid hormone receptors to thyroid hormone response elements. *Mol. Endocrinol.* **3**:1434-1442.

Myal, Y., Gregory, C., Wang, H., Hamerton, J. and Shiu, R.P.C. (1989). The gene for prolactin-inducible protein (PIP), uniquely expresses in exocrine organs, maps to chromosome 7. *Som. Cell Mol. Genet.* **15**:265-270.

Myal, Y., Robinson, D.B., Iwasiow, B., Tsuyuki, D., Wong, P. and Shiu, R.P.C. (1991). The prolactin-inducible protein (PIP/GCDFP-15) gene: cloning, structure and regulation. *Mol. Cell. Endocrinol.* **80**:165-175.

Näär, A.M., Boutin, J-M., Lipkin, S.M., Yu, V.C., Holloway, J.M., Glass, C.K. and Rosenfeld, M.G. (1991). The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* **65**:1267-1279.

Nagpal, S., Zelent, A. and Chambon, P. (1992a). RAR-β4, a retinoic acid receptor isoform is generated from RAR-β2 by alternative splicing and usage of a CUG initiator codon. *Proc. Natl. Acad. Sci. USA* **89**:2718-2722.

Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H. and Chambon, P. (1992b). Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. *Cell* **70**:1007-1019.

Nardulli, A.M. and Katzenellenbogen, B.S. (1988). Progesterone receptor regulation in T-47D human breast cancer cells: analysis by density labeling of progesterone receptor synthesis and degradation and their modulation by progestin. *Endocrinology* **122**:1532-1540.

Nettesheim, P. (1980). Inhibition of carcinogenesis by retinoids. *Can. Med. Assoc. J.* **122**:757-766.

Newmark, J.R., Hardy, D.O., Tonb, D.C., Carter, B.S., Epstein, J.I., Isaacs, W.B., Brown, T.R. and Barrack, E.R. (1992). Androgen receptor gene mutations in human prostate cancer. *Proc. Natl. Acad. Sci. USA* **89**:6319-6323.

Niall, H.D., Hogan, M.L., Sayer, R., Rosenblum, I.Y. and Greenwood, I.C. (1971). Sequences of pituitary and placental lactogenic and growth hormones. Evolution fron a primordial peptide by gene duplication. *Proc. Natl. Acad. Sci. USA* **68**:866-870.

Nicoll, C.S., Mayer, G.L. and Russell, S.M. (1986). Structural features of prolactins and growth hormones that can be related to their biological properties. *Endocr. Rev.* 7:169-203.

Nikolov, D.B., Hu, S-H., Lin, J., Gasch, A., Hoffman, A., Horikoshi, M., Chua, N-H., Roeder, R. and Burley, S.K. (1992). Crystal structure of TFIID TATA-box binding protein. *Nature* **360**:40-46.

Ofenstein, J.P. and Rillema, J.A. (1987). Possible involvement of the phospholipases in the mitogenic actions of prolactin (PRL) on Nb2 node lymphoma cells. *Proc. Soc. Exp. Biol. Med.* **185**:147-152.

O'Malley, B.W. and Conneely, O.M. (1992). Orphan receptors: in search of a unifying hypothesis for activation. *Mol. Endocrinol.* **6**:1359-1361.

O'Malley, B.W. (1993). Cellular mechanisms for activation of steroid receptor superfamily members. In: Mechanism of action of retinoids, vitamin D, and steroid hormones, March 15-20, Banff, Canada.

Ong, D.E. (1987). Cellular retinoid-binding proteins. *Arch. Dermatol.* **123**:1693a-1695a.

Ono, M. and Oka, T. (1980). α-lactalbumin-casein induction in virgin mouse mammary explants: dose-dependent differential action of cortisol. *Science* **207**:1367-1369.

Oro, A.E., McKeown, M. and Evans, R.M. (1990). Relationship between the product of the *Drosophila* ultraspiracle locus and the vertebrate retinoid X receptor. *Nature* **347**:298-301.

Ortí, E., Bodwell, J.E. and Munck, A. (1992). Phosphorylation of steroid hormone receptors. *Endo. Rev.* **13**:105-128.

O'Shea, E.K., Rutkowski, R. and Kim, P.S. (1989). Evidence that the leucine zipper is a coiled coil. *Science* **243**:538-541.

Osumi, T., Wen, J-K. and Hashimoto, T. (1991). Two *cis*-acting regulatory sequences in the peroxisome-proliferator-responsive enhancer region of rat acyl-CoA oxidase gene. *Biochem. Biophys. Res. Commun.* **175**:866-871.

Paulsen, R.E., Weaver, C.A., Fahrner, T.J. and Milbrandt, J. (1992). Domains regulating transcriptional activity of the inducible orphan receptor NGF1-B. *J. Biol. Chem.* **267**:16491-16496.

Pearson, O.H. and Ray, B.S. (1959). Results of treatment of metastatic mammary carcinoma. *Cancer (Phila.)* **12**:85-92.

Petkovich, M., Brand, N.J., Krust, A. and Chambon, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330**:444-450.

Pham, T.A., Elliston, J.F., Nawaz, Z., McDonnell, D.P., Tsai, M-J. and O'Malley, B.W. (1991). Antiestrogen can establish nonproductive receptor complexes and alter chromatin structure at target enhancers. *Proc. Natl. Acad. Sci. USA* **88**:3125-3129.

Pham, T.A., Hwung, Y-P., Santiso-Mere, D., McDonnell, D.P. and O'Malley, B.W. (1992). Ligand-dependent and -independent function of the transactivation regions of the human estrogen receptor in yeast. *Mol. Endocrinol.* **6**:1043-1050.

Posner, B.I., Kelly, P.A. and Friesen, H.G. (1974). Induction of a lactogenic receptor in rat liver: influence of estrogen and the pituitary. *Proc. Natl. Acad. Sci. USA* **71**:2407-2410.

Posner, B.I., Kelly, P.A. and Friesen, H.G. (1975). Prolactin receptors in rat liver: possible induction by prolactin. *Science* **188**:57-69.

Poulin, R. and Labrie, F. (1986). Stimulation of cell proliferation and estrogenic response by adrenal C_{19}-Δ^5 steroids in the ZR-75-1 human breast cancer cell line. *Cancer Res.* **46**:4933-4937.

Poulin, R., Baker, D. and Labrie, F. (1988). Androgens inhibit basal and estrogen-induced cell proliferation in the ZR-75-1 human breast cancer cell line. *Breast Cancer Res. Treat.* **12**:213-225.

Poulin, R., Simard, J., Labrie, C., Petitclerc, L., Dumont, M., Lagacé, L. and Labrie, F. (1989). Down regulation of estrogen receptors by androgens in the ZR-75-1 human breast cancer cell line. *Endocrinology* **125**:392-399.

Power, R.F., Lydon, J.P., Conneely, O.M. and O'Malley, B.W. (1991). Dopamine activation of an orphan of the steroid receptor superfamily. *Science* **252**:1546-1548.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. *Nature* **335**:683-689.

Ptashne, M. and Gann, A.A.F. (1990). Activators and targets. *Nature* **346**:329-331.

Quarmby, V.E., Yarbrough, W.G., Lubahn, D.B., French, F.S. and Wilson, E.M. (1990a). Autologous down-regulation of androgen receptor messenger ribonucleic acid. *Mol. Endocrinol.* **4**:22-28.

Quarmby, V.E., Kemppainen, J.A., Sar, M., Lubahn, D.B., French, F.S. and Wilson, E.M. (1990b). Expression of recombinant androgen receptor in cultured mammalian cells. *Mol. Endocrinol.* **4**:1399-1407.

Radanyi, C., Renoir, J.M., Sabbah, M. and Baulieu, E-E. (1989). Chick heat shock protein of Mr = 90,000, free or released from progesterone receptor, is in a dimeric form. *J. Biol. Chem.* **264**:2568-2573.

Redeuilh, G., Moncharmont, B., Secco, C. and Baulieu, E-E. (1987). Subunit composition of the molybdate-stabilized "8-9S" nontransformed estradiol receptor purified from calf uterus. *J. Biol. Chem.* **262**:6969-6975.

Reiss, M.D., Dibble, C.L. and Sartorelli, A.C. (1988). Down regulation of glucocorticoid binding sites by retinoic acid in squamous carcinoma cells resistant to the induction of keritinization by hydrocortisone. *J. Invest. Dermatol.* **91**:344-348.

Rennie, P.S., Bruchovsky, N., Leco, K.J., Sheppard, P.C., McQueen, S.A., Cheng, H., Snoek, R., Hamel, A., Bock, M.E., MacDonald, B.S., Nickel, B.E., Chang, C., Liao, S., Cattini, P. and Matusik, R.J. (1993). Characterization of two *cis*-acting DNA elements involved in the androgen regulation of the probasin gene. *Mol. Endocrinol.* 7:23-36.

Renoir, J-M., Buchou, T. and Baulieu, E-E. (1986). Involvement of a nonhormone-binding 90-kilodalton protein in the nontransformed 8S form of the rabbit uterus progesterone receptor. *Biochemistry* **25**:6405-6413.

Rheaume, C., Barbour, K.W., Tseng-Crank, J. and Bergerm F.G. (1989). Molecular genetics of androgen-inducible RP2 gene transcription in the mouse kidney. *Mol. Cell. Biol.* **9**:477-483.

Rhee, M., Dimaculangan, D. and Berger, F.G. (1991). Androgen modulation of DNA-binding factors in the mouse kidney. *Mol. Endocrinol.* **5**:564-572.

Richard, S. and Zingg, H.H. (1991). Identification of a retinoic acid response element in the human oxytocin promoter. *J. Biol. Chem.* **266**:21428-21433.

Richards, J.F. (1975). Ornithine decarboxylase activity in tissues of prolactin treated rats. *Biochem. Biophys. Res. Commun.* **63**:292-299.

Riegman, P.H.J., Vlietstra, R.J., van der Korput, J.A.G.M., Brinkmann, A.O. and Trapman, J. (1991). The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Mol. Endocrinol.* **5**:1921-1930.

Rittmaster, R.S., Magor, K.E., Manning, A.P., Norman, R.W. and Lazier, C.B. (1991). Differential effect of 5α-reductase inhibition and castration on androgen-regulated gene expression in rat prostate. *Mol. Endocrinol.* **5**:1023-1029.

Robins, D.M., Adler, A.J., Scheller, A. and Scarlett, C.O. (1992). Specificity of hormonal response of the androgen dependent enhancer of the mouse Slp gene. In: The 74th annual meeting of the Endocrine Society, #1513, San Antonio, Texas.

Rochette-Egly, C., Lutz, Y., Saunders, M., Scheuer, I., Gaub, M-P. and Chambon, P. (1991). Retinoic acid receptor γ: specific immunodetection and phosphorylation. *J. Cell Biol.* **115**:535-545.

Roeder, R.G. (1993). Structure and functional interactions of general transcription initiation factors, regulatory factors, and cofactors. In: Mechanism of action of retinoids, vitamin D, and steroid hormones, March 15-20, Banff, Canada.

Roman, S.D., Clarke, C.L., Hall, R.E., Alexander, I.E. and Sutherland, R.L. (1992). Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.* **52**:2236-2242.

Romrell, L.J. and Bland, K.I. (1991). Anatomy of the breast axilla, chest wall and related metastatic sites. In: *The Breast: comprehensive management of benign and malignant diseases.* (Bland, K.I. and Copeland, E.M., eds.), pp. 17-35, Saunders, Philadelphia.

Rosen, J.M., Jones, W.K., Rodgers, J.R., Compton, J.R., Bisbee, C.A., David-Inouye, Y. and Yu-Lee, L-Y. (1986). Regulatory sequences involved in the hormonal control of casein gene expression. *Ann. NY Acad. Sci.* **464**:87-99.

Rosen, J.M., Poyet, P., Goodman, J. and Lee, K-F. (1989). Mechanisms by which prolactin and glucocorticoids regulate casein gene expression. *Biochem. Soc. Symp.* **55**:115-123.

Rosen, E.D., O'Donnell, A.L. and Koenig, R.J. (1991). Protein-protein interactions involving *erbA* superfamily receptors: through the TRAPdoor. *Mol. Cell. Endocrinol.* **78**:C83-C88.

Rosinski-Chupin, I. and Rougeon, F. (1990). A new member of the glutamine-rich protein gene family is characterized by the absence of internal repeats and the androgen control of its expression in the submandibular gland of rats. *J. Biol. Chem.* **265**:10709-10713.

Rottman, J.N., Widom, R.L., Nadal-Ginard, B., Mahdavi, V. and Karathanasis, S.K. (1991). A retinoic acid-responsive element in the apolipoprotein A1 gene distinguishes between two different retinoic acid response pathways. *Mol. Cell. Biol.* **11**:3814-3820.

Rowinsky, E.K., Cazenave, L.A. and Donehower, R.C. (1990). Taxol: a novel investigational antineoplastic agent. *J. Natl. Cancer Inst.* **82**:1247-1259.

Rozakis-Adcock, M. and Kelly, P.A. (1991). Mutational analysis of the ligand-binding domain of the prolactin receptor. *J. Biol. Chem.* **266**:16472-16477.

Rozakis-Adcock, M. and Kelly, P.A. (1992). Identification of ligand binding determinants of the prolactin receptor. *J. Biol. Chem.* **267**:7428-7433.

Rundlett, S.E., Wu, X-P. and Miesfeld, R.L. (1990). Functional characterizations of the androgen receptor confirm that the molecular basis of androgen action is transcriptional regulation. *Mol. Endocrinol.* **4**:708-714.

Rush, M.G., Ul-Haq, R. and Chytil, F. (1991). Opposing effects of retinoic acid and dexamethasone on cellular retinol-binding protein ribonucleic acid levels in the rat. *Endocrinology* **129**:705-709.

Rushmere, N.K., Claessens, F., Weir, A.M.K., Verhoeven, G., Rombauts, W.A. and Davies, P. (1990). Functional analysis of intronic androgen response elements. In: The American Association for Cancer Research Special Conference: Steroid Receptors, Transcription Factors and Gene Expression, #A-19, San Diego, California.

Sagami, I., Tsai, S.Y., Wang, H., Tsai, M-J. and O'Malley, B. (1986). Identification of two factors required for transcription of the ovalbumin gene. *Mol. Cell. Biol.* **6**:4259-4267.

Sanchez, E.R., Toft, D.O., Schlesinger, M.J. and Pratt, W.B. (1985). Evidence that the 90-kdalton phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. *J. Biol. Chem.* **260**:12398-12401.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H. and Vennstrom, B. (1986). The *c-erbA* protein is a high affinity receptor for thyroid hormone. *Nature* **124**:635-640.

Sauer, R.T. (1990). Scissors and helical forks. *Nature* **347**:514-515.

Schaller, J., Akiyama, A., Kimura, H., Hess, D., Affolter, M. and Rickli, E.E. (1991). Primary structure of a new actin-binding protein from human seminal plasma. *Eur. J. Biochem.* **196**:743-750.

Schüle, R., Muller, M., Otsuka-Murakami, H. and Renkawitz, R. (1988). Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. *Nature* **332**:87-90.

Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M. and Evans, R.M. (1990a). Functional antagonism between oncoprotein *c-Jun* and the glucocorticoid receptor. *Cell* **62**:1217-1226.

Schüle, R., Umesono, K., Mangelsdorf, D.J., Bolado, J., Pike, J.W. and Evans, R.M. (1990b). *Jun-fos* and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell* **61**:497-504.

Schüle, R., Rangarajan, P., Yang, N., Kliewer, S., Ransone, L.J., Bolado, J., Verma, I.M. and Evans, R.M. (1991). Retinoic acid is a negative regulator of AP-1 responsive genes. *Proc. Natl. Acad. Sci. USA* **88**:6092-6096.

Schwabe, J.W., Neuhaus, D. and Rhodes, D. (1990). Solution structure of the DNA-binding domain of the oestrogen receptor. *Nature* **348**:458-461.

Secreto, G., Toniolo, P., Berrino, F., Recchione, C., Cavalleri, A., Pisani, P., Totis, A., Fariselli, G. and Di Pieto, S. (1991). Serum and urinary androgens and risk of breast cancer in postmenopausal women. *Cancer Res.* **51**:2572-2576.

Serghini, M.A., Ritzenthaler, C. and Pinck, L. (1989). A rapid and efficient "miniprep" for isolation of plasmid DNA. *Nucleic Acids Res.* **17**:3604.

Sharif, M. and Privalsky, M.L. (1991). *v-erbA* oncogene function in neoplasia correlates with its ability to repress retinoic acid receptor function. *Cell* **66**:885-893.

Shirota, M., Banville, D., Ali, S., Jolicoeur, C., Boutin, J.M., Edery, M., Djiane, J. and Kelly, P.A. (1990). Expression of two forms of prolactin receptor in rat ovary and liver. *Mol. Endocrinol.* **4**:1136-1143.

Shiu, R.P.C. (1979). Prolactin receptors in human breast cancer cells in long term tissue culture. *Cancer Res.* **39**:4381-4386.

Shiu, R.P.C., Elsholtz, H.P., Tanaka, T., Friesen, H.G., Gout, P.W., Beer, C.T. and Noble, R.L. (1983). Receptor-mediated mitogenic action of prolactin in a rat lymphoma cell line. *Endocrinology* **113**:159-165.

Shiu, R.P.C. and Paterson, J.A. (1984). Alteration of cell shape, adhesion and lipid accumulation by prolactin in human breast cancer cells. *Cancer Res.* **44**:1178-1186.

Shiu, R.P.C. and Iwasiow, B.M. (1985). Prolactin-inducible proteins in human breast cancer cells. *J. Biol. Chem.* **260**:11307-11313.

Shiu, R.P.C., Murphy, L.C., Tsuyuki, D., Myal, Y., Lee-Wing, M. and Iwasiow, B. (1987). Biological actions of prolactin in human breast cancer. *Recent Prog. Horm. Res.* **43**:277-303.

Simard, J., Luthy, I., Guay, J., Belanger, A. and Labrie, F. (1986). Characteristics of interaction of the antiandrogen flutamide with the androgen receptor in various target tissues. *Mol. Cell. Endocrinol.* **44**:261-270.

Simard, J., Hatton, A-C., Labrie, C., Dauvois, S., Zhao, H.F., Haagensen, D.E. and Labrie, F. (1989). Inhibitory effect of estrogens on GCDFP-15 mRNA levels and secretion in ZR-75-1 human breast cancer cells. *Mol. Endocrinol.* **3**:694-702.

Simental, J.A., Sar, M., Lane, M.V., French, F.S. and Wilson, E.M. (1991). Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J. Biol. Chem.* **266**:510-518.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P.W., Boncinelli, E. and Mavilio, F. (1990). Sequential activation of *Hox2* homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature* **346**:763-766.

Simons, S.S., Oshima, H. and Szapary, D. (1992). Higher levels of control: modulation of steroid hormone-regulated gene transcription. *Mol. Endocrinol.* **6**:995-1002.

Skidmore, J.R., Walpole, A.L. and Woodburn, J. (1972). Effect of some triphenylethylenes on oetradiol binding *in vitro* to macromulecules from uterus and anterior pituitary. *J. Endocrinol.* **52**:289-298.

Sladek, F.M., Zhong, W., Lai, E. and Darnell, J.E. (1990). Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* **4**:2353-2365.

Smale, S.T. and Baltimore, D. (1989). The "initiator" as a transcription control element. *Cell* **57**:103-113.

Smith, W.C., Nakshatri, H., Leroy, P., Rees, J. and Chambon, P. (1991). A retinoic acid response element is present in the mouse cellular retinol binding protein I (CRBPI) promoter. *EMBO J.* **10**:2223-2230.

Soares, M.J., Faria, T.N., Roby, K.F. and Deb, S. (1991). Pregnancy and the prolactin family of hormones: coordination of anterior pituitary, uterine and placental expression. *Endocrine Rev.* **12**:402-423.

Sonnenschein, C., Olea, N., Pasanen, M.E. and Soto, A.M. (1989). Negative controls of cell proliferation: human prostate cancer cells and androgens. *Cancer Res.* **49**:3474-3481.

Spence, A.M., Sheppard, P.C., Davie, J.R., Matsuo, Y., Nishi, N., Mcheehan, W.L., Dodd, J.G. and Matusik, R.J. (1989). Regulation of a bifunctional mRNA in synthesis of secreted and nuclear probasin. *Proc. Natl. Acad. Sci. USA* **86**:7843-7847.

Sporn, M.B., Dunlop, N.M., Newton, D.L. and Smith, J.M. (1976). Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Fed. Proc.* **35**:1332-1338.

Stoner, C.M. and Gudas, L.J. (1989). Mouse cellular retinoic acid binding protein: cloning, complementary DNA sequence, and messenger RNA expression during the retinoic acid-induced differentiation of F9 wild type and RA-3-10 mutant teratocarcinoma cells. *Cancer Res.* **49**:1497-1504.

Strähle, U., Klock, G. and Schutz, G. (1987). A DNA sequence of 15 base pairs is sufficient to mediate both glucocorticoid and progesterone induction of gene expression. *Proc. Natl. Acad. Sci. USA* **84**:7871-7875.

Strähle, U., Schmid, W. and Schutz, G. (1988). Synergistic action of the glucocorticoid receptor with transcription factors. *EMBO J.* 7:3389-3395.

Stred, S.E., Stubbart, J.R., Argetsinger, L.S., Shafer, J.A. and Carter-Su, C. (1990). Demonstration of growth hormone (GH) receptor-associated tyrosine kinase activity in multiple GH-responsive cell types. *Endocrinology* **127**:2506-2516.

Struhl, K. (1991). Acid connections. *Current biol.* **1**:188-191.

Sucov, H.M., Murakami, K.K. and Evans, R.M. (1990). Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. *Proc. Natl. Acad. Sci. USA* **87**:5392-5396.

Tabin, C.J. (1991). Retinoids, homeoboxes and growth factors: toward molecular models for limb development. *Cell* **66**:199-217.

Tan, J-A., Marschke, K.B., Ho, K-C. and Perry, S.T. (1992). Response elements of the androgen-regulated C3 gene. *J. Biol. Chem.* **267**:4456-4466.

Tanaka, H., Dong, Y., Li, Q., Okret, S. and Gustafsson, J-Å. (1991). Identification and characterization of a *cis*-acting element that interferes with glucocorticoid-inducible activation of the mouse mammary tumor virus promoter. *Proc. Natl. Acad. Sci. USA* **88**:5393-5397.

Tasset, D., Tora, L., Fromental, C., Scheer, E. and Chambon, P. (1990). Distinct classes of transcriptional activating domains function by different mechanisms. *Cell* **62**:1177-1187.

Tellam, R.L., Morton, D.J. and Clarke, F.M. (1989). A common theme in the amino acid sequences of actin and many actin-binding proteins. *Trends Biochem. Sci.* **14**:130-133.

Thompson, J.N., Howell, J.M., Pitt, G.A.J. and McLaughlin, C.I. (1969). The biological activity of retinoic acid in the domestic fowl and the effects of vitamin A deficiency on the chick embryo. *Br. J. Nutr.* **23**:471-490.

Thompson, C.C., Weinberger, C., Lebo, R. and Evans, R.M. (1987). Identification of a novel thyroid hormone receptor expressed in the mammalian central nervous system. *Science* **237**:1610-1614.

Thompson, K.L. and Rosner, M.R. (1989). Regulation of epidermal growth factor receptor gene expression by retinoic acid and epidermal growth factor. *J. Biol. Chem.* **264**:3230-3234.

Thoreau, E., Petridou, B., Kelly, P.A., Djiane, J. and Morton, J.P. (1991). Structural symmetry of extracellular domain of the cytokine/growth hormone/prolactin receptor family and interferon receptors revealed by hydrophobic cluster analysis. *FEBS Lett.* **282**:26-31.

Tilley, W.D., Marcelli, M., Wilson, J.D. and McPhaul, M.J. (1989). Characterization and expression of a cDNA encoding the human androgen receptor. *Proc. Natl. Acad. Sci. USA* **86**:327-331.

Tilley, W.D., Marcelli, M. and McPhaul, M.J. (1990a). Expression of the human androgen receptor gene utilizes a common promoter in diverse human tissues and cell lines. *J. Biol. Chem.* **265**:13776-13781.

Tilley, W.D., Wilson, C.M., Marcelli, M. and McPhaul, M.J. (1990b). Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res.* **50**:5382-5386.

Tong, P.S., Horowitz, N.N. and Wheeler, L.A. (1990). Transretinoic acid enhances the growth response of epidermal keratinocytes to epidermal growth factor and transforming growth factor β. *J. Invest. Dermatol.* **94**:126-131.

Too, C.K.L., Shiu, R.P.C. and Friesen, H.G. (1990). Cross-linking of G-proteins to the prolactin receptor in rat Nb2 lymphoma cells. *Biochem. Biophys. Res. Commun.* **173**:48-52.

Topper, Y.J., Sankaran, L., Chomczynski, P., Prosser, C. and Qasba, P. (1986). Three stages of responsiveness to hormones in the mammary cell. *Ann. NY Acad. Sci.* **464**:1-10.

Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E. and Chambon, P. (1989). The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59**:477-487.

Trapman, J., Klaassen, P., Kuiper, G.G.J.M., van der Korput, J.A.G.M., Faber, P.W., van Rooij, H.C.J., Geurts van Kessel, A., Voorhorst, M.M., Mulder, E. and Brinkmann, A.O. (1988). Cloning, structure and expression of a cDNA encoding the human androgen receptor. *Biochem. Biophys. Res. Commun.* **153**:241-248.

Tugwood, J.D., Isseman, I., Anderson, R.G., Bundell, K.R., McPhe, W.L. and Green, S. (1992). The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *EMBO J.* **11**:433-439.

UCLA Colloquium Meeting Report (1990). New insights into breast cancer: the molecular biochemical and cellular biology of breast cancer. *Cancer Res.* **50**:4446-4447.

Umesono, K., Giguere, V., Glass, C.K., Rosenfeld, M.G. and Evans, R.M. (1988). Retinoic acid and thyroid hormone induce gene expression through a common responsive element. *Nature* **336**:262-265.

Umesono, K. and Evans, R.M. (1989). Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57**:1139-1146.

Umesono, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D_3 receptors. *Cell* **65**:1255-1266.

Urbanek, M., MacLeod, J.N., Cooke, N.E. and Liebhaber, S.A. (1992). Expression of a human growth hormone (hGH) receptor isoform is predicted by tissue-specific alternative splicing of exon 3 of the hGH receptor gene transcript. *Mol. Endocrinol.* **6**:279-287.

Vallette, F., Mege, E., Reiss, A. and Adesnik, M. (1989). Construction of mutant and chimeric genes using the polymerase chain reaction. *Nucleic Acids Res.* **17**:723-733.

van Laar, J.H., Berrevoets, C.A., Trapman, J., Zegers, N.D. and Brinkmann, A.O. (1991). Hormone-dependent androgen receptor phosphorylation is accompanied by receptor transformation in human lymph node carcinoma of the prostate cells. *J. Biol. Chem.* **266**:3734-3738.

Vasios, G., Gold, J.H., Petkovich, M., Chambon, P. and Gudas, L.J. (1989). A retinoic acid-responsive element is present in the 5'-flanking region of the laminin B1 gene. *Proc. Natl. Acad. Sci. USA* **86**:9099-9013.

Vasios, G., Mader, S., Gold, J.D., Leid, M., Lutz, Y., Gaub, M-P., Chambon, P. and Gudas, L. (1991). The late retinoic acid induction of laminin B1 gene transcription involves RAR binding to the responsive element. *EMBO J.* **10**:1149-1158.

Veldscholte, J., Ris-Stalpers, C., Kuiper, G.G.J.M., Jenster, G., Berrevoets, C., Claessen, E., van Rooij, H.C.J., Trapman, J., Brinkmann, A.O. and Mulder, E. (1990). A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **173**:534-540.

Vogelstein, B. (1990). A deadly inheritance. *Nature* **348**:681-682.

von der Ahe, D., Renoir, J-M., Baulieu, E.E. and Beato, M. (1986). Receptors for glucocorticosteroid and progesterone recognize distinct features of a DNA regulatory element. *Proc. Natl. Acad. Sci. USA* **83**:2817-2821.

Vonderhaar, B.K. (1977). Studies on the mechanism by which thyroid hormones enhance α-lactalbumin activity in explants from mouse mammary glands. *Endocrinology* **100**:1423-1431.

Vonderhaar, B.K. (1984). Hormones and growth factors in mammary gland development. In: *Control of cell growth and proliferation.* (Venziale, C.M., ed.) pp. 11-33, Van Vostrand Reinhold, New York, NY.

Vonderhaar, B.K. and Ziska, S.E. (1989). Hormonal regulation of milk protein gene expression. *Annu. Rev. Physiol.* **51**:641-652.

Wang, S-Y., LaRosa, G.J. and Gudas, L.J. (1985). Molecular cloning of gene sequences transcriptionally regulated by retinoic acid and dibutyryl cyclic AMP in cultured mouse teratocarcinoma cells. *Dev. Biol.* **107**:75-86.

Wang, S-Y. and Gudas, L.J. (1988). Protein synthesis inhibitors prevent the induction of laminin B1, collagen IV (alpha 1) and other differentiation-specific mRNAs by retinoic acid in F9 teratocarcinoma cells. *J. Cell. Physiol.* **136**:305-311.

Warrell, R.P., Frankel, S.R., Miller, W.H., Scheinberg, D.A., Itri, L.M., Hittelman, W.N., Vyas, R., Andeff, M., Tafuri, A. and Jakubowski, A. (1991). Differentiation therapy of acute promyelocytic leukaemia by tretinoin (all *trans*-retinoic acid). *New Eng. J. Med.* **324**:1385-1393.

Watson, G. and Paigen, K. (1990). Progressive induction of mRNA synthesis for androgen-responsive genes in mouse kidney. *Mol. Cell. Endocrinol.* **68**:67-74.

Watt, K.W., Lee, P.J., M'Timkulu, T., Chan, W.P. and Loor, R. (1986). Human prostate-specific antigen: structural and functional similarity with serine protease. *Proc. Natl. Acad. Sci. USA* **83**:3166-3170.

Wei, L.L., Krett, N.L.L., Francis, M.D., Gordan, D.F., Wood, W.M., O'Malley, B.S. and Horwitz, K.B. (1988). Multiple human progesterone receptor messenger ribonucleic acids and their autoregulation by progestin agonists and antogonists in breast cancer cells. *Mol. Endocrinol.* **2**:62-72.

Weigel, N.L., Beck, C.A., Estes, P.A., Prendergast, P., Altmann, M., Christensen, K. and Edwards, D.P. (1992). Ligands induce conformational changes in the carboxy-terminus of progesterone receptors which are detected by a site-directed antipeptide monoclonal antibody. *Mol. Endocrinol.* **6**:1585-1597.

Welsch, C.W. (1985). Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. *Cancer Res.* **45**:3415-3443.

Wenderoth, U.K., George, F.W. and Wilson, J.D. (1983). The effect of a 5α-reductase inhibitor on androgen-mediated growth of the dog prostate. *Endocrinology* **113**:569-574.

Wetherall, N.T. and Taylor, C.M. (1986). The effects of retinoid treatment and antiestrogens on the growth of T-47D human breast cancer cells. *Eur. J. Cancer Clin. Oncol.* **22**:53-59.

Wikstrom, A-C., Bakke, O., Okret, S., Bronnegard, M. and Gustafsson, J-A. (1987). Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology* **120**:1232-1242.

Willett, W.C., Stampfer, M.J. and Colditz, G.A. (1987). Dietary fat and the risk of breast cancer. *N. Engl. J. Med.* **316**:22-28.

Wilson, E.M. (1992). Molecular mechanisms of androgen action. In: The 74th annual meeting of the Endocrine Society, p.38, San Antonio, Texas.

Wilson, T.E., Paulsen, R.E., Padgett, K.A. and Milbrandt, J. (1992). Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. *Science* **256**:107-110.

Windass, J.D., Mullins, J.J., Beecroft, L.J., George, H., Meacock, P.A., Williams, B.R.G. and Brammer, W.J. (1984). Molecular cloning of cDNAs from androgen-independent mRNA species of DBA/2 mouse submaxillary glands. *Nucleic Acid Res.* **12**:1361-1376.

Wolbach, S.B. and Howe, P.R. (1925). Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.* **42**:753-777.

Xie, Y-B., Sui, Y-P., Shan, L-X., Palvimo, J.J., Philips, D.M. and Jänne, O.A. (1992). Expression of androgen receptor in insect cells. *J. Biol. Chem.* **267**:4939-4948.

Yang, N., Schüle, R., Mangelsdorf, D.J. and Evans, R.M. (1991). Characterization of DNA binding and retinoic acid binding proterties of retinoic acid receptor. *Proc. Natl. Acad. Sci. USA* **88**:3559-3563.

Yang-Yen, H-F., Chambard, J-C., Sun, Y-L., Smeal, T., Schmidt, T.J., Drouin, J. and Karin, M. (1990). Transcriptional interference between *c-Jun* and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**:1205-1215.

Yarbrough, W.G., Quarmby, V.E., Simental, J.A., Joseph, D.R., Sar, M., Lubahn, D.B., Olsen, K.L., French, F.S. and Wilson, E.M. (1990). A single base mutation in the androgen receptor gene causes androgen insensitivity in the testicular feminized rat. *J. Biol. Chem.* **265**:8893-8900.

Yin, H.L. and Hartwig, J.H. (1988). The structure of the macrophage actin skeleton. *J. Cell Sci. Suppl.* **9**:169-184.

Young, C.Y-F., Montgomery, B.T., Andrews, P.E., Qui, S., Bilhartz, D.L. and Tindall, D.J. (1991). Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. *Cancer Res.* **51**:3748-3752.

Young, C.Y-F. and Andrews, P.E. (1992). Modulation of androgenic regulation of gene expression by retinoic acid in the human prostate cancer cell line, LNCaP. In: The 74th annual meeting of the Endocrine Society, #1512, San Antonio, Texas.

Yu, V.C., Delsert, C., Andersen, B., Holloway, J.M., Devary, O.V., Näär, A.M., Kim, S.Y., Boutin, J-M., Glass, C.K. and Rosenfeld, M.G. (1991). RXRβ: A coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements. *Cell* **67**:1251-1266.

Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P. (1989). Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. *Nature* **339**:714-717.

Zelent, A., Mendelsohn, C., Kastner, P., Garnier, J.M., Ruffenach, F., Leroy, P. and Chambon, P. (1991). Differentially expressed isoforms of the mouse retinoic acid receptor beta are generated by usage of two promoters and alternative splicing. *EMBO J.* **10**:71-81.

Zhang, X-K., Tran, P.B-V. and Pfahl, M. (1991). DNA binding and dimerization determinants for thyroid hormone receptor α and its interaction with a nuclear protein. *Mol. Endocrinol.* **5**:1909-1920.

Zhang, X-K., Hoffmann, B., Tran, P.B-V., Graupner, G. and Pfahl, M. (1992). Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* **355**:441-446.

Zoppi, S., Marcelli, M., Deslypere, J-P., Griffin, J.E., Wilson, J.D. and McPhaul, M.J. (1992). Amino acid substitutions in the DNA-binding domain of the human androgen receptor are a frequent cause of receptor-binding positive androgen resistance. *Mol. Endocrinol.* **6**:409-415.