Effects of Epidermal Growth Factor (EGF) and Androgens on Prostatic Gene Regulation in a Transgenic Animal Model

By

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EFFECTS OF EPIDERMAL GROWTH FACTOR (EGF) AND ANDROGENS ON PROSTATIC GENE REGULATION IN A TRANSGENIC ANIMAL MODEL

BY

LIMEI HU

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

ABSTRACT

Normal prostate function involves the continual interaction of androgens and peptide growth factors including epidermal growth factor (EGF). EGF expression is under androgenic regulation in the mouse ventral prostate and submandibular gland. Surgical removal of the salivary glands (sialoadenectomy, Sx) results in elevation of circulating androgens over time. We hypothesize the EGF effects gene regulation in the prostate through androgen receptor-dependent mechanisms. To study the effect of this interaction on gene expression in the prostate both *in vivo* and *in vitro,* we used the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the minimal rat probasin (PB) gene promoter. Expression of this transgene is targeted specifically to the prostate in transgenic mice and is developmentally and hormonally regulated. The effects of castration-induced androgen withdrawal and sialoadenectomy-induced EGF withdrawal on the PB-CAT expression were examined *in vivo* using PB-CAT transgenic mice and *in vitro* using transient transfection assays. In addition, the effects of hormonal manipulation on EGF, epidermal growth factor receptor (EGF-R), transforming growth factor alpha (TGF-α) and androgen receptor (AR) gene transcription in the different lobes of the normal mouse prostate were also investigated using reverse transcriptase-polymerase chain reaction (RT-PCR).

In adult transgenic mice, sialoadenectomy(Sx) resulted in a 10-fold reduction in PB-CAT expression in the dorsolateral prostate (DLP) by day 14. However by 28 days post-Sx, PB-CAT levels had returned to pre-Sx levels without further treatment. Daily administration of EGF(100μg/kg/day) following Sx blocked the initial decrease in PB-

CAT expression in the DLP. In contrast, expression of PB-CAT in the ventral prostate (VP) was not altered significantly by Sx with or without EGF administration. Following castration (Cx), PB-CAT levels declined to 1% or less of the pre-Cx level in both DLP and VP. Expression was restored in both lobes by administration of dihydrotestosterone (DHT) alone but not by EGF alone. Treatment with both DHT and EGF resulted in the maximum level of PB-CAT expression in both DLP and VP.

A somewhat different response is observed when PB-CAT is transferred into the human prostate cancer cell line-(DU145). EGF alone can increase the CAT activity in the presence or absence of the androgen receptor and under conditions with or without DHT ($p<0.001$). The PB-CAT expression can be blocked partially by adding anti-human EGF-receptor antibody and this reduction can be reversed by adding EGF ($p<0.005$). As with the transgenic model, the maximum expression of PB-CAT occurred with both EGF and DHT.

Examination of the expression of the EGF-receptor and its ligands in the normal mouse prostate revealed the following: compared to the dorsal prostate (DP), the EGF and EGF-R mRNA levels of the ventral prostate (VP) were lower ($p<0.001$) but TGF-α and AR mRNA levels were higher ($p<0.001$) in the VP of normal mice. After 14 days post- Sx, EGF, EGF-R, TGF-α and AR mRNA levels were increased in DP compared to the controls. By 28 days post-Sx however, EGF-R and AR mRNA levels had returned to the control levels but EGF mRNA level was decreased whereas TGF-α mRNA level remained higher than control. In contrast, the TGF-α mRNA level was decreased after 14 days post- Sx and all the mRNA levels except EGF-R were decreased by 28 days post-Sx

in VP. In the Cx groups, the mRNAs showed similar patterns as the Sx groups in DP. In VP after Cx, the mRNAs showed similar patterns as the Sx group except that EGF mRNA level was lower by 14 day post-Cx. In lateral prostate (LP), all the mRNAs were decreased compared to the controls in both Cx and Sx groups.

In summary, we confirmed the following in this project:

(1). The -426/+28 PB-CAT transgene was expressed in the prostate tissues of the transgenic mice *in vivo* and both castration(Cx) and sialoadenectomy(Sx) affected the transgene expression. Cx affected the expression in all lobes of the prostate, however, Sx only affected the expression in dorsolateral but not in ventral prostate.

(2). In normal mouse prostate, relative mRNA levels of both EGF and EGF-R were higher in dorsal and lateral prostate than in ventral prostate. The dorsal prostate possessed the lowest AR mRNA level compared with lateral and ventral prostate.

(3). Both Cx and Sx affected EGF, EGF-R, TGF-α and AR mRNA expression in the mouse prostate. However, patterns of the effects were different among different lobes and after different periods post- Cx or Sx.

(4). Both EGF and DHT induced the PB-CAT expression in DU145 cells. Under conditions with or without cotransfection with the AR expression vector , EGF induced the CAT activity which indicated that the EGF induction was androgen-receptor independent in the cells.

(5). A series of deletions from both 5' and/or 3' of the PB 5'-flanking DNA fragment from -426 to +28 bp did not significantly affect EGF induction of the CAT activity. The smallest construct tested contains a PB 5'-flanking fragment from -141 to

-91 bp, which still is EGF-responsive; we propose that a putative EGF regulatory element exists in this region, possibly an AP1-like sequence.

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From bottom of my heart, I am deeply grateful to my advisor Dr. Janice G. Dodd for her constant guidance, encouragement and constructive suggestions through all phases of my research project. I have benefited greatly in many ways by studying and working with her.

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LIST OF ABBREVIATIONS

A_{260}	absorbance at 260nm
Ab-hEGF-R	anti-human EGF-R antibody
AR	androgen receptor
ARE	androgen response element
bp	base pairs
BPH	benign prostatic hyperplasia
^0C	degrees centigrade
CaP	prostatic carcinoma
CAT	chloramphenical acetyltransferase
cDNA	complementary DNA
cpm	counts per minute
CO_2	carbon dioxide
ddH_2O	double distilled water
DHT	dihydrotestosterone
DLP	dorsolateral prostate
DNA	deoxyribonucleic acid
dpm	degradation per minute
DTT	dithiothreitol
DU-145	human prostatic adenocarcinoma cells
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor

EGF-R	epidermal growth factor receptor
ER	estrogen receptor
erb	erythroblastoma
EtBr	ethidium bromide
FCS	fetal calf serum
GAP	GTPase-activating protein
G3PDH	mouse glyceraldehyde 3-phosphate dehydrogenase
HCl	hydrochloric acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IGF	insulin like growth factor
KCl	potassium chloride
Kd	kilodaltons
Kb	kilobase
LNCaP	human prostatic adenocarcinoma cells derived from the lymph nodes
LP	lateral prostate
µg	microgram
µl	microliter
MAP	mitogen-activated protein
MEM	minimum essential medium

mg	milligram
ml	milliliter
$MgCl_2$	magnesium chloride
mm	millimeter
M-MLV RT	moloney murine leukemia virus reverse transcriptase
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
nAR	nuclear androgen receptor
PB	probasin
PB-CAT	probasin CAT chimaeric construct
PBS	phosphate buffered saline
PC-3	human prostatic adenocarinoma cells
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3-kinase	phosphatidylinositol 3'-kinase
PKC	protein kinase C
rAR	rat androgen receptor
RNA	ribonucleic acid
S.E.	standard error
SV40	simian virus 40 antigen
TGF-α	transforming growth factor alpha

TGF-β	**transforming growth factor Beta**
TK	**thymidine kinase**
TK-CAT	**thymidine kinase CAT chimaeric construct**
Tris	**tris(hydroxymethyl)aminomethane**
v/v	**volume/volume**

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INTRODUCTION

Prostate gland and prostatic cancer

The prostate gland is the major accessory organ of the male reproductive tract which is located at the base of the urethra in mammalian males. In the rodents the prostate is composed of four anatomically distinct lobes: ventral (VP), dorsal (DP), lateral (LP), and anterior (coagulating gland). Because of size and proximity, the dorsal and lateral lobes are often examined collectively as dorsolateral (DLP) prostate. The ventral lobe is located below the ventral aspect of the bladder neck. The dorsolateral lobes lie in the triangular area between urethra and seminal vesicle (Cunha et al., 1987). In the human the prostate surrounds the urethra immediately below the bladder and lies between the symphysis pubis and the rectum. Although human prostate is not distinct in lobes as the rodent prostate, four morphologically discrete zones have been defined: the anterior fibromuscular stroma, the peripheral zone, the central zone and the transition zone (McNeal, 1983). The peripheral zone is considered the most homologous to the dorsolateral lobes and it has also been reported to be the place where the most carcinomas arise (McNeal, 1988).

Histological studies demonstrate that the prostate gland is composed of a complex array of ductal-acinar structures. Cunha et al. summarized the results obtained by McNeal, Franks and Bloom et al. and described the morphology of the prostate as follows: " In humans, the prostate is composed of compound tubuloalveolar or tubosaccular glands. The epithelium of the acini ranges from pseudostratified columnar to cuboidal. The distal ducts are lined by simple cuboidal or columnar epithelium, while transitional epithelium is seen in the proximal ducts as they emerge from the urethra. The rodent prostate is a compound

ductal gland completely lacking true acini and each lobe has a distinctive ductal branching pattern" (Bloom and Fawcett, 1975; Franks, 1976; McNeal, 1983; Cunha et al., 1987). The stromal component of the prostate is composed of all cellular and extracellular elements outside of the epithelial basal lamina and includes the smooth muscle cells, fibroblasts, blood vessels and their associated pericytes, wandering connective tissue cells, nerve terminals, and lymphatics (Aumuller, 1983). The relative numbers of epithelial and stromal cells within the prostate differ between species (Cunha et al., 1987). DeKlerk and Coffey have reported that the epithelial-stromal ratio in the adult rat is about 5:1 (DeKlerk and Coffey, 1978). However, Bartsch and coworkers have reported that in man the stromal and epithelial cells are present in approximately equal numbers (Bartsch and Rohr, 1980). It has been demonstrated that castration elicited a disproportionate loss of epithelial *vs.* stromal cells which changed the epithelial-stromal ratio by a factor of 10 (4.54:0.43) [DeKlerk and Coffey, 1978].

In rodents, branching of the prostatic ducts largely occurs postnantally and growth of the prostate essentially continuous processes which extend from late fetal life until sexual maturity (Cunha et al., 1987). However, in normal human prostate, the ductal morphogenesis and growth occur in two separate periods, prenatally and pubertally. While the pathological growth in benign prostate hyperplasia (BPH) is initiated in the fourth decades (Zondek and Zondek, 1975; Berry et al., 1985). The development of the prostate is androgen dependent (Cunha et al., 1987). Androgen production by the developing fetal testes begins before and continues throughout periods of prostatic morphogenesis (Siiteri et al., 1974; Resko, 1978; Pointis et al., 1980). Except androgens peptide growth factors are also identified to play roles in the control of normal and abnormal prostate growth. The

importance of androgens and growth factors in the control of prostate growth will be addressed later.

Prostate cancer is the most frequently diagnosed malignancy (other than that of skin) in American males and the second leading cause of cancer-related deaths in that group (Garnick, 1994). The etiology of prostate cancer remains unclear. According to the epidemiologic studies, Zaridze et al. have reported that there is no consistent correlation of prostatic cancer with diet, venereal disease, sexual habits, smoking, or occupational exposure (Zaridze and Boyle, 1987). However, Ross and coworkers have proposed that higher serum testosterone levels could be a major determinant of the risk of prostatic cancer (Ross et al., 1988). Based on the discovery that androgen withdrawal could significantly reduce the growth rate of the prostate, Huggins and his associates introduced the endocrine therapy for the treatment of the prostate cancer in 1940s (Huggins et al., 1940; Huggins et al., 1941a.b). Until now, it is still the best palliative treatment available for patients with prostatic cancer (Schroder, 1991). Unfortunately, this treatment can not prolong the life because the androgen independent cells will progress rapidly at some point. This evidence indicates that a critical dilemma facing clinicians treating patients with prostate cancer is what to do with patients with androgen independent cancer (Wilding, 1992).

Epidermal growth factor, epidermal growth factor receptor and transforming growth factor-α

Epidermal growth factor (EGF) is one of the best-known and characterized growth factors. It was originally isolated from the male mouse submaxillary gland by Cohen in 1962 (Cohen, 1962) and it was later sequenced (Savage et al., 1972). The location of three intramolecular disulphide bonds of the molecule was determined in 1973 (Savage et al.,

1973). The mouse EGF contains 53 amino acid residues and disulphide bonds between residues 5 and 20, 14 and 31, and 33 and 42 produce three disulphide loops in the secondary structure of the molecule (Carpenter, 1985). In the mid-1970s EGF was detected in(Starkey et al., 1975) and isolated from human urine (Cohen and Carpenter, 1975) and initially termed urogastrone. The primary amino acid sequence of the human form of this growth factor was also determined (Gregory, 1975). In humans, EGF has been detected in nearly all body fluids and multiple sites of synthesis of EGF are inferred. In the mouse, EGF is made predominantly in the submaxillary glands (Cohen, 1962), but secondary sites of synthesis are evident when the submaxillary glands are removed (Byyny et al., 1974). The mRNA for EGF is remarkably large with the 53 amino acid EGF peptide translated from an mRNA of approximately 4750 base pairs (Gray et al., 1983 and Scott et al., 1983). Ten sequences with a high degree of homology to EGF, including the EGF coding region itself are contained within this long coding region. Subsequent studies with transfected cells have demonstrated that prepro-EGF can exist as a glycosylated membrane protein (Mroczkowski et al., 1989). The observation that the EGF precursor often is not processed into mature EGF suggests that the EGF precursor may be a receptor for an unknown ligand (Pfeffer and Ullrich, 1985; Rall et al., 1985).

The epidermal growth factor receptor is the proto-oncogene of erb B1; both possess intrinsic protein tyrosine kinase activity, a property shared by several retroviral onc gene. The EGF receptor is detectable on a large variety of cell types or tissues with the exception of hemopoietic cells (Carpenter, 1987). The EGF receptor from A431 cells was first purified to near homogeneity in 1980 by the use of affinity chromatography. The mature EGF receptor, Mr =170,000, is composed of a single polypeptide chain of 1186 amino acid

residues. It is a transmembrane glycoprotein with an external EGF binding domain (Thompson and Gill, 1985) and a cytoplasmic kinase domain which has both the kinase activity and the autophosphorylation site (Yarden and Ullrich, 1988). The nucleotide sequence of the EGF receptor predicts that the two principal domains of the receptor are separated by a single hydrophobic membrane-spanning sequence (Ullrich et al., 1981). The cytoplasmic 542 amino acid carboxyl terminus contains a tyrosine kinase domain with structural features similar to those of other retroviral onc genes with tyrosine kinase activity (Ullrich et al., 1981; Downward et al., 1984; Lin et al., 1984; and Xu et al., 1984). The extracellular domain of the EGF receptor is characterized by its capacity to bind EGF and EGF-like ligands with high affinity (Carpenter et al., 1990). It has been demonstrated that the EGF receptor-mediated signal transduction is a direct protein-tyrosine kinase coupling pathway. The ligand binding to the extracellular receptor domain activates its tyrosine kinase activity that is intrinsic to the receptor and leads to increased intracellular phosphorylation as well as self-phosphorylation (Hunter and Cooper, 1981).

Recently an EGF-R gene knock-out animal model has been established (Threadgill et al., 1995; Sibilia and Wagner, 1995). Most of the embryos of the EGF-R null mutants had died in utero; those that survived until postnatal day 20 showed abnormal growth such that they were only 40-50% of the size and weight of control littermates. The investigators also observed: that the mutant placentas were structurally abnormal; the spongiotrophoblast layer was reduced in size; the surviving mutant fetuses had open eyes after embryonic day 16.5 and both skin and lungs of the null mutants were abnormal, too. In the skin, the mutant epidermis was thin, the layers not well distinguished and keratinization of the stratum corneum was almost absent. The lung tissue of the mutants showed undifferentiated

epithelium in the respiratory bronchioles and alveoli and increased amounts of cells in the alveolar septae. All these findings imply that the EGF-R plays an important role in the proliferation and differentiation of the epithelial compartments of these organs.

Transforming growth factor-α (TGF-α) was originally discovered in the conditioned medium from tumor cell cultures (De Larco and Todaro, 1978; Todaro et al., 1980). It was a member of a family of structurally related growth factors, of which EGF was the first member to be isolated and biochemically characterized (Savage et al., 1972, 1973). All members of the family interact with the same receptor, usually referred to as the EGF receptor (Massague, 1983; Stroobant et al., 1985 and Higashiyama et al., 1991). TGF-α is a 50-amino acid polypeptide synthesized as an internal segment of a 160-amino acid precursor (Derynck et al., 1984 and Lee et al., 1985). Based on the observation that TGF-α is highly expressed in fetal stages, Goustin and co-workers proposed that EGF may be the adult form of the embryonic growth factor TGF-α (Goustin et al., 1986).

The EGF/TGF-α responsiveness is mediated by the EGF receptor expressed by cells. It has been demonstrated that EGF and TGF-α play roles in the physiology of normal cells and tissues. EGF inhibits gastric acid secretion and protects the gastric mucosa from damage by the acid (Kirkegaard et al., 1983; Olsen et al., 1984). EGF is also a potent stimulator of cell multiplication and a modulator of the differentiation and function of cells of various types (Gregory, 1975; Hommel et al., 1991). Fisher and Lakshmanan summarized EGF's functions as follows:" EGF provokes precocious eyelid opening and tooth eruption in neonatal rodents; stimulates lung maturation in fetal rats, lambs, and rabbits; promotes palatal development in organ culture; stimulates gastrointestinal, liver, and pancreatic

maturation; promotes thyroid and adrenal gland growth; stimulates wound healing and mammary gland development; inhibits gastric acid secretion; evokes pituitary hormone secretion including GH, PRL, and ACTH; and stimulates CG and placental lactogen secretion from placental tissue" (Fisher and Lakshmanan, 1990). Epithelial cells are the major source of TGF-α synthesis under normal conditions (Derynck, 1992). Besides a presumed major role of TGF-α in proliferation of epithelia, TGF-α may also play a role in several other tissues. For example, TGF-α synthesis has been reported in activated macrophages (Madtes et al. 1988; Rappolee et al., 1988). The abundant presence of macrophages at sites of inflammation and wound healing and the effects of growth factor on cell proliferation strongly suggest that the role of the macrophage-released TGF-α is to participate in the wound-healing process and to stimulate the proliferation of epithelial cells. In addition, TGF-α is also able to induce proliferation of other cell types that have the EGF/TGF-α receptors, such as fibroblasts or endothelial cells (Derynck, 1992).

EGF, EGF-R and androgens in the control of prostate growth

Growth and the development of adult differentiated function in the prostate gland involves processes critically dependent on the presence of circulating androgens (Eaton et al., 1991). Immunohistochemical analysis of human prostate tissue sections with polyclonal or monoclonal antibodies has localized the androgen receptor (AR) protein predominantly in the nuclei of glandular epithelial cells (Lubahn et al, 1988a; Chang et al, 1989; Takeda et al, 1991). Embryologically, the AR mediates androgenic regulation of prostate epithelial differentiation via stromal-epithelial interactions (Wilding, 1992). Using tissue recombinant studies of the urogenital sinus and its epithelial and stromal components, it has been shown

that androgens regulate the growth, differentiation and morphogenesis of the prostate epithelium indirectly via the surrounding stroma (Cunha et al., 1983). Analysis of the AR in these tissue components demonstrates that a functional AR in the stroma, but not the epithelium during embryogenesis is the crucial factor for normal prostate development. The manner in which the androgen stimulated stroma mediates its influence on the developing epithelium is unclear, although extracellular matrix components and soluble growth factors are potential candidates (Wilding, 1992). The regulation of the adult prostate epithelium appears to be more complex, with both direct and indirect androgneic actions taking place.

The major circulating androgen in the male is testosterone of which 90-95% is testicular in origin (Lipsett, 1970). The testicular androgen is synthesized by the Leydig cells of the testis and its synthesis and release are regulated by luteinizing hormone (LH) (Berne and Levy, 1993). This steroid is metabolized to 5α-dihydrotestosterone (5α-DHT) in the prostatic tissues of most species and it is predominantly this androgen which interacts with prostatic androgen receptors, cellular proteins which mediate the specific activities of this class of steroid in the prostate (Davies and Griffiths, 1973). The importance of androgens in normal development of the prostate is shown by the evidence: (a) in congenital syndromes of androgen resistance, such as 5α-reductase deficiency or defects in androgen receptor function, are characterized by absent or deficient prostatic growth (Wilson, 1992) and (b) depletion of androgens from the circulation of experimental animals by orchidectomy results in a severe reduction in prostatic size and cellular content which can be restored by administration of exogenous androgen to these animals (Bruchovsky et al., 1975).

In addition to the crucial role of androgens in the normal development of the prostate gland and in maintaining its functional state in the adult, androgenic hormones have

long been recognized as an integral component of the development of the common prostatic diseases, benign prostatic hyperplasia (BPH) and prostate cancer (PCa)(Montie and Pienta, 1994). It has been demonstrated that androgens increase the incidence of N-methyl-nitrosourea (NMU) induced prostate cancer in rats (Bosland et al., 1990). It is also reported that the risk of developing prostate cancer for Nb rat was significantly increased by prolonged testosterone administration (Noble, 1977). There was noted to be a significant accumulation of testosterone in the PCa, relative to values in BPH (Montie and Pienta, 1994). Present evidence suggest that the roles of androgens and the AR in prostate carcinogenesis have two dimensions: (a) androgens and the AR play a seminal part in prostate carcinogenesis, suggesting a permissive role for androgens and (b) the actions of the AR as a transcription factor regulating the expression of proto-oncogenes, TGF-α and growth factor receptors in prostate epithelial cells (Wilding, 1992).

Based on the discovery that androgens can markedly accelerate the growth of prostate cancer and that withdrawal of such hormones can retard its growth, Huggins and his associates introduced endocrine therapy into the treatment of human prostate cancer beginning in the 1940s (Huggins et al., 1940 and 1941). All types of endocrine management of prostate cancer make use of mechanisms that are related either to hypothalamic, pituitary, testicular feedback mechanisms or to the capability of anti-androgens to counteract the effect of androgens at target cells (Schroder, 1991). Unfortunately, almost all metastatic tumors become resistant to hormonal therapy at some point and then progress rapidly. The transition from androgen dependent to independent growth in prostatic tumors is attributed to intrinsic heterogeneity in cellular responsiveness to androgen ablation at the outset of therapy, insensitive cells being able to continue growing and perhaps favored by the

selection pressure of androgen withdrawal (Eaton et al., 1991). The mechanisms by which the growth of these cells is sustained in the absence of androgens are unknown, but it is probable that these involve a combination of reduced sensitivity to normal growth constraints and dependence on other non-steroidal growth modulators (Eaton et al., 1988).

It is obvious that development of the prostate and maintenance of adult structure and function, as well as its pathological disturbances, such as human benign prostatic hyperplasia (BPH) and prostate cancer, are strongly influenced by testicular androgens. There is abundant evidence, however, that androgens are not the only substances capable of influencing these events in the prostate. The past decade of investigation has identified several polypeptides that either stimulate or inhibit growth (Story, 1991). Growth-promoting polypeptides related to epidermal growth factor (EGF) [Elson et al., 1984, Fowler et al., 1988], fibroblast growth factor (FGF) [Story et al., 1987, Mydlo et al., 1988, Nakamoto et al., 1992], insulin like growth factor (IGF) [Fiorelli et al., 1991, Knabbe et al., 1991] and transforming growth factor alpha (TGF-α) [Derynck, 1992] have been implicated in the control of prostate growth.

It has been identified that low levels of IGF-I are sufficient to support proliferation of both normal and tumor derived cultured prostate epithelial and mesenchymal cells (McKeehan et al., 1984). Fiorelli and co-workers have also reported that specific receptors for IGF-I are present in BPH tissues and that IGF-I binding capacity was significantly increased in prostatic tissue from patients affected by BPH treated with a long-acting luteinizing hormone-releasing hormone analog (Fiorelli et al., 1991). These observations further indicated a potential role for IGF-I in the mechanism of response to the castration-induced regression of androgen-dependent prostatic tissue.

The evidence that FGF is implicated in prostate cell growth includes: (a) epithelial and mesenchymal cells from normal prostate and various model rat prostate tumors exhibit specific membrane recpetor sites for FGF (Mansson et al., 1989) and (b) FGF expression has been detected in both BPH and prostate cancer specimens (Mydlo et al., 1988, Nakamoto et al., 1992).

EGF/TGF-α and EGF-R have been identified in the prostate and in cultured cells derived from the prostate and it has been demonstrated that they are important for maintenance of the structural and functional integrity of the benign prostatic epithelium (Sherwood and Lee, 1995). More and more evidence suggest that EGF/TGF-α and EGF-R play important roles in control of growth, especially abnormal growth, of prostate. The evidence which suggests a role for EGF in the biology of the prostate gland includes: (1) high concentrations of EGF have been detected in human prostatic fluid, seminal fluid and in the cytoplasm of prostatic cells (Elson et al., 1984; Gregory et al., 1986; Maddy et al., 1987; Fowler et al., 1988); (2) High levels of EGF and EGF-R were demonstrated to be present in both of human benign prostatic hyperplasia (BPH) and prostate cancer specimens (Fowler et al., 1988; Habib, 1990; Shaikh et al., 1990; Morris and Dodd, 1990, Ching et al., 1993); (3) Elevated levels of EGF-R have been identified in a variety of human prostate cancer cell lines (Schuurmans et al., 1988a; Morris and Dodd, 1990, Ching et al., 1993); (4) EGF-R and TGF-α transcripts have been identified in LNCaP cells (Derynck et al., 1987; Schuurmans et al., 1988b) and TGF-α peptide has been identified in the medium conditioned by DU-145 cells (Connolly and Rose, 1989; Habib, 1990); (5) EGF and TGF-α have been identified to

stimulate the growth of human prostate cancer cells (Wilding et al., 1989; Connolly and Rose, 1991).

Taylor and Ramsdel have reported that although the rat prostate is in a proliferative state at birth; TGF-α expression did not appear until 4 weeks of age and corresponds to the early pubertal stage (Taylor and Ramsdell, 1993). Then the expression of this growth factor in the prostatic epithelium subsequently increased and persisted into adulthood. No TGF-α was identified in the stroma of the adult rat prostate (Sherwood and Lee, 1995). The evidence that TGF-α appears in the developing rat prostate at a time when differentiated function is beginning and then persists into adulthood suggests that TGF-α may play a role in prostatic differentiation and in the maintainence of epithelium integrity rather than proliferation (Sherwood and Lee, 1995).

The presence of EGF in human, mouse, and rat prostate has been well established by radioimmunoassay, Western blotting and competitive binding studies (Nishi et al., 1991; Liu et al., 1992; Myers et al., 1993; Wu et al., 1993). Preliminary data indicated that the normal prostate expresses higher levels of EGF than of TGF-α (Myers et al., 1993; Wu et al., 1993). EGF has been identified in prostatic secretions and seminal plasma (Elson et al., 1984; Fuse et al., 1992). It has been demonstrated that the prostate is the primary source of EGF in seminal plasma (Fuse et al., 1992) and that EGF is an important factor in the maintainence of the structural integrity of the adult mouse prostate (Liu et al., 1992).

Both EGF and TGF-α have been identified in human prostate cancer. For example, EGF and TGF-α have been observed in human prostatic tumor cell lines, such as LNCaP and DU145 (Connolly and Rose, 1990, Connolly and Rose, 1991; Hofer et al., 1991; Ching

et al., 1993) and tissues (Fowler et al., 1988; Myers et al., 1993). Compared with BPH, higher EGF and TGF-α mRNA levels have been identified in human prostate cancer specimens (Davies and Eaton, 1989; Ching et al., 1993; Harper et al., 1993; Myers et al., 1993). It has been reported that higher percentage of EGF expression was observed in metastatic tumors and in metastatic lesions following androgen ablation than in primary prostatic cancer (Fowler et al., 1988). It has also been observed that EGF staining is higher in more poorly differentiated tumors as compared with well-differentiated prostate cancers (Fowler et al., 1988; Davies and Eaton, 1989; Yang et al., 1993). In addition, Jarrard and co-workers have reported that EGF can enhance the invasive properties of PC3 cells (Jarrard et al., 1994).

EGF-R is expressed primarily on the epithelial cells of a variety of tissues. Like EGF, EGF-R has been identified in normal rodent and human prostate (Maygarden et al., 1992; Ibrahim et al., 1993; Taylor et al., 1993). It has recently been reported that a high level of EGF-R expression occurs in neuroendocrine (NE) cells of the human prostate (Iwamura et al., 1994). Studies have shown that castration results in an increase of the EGF-R expression in rat prostate (St Arnaud et al., 1988). This evidence suggests that the expression of EGF-R is negatively regulated by androgens.

EGF-R has been identified to be predominantly localized to the basal component of the BPH epithelium (Maygarden et al., 1992; Ibrahim et al., 1993). Sherwood et al. have recently demonstrated constitutive activation of EGF-R in PC3, DU145, and LNCaP cells but not in normal prostatic epithelial cells (Sherwood and Lee, 1995). This evidence indicates that "constitutive phosphorylation of EGF-R is a common feature of prostate cancer cells and appears to be a defining characteristic of the transformed phenotype"

(Sherwood and Lee, 1995). The evidence that the androgen-independent cell lines PC3 and DU145 exhibited higher levels of EGF-R expression and phosphorylation than did the androgen-responsive cell line LNCaP also suggests that increased EGF-R activation appeared to correlate with more aggressive androgen-independent behavior. In addition, PC3 and DU145 also exhibited higher levels of autonomous growth than did LNCaP and were more aggressive when xenografted into nude mice (Sherwood and Lee, 1995). Overexpression of EGF-R has been identified to be associated with a poor prognosis in breast cancer (Nicholson et al., 1988; Koenders et al., 1992), however, such an association has not been established in prostate cancer. Sherwood et al. have recently identified that blocking antibodies to EGF-R will reduce prostate cancer cell growth and enhance the sensitivity of prostate cancer cells to cyto-toxic agents *in vitro* (Sherwood and Lee, 1995), although the effect of anti-EGF-R on solid prostate cancers *in vivo* has not been determined.

In addition, there is at least ten times as much EGF in the submandibular glands and two times as much EGF in the serum of male mice than in those of female mice (Kurachi et al., 1985; Perheentupa et al., 1984) and the increase of EGF production in submandibular gland is in parallel with sexual maturation (Byyny et al., 1972; Walker et al., 1981). The number of mature sperm in the epididymis decreased by as much as 55 percent in the mouse after withdrawal of circulating EGF by sialoadenectomy (Tsutsumi et al. 1986), further suggesting that EGF may play more important roles in males, especially in regulating growth of the sex organs.

Regulation of expression of EGF, EGF-R and AR

In humans, EGF has been detected in nearly all body fluids, however, the site(s) of its synthesis is not clear (Carpenter, 1985). In the mouse, the salivary glands are the major

source of EGF (Cohen, 1962) although secondary sites of synthesis are evident when the animals are sialoadenectomized (Byyny et al., 1974).

EGF precursor mRNA levels are absent or low in the fetal rodent. However, low EGF levels have been demonstrated in most mouse tissues during the early neonatal period (Fisher and Lakshmanan, 1990) and it was shown to increase during the first two months of postnatal life (Walker et al., 1982; Perheentupa et al., 1985; Lakshmanan et al., 1985; Laborde et al., 1988). EGF concentrations in mouse salivary gland tissue were increased exponentially between 15 and 70 days. In male mouse, the highest EGF concentrations in salivary glands occurred between 30 and 70 days (Walker et al., 1981). The increases in EGF concentrations in tissues of the neonatal rodent correlate with the increases in sex hormone levels (Fisher and Lakshmanan, 1990). Both EGF and EGF pro-mRNA concentrations in mouse salivary glands are stimulated by testosterone (Byyny et al., 1974; Gubits et al., 1986; Schaudies et al., 1989). It has been demonstrated that androgen can increase the production of EGF in the salivary glands and its level in the circulation in mouse (Byyny et al., 1974; Bullock et al., 1975). Jacobs and co-workers have also reported that EGF synthesis in the rat ventral prostate requires androgens (Jacobs et al., 1987). Pascall and colleagues observed that castration of mice at 8 weeks of age reduced salivary gland pro-EGF mRNA to female levels within 7 days (Pascall et al., 1989). Besides androgens, growth hormone and insulin can also affect EGF expression. For example, urine EGF was increased by growth hormone stimulation and decreased salivary gland EGF and pro-EGF mRNA concentrations was observed in insulin deficiency in diabetic mice (Perheentupa et al., 1984; Kasayama et al., 1989a.b). These observations suggest that EGF may be important mediator of the growth and developmental effects of several hormones. Besides

the hormones, the increase of circulating EGF was also obtained by acute administration of α-adrenergic agents (Gresik et al., 1980).

Synthesis and metabolism of the EGF-R are extensively regulated to modulate cellular responses to ligand (Hudson et al., 1989). The EGF-R activity is stimulated by ligand binding and self-phosphorylation and inhibited by heterologous phosphorylation (Thompson and Gill, 1985). Both EGF and TGF alpha have been found to downregulate the EGF-R gene expression (James and Bradshaw, 1984; Thompson and Gill, 1985). Retinoids can increase EGF-R number in epidermal, fibroblastic and embryonal carcinoma cells (Jetten, 1980). TGF beta also has been shown to increase the number of EGF-R in NRK cells (Assoian et al., 1984). Insulin has been found to increase EGF-R expression in the liver (Kasayama et al., 1989b).

Androgens have been reported to stimulate EGF-R expression in the liver tissue. For example, castration of adult male rats has been found to significantly decrease the EGF-R level in the liver and that was completely restored by treatment with testosterone (Kashimata et al., 1988). In studies of androgen regulation of EGF-R expression in the prostate tissues, equivocal results have been reported. Androgens have been found to inhibit EGF-R expression in rat ventral prostate (St. Arnaud et al., 1988), but stimulate the gene expression in human prostate tumor cells-LNCaP. Androgens have been reported to increase both the number and the activity of EGF-R in LNCaP cells (Schuurmans et al., 1988b; Schuurmans et al., 1989). Ligand binding and immunochemical analysis suggest that the EGF receptor family is a candidate for regulation by androgens, but whether the family is a mediator of androgen regulation of prostate cell growth and a factor in unregulated growth of prostate tumor cells remains to be established (McKeehan, 1991).

There is an autoregulation mechanism for the regulation of steroid hormone-receptor expression. It has been reported that the expression of glucocorticoid and estrogen receptors is down-regulated by the receptor ligand (Okret et al, 1986; Saceda et al, 1988; Saceda et al, 1989), however, the results of autoregulation of AR expression are controversial. It has been observed that androgen administration to castrated rats down-regulated AR mRNA expression in kidney, brain and epididymis (Quarmby et al., 1990; Blok et at., 1992). On the other hand, Chadha et al have reported that there is a stable and persistent androgen-induced up-regulation of AR expression in human ovaries (Chadha et al., 1994). The up-regulation has also been shown in human genital skin fibroblasts by ligand binding assay (Gad et al, 1988). Androgen down-regulation of AR mRNA level has been observed in rat ventral prostate (Lubahn et al., 1988a; Quarmby et al., 1990; Blok et al., 1992); coagulational glands (Quarmby et al., 1990) and in LNCaP cells (Blok et al., 1992; Henttu and Vihko, 1993). In contrast, using immunohistochemical and *in situ* hybridization techniques, Takeda and colleagues have reported an up-regulation of AR expression by androgen in both mouse and rat prostate (Takeda et al, 1991).

Recently, Prins and Woodham have investigated the autoregulation of AR mRNA in the separate regions of the rat prostate gland (Prins and Woodham, 1995). They demonstrated that AR mRNA levels are down-regulated by androgens in all prostate lobes by Northern blot analysis. Using *in situ* hybridization, they further confirmed that the increase in AR mRNA levels immediately following androgen withdrawal is due to increased transcripts per cell. However, they found that the AR mRNA elevation upon androgen withdrawal was transient when normalized to DNA content and the value returned to control levels in the ventral and dorsal lobes within three days while the elevation of AR

message in the lateral lobe was prolonged. Based on these observations, they concluded that differences exist in AR mRNA regulation within the different regions of the rat prostate gland and the differential autoregulation of the AR protein in the separate prostate lobes may be due to these differences.

Besides androgen itself, follicle-stimulating hormone (FSH) has been reported to increase AR expression in Sertoli cells obtained from immature rat testis (Blok et al, 1992).

EGF- and androgen-signal transduction

Because EGF-R is initially localized to the plasma membrane, the ligand binding elicits a cellular response mediated by an intracellular second messenger pathway. Currently, two general mechanisms of signal transduction are relatively well understood: coupling of receptors to various transcriptional factors by means of G proteins or coupling through the activation of tyrosine kinase activity that is intrinsic to the receptor molecule (Yarden and Ullrich, 1988). It has been demenstrated that the EGF receptor-mediated signal transduction is a direct protein-tyrosine kinase coupling pathway.

The EGF-R molecule contains three domains: a 621 amino acid extracellular domain responsible for ligand recognition; a 23 amino acid hydrophobic transmembrane region; and a 542 amino acid intracellular region containing a highly conserved tyrosine kinase domain (Prigent and Lemoine, 1992). The ligand binding to the extracellular receptor domain stimulates its tyrosine kinase activity and leads to increased intracellular phosphorylation as well as self-phosphorylation (Hunter and Cooper, 1981). Binding of ligand also leads to receptor dimerisation which increases the catalytic activity of its tyrosine kinase (Yarden et al., 1987). It has been identified that ligand binding followed by tyrosine kinase activition is essential and the first step in the EGF signal transduction pathway. Following activation, the

EGF-R tyrosine kinase phosphorylates a number of cellular substrates including phospholipase C gamma, MAP kinase, PI-3 kinase and the ras GTP-ase-activating protein GAP (Margolis et al., 1989, Wahl et al., 1989, Ellis et al., 1990). Recently, it has been reported that growth factor-stimulated phosphorylation increases the catalytic activity of the serine kinase *raf* which is the proto-oncogene of the transforming gene of murine sarcoma virus 3611 and a tyrosine phosphorylation substrate of PDGF receptor (Morrison et al.,1988, Morrison et al., 1989).

Phosphorylation of the tyrosine kinase substrates rapidly affects a number of processes involved in the growth response. These include the transcriptional activition of various genes encoding early response factors (such as *fos, jun, myc* etc.), receptors, structural proteins and hormones. There are multiple mechanisms which mediate EGF stimulation of gene transcription (Merchant et al., 1991).

Only a few EGF response elements and their corresponding DNA-binding proteins have been identified to date. The characterized EGF response elements include: (1) AP1 binding site (5'-TGACTCA-3') for *c-fos* gene (Fisch et al., 1989), (2) a 49bp DNA fragment (located at -78 to -30 of the promoter) for rat prolactin (rPRL) gene (Elsholtz et al., 1986), (3) a sequence of 5'-GACATGG-3' (located at -162 to -156 of the promoter) for H^+, K^+-ATPase alpha-subunit gene (Kaise et al., 1995), (4) a fragment located between -68 and -53 of the promoter for human gastrin gene (Merchant et al., 1991), (5) a 21bp fragment located between -80 to -60 of 5'-flanking region for mouse lactoferrin gene (Shi and Teng, 1994). In addition, EGF response elements for Moloney murine leukemia virus (Elsholtze et al., 1986), tyrosine hydroxylase (Lewis and Chikaraishi, 1987), and transin promoters (Kerr et al., 1988) also have been identified. Nevertheless, the present evidence suggests that different *cis* regulatory elements mediate

the transcriptional responses of different promoters to EGF stimulation (Elsholtz et al., 1986; Lewis and Chikaraishi, 1987; Fisch et al., 1989). For example, as described above, EGF stimulation of *c-fos* transcription is mediated by an AP1 *cis* regulatory element, whereas, EGF stimulation of prolactin gene transcription is mediated by a different *cis* regulatory sequence (Elsholtz et al., 1986; Fisch et al., 1989).

We need to recognize that EGF, like other growth factors, elicits biologic responses unrelated to mitogenesis and signaling for these responses may involve new pathways or some subset of the pathways depicted above (Carpenter and Cohen, 1990).

In deed, it has been demonstrated that there is interaction between the EGF-signaling pathway and the estrogen receptor (Grove and Korach, 1987; Ignar-Trowbridge et al., 1991; Ignar-Trowbridge et al., 1992). Moreover, that peptide growth factors including IGF-I, KGF and EGF could directly activate the AR in human prostate cancer cells-DU145 in the absence of androgens also has been reported recently (Culig et al., 1994).

The AR, like other steroid receptors and receptors for thyroid hormones, retinoids, and vitamin D, belongs to a family of gene regulatory proteins. The cDNA sequence of the AR was initially reported in 1988 (Chang et al, 1988; Lubahn et al, 1988b; Tillery et al, 1989; Faber et al., 1989) and the rat AR and human AR have been identified to share complete sequence identity in their DNA binding and steroid-binding domains (Tan et al., 1988). It has been demonstrated that human AR protein is present predominantly in the nuclei of glandular epithelial cells in the prostate tissue (Lubahn et al., 1988a; Chang et al., 1989; Takeda et al., 1991).

DNA sequence analysis indicates that all members of the gene regulatory protein family are structured in a similar way: they exhibit a variable N-terminal region, a short and

well-conserved cysteine-rich central domain, and a relatively well-conserved C-terminal domain (Beato, 1989). The heterologous N-terminal domains of receptors have been identified to contain an additional transcriptional modulation domain. The central domain has been identified to be able to form two so-called zinc fingers which promote the interactions of receptors with target genes by binding with zinc and this domain is also responsible for the DNA binding activity of the receptors. The C-terminal domain has been identified to play role in ligand binding and nuclear translocation. In addition, evidence suggests that the C-terminal domain may also contain trans-activation and dimerization functions (Evans and Hollenberg, 1988; Beato, 1989; O'Malley et al., 1991).

Extensive investigations on steroid regulation of gene expression have been done. It has been identified that the steroid hormones (glucocorticoid, progesterone, estrogen, aldosterone, and androgen) all act by a similar mechanism in which the hormones diffuse directly across the plasma membrane of target cells and bind to intracellular receptor proteins. Ligand binding activates the receptors, which then directly regulate the transcription of specific genes (Alberts et al., 1994). O'Malley et al. have summarized the primary pathway for steroid hormone action as follows: steroid $\rightarrow$ (steroid-receptor) $\rightarrow$ (steroid-receptor-DNA) $\rightarrow$ mRNA $\rightarrow$ protein $\rightarrow$ functional response (O'Malley et al., 1991).

The receptor binding regions of regulated genes are collectively termed steroid response element (or SREs) (Yamamoto, 1985). In general, the steroid response elements (SREs) are located in the 5' flanking sequences of regulated genes and function as receptor-dependent transcriptional enhancer (Yamamoto, 1985). Using gene transfer and DNA foot-printing techniques, numerous cis-acting SREs have been characterized (Yamamoto, 1985;

Maniatis et al., 1987; Evans and Hollenberg, 1988; Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Struhl, 1989; Vinson et al., 1989). It has been demonstrated that the SREs for various receptors share similarities in sequence and, in fact, the identical sequence allows activation by glucocorticoid, progesterone, and androgen receptors (O'Malley et al., 1991).

Estrogen and progesterone regulation of ovalbumin gene expression is the classic example for steroid regulation of gene expression (Dean et al., 1984). Regulation by glucocorticoids, estrogen and progesterone are defined for a large number of genes (Cato et al., 1984; Karin et al., 1984; Addison and Kurtz, 1986; Danesch et al., 1987; Kumar and Chambon, 1988; Tsai et al., 1988; Fawell et al., 1990; Klein-Hitpass et al., 1990). However, compared to other steroid hormones, androgen regulation of gene expression remains an enigma. Few model systems for androgen action have been reported (Parker et al., 1980; Catterall and Leary, 1983; Soares et al., 1987; Matusik et al., 1991). Regulation of the rat prstatic probasin gene (PB) by androgens gives us an excellent model system for androgen action. In this system, not only transcriptional and translational regulations of the mRNA by androgens have been elucidated, but also a cis-acting androgen response element (ARE) within the PB 5'-flanking region has been identified (Spence et al., 1989; Rennie et al., 1993). The details of this system will be described later.

In summary, EGF-signal and androgen-signal are transduced by different mechanisms. Binding of EGF to its receptor elicits a cellular response mediated by a intracellular kinase cascade pathway. However, androgen-receptor complexes directly regulate the transcription of specific genes. Nevertheless, the existence of interactions between EGF and androgen signal transduction have also been reported. The signal transductions for both systems are summarized in Figure 1.

The rat PB gene serves as a model system for study gene regulation and for targeting gene specific expression in the prostate

To study androgen regulation of prostatic gene expression, several model systems have been established. To date the regulatory regions of three rat prostatic genes including: prostatic steroid-binding protein (PSBP)(Heyns and DeMoor, 1977), seminal vesicle secretory protein II (SVSII) and probasin (PB) have been characterized (Dodd et al., 1983; Dodd et al., 1986; Matusik et al., 1986).

PSBP, which is also known as prostatein (Lea et al., 1979), α-protein (Chen et al, 1979; Chen et al., 1982), prostatic secretory protein (Pousette et al., 1980), or estramustine-binding protein (Forsgren et al., 1979), is synthesized almost entirely in rat ventral prostate (Forsgren et al., 1979). It is a glycoprotein and consists of two nonidentical subunits each containing a 13,000 Mr glycopeptide (C3) disulfide-bonded to either an 8,000 Mr (C1) or 11,000 Mr (C2) peptide (Heyns et al., 1978; Peeters et al., 1981; Peeters et al., 1982). There are two nonallelic genes for the C3 polypeptide: C3(1), which is expressed in the ventral prostate, and C3(2) (Hurst et al., 1983; Parker et al., 1983). PSBP gene has been shown to be regulated by androgens. Castration results in a coordinated 1000-fold decrease in all three mRNAs and the mRNA levels could be restored by testosterone replacement (Parker et al., 1980). Although the androgen regulation of PSBP C3(1) gene has been identified, the precise sequence of androgen regulatory elements (AREs) within the gene was unknown (Rushmere et al., 1987). In addition, even though the genes were accurately transcribed after transfection into a number of cell lines that contain androgen receptors, it

failed to show expression regulated by testosterone (Page and Parker, 1983; Parker et al., 1988).

SVS II is synthesized in the rat dorsolateral prostate and seminal vesicles and it is also shown to be regulated by androgens (Dodd et al., 1983; Dodd et al., 1986).

PB mRNA is abundant in rat dorsolateral prostate and is androgen (Dodd et al., 1983) and zinc regulated (Matusik et al., 1986). The PB gene has been cloned (Dodd et al., 1983; Spence et al., 1989) and has been widely used as a model system to study transcriptional regulation by androgens and glucocorticoids (Rennie et al., 1993) and translational regulation of a mRNA (Spence et al., 1989).

Both androgens and glucocorticoids induced CAT activity when a PB-CAT construct [a hybrid PB 5'-flanking region fused to a bacterial chloramphenicol acetyl-transferase (CAT) gene] were co-transfected with a eukaryotic rat androgen receptor (rAR) expression vector into HeLa (human cervical carcinoma) cells. This indicated that the PB 5'-flanking DNA has a functional ARE (androgen response element) and a GRE (glucocorticoid response element) that transcriptionally activated the gene (Matusik et al., 1991). Deletion mapping has located both hormone response elements within the same DNA fragment of the PB 5'-flanking region (Rennie et al., 1993). The precise sequences and positions of AREs in the PB 5'-flanking region has been determined using DNase I footprinting. The two identified AREs are: one between positions -236 and -223 (named ARE-1) and the other between -140 and -117 (termed ARE-2). The sequences of ARE-1 and ARE-2 are : 5'-ATCTTGTTCTTAGT-3' and 5'-GTAAAGTACTCCAAGAACCTA-TTT-3' respectively (Rennie et al., 1993). 5'- and 3'- deletion mapping of the PB 5'-

flanking DNA has demonstrated that both ARE-1 and ARE-2 were required for androgen regulation (Rennie et al., 1993).

Several promoters have been employed in the production of transgenic mice, and some of these have resulted in expression of the transgene in the male accessory sex glands. The MMTV-LTR has been used to target gene expression to the mammary gland; however, the MMTV-LTR transgenes are also expressed in salivary gland, lung, kidney, thymus, spleen, testes and prostate (Ross and Solter, 1985; Leder et al., 1986; Choi et al., 1987; Sinn et al., 1987; Choi et al., 1988; Stewart et al., 1988; Ornitz et al., 1991).

The promoter from the metallothionein (MT) gene has also been used to construct transgenes. The MT promoter coupled to TGF-alpha produced transgenic mice with mammary carcinomas, pancreatic metaplasias, and dramatic epithelial hyperplasia of the coagulating gland (Sandgren et al., 1990). Skalnik et al. have generated another transgenic mouse model by using gp91-phox gene promoter to drive the early region of the SV40 genome (Skalnik et al., 1991). However, the tumors of the prostate gland formed in the transgenic mice are neuroblastomas, and do not arise in the epithelia but are of neuroectodermal origin.

Allison and coworkers have generated a transgenic mouse model by microinjecting a 9.5 kb BamHI fragment of C3(1) gene into the fertilized eggs of C57 x SJL mice (Allison et al., 1989). The transgene contains the coding sequences for the C3 subunit, 4.3 kb of 5'-flanking sequence, and 2.2 kb of downstream flanking sequence. Unfortunately, besides showing selective expression in the ventral prostate, the gene also displayed nonuniform tissue distribution including expression in the testes in one line and in the pancreas, salivary gland and heart in another line (Allison et al., 1989).

Recently, a fragment of the PB promoter including 426 bp of the 5'-flanking DNA and 28 bp of the 5'-untranslated region (-426/+28) was shown to target expression of an heterologous gene in transgenic mice and the transgene displays androgen-responsive expression which is restricted to the prostatic epithelium (Greenberg et al., 1994). A transgenic animal model of prostate cancer has also been successfully generated by using the prostate-specific rat minimal PB promoter to drive expression of the simian virus 40 large tumor antigen-coding region. The transgenic mice expressing high levels of the transgene display progressive forms of prostatic disease that histologically resemble human prostate cancer, ranging from mild intraepithelial hyperplasia to large multinodular malignant neoplasia (Greenberg et al., 1995). All those results indicated that the PB gene serves as the best model system currently available for studying gene regulation in the prostate both *in vivo* and *in vitro*.

Hypothesis and Objectives

It has been demonstrated that the rat probasin (PB) gene is androgen-responsive and specific to the prostatic epithelial cells. According to the evidence that both androgens and EGF are implicated in regulation of the prostate growth, our hypothesis is that EGF effects the gene regulation in the prostate through androgen receptor-dependent mechanisms. The PB gene was chosen as a model to study the possible interactions between EGF and androgens in the gene regulation.

Our objectives are:

1. To demonstrate the effects of EGF and possible interactions between EGF and androgens in the prostatic gene regulation *in vivo*. The transgenic mouse model generated by using the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the

prostate-specific promoter of rat probasin (PB) gene would be used. The sexually mature PB-CAT transgenic mice would be sialoadenectomized or castrated and followed by the EGF and androgen administration. PB-CAT expression at each stage would be compared.

2. To study the effects of EGF and the possible interactions between EGF and androgens in prostatic gene regulation *in vitro*. The PB-CAT construct would be transfected into the human prostatic cancer cell line-DU145. The possible interactions between EGF and androgens in the gene regulation would be studied by co-transfection of the PB-CAT with or without the rAR expression vector and followed by the treatment with EGF and androgen. PB-CAT induced expression by EGF and/or androgen would be compared.

3. To localize the putative EGF response element(s). First, a series of PB-CAT chimaeric constructs containing PB 5'-flanking sequences (-426,-286, -244, -158, and -141 bp) would be transfected into DU145 cells and the cells would be treated with EGF. PB-CAT expression induced by EGF would be compared for each PB-CAT construct. Second, the constructs with point mutation within either ARE and a construct with deletion of ARE-1 would be transfected into DU145 cells. PB-CAT induced expression by EGF and DHT for each construct would be compared with the wild type of PB-CAT construct.

MATERIALS AND METHODS

Materials

Transgenic lines

All Swiss Webster (S.W) mice used in this project were purchased from University of Manitoba animal center. The lines of transgenic mice were generated by microinjection of a 2.1-kb transgene carrying the -426/+28 PB 5'-flanking sequences fused to the CAT reporter gene (Greenberg et al., 1994). The details about the structure of the transgene are shown in Fig. 2. Four male founder animals were identified to be transgenic by polymerase chain reaction (PCR). All transgenic mice used in the experiments are from Line 4248.

Prostate tissue

Prostate tissues used for CAT assay and RT-PCR were obtained from transgenic and non-transgenic male mice respectively. The tissues were stored at -70^{0}C before protein and RNA extraction was carried out.

Cell line and reagents for cell culture

The established human prostate cancer cell line in this study (DU145) was obtained from the American Type Culture Collection (ATCC) (ATCC, Rockville, MD). DU145 was derived from brain of metastasized prostate cancer (Stone et al., 1978). Stock culture of the cell was maintained at 37^0 C in 5% carbon dioxide (CO_2) incubator and grown as monolayers in 10% (v/v) heat-inactivated fetal bovine serum (FBS) (UBI, Upstate Biotechnology Inc., Lake Placid, NY) in Minimal essential Media (MEM) (Gibco BRL, Burlington, ON, CA) with 1% antibiotic-antimycotic solution (Gibco Laboratories, Grand

Island, New York). Transferin and insulin were purchased from Sigma (Sigma, St.Louis, MO) and the stock (1mg/ml in MEM) was stored at -20^{0}C. Trypsin-EDTA was purchased from Gibco GRL (Gibco BRL, Burlington, Ontario).

Antibody and growth factors

The following antibody and growth factors were purchased from Upstate Biotechnology Inc. (UBI Lake Placid, New York) and stored at -70^0 C : (1) anti-human epidermal growth factor receptor (EGF-R) antibody (0.125μg in PBS with 5mg/ml BSA, pH7.4) reacts with external domain of EGF-R on all human cells. (2) human epidermal growth factor (hEGF) (10^{-5} M in MEM), and (3) mouse epidermal growth factor (mEGF)(20μg /ml in 0.85% sodium chloride solution).

Hormones

The 5α - Dihydrotestosterone (DHT) was purchased from Sigma (Sigma, St. Louis, MO) and the stock was stored at -70^0 C (10^{-3} M in ethanol).

Reagents for CAT-assay

The following items were stored at 4^0 C : (1). [^{3}H]-Acetyl-Coenzyme A (0.25μCi / μl) purchased from Amersham (Amersham, Oakville, ON, CA) ; (2). BioRad Protein Assay Reagent purchased from Bio-Rad Laboratories (Bio-Rad Laboratories, Richmond, CA). Scintillene was purchased from Fisher (Fisher, Fairlaom, N. J.) and stored at room temperature.

Reagents for PCR and RT-PCR

TRIzol® Reagent -total RNA isolation reagent was purchased from Gibco BRL and stored at 4^0 C. The following reagents were stored at -20^0 C :(1) moloney murine leukemia

virus reverse transcriptase (M-MLV RT) purchased from Gibco BRL, (Gibco BRL, Bulington, ON) and Taq DNA polymerase, RNase inhibitor (RNAguard®), and ultrapure dNTP set 2'-deoxynucleoside 5'-triphosphate (dNTPs) were all purchased from Pharmacia Biotech (Pharmacia Biotech Inc. Quebec, CA).

Reagents for plasmid DNA preparation

Tryptone and yeast extract for growing bacteria were purchased from Fisher (Fisher Scientific Limited, Ottawa, ON) and stored at room temperature. The QIAGEN® Plasmid Maxi Kit which was used for plasmid purification was purchased from QIAGEN Inc. (QIAGEN Inc. Chatsworth, USA).

Primers

All primers used in the studies were purchased from the DNA Laboratory, University of Manitoba and stored at -20^0 C.

Primers specific for the PB-CAT:

5'- TAGCATCTTGTTCTTAGTCTT - 3' (upstream);

5'- CAACGGTGGTATATCCAGTG - 3' (downstream);

Primers specific for mouse β-casein:

5'- GATGTGCTCCAGGCTAAAGTT -3' (upstream);

5'- AGAAACGGAATGTTGTGGAGT -3' (downstream);

Primers specific for mouse androgen receptor:

5'- AGAAAGAATCCCACATCCTGC - 3' (upstream);

5'- ATCTGGAAAGGGAACAAGGTG - 3' (downstream);

Primers specific for mouse EGF:

5'- GACAGAGGACCCCAGGAAATA - 3' (upstream);

5'- GTTCCAAGCGTTCCTGAGAGT - 3' (downstream);

Primers specific for mouse EGF-R:

5'- AAAAAACTCTTCGGGACACC - 3' (upstream);

5'- GTGGCAGACATTATTGGCAT - 3' (downstream);

Primers specific for mouse TGF-α :

5'- CAACAAGTGCCCAGATTCCC - 3' (upstream);

5'- AGAGTGGCAGCAAGCAGTCC -3' (downstream);

Primers specific for mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH):

5'- TGAAGGTCGGTGTGAACGGATTTGGC -3' (upstream);

5'- CATGTAGGCCATGAGGTCCACCAC -3' (downstream);

Plasmid constructs

A series of plasmid constructs which contain different sizes of rat probasin 5'-flanking sequences (from -246 to +28) fused to the CAT reporter gene were gifts from Dr. R. J. Matusik. The constructs used in the project were as follows:

(1) a series of 5' deletion constructs including: p(-426/+28)PB-CAT; p(-286/+28)PB-CAT and p(-158/+28)PB-CAT;

(2) two point mutation PB-CAT constructs: p(-286/+28)M.1PB-CAT containing a point mutation in ARE-1 (G to A at position -231, referred to as mut-1) and p(-286/+28)M.2PB-CAT containing a point mutation in ARE-2 (C to A at position -123, named mut-2); Both constructs have been demonstrated to lead to > 90% loss in CAT activity in transient transfection assays (Kasper et al., 1994);

(3) constructs with both 5' and 3' deletions including: p(-244/-96)TK-CAT and p(-141/-91)TK-CAT.

Schematic drawing of the plasmid constructs and the sequence of PB 5'-flanking DNA from -426 to +28 bp are shown in Fig. 3 and Fig. 4, respectively.

Methods

Genomic DNA preparation and polymerase chain reaction (PCR) analysis

To screen positive transgenic mice, tails were cut from transgenic mice (frozen at -70^0 C) and genomic DNA was isolated by proteinase K digestion with final concentration of 50μg /ml (stock concentration of 20mg /ml , stored at -20^0 C) in reaction buffer [10mM Tris-HCl, pH8.0, 25mM ethylenediamine-tetraacetic acid (EDTA), 75mM NaCl and 1% sodium dodecyl sulfate (SDS)] at 55^0 C overnight (Greenberg et al.,1994). The samples were then extracted by 1:1 mixture of TE(10mM Tris-HCl and 1 mM EDTA pH 8.0)-saturated phenol and chloroform : isoamyl alcohol (24:1) once, followed by ethanol precipitation. The DNA was stored at 4^0 C. Positive transgenic mice were screened by PCR. The primer pairs used in PCR screening were : A and B for PB-CAT , C and D for mouse β-casein (MBC). The sequences of the synthetic oligonucleotides were described above (see Materials, page 32). The sequence of primer A is identical to nucleotides -240 to -219 of rat probasin gene, while the sequence of primer B represents the reverse complement of nucleotides of CAT. PCR amplification of the sequence between these primers was designed to produce a 320bp fragment. The location and orientation of the primers are shown. The C and D primers generated a 540 bp fragment of PCR product from mouse β-casein gene and served as positive control. The condition of PCR amplification was as follows: For each reaction, in total 50-μl reaction buffer contained about 50ng tail DNA, 100ng of each

primer, 0.2 units Taq DNA polymerase, 50mM potassium chloride (KCl), 1.5mM magnesium chloride ($MgCl_2$), 10mM Tris-HCl (pH9.0),0.2mM each of dNTPs (dCTP, dGTP, dATP and dTTP). The samples were topped with light mineral oil. Amplification was performed by using a thermal cycler apparatus (PTC-100, Atomic Energy of Canada limited, Chalk River, ON). Thirty cycles of amplification were carried out beginning with DNA denaturation at 95^0 C for 1 minute, primer annealing at 50^0 C for 45 seconds followed by primer extension at 72^0 C for 45 seconds. The reaction was finished after another 50^0 C for 1 minute and 72^0 C for 7 minutes. Then the PCR products were electrophoresed on 2% argarose gel with 1μg /ml ethidium bromide.

Animal experiment and prostate tissue preparation

Ten-week old transgenic S.W. male mice were randomly divided into three groups : (1) untreated; (2) castrated (Cx); and (3) sialoadenectomied (Sx). For Cx group, mice were castrated by the scrotal route and divided to Cx1 and Cx2 groups. For Cx1 group, the animals were killed on day 14 post-Cx. For Cx2 group, the mice received DHT (3mg / kg / day in 0.2ml 10% ethanol -90% peanut oil as vehicle) on the day 14 after surgery until the mice were killed on day 28. Delivery was s.c. in the scruff of the neck. For both groups the control mice received injections of vehicle only. For sialoadenectomy group, the mice had salivary glands removed by surgery and were also divided into two groups: Sx1 and Sx2. The mice in Sx1 group received mouse EGF (100μg / kg / day) beginning the day after surgery until mice were killed on day 14. However, the mice in Sx2 group received the same amount of mouse EGF daily as in Sx1 group on the day 14 after surgery until mice were killed on day 28. For both groups the EGF was given by I. P. injection. The mice were killed under anesthesia and the prostate tissues removed from the mice were put on dry ice

immediately and stored at -70^0 C. Dorsal and lateral prostate, ventral prostate, coagulating glands and seminal-vesicles were collected separately from each mouse. In accordance with the Canadian Council for Animal Care for the Care and Use of Laboratory Animals, all experiments were conducted using the highest standard for animal care.

Protein preparation and transgene expression analysis

The tissue extracts were prepared by homogenization in 0.1M Tris-HCl / 0.1% TritonX-100 (pH7.8) followed by centrifuging at 12,000xg / min for 10 minutes at 4^0 C. The supernatant was collected and the protein concentration was determined by colorimetric analysis using the BioRad Protein Assay (Richmond, Ca.). Expression of the transgene was determined by measuring CAT activity in the extract by a two phase fluor diffusion assay (CAT assay) (Nachtigal et al., 1989). Briefly, 200µg protein from each sample was taken and adjusted to 200µl with 0.1M Tris-HCl / 0.1% Triton X-100 (pH7.8), followed by heating to 65^0 C for 10 minutes. After cooling, each sample received 75µl of CAT reaction buffer including 0.5µCi [^{3}H]- Acetyl Coenzyme A(Amersham Canada Lt., Oakville, Ont.), 0.1M Tris-HCl (pH7.8),0.5M chloramphenicol (Sigma, St. Louis, Mo.) and ddH_2O. This solution was overlaid with 3ml of Scintillene (Fisher, Fairlaom, N. J.) and left to incubate, in the dark, for 30 minutes at room temperature. The radiolabel was measured by using a Gamma Spectrometer (80000 Gamma Sample Counter, LKB, Wallac) and cycled for at least 3 times of counting. Each cycle was timed and the CAT activity was calculated at each time point. CAT activity was expressed as dpm/min/mg protein.

To determine the slope of the line for CAT activity, the counts per minute (cpm) generated from each protein sample was divided by the period of time that each sample was cyclically counted. This would give you cpm/min/mg protein. To convert cpm/min to dpm/min/mg protein, each slope of the line (cpm/min) is multiplied by a conversion factor (1.4)(according to the manufacturer's instruction, the machine's conversion factor is 1.4) and divided by the amount of protein assayed. For each sample, 200μg of protein was assayed, therefore, the formula becomes:

{ (1.4) x [slope of the line (cpm/min)] x (1000μg/mg) }/ 200μg protein

An example of converting (cpm/min /200μg protein) to (dpm/min/mg protein) is given:

Sample	A	B	C
CAT Activity (cpm/min/200μg protein)	30	40	35
CAT Activity (dpm/min/mg protein)	210	280	245

Plasmid DNA preparation

I. Bacterial Cultures

The bacteria (E. coli) containing appropriate plasmid were from a glycerol stock and grown in 5ml of LB (10g NaCl, 10g tryptone and 5g yeast extract / L) as described by Sambrook et al., (1989) with Ampicillin 400μg/ml (Sigma, St., Louis, MO.) and placed overnight in a shaking incubator at 37^{0}C. The following day 250ml cultures of LB containing Ampicillin were inoculated with 2.5ml of overnight culture and put into the shaking incubator at 37^{0}C for 3 to 4 hours (to OD_{600} of ~ 0.5), then chloramphenicol (170μg/ml) was added to the culture and continually incubated at 37^{0}C overnight. The

cultures were poured into Beckmann 500ml plastic bottles and centrifuged at 4000rpm (Beckmann, model J2-21, JA10 rotor, Paulo Alto, Ca.) at 4^0C for 10 minutes.

II. QIAGEN Plasmid Maxi Preparation

Purification of the plasmid DNA was carried out using commercially prepared QIAGEN Plasmid Maxi Preparation Kits and the manufacturer's protocol was followed. Briefly, the bacterial pellet (grown in 250ml of LB) was resuspended in 10ml of Resuspension Buffer (50mM Tris-HCl, pH8.0; 10mM EDTA; 100µg /ml RNase A) and after complete resuspension, 10ml of lysis buffer (200mM NaOH; 1% SDS) was added and followed by incubation for 5 minutes at room temperature. Then 10ml of chilled Neutralization Buffer was added to the mixture and followed by incubation for 20 minutes on ice. After centrifuging at 20,000 xg for 15 minutes at 4^0 C, the supernatant was applied to the QIAGEN-tip which had been equilibrated by applying 10ml of Equilibration Buffer (750 mM NaCl; 50mM MOPS, pH7.0; 15% ethanol; 0.15% Triton X-100).Then the tip was washed by applying 2 x 30ml of Wash Buffer (1.0M NaCl; 50mM MOPS, pH7.0; 15% ethanol) and the plasmid DNA was eluted by using 15ml of Elution Buffer (1.25M NaCl; 50mM Tris-HCl, pH8.5; 15% ethanol) and finally precipitated with 0.7 volumes of room-temperature isopropanol followed by centrifuging at 15,000 xg for 30 minutes at 4^0 C. The DNA was washed once with 70% ethanol, air-dried for about 5 min and redissolved in 300-600µl of TE (10mM Tris-HCl, pH8.0; 1mM EDTA) depending on the size of the pellet and stored at -20^0C. The absorbance at A_{260} was read to determine the DNA concentration and ratio of A_{260}/A_{280} was calculated to confirm the relative purity of the plasmid DNA prepared.

III. Restriction Endonuclease Analysis

In order to confirm the identity of the plasmid DNA prepared, 1μg of each plasmid preparation was digested for at least 3 hours at 37^0C in a total of 20μl reaction buffer including 5 units of the appropriate restriction endonuclease (Pharmacia, Quebec) and 1x or 2x of 10x One-Phor-All Buffer PLUS (Pharmacia) depending on the enzyme working condition. A BioRad Mini Sub DNA cell containing a 1% agarose gel with 10μg/ml EtBr was then filled with 0.5x TBE. The restriction products were loaded into the individual wells within the agarose gel. To estimate the resulting digested fragment sizes, one lane of the gel was loaded with 0.5μg of commercially prepared DNA marker (Lambda/Hind III)(Pharmacia, Quebec). Electrophoresis was performed at 70 volts until the DNA fragment had migrated sufficiently as visualized with ultraviolet light. A photograph was taken of the gel using DNA Transilluminator (Fotodyne In., New Berlin, Wisc.). This procedure was followed for each of the plasmids prepared. Specifically, the expression vector for rat androgen receptor was digested with EcoRI giving 3 bands of 3.3, 2.5 and 0.5 kb. Double digestion of all the 5' -PB-CAT chimaeric constructs with Bam HI and Hind III yielded 2 bands of 3.2 and 1.45 kb. The chimaeric PB-CAT constructs containing 5'-flanking DNA sequences from -244 bp to -96 bp and -141 bp to -91 bp in front of TK gene promoter, were double digested with Hind III/Acc I respectively, and resulted in bands of 3200 and 148 bp for -244/-96 TK-CAT and 3200 and 50 bp for -141/-91 TK-CAT.

Cell culture and DNA transfections

DU145 cells were plated at initial density of 2 x 10^5 / 60-mm dish in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1%

antibiotics(v/v). Transient transfection of the DU145 cells with plasmid DNA was performed using a calcium phosphate / DNA precipitation method (Sambrook et al., 1989). Briefly, after growing 16-18 hours, each plate received 3.5 μg of plasmid DNA including: 1.5μg of PB-CAT, 1.5μg of pAR (androgen expression vector) or promoterless plasmids-pUC119 or pXP1, and 0.5μg of pCH110-β-gal (β-galactosidase expression vector). Calcium chloride (2M) was added to the DNA solution to a final concentration of 125mM and the mixture was slowly added to same amount of 2 x HEBS solution (280mM NaCl, 50mM Hepes, 1.5mM Ma_2HPO_4 , pH7.1) with gentle mixing by bubbling the solution for a period of ~ 2 minutes. Then the solution was left for half an hour, at room temperature, in order to allow time for the plasmid DNA to form calcium phosphate precipitates. Before the solution was put on cells, the media from the cells was drawn off and replaced with 2 ml of fresh serum-free media. Then 150μl of calcium phosphate / DNA solution was added to each plate. The cells were then incubated for 6 hours at 37^0C. The media was then removed and the cells were treated with 1 ml of 20% glycerol in MEM for 2 minutes. The cells were rinsed twice with 3 ml Phosphate Buffered Saline (PBS), pH7.1. Then the cells were subsequently grown in serum free MEM plus 1% antibiotics (v/v) , 2μg / ml of insulin and transferin with or without DHT , EGF and Ab-hEGF-R. The cells were incubated at 37^0 C with 5% CO_2 for about 40-48 hours before harvesting.

DNA expression analysis

A.CAT Assays

Cells, which had undergone transient transfection, were rinsed twice with 3 ml of PBS (137mM NaCl, 2.7mM KCl, 1.8mM KH_2PO_4 , and 10mM Na_2HPO_4) and harvested in

Trypsin-EDTA. After centrifuging at 2000 xg for 4 minutes at room temperature, cells were lysed in 0.1M Tris-HCl / 0.1% Triton X-100, (pH7.8) for 15 minutes on ice. Insoluble material was removed by centrifugation at 14,000 xg for 15 minutes at 4^0 C. The CAT activity in the cell extract was determined as described above (see page 35 and 36).

B. Transfection Efficiency

All transfection experiments were corrected for transfection efficiency by β-galactosidase (β-gal) activity. The β-gal activity was measured by the Galacto-Light™ analysis kit and performed as described by the manufacturer (TROPIX, Inc. Bedford, Massachusetts, USA). Briefly, 20µl of cell lysate was added into Baxter 12 x 75mm tube and then 200µl of Reaction Buffer [substrate diluted to 1-fold with Galacto-Light™ Reaction Buffer Diluent (100mM sodium phosphate pH8.0, 1mM magnesium chloride)] was added to each tube. The interval between each adding is 15 second. The samples were incubated at room temperature for 60 minutes. Then 300µl of Accelerator was automatically injected into each sample while β-gal activity was measured by OPTOCOMP I Luminometer (MGM Instruments, Inc. Hamden, CT. USA). All reagents used for the assay were warmed to room temperature before the assay was carried out. The highest value of β-gal activity was given the value of 100% and each of the other values were expressed as a percentage of the highest value. Each value then represents the percentage of CAT DNA incorporated per plate of transfected cells.

An example of a correction for transfection efficiency is given:

Sample	A	B	C
β-Gal Activity	101641 (100%)	99745 (98%)	99583 (98%)
CAT Activity (before normalized)	30	50	40
CAT Activity (after normalized)	30	51.2	40.82

C. Slope of the Line (cpm/min)

To determine the slope of the line for CAT activity, the counts per minute (cpm) generated from each protein sample (from 1 plate of transfected and harvested cells) was divided by the period of time each sample was cyclically counted. The CAT activity was calculated and expressed as described before (see page 36).

RNA preparation and reverse transcription polymerase chain reaction (RT-PCR) analysis

The RNAs used for RT-PCR analysis were extracted from the prostate tissues of 10-week old untreated , castrated and sialoadenectomized S.W mice respectively. For castration and sialoadenectomy groups, the mice were divided into Cx_{RT} 1, Cx_{RT} 2, Sx_{RT} 1 and Sx_{RT} 2. The mice were sacrificed on the day 14 (Cx_{RT} 1 and Sx_{RT} 1) and day 28 (Cx_{RT}2 and Sx_{RT} 2) after the surgery respectively. The tissues obtained from dorsal, lateral and ventral prostate were separately stored at -70^0 C before RNA extraction was carried out. For each group, the tissues were pooled from three prostates. Intact males served as controls. The TRIzol total RNA isolation reagent was used for total RNA extraction and the manufacturer's instruction was followed. Briefly, 1ml of the reagent was added to 50-100

mg of tissues, followed by homogenizing using Brinkmann Homogenizer (Brinkmann Instruments Inc. Rexdale, ON). The homogenized samples were incubated at room temperature for 5 minutes and then 0.2 ml of chloroform per 1ml of the TRIzol reagent was added to each sample. The mixtures were then incubated at room temperature for 2-3 minutes and followed by centrifuging at 12,000 xg for 15 minutes at 4^0 C. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by mixing with isopropyl alcohol (0.5 ml of isopropyl alcohol per 1 ml TRIzol reagent) and followed by an incubation for 10 minutes at room temperature. Then the samples were centrifuged at 12,000 xg for 10 minutes at 4^0 C. The RNA was washed once with 75% ethanol and redissolved in deionized distilled water with 0.1% DEPC (diethyl pyrocarbonate).

An aliquot of 1μg of total RNA from each sample, as determined by absorbance at 260nm, was used as template for the random hexamer [$pd(N)_6$, Phamacia] to synthesize first strand of cDNA. Each 20-μl reaction contained 200 units of M-MLV RT; 50mM Tris-HCl (pH8.3); 75mM KCl; 10mM DTT; 3mM$MgCl_2$; 1mM each of dNTPs; 500ng $pd(N)_6$; 39 units of RNAguard®. The reverse transcription was carried out at 37^0 C for 60 minutes and followed by incubation at 65^0 C for 5 minutes, cooled on ice, and then amplified by PCR. All primers which were used in studies were designed according to the sequences of specific mouse cDNAs. The sequences of all primers were shown in Materials and Methods. To confirm that the DNA fragments from PCR are EGF, TGF-α, EGF-R and AR, all PCR products generated by the primers for these factors were analyzed by restriction enzyme digestion. The primer annealing temperature, position and size of DNA fragment generated by PCR from each pair of primers and restriction enzyme digestion are shown in Table1.

To compare differences of the gene transcription among different lobes of the prostate, each pair of primers was tested to establish the linear range of amplification. Varying amounts (0.5, 1.0, 2.0, 3.0 or 4.0μl) of RT reaction were used as template for PCR amplification. These tests demonstrated amplification in the linear range and accordingly, 1μl of RT reaction was chosen for each pair of primers in later PCR analysis. For each pair of primers, PCR amplification conditions were the same except for the annealing temperatures (see Table1). Each 50-μl of PCR reaction included: 1μl of RT reaction; 50mM KCl; 1.5mM $MgCl_2$; 10mM Tris-HCl (pH9.0); 0.1mM each of dNTPs; 50ng of each primer and 1unit of Taq DNA polymerase. The PCR amplification was performed as described above. To confirm that total RNA from the prostate tissues was suitable for amplification, primers specific for mouse G3PDH sequence were used in control PCR. The relative expression of mouse EGF, EGF-R , TGF-α, and AR in total RNA from prostate tissues were demonstrated by relative absorbance units of bands from these factors versus G3PDH bands determined by densitometic scanning of pictures of RT-PCR argarose electrophoresis (IBM ScanPlot program).

Table 1. Primer annealing temperature, position and size of DNA fragment generated by each pair of primers and restriction enzyme digestion.

RNA	Position (in cDNA sequence)	Size of PCR Product (bp)	Annealing Temperature (°C)	DNA fragment from restriction enzyme digestion (bp)
EGF-R	1591-2014	424	56	359,65 (Bgl I)
EGF	3858-4414	557	56	159,398 (AccI)
TGF-α	134-470	337	58	228,109 (Sau3AI)
AR	2508-2781	274	55	117,157 (NdeI)
G3PD	51-1033	983	55-58	Not tested

RESULTS

PB-CAT expression in the transgenic mouse prostate

To study the *in vivo* regulation of gene expression in the prostate gland, we made use of a line of transgenic mice expressing the construct (-426/+28) PB-CAT. To determine whether the PB-CAT transgene was expressed in the prostate, the male transgenic mice were killed at 10 weeks of age, and extracts were prepared from dorsolateral and ventral prostate as well as coagulating gland and seminal vesicle as described in Materials and Methods. As shown in Fig 5, PB-CAT expression was detected mainly in extracts prepared from dorsolateral and ventral prostate tissue. However, extremely low level of CAT activity, compared to dorsolateral and ventral prostate, was detected in extracts prepared from coagulating gland and seminal vesicle. This expression pattern was consistent with the results observed before (Greenberg et al., 1994). The highest PB-CAT expression was detected in extracts prepared from ventral prostate tissue is in contrast to the endogenous gene in the rat (Matusik et al., 1986).

Effects of EGF and DHT in regulation of the PB transgene as well as the effects of castration and sialoadenectomy on the weight of the prostate tissues

The rat PB gene is regulated by androgens (Dodd et al., 1983; Matusik et al., 1986 and Matusik et al., 1991), but whether it is regulated by EGF is unknown. To determine whether the PB-CAT construct is regulated by EGF and whether there is any interaction between androgen and EGF for the gene regulation *in vivo*, sexually mature male mice were either castrated or sialoadenectomized, and levels of CAT activity were determined after

androgen and/or EGF replacement. As shown in Fig. 6, CAT activity decreased dramatically in ventral and dorsolateral (DLP) lobes (to 1% or less of pre-Cx level) after castration and returned to pre-castration levels after the administration of dihydrotestosterone (DHT) alone or DHT plus EGF. However, CAT activity was not restored by administration of EGF alone. Treatment with both DHT and EGF resulted in the maximum level of PB-CAT expression. As shown in Fig. 7 (A), sialoadenectomy (Sx) resulted in a 10-fold reduction in PB-CAT expression in DLP by day 14 (Sx1) and CAT activity returned proportionally to pre-Sx levels after the administration of EGF (Sx1 + EGF). However by 28 days post-Sx, CAT activity had returned to pre-Sx levels without further treatment (Sx2). In contrast, expression of PB-CAT in the ventral prostate (VP) was not altered significantly by Sx with or without EGF administration (Fig. 7 B). The evidence that EGF treatment alone could not restore the PB-CAT expression in the prostate suggests that EGF may need the presence of androgen to elicit its function.

In addition, the weight of the prostate tissues were altered much more by Cx than by Sx, further indicating that androgen is the main factor for the regulation of the prostate growth (see Table 2). As shown in Table 2, after 14 days post-Cx, the weight from the DLP decreased to ~70% of that from the controls (Cx1). In contrast, the weight of VP did not decrease after 14 days castration. However, after 28 days post-Cx, the weights from both DLP and VP were decreased to ~40% of that from the controls (Cx2). EGF treatment alone could not restore the PB-CAT expression in the castrated mice; EGF treatment alone also could not restore the weight of the prostate tissues in those mice (Table 2, Cx2+EGF). The weight of the prostate tissues both in DLP and VP were restored by either DHT treatment alone or treatment with DHT & EGF (see Table 2, Cx2+DHT and Cx2+DHT/EGF).

As shown in Table 2, the weight from DLP was slightly decreased (to ~85%) after 14 days post-Sx. In contrast, the weight from the VP were increased after 14 days post-Sx. This could be due to the increase of the circulating androgen level [It has been reported that EGF inhibits the testicular hormone secretion (Fisher and Lakshmanan, 1990)]. However, the weight of DLP after 28 days sialoadenectomy had returned to the control level (Sx2). The weight of VP also returned to the control level. These results indicated that androgens are the main factor in the control of the prostate in both gene regulation and the growth. However, EGF also showed effects; the highest levels of both PB-CAT expression and prostate weight were obtained from the DHT & EGF treatment groups (see Fig. 6, Cx2 EGF+DHT and Table 2, Cx2 +DHT/EGF).

Relative expression of EGF, EGF-R, TGF-α and AR mRNA in normal mouse prostate as well as the effects of EGF and androgen

We have shown that both EGF and androgens could affect the PB-CAT transgene expression in the transgenic mice. However, EGF withdrawal only affected the transgene expression in the dorsolateral prostate and the PB-CAT expression was restored by 28 days post-Sx without further EGF treatment (see Fig. 7). In addition, EGF replacement alone could not restore the PB-CAT expression in the castrated mice (see Fig. 6). These data raised several questions: (1) Is EGF and EGF-R are expressed differentially in dorsolateral and ventral prostate? (2) Does EGF and androgen withdrawal affect the EGF, TGF-α and EGF-R expression? (3) Does castration (Cx) and sialoadenectomy (Sx) affect the expression of the AR? To answer these questions, the relative EGF, TGF-α, EGF-R, and AR mRNA expression in the different lobes of normal mouse prostate as well as the effects of castration

and sialoadenectomy on the expression of the mRNAs were tested using RT-PCR techniques.

To confirm that the DNA fragments from PCR are EGF, EGF-R TGF-α and AR, all PCR products generated by the primers for these factors were analyzed by restriction enzyme digestion. As shown in Fig. 8, PCR product of EGF is a 557 bp fragment and digested into two bands of 159 bp and 398 bp by AccI. The PCR product of EGF-R is a 424 bp fragment and digested into two bands of 65 bp and 359 bp by Bgl I. PCR products of TGF-α and AR are 337 bp and 274 bp fragments, respectively. Two fragments of restriction enzyme digestion of TGF- α are 228 bp and 109 bp in length digested by Sau3AI, and AR was digested into two fragments of 117 bp and 157 bp in length by restriction enzyme NdeI.

To compare differences of the gene expression among different lobes of the prostate, each pair of primers was used to establish linear range of amplification for the analysis (Fig.9). According to the result, 1μl of RT reaction was chosen for each pair of primers to generate PCR in all RT-PCR analysis.

To determine relative mRNA levels of EGF, EGF-R, TGF-α and AR in prostate tissues of normal male mice, sexually mature male mice were killed and total RNA was prepared from dorsal, lateral and ventral prostate respectively as described in Materials and Methods. As shown in Fig. 10, EGF-R mRNA level is higher in dorsal lobe than in ventral lobe. In contrast, ventral lobe contains higher AR mRNA level than dorsal lobe. All the mRNAs , except EGF, are shown in highest levels in lateral prostate compare to dorsal and ventral prostates. These observations suggest that under normal conditions, different lobes

of the prostate are regulated differentially by androgens and EGF. In addition, all lobes express all mRNAs; however VP , which is not responsive to Sx, expresses lowest EGF and EGF-R mRNAs. This evidence indicates that EGF may play more important roles in dorsal and lateral lobes than in ventral prostate.

To answer the questions of whether EGF produced by the prostate itself could contribute to the rebound of PB-CAT activity by 28 days post-Sx and whether Cx and Sx affect EGF, EGF-R, TGF-α and AR mRNA levels in the prostate, the effects of androgen and EGF withdrawal on relative expression of EGF, EGF-R, TGF-α and AR mRNA in dorsal, lateral and ventral lobes of mouse prostate were identified. As shown in Fig. 11 and Fig 12, 10-week old male mice were either castrated or sialoadenectomized for 14 days or 28 days as described. According to the observations obtained from these experiments, the following conclusions were formed: (1) In DP, all the mRNAs are elevated at 14 days post-Sx but have returned to normal levels by 28 days post-Sx. This result suggested that EGF/TGF-α produced locally could contribute to the rebound of the PB-CAT activity by 28 days post-Sx. (2) In VP, mRNAs are less effected by Sx than in the DP. This may explain the failure of EGF regulation of PB-CAT expression in the VP. (3) Cx caused EGF mRNA to decrease in all lobes. (4) In DP, all tested mRNA (except EGF) are elevated at 14 and 28 days post-Cx. (5) All treatments cause mRNAs to decrease in LP. In summary, the effect of androgen and EGF withdrawal on the relative expression of EGF, EGF-R, TGF-α and AR mRNA in different lobes of mouse prostate was not uniform, confirming that different lobes of the mouse prostate are differentially regulated by androgens and EGF.

Effects of EGF and DHT on the p(-426/+28)PB-CAT expression in DU-145 Cells

DU145 cell line was derived from brain of metastasized prostate cancer and has been demonstrated to be androgen-independent (Stone et al., 1978; Connolly and Rose, 1990). Several studies have shown that the DU145 cell line specifically binds EGF and produces both EGF and TGF-α (Connolly and Rose, 1989; Ching et al., 1993). EGF-R mRNA has also been detected by using RNase protection assay in this cell line (Morris and Dodd, 1990).

We have shown that both EGF and DHT affected the PB-CAT transgene expression in the transgenic mice. However, EGF showed an androgen-dependent pattern in the regulation of the PB transgene *in vivo* since EGF replacement alone could not restore the PB-CAT expression in the castrated mice (see Fig. 4). To determine whether EGF regulation of the PB-CAT expression is the same *in vitro* as *in vivo*, and whether EGF needs the presence of androgen receptor to display regulation of PB gene, we transfected the same PB-CAT construct as the one that has been used to generate the transgenic mice into the human prostate cancer cell line-DU145. Because DU145 cells do not contain endogenous AR (Culig et al., 1994), to demonstrate the function of the AR in the PB gene regulation, the rat androgen expression vector (rAR) was co-transfected with the constructs tested.

As shown in Fig. 13, without co-transfection with the rAR, addition of EGF resulted in ~ four-fold increase in the PB-CAT expression compared to the controls (Fig. 13 A). In contrast, treatment with DHT did not increase the CAT activity in the absence of the rAR (Fig. 13 A). However, co-transfection with rAR and treatment with either EGF or DHT resulted in an increase of PB-CAT expression (Fig. 13 B). This evidence indicated that EGF does not need the presence of androgen or AR to regulate the PB-CAT expression. We

should note that the CAT activity from the transfection experiments were obtained from the extracts of a homogeneous cell population, but the CAT activity from the mouse prostate tissues were obtained from the extracts of whole tissues including epithelial and stromal cells. Because the PB-CAT expression has been demonstrated to be restricted in the prostate epithelial cells (Greenberg et al., 1994), the observation that EGF treatment alone could not restore the PB-CAT expression after androgen withdrawal *in vivo* experiments could be due to the lack of epithelial cells. We will further discuss this later.

Effects of EGF and DHT on 5'-deletion PB-CAT expression in DU145 cells

Both *in vivo* and *in vitro* experiments have demonstrated the effects of EGF on the PB-CAT expression. To localize the putative EGF response element(s) (ERE), and also to further investigate the possible interactions between EGF and androgens, a series of 5'-deletion PB-CAT constructs containing PB 5'-flanking sequences (-426, -286, -158, and -141, see Fig. 3) were transfected into DU145 cells. Fig. 4 shows the PB 5'-flanking DNA sequence commencing at nucleotide -426. The stars above the nucleotides indicate the 5' end of particular chimaeric PB-CAT constructs: -426, -286, -158 PB-CAT. The results of transient transfection of these constructs in the absence or presence of the rAR expression vector in DU145 cells treated with EGF and/or DHT are shown in Fig. 14. As shown in Fig.14 (A), in the absence of the rAR, addition of EGF resulted in an increase of PB-CAT expression from all three constructs. In contrast, treatment with DHT did not increase the CAT activity. Treatment with both EGF and DHT resulted in the same effect as addition of EGF alone. As shown in Fig14 (B), in the presence of the rAR, treatment with either EGF or DHT resulted in an increase of PB-CAT expression for -426 and -286 PB-CAT constructs and the maximal PB-CAT expression was obtained by adding both EGF and

DHT. However, for -158 PB-CAT construct, addition of DHT alone did not induce the PB-CAT expression, presumably because of the lack of ARE-2 sequences (-242 to -223). Treatment with both EGF and DHT resulted in the same effect as addition of EGF alone. This result indicated that both ARE-1 and ARE-2 were required for androgen regulation for PB gene and this is also consistent with the previous reports in literature (Rennie et al., 1993; Kasper et al., 1994). In contrast, deletion of ARE-2 sequence did not affect EGF's ability to induce expression regardless of presence or absence of the rAR (see Fig. 14). These observations further confirm that the induction of the PB-CAT expression by EGF is androgen receptor independent. As shown in Fig.15, the deletion from -426 to -158 did not affect very much the function of EGF in induction of PB-CAT expression. This result suggested that the EGF regulatory element could be present in the PB 5'-flanking region from -158 to +28 bp.

Effects of EGF and DHT on expression from heterologous promoter constructs [PB-TK-CAT]

To further determine possible EGF response element(s) within the PB 5' -flanking sequence, two other chimaeric CAT constructs were transfected into DU145 cells. The p(-244/-96) TK-CAT contained both ARE-1 (-241 to -223) and ARE-2 (-140 to -117) in the PB 5'-flanking DNA fragment and was adjacent to the heterologous promoter TK. A second construct, p(-141/-91)TK-CAT, only contained ARE-2 in the PB 5'-flanking DNA fragment. As shown in Fig. 16, EGF induced the CAT activity when co-transfected without (A) and with (B) rAR for both constructs. However, for the -141/-91 construct, the same result as that of -158 construct for DHT stimulation was obtained (Fig. 16 B and 17 B). Fig. 17 shows the fold change in CAT activity of -244/-96 and -141/-91 TK-CAT constructs.

Compared with the -286/+28 PB-CAT construct, both 5'- deletion to -141 bp and 3'-deletion to -91 bp within the 5'-flanking region of the PB gene did not affect very much the inducibility by EGF in the PB-CAT and TK-CAT expression (Fig. 17). This evidence suggested that an EGF response regulatory element could be in -141/-91 bp region of the PB 5'-flanking sequence.

Effects of EGF and DHT on point mutation PB-CAT constructs

To test further whether ARE sequences influence EGF effects, another two constructs with individual point mutations within ARE-1 and ARE-2, respectively, were tested by transient transfection. Within ARE-1, base-231(G) was changed to an A [mutation 1(M.1)]; within ARE-2, base -123 (C) was changed to an A (M.2). These two point mutations within AREs have been proven to reduce PB-CAT activities induced by DHT (Rennie et al., 1993; Kasper et al., 1994). To identify whether these mutations affect PB-CAT activities induced by EGF, the constructs were co-transfected with or without the AR expression vector into DU145 cells. As shown in Fig. 18 & 19, a single mutation in either ARE reduced the level of CAT activity from about 11 fold to a less than 5 fold by DHT treatment when co-transfected with the AR expression vector compared to the wt PB-CAT. In contrast, these mutations did not affect CAT activity induced by EGF under the circumstances with (Fig. 18B) or without (Fig. 18A) co-transfection with the AR expression vector compared to the wt PB-CAT. These observations further indicated that EGF and DHT regulated the PB promoter through different sequences.

Effect of Ab-hEGF-R on the PB-CAT expression in DU145 cells.

Because DU145 cells possess EGF-R and synthesize both EGF and TGF-α (Ching et al., 1993), the basal level of the CAT activity may due to the endogenous EGF. To

confirm the specificity of the induction of CAT activity by EGF, anti-human EGF-R monoclonal antibody(Ab-hEGF-R) was added into the cell culture during DNA transfection. Transient transfections were performed in DU145 cells using -286/+28 PB-CAT construct and treated with Ab-hEGF-R at concentrations ranging from 0.01875 to 0.3 μg/ml. As shown in Fig. 20, at the concentration of 0.3μg/ml, the CAT activity induced by EGF was inhibited to ~ 50% compared with the control. The inhibition was reversed by addition of EGF(10nM) to the cell culture. The observation that the antibody could not completely block the CAT activity suggested that either the amount of the antibody used was not enough or other factor(s) produced by the cells also play a role in regulation of the PB promoter.

Summary

In summary, the data shows:

(1) The -426/+28 PB-CAT transgene was expressed in the prostate tissues of the transgenic mice *in vivo*.

(2) Both castration(Cx) and sialoadenectomy(Sx) affected the transgene expression in the prostate of the transgenic mice. Cx affected the expression in all lobes of the prostate, however, Sx only affected the expression in dorsolateral but not in ventral prostate.

(3) In normal mouse prostate, relative mRNA levels of both EGF and EGF-R were higher in dorsal and lateral prostate than in ventral prostate. The dorsal prostate possessed the lowest AR mRNA level compared with lateral and ventral prostate.

(4) Both Cx and Sx affected EGF, EGF-R, TGF-α and AR mRNA expression in the mouse prostate. However, patterns of the effects were different among different lobes and different periods post- Cx or Sx.

(5) Both EGF and DHT induced the PB-CAT expression in DU145 cells. Under conditions with or without cotransfection with the AR expression vector , EGF induced the CAT activity which suggested that the EGF induction was androgen-receptor independent in the cells.

(6). A series of deletions from both 5' and/or 3' directions of the PB 5'-flanking DNA fragment from -426 to +28 bp did not significantly affect EGF induction of the CAT activity. Because the minimal construct tested in the experiments contains PB 5'-flanking sequence from -141 to -91 bp, it is suggested that an EGF-response element could exist in this region of the PB promoter.

SAMPLES	WEIGHT OF THE TISSUES (gm)	
	DLP	VP
control (n=10)	0.0177 ± 0.0046	0.0189 ± 0.0042
Sx 1 (n=4) % control	0.0151 ± 0.0011 85	0.0266 ± 0.0103 140
Sx 1 + EGF (n=4) % control	0.0176 ± 0.0027 99	0.0251 ± 0.0029 132
Sx 2 (n=3) % control	0.0188 ± 0.0061 106	0.0171 ± 0.0026 91
Sx 2 + EGF (n=4) % control	0.0203 ± 0.0045 114	0.0169 ± 0.0017 89
Cx 1 (n=4) % control	0.0125 ± 0.0033 71	0.0214 ± 0.0106 113
Cx 2 (n=4) % control	0.0076 ± 0.0015 43	0.0065 ± 0.0016 34
Cx 2 + EGF (n=5) % control	0.0099 ± 0.0010 56	0.0078 ± 0.0012 41
Cx 2 + DHT (n=5) % control	0.0165 ± 0.0020 93	0.0156 ± 0.0036 83
Cx 2 + EGF&DHT (n=5) % control	0.0189 ± 0.0034 106	0.0183 ± 0.0030 97

Table 2. Effects of Sx and Cx on the weight of the prostate tissues of the transgenic mice. The prostate tissues were obtained as described in Materials and Methods. For Sx 1 and Cx 1 groups, the mice were killed on day 14 after the surgery. For Sx 2 and Cx 2 groups, the mice were killed on day 28 after the surgery. For Sx 1 + EGF group, the mice were received EGF (100μg/kg/day) delivered by intra-abdominal injection daily until they were killed on day 14. For Sx 2 group, the mice received EGF on day 14 post-Sx and EGF was given daily until the mice were killed on day 28. For Cx 2 group, the mice received EGF and/or DHT on day 14 post-Cx. DHT (3mg/kg/day) were delivered by subcutaneous injection daily until the mice were killed on 28 day post-Cx. Intact transgenic mice were served as controls.

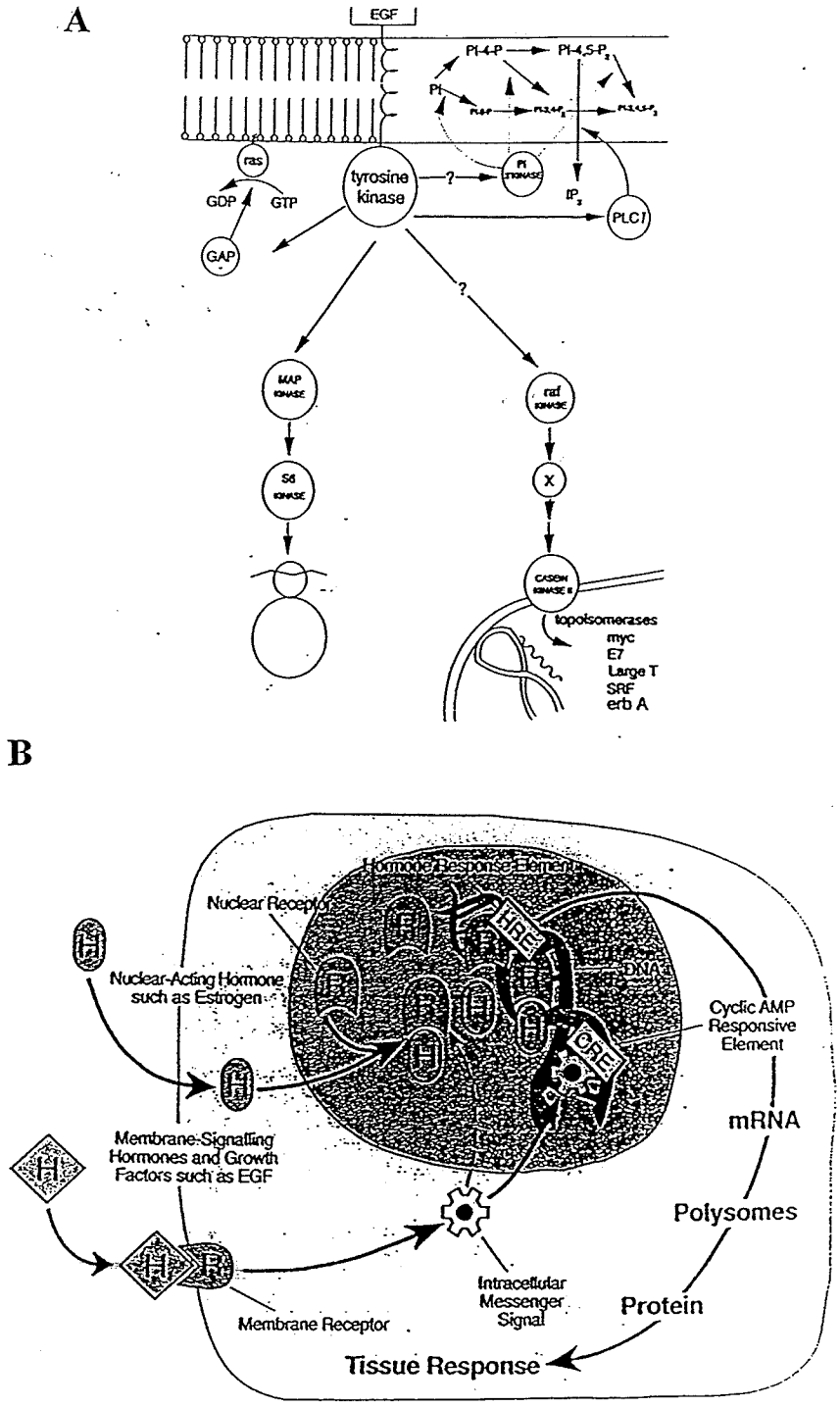


Figure 1. EGF- and androgen- signal transduction pathways. A. Tyrosine kinase substrates and potential pathways for EGF signal transduction (adopted from Carpenter and Cohen, 1990). **B.** Examples of cellular mechanisms for hormonal stimulation. Steroid, thyroid, and retinoid hormones diffuse into cells in which they interact with nuclear receptor proteins that function as ligand-activated transcription factors. The receptor ligand complex dimerizes and binds to specific DNA sequences (HRE) upstream of genes regulated by the hormone. Regulation results in an increase in specific gene transcription that influences responses within target cells. Protein hormones and growth factors are examples of stimulants which interact with membrane receptors eliciting a cellular response mediated by an intracellular second messenger signaling pathway (adopted from Korach, 1994).

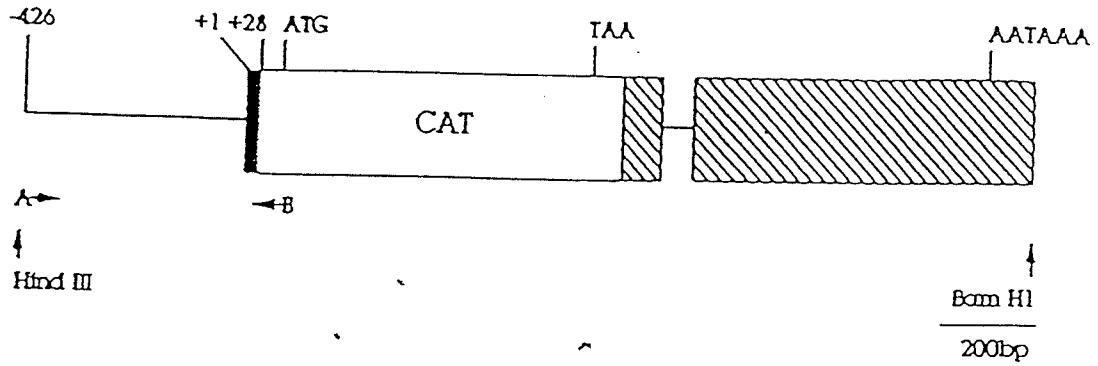


Figure 2. Structure of the PB-CAT Fusion Gene Construct Used to Generate Transgenic Mice (adopted from Greenberg et al. 1994). The elements are: -426 to 1, the 5'-flanking region of the rat PB gene; 1-28, portion of the noncoding first exon of PB; ATG and TAA, the CAT open reading frame; hatched region, simian virus-40 sequences; and AATAAA, polyadenylation signals. The location and orientation of the primers used for PCR are denoted A and B.

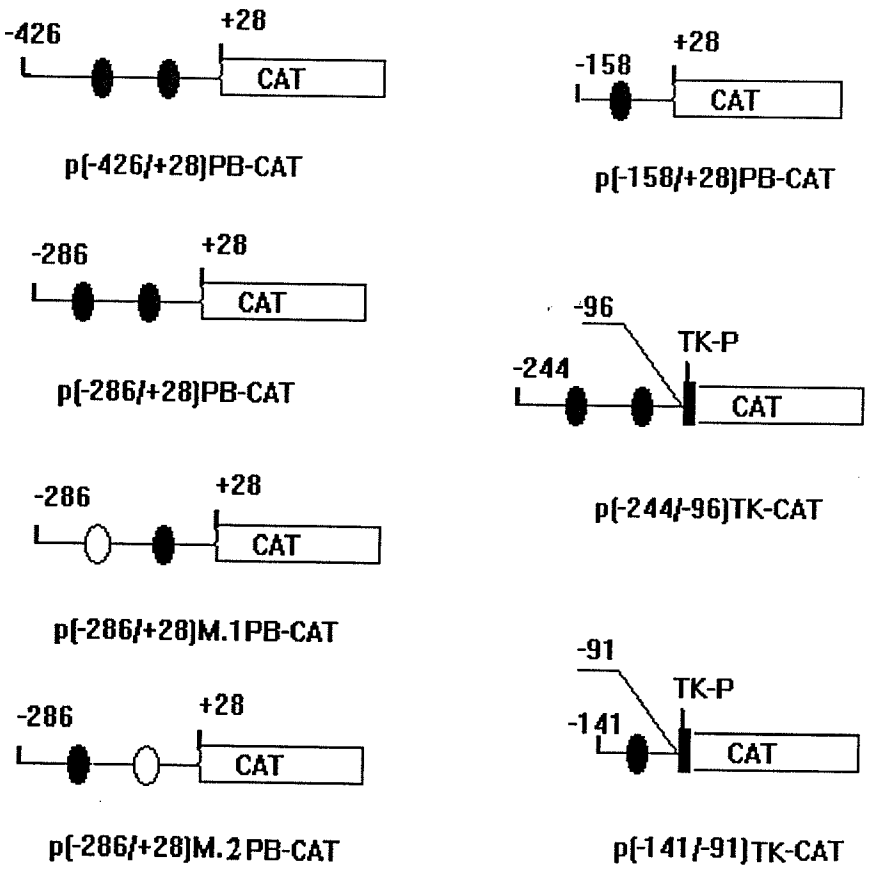


Figure 3. Schematic drawing of the plasmid constructs used in the transfection experiments.

The androgen regulatory elements are indicated as solid ovals (ARE-1 and ARE-2). The position and sequence of the two regulatory elements in the 5'-flanking region of the rat probasin gene are: ARE-1: 5'-ATCTTGTTCTTAGT (from -236 to -223) and ARE-2: 5'-GTAAAGTACTCCAAGAACCTATTT (from -140 to -117) (Rennie et al. 1993). The mutated AREs are indicated by open ovals. The G in ARE-1 at - 231 was replaced by A for p(-286/+28) M .1 PB-CAT and the C in ARE-2 at -123 was replaced by A for p(-286/+28)M.2 PB-CAT . TK-P represents thymidine kinase promoter that was fused to CAT reporter gene.

```
-426
*
AAGCTTCCACAAGTGCATTTAGCCTCTCCAGTATTGCTGATGAATCCACAGTTCAGGTTC

AATGGCGTTCAAAACTTGATCAAAAATGACCAGACTTTATATTCTTACACCAACATCTAT

                    -286
                    *
CTGATTGGAGGAATGGATAATAGTCATCATGTTTAAACATCTACCATTCCAGTTAAGAAA

  -244
  *
ATATGATAGCATCTTGTTCTTAGTCTTTTTCTTAATAGGGACATAAAGCCCACAAATAAA

                              -158
                              *
AATATGCCTGAAGAATGGGACAGGCATTGGGCATTGTCCATGCCTAGTAAAGTACTCCAA

GAACCTATTTGTATACTAGATGACACAATGTCAATGTCTGTGTACAACTGCCAACTGGGA

                                        *
TGCAAGACACTGCCCATGCCAATCATCCTGAAAAGCAGCTATAAAAAGCAGGAAGCTACT

      +1                          +28
      *                           *
CTGCACCTTGTCAGTGAGGTCCAGATACCTACAGAGCTCACACACG ATG AGG GTC
                                                Met Arg Val

ATC CTC CTC CTG CTC ACA CTG GAT GTG CTA GGT GTC TCC AGT
Ile Leu Leu Leu Leu Thr Leu Asp Val Leu Gly Val Ser Ser

ATG ATG ACA GAC AAG AAT CTC AAA AAG AAG CTAGCAGAC
Met Met Thr Asp Lys Asn Leu Lys Lys Lys
```

Figure 4. Sequence of Probasin 5' -Flanking DNA. The start of transcription is as +1. All negative numbering is relative to the start site of transcription. The stars above the nucleotides indicate the 5' end of particular chimaeric PB-CAT or TK-CAT construct. The ARE-1 and ARE-2 sequences are boxed and the CAAT box, and TATAA box are underlined(adopted from Rennie et al., 1993)

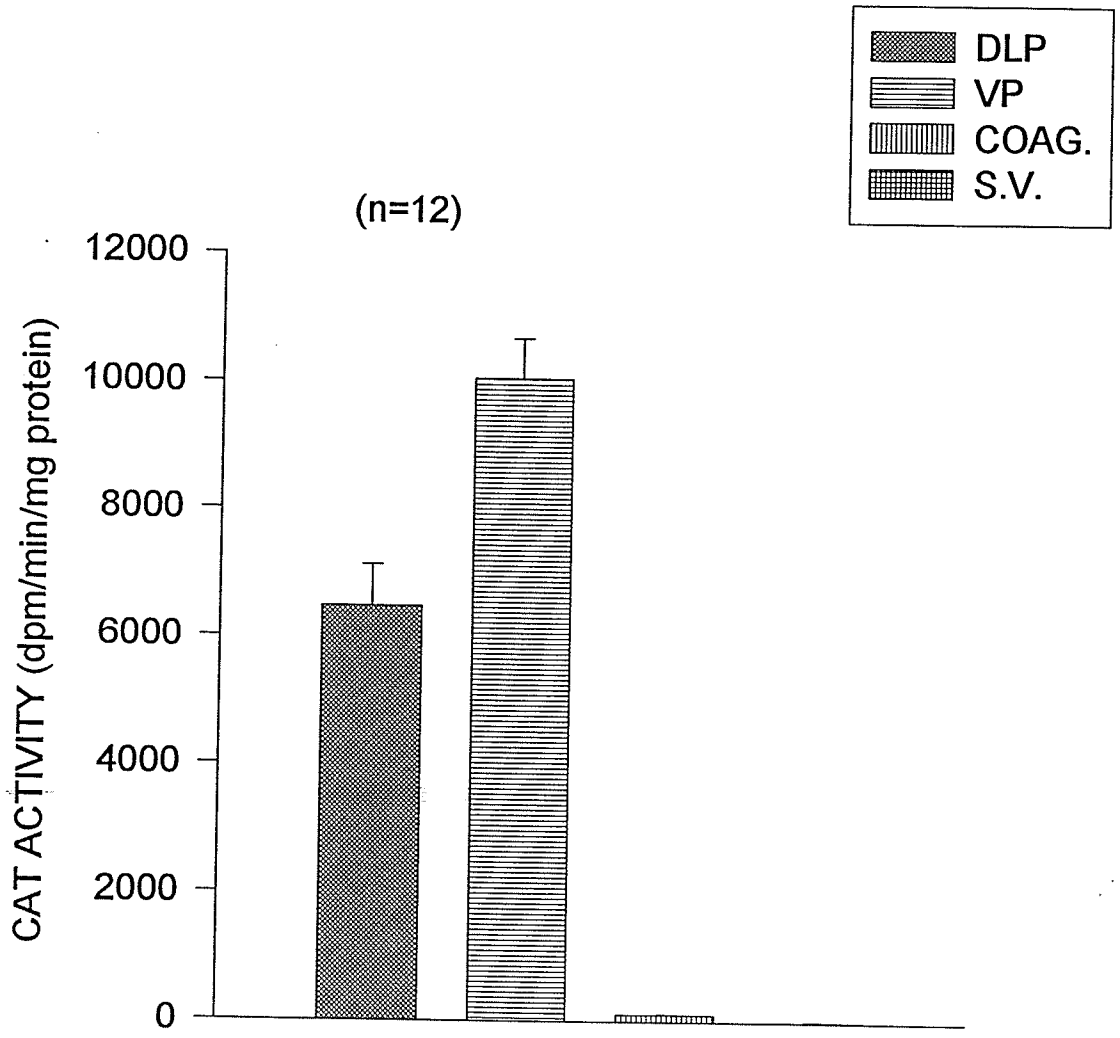

DLP
VP
COAG.
S.V.
(n=12)
CAT ACTIVITY (dpm/min/mg protein)
12000
10000
8000
6000
4000
2000
0

Figure 5. PB-CAT Expression in the Transgenic Mice. The tissues of dorsolateral, ventral prostate, coagulating gland and seminal vesicle were obtained from 10-week old S.W. male transgenic mice. The CAT assay was performed as described and the data represents mean ± S.E. from 12 mice (n=12).

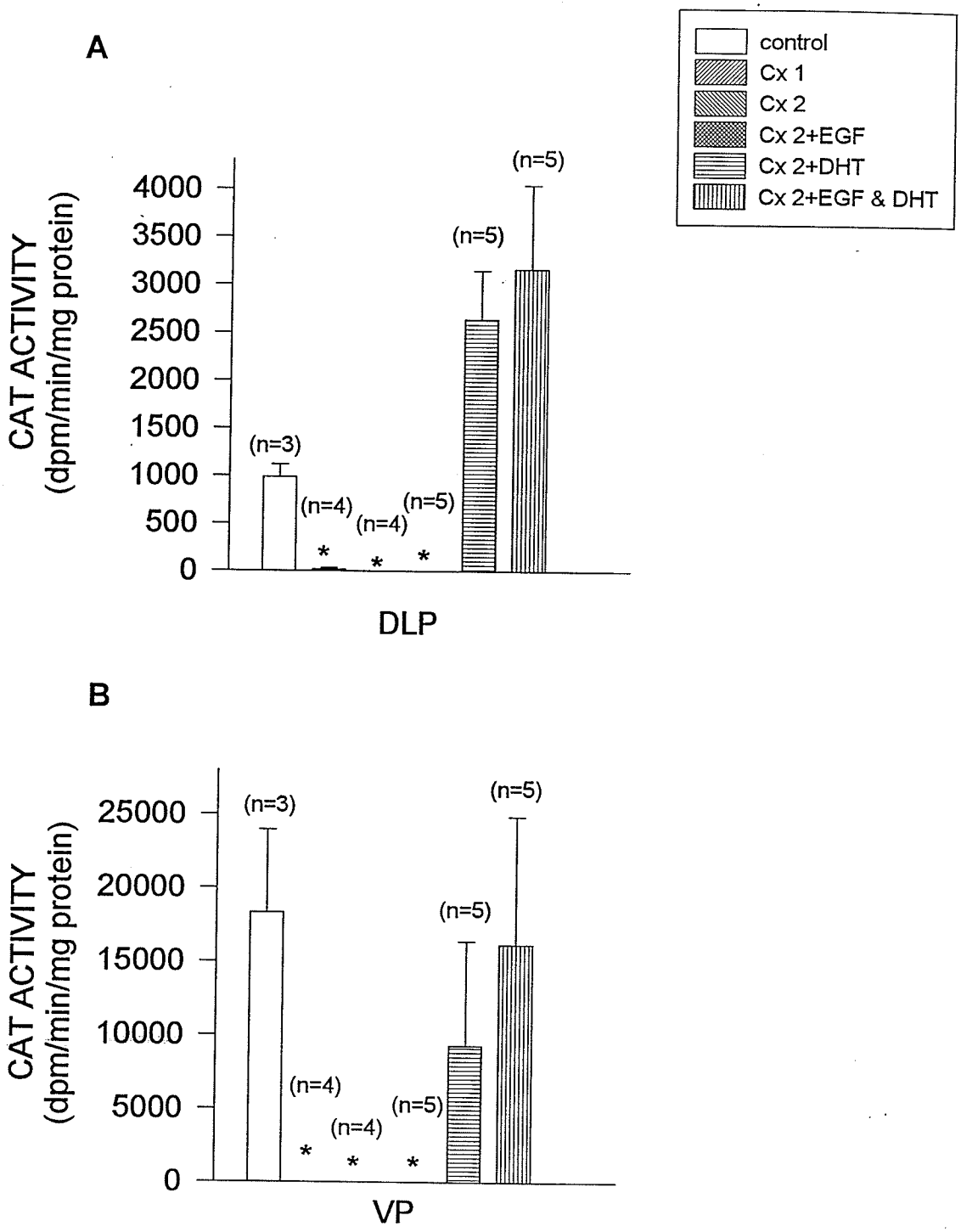
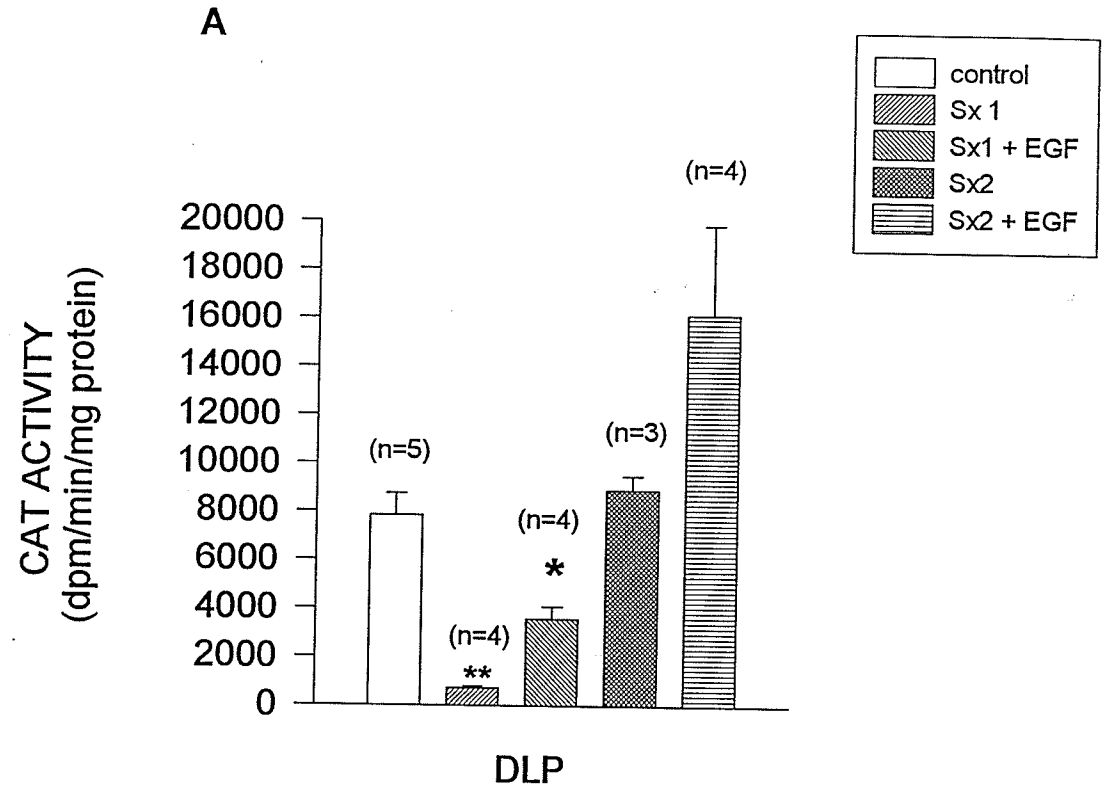

A
control
Cx 1
Cx 2
Cx 2+EGF
Cx 2+DHT
Cx 2+EGF & DHT
CAT ACTIVITY
(dpm/min/mg protein)
4000
3500
3000
2500
2000
1500
1000
500
0
(n=3)
(n=4)
(n=4)
(n=5)
(n=5)
(n=5)
*
*
*
DLP
B
CAT ACTIVITY
(dpm/min/mg protein)
25000
20000
15000
10000
5000
0
(n=3)
(n=4)
(n=4)
(n=5)
(n=5)
(n=5)
*
*
*
VP

Figure 6. Effect of Castration on PB-CAT Expression in the Transgenic Mice (A: DLP; B: VP) 10-week old S.W. male transgenic mice were castrated (Cx); the intact mice were served as controls. The dorsolateral and ventral prostate tissues were obtained as described. For the Cx1 and Cx2 groups, the mice were killed on day 14 and day 28 after the surgery respectively. For Cx2 group, the mice received EGF and /or DHT on day 14 after the surgery. EGF (100μg/kg/day) and DHT (3mg/kg/day) were delivered by intra-abdominal and subcutaneous injection respectively each day until the mice were killed. For the controls, mice received injections of vehicles only. CAT assay was performed as described and the data represents mean ± S.E. (n= number of mice). Based on SigmaPlot Student's *t* test (unpaired group), significant difference from the level of intact control is shown by * ($p<0.01$).


A
control
Sx 1
Sx1 + EGF
Sx2
Sx2 + EGF
CAT ACTIVITY
(dpm/min/mg protein)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
(n=5)
(n=4)
**
(n=4)
*
(n=3)
(n=4)
DLP

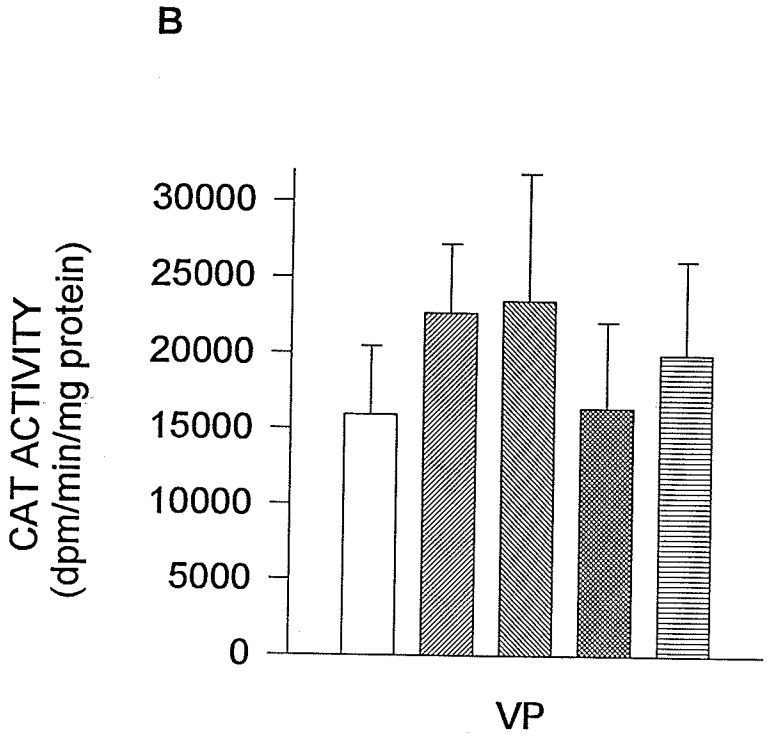
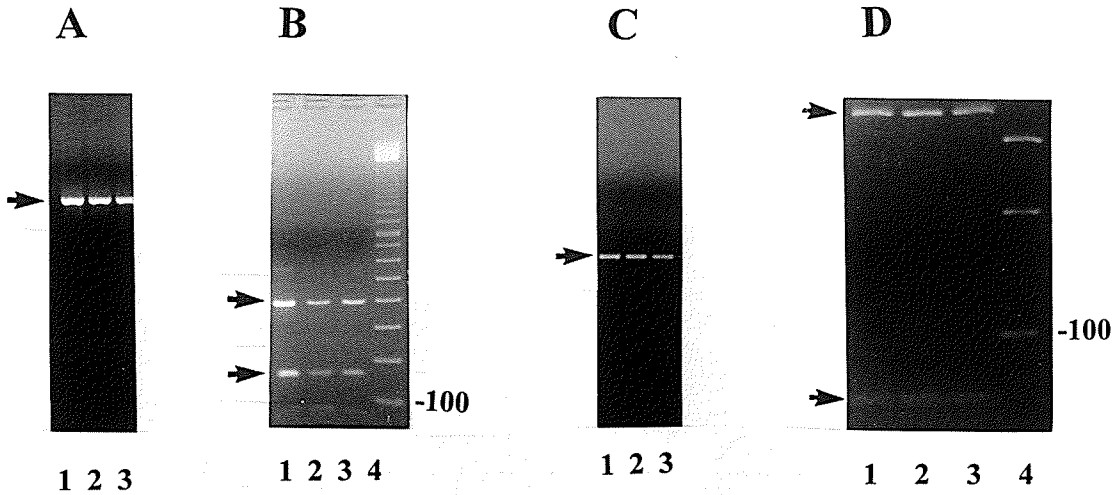

B
CAT ACTIVITY
(dpm/min/mg protein)
30000
25000
20000
15000
10000
5000
0
VP

Figure 7. Effect of Sialoadenectomy on PB-CAT Expression in the Transgenic Mice (A: DLP; B: VP). 10-week old S.W. male transgenic mice were sialoadenectomized (Sx) and the intact mice served as controls. The dorsolateral prostate tissues were obtained as described. For the Sx1 and Sx2 groups, the mice received EGF on the next day and the day 14 after the surgery, respectively. EGF (100μg/kg/day) was delivered by intra-abdominal injection each day until the mice were killed either on the day 14 (Sx1) or on the day 28 (Sx2) after the surgery. CAT assay was performed as described and the data represents mean ± S.E. (n=number of mice). Based on SigmaPlot Student's *t* test (unpaired group), significant difference from the level of intact control and Sx1 is shown by * *($p<0.001$) and by * ($p<0.001$), respectively.


A
B
C
D
-100
1 2 3
1 2 3 4
1 2 3
1 2 3 4

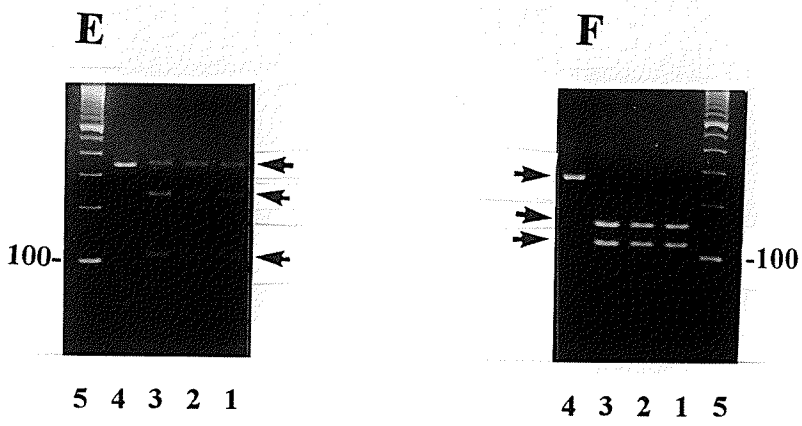

E
F
100-
-100
5 4 3 2 1
4 3 2 1 5

Figure 8. Ethidium Bromide Stained Gels of RT-PCR Products for EGF, EGF-R, TGF-α and AR. RNA extraction and RT-PCR were performed as described. Lanes 1,2 and 3 are RT-PCR products obtained from dorsal, lateral and ventral prostate mRNA, respectively. EGF (A, 557bp), EGF-R (C, 424bp), TGF-α (E, 337bp, lane 4), AR (F, 274bp, lane 4) and two bands of 159bp and 398bp for EGF by AccI(B); 65bp and 359bp for EGF-R by BglI(D); 228bp and 109bp for TGF-α by Sau3AI(E); 117bp and 157bp for AR by NdeI(F) restriction enzyme digestion are indicated by the arrows. Lanes 4 (B, D) and 5 (E , F) are 100 base-pair ladder marker. Picture D was taken from 4% agarose gel and the others were taken from 2% agarose gel.

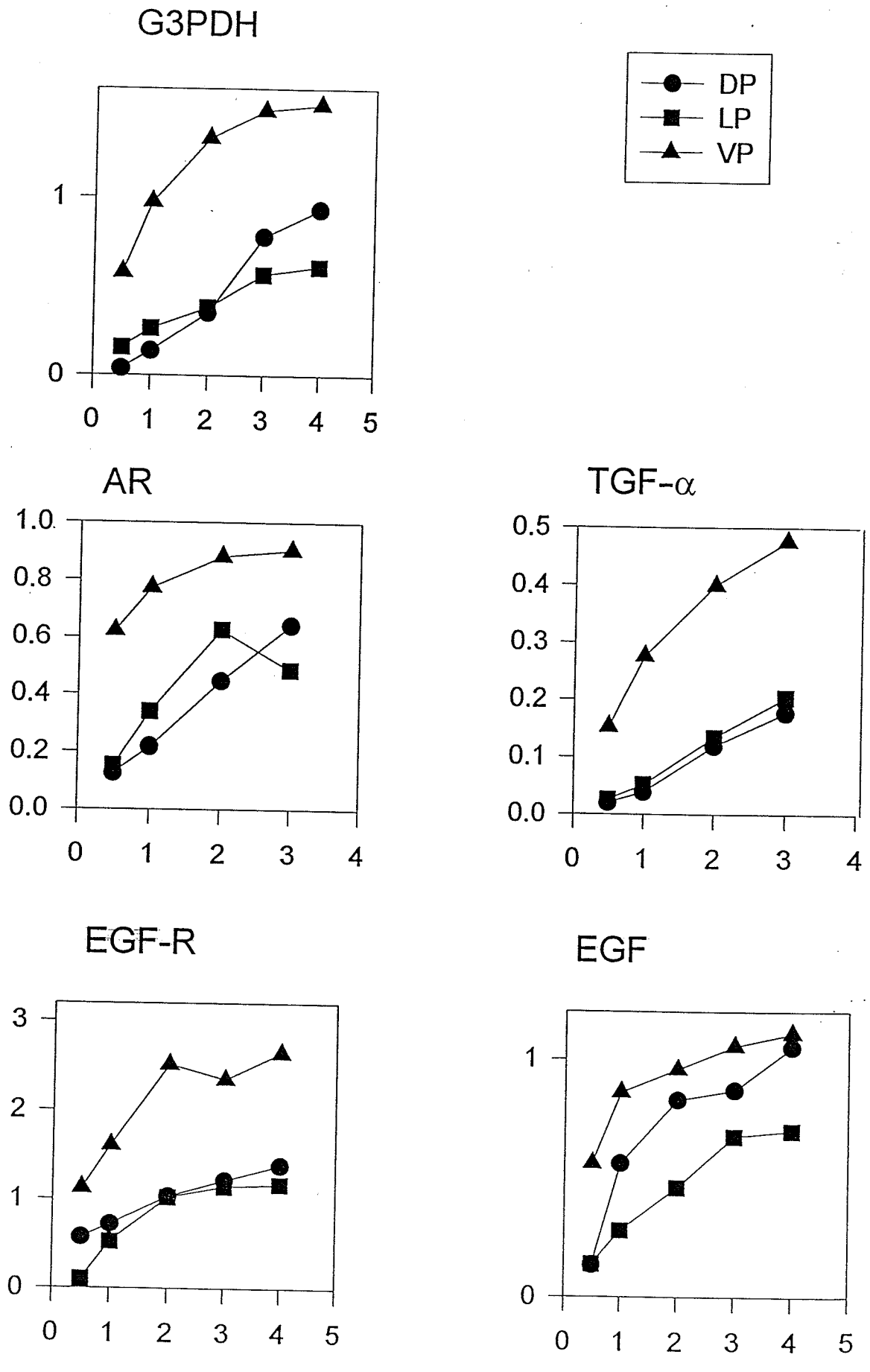

G3PDH
DP
LP
VP
AR
TGF-α
EGF-R
EGF
Amount of RT Reaction(ul)

Figure 9. Linear Ranges of RT-PCR Generated by the Primers for AR, TGF-α, EGF and EGF-R. The prostate tissues were obtained from normal S.W. male mice and RT-PCR was performed as described. 1μg of total RNA, as determined by absorbance at 260 nm, was used as template for the random hexamer [$pd(N)_6$] to synthesize first strand of cDNA. Varying amounts (0.5,1.0, 2.0, 3.0, 4.0 μl for EGF, EGF-R and G3PDH; 0.5, 1.0, 2.0, 3.0 μl for AR and TGF-α) of RT reaction were used as template for PCR amplification. The relative expression of mouse EGF, EGF-R, TGF-α, AR and G3PDH in total RNA was demonstrated by relative absorbance units of bands determined by densitometic scanning of pictures of RT-PCR ethidium bromide stained agarose electrophoresis (IBM ScanPlot program).

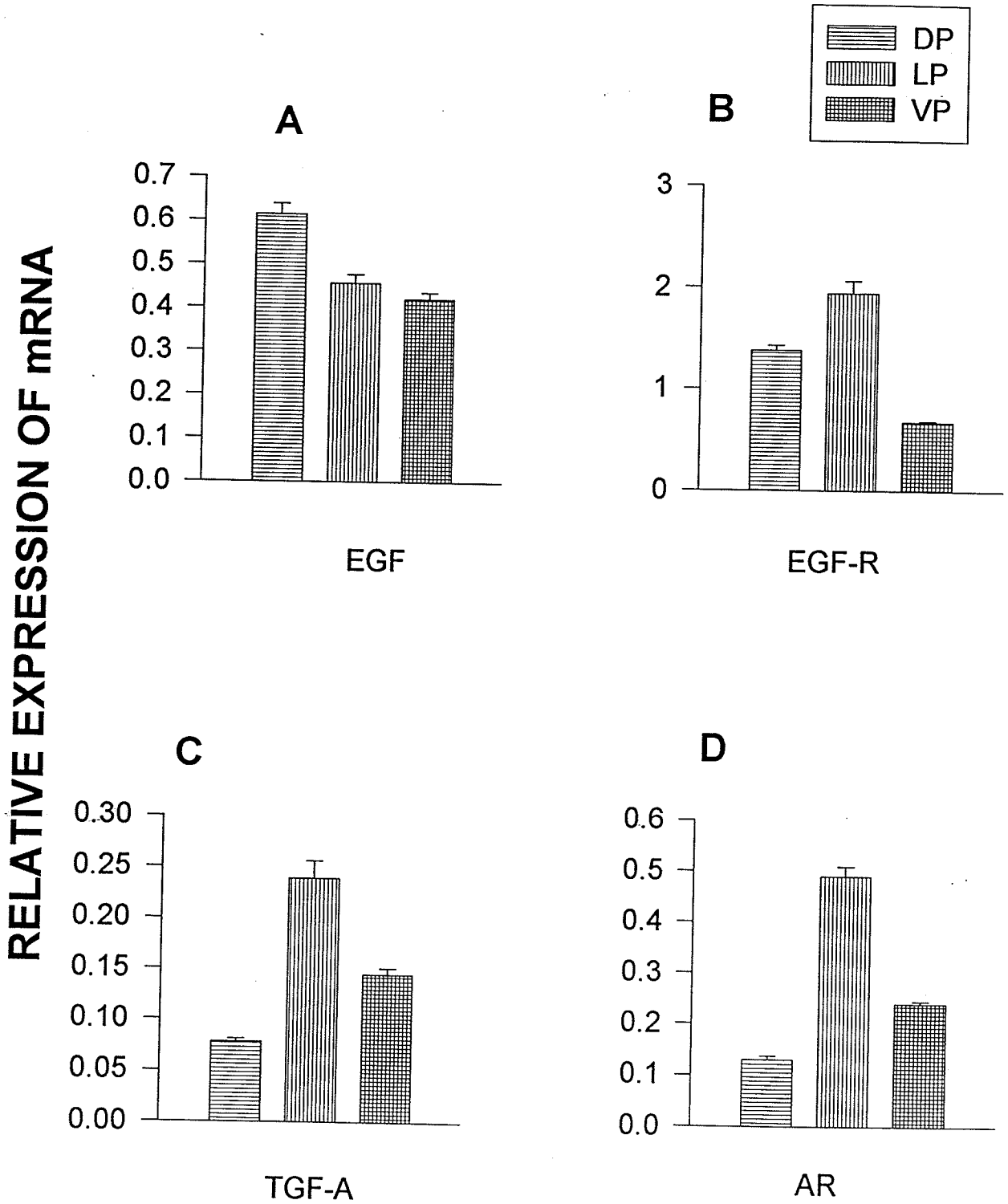

RELATIVE EXPRESSION OF mRNA
A
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
EGF
B
DP
LP
VP
3
2
1
0
EGF-R
C
0.30
0.25
0.20
0.15
0.10
0.05
0.00
TGF-A
D
0.6
0.5
0.4
0.3
0.2
0.1
0.0
AR

Figure 10. Relative Expression of EGF, EGF-R, TGF-α and AR mRNA in Total RNA from Dorsal, Lateral and Ventral Prostate Tissues of Normal S.W. Male Mice. The prostate tissues were obtained from 10-week old S.W. male mice and total RNA extraction and RT-PCR were performed as described. The relative abundance of each mRNA was demonstrated by relative absorbance units of bands from these factors vs G3PDH bands determined by densitometric scanning of RT-PCR ethidium bromide stained agarose electrophoresis pictures. The bar graph shows mean ± S.E. from 5 determinations (n=5). In (A) DP is significantly higher than LP and VP ($p<0.001$). In (B), (C), and (D)DP, LP and VP are each significantly different from the others ($p<0.01$).

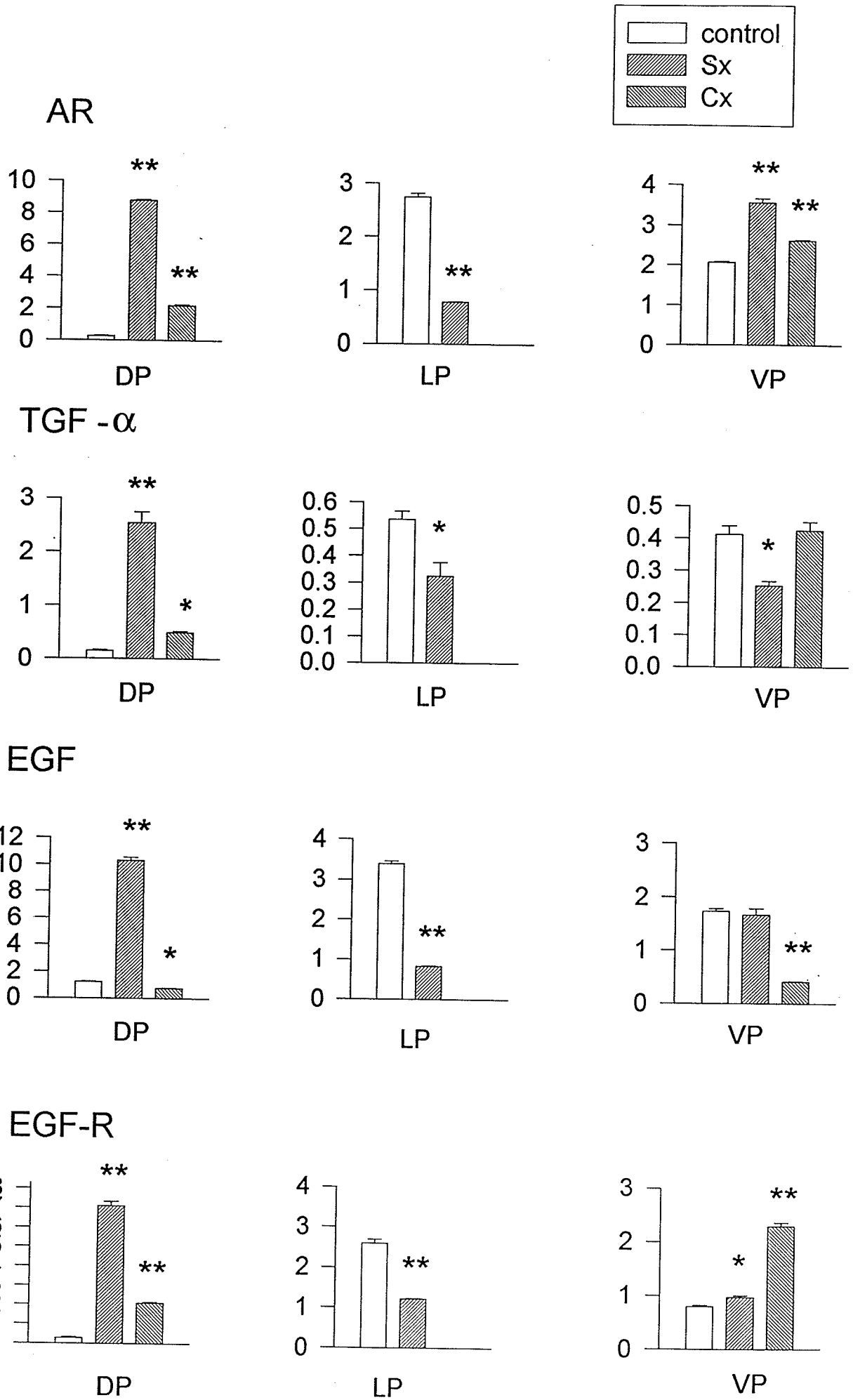

control
Sx
Cx
AR
10
8
6
4
2
0
**
**
DP
3
2
1
0
**
LP
4
3
2
1
0
**
**
VP
TGF -α
3
2
1
0
**
*
DP
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
LP
0.5
0.4
0.3
0.2
0.1
0.0
*
VP
EGF
12
10
8
6
4
2
0
**
*
DP
4
3
2
1
0
**
LP
3
2
1
0
**
VP
EGF-R
8
7
6
5
4
3
2
1
0
**
**
DP
4
3
2
1
0
**
LP
3
2
1
0
*
**
VP

Figure 11. Relative Expression of AR, TGF-α, EGF and EGF-R mRNA in Total RNA from Dorsal, Lateral and Ventral Prostate Tissues of the Mice on the Day 14 after Castration and Sialoadenectomy. 10-Week old S.W. male mice were castrated (Cx) or sialoadenectomized (Sx). The animals were killed on day 14 after the surgery and the dorsal, lateral and ventral prostate tissues were obtained as described. For the LP, because of lack of sufficient tissues, the relative expression of the mRNAs was not determined in this lobe after Cx. Intact mice served as controls. Total RNA extraction and RT-PCR were performed as described. The relative abundance of each mRNA was demonstrated by relative absorbance units of bands from these factors vs G3PDH bands determined by densitometric scanning of RT-PCR ethidium bromide stained agarose electrophoresis pictures. The bar graph shows mean ± S.E. from four determinations (n=4). Based on SigmaPlot Student's *t* test (unpaired group), significant difference from the levels of controls is shown by * ($p<0.05$) and ** ($p<0.01$).

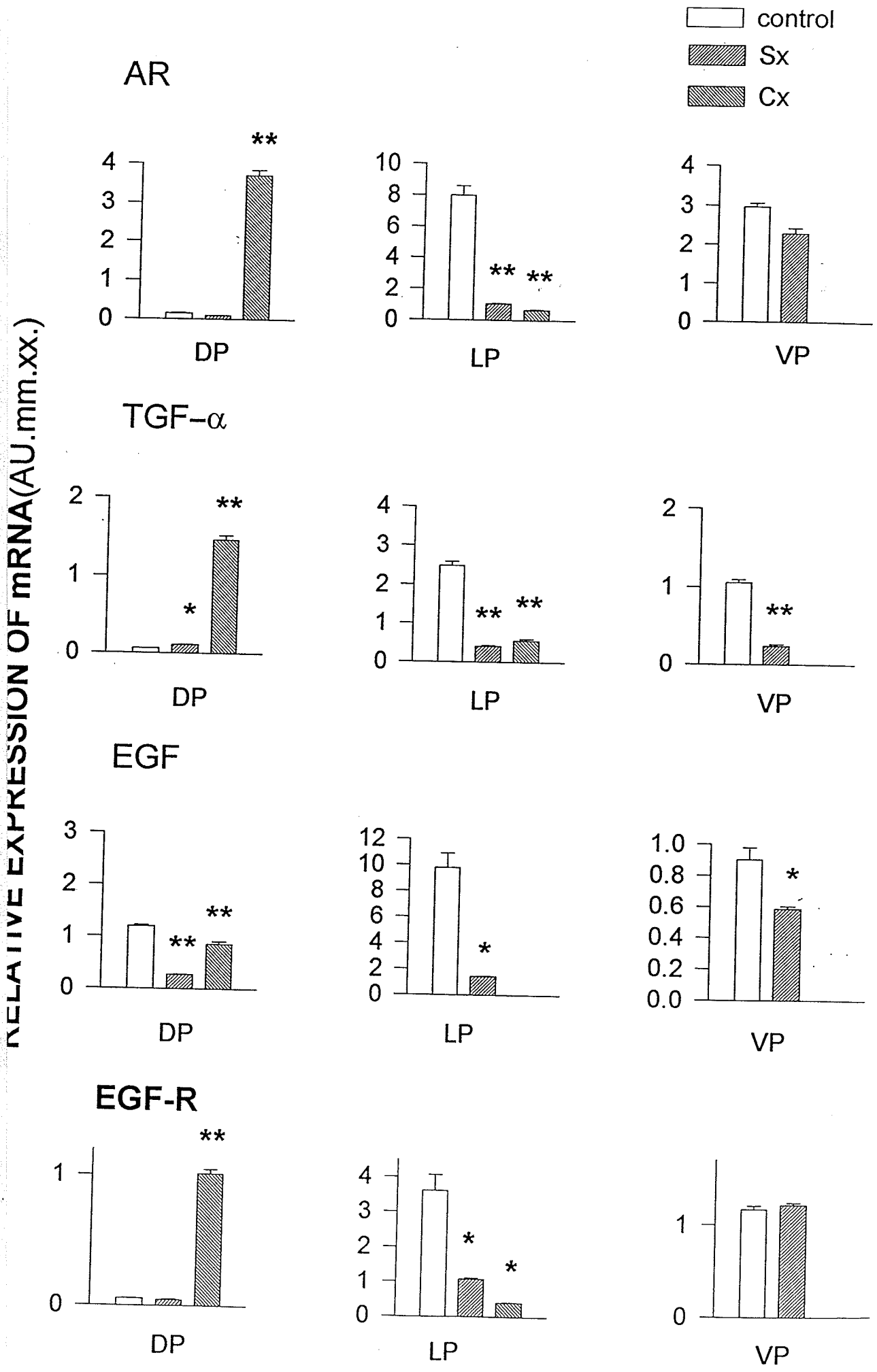
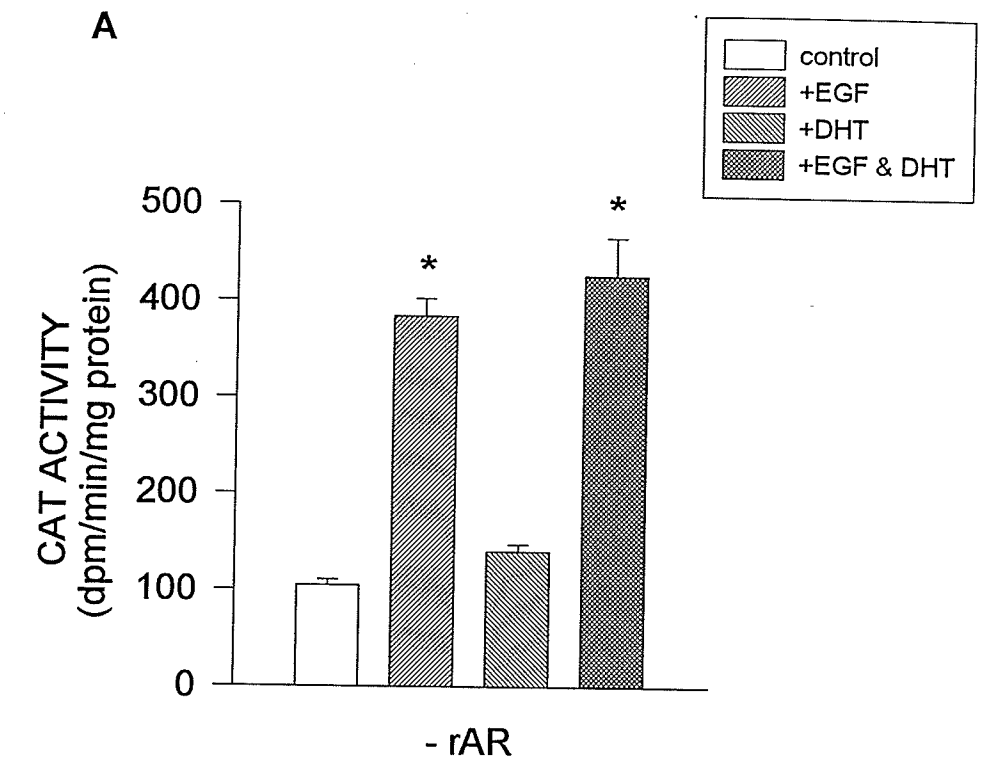

control
Sx
Cx
AR
TGF-α
EGF
EGF-R
RELATIVE EXPRESSION OF mRNA(AU.mm.xx.)
DP
LP
VP

Figure 12. Relative Expression of AR, TGF-α, EGF and EGF-R mRNA in Total RNA from Dorsal, Lateral and Ventral Prostate Tissues of the Mice on the Day 28 after Castration and Sialoadenectomy. 10-Week old S.W. male mice were castrated (Cx) and sialoadenectomized (Sx). The animals were killed on day 28 after the surgery and the dorsal, lateral and ventral prostate tissues were obtained as described. Total RNA extraction and RT-PCR were performed as described. The relative abundance of each mRNA was demonstrated by relative absorbance units of bands from these factors vs G3PDH bands determined by densitometric scanning of RT-PCR ethidium bromide stained agarose electrophoresis pictures. Because of lack of tissues for VP, the relative expression of the mRNAs was not detected for this lobe after Cx. The bar graph shows mean ± S.E. from four determinations (n=4). Based on SigmaPlot Student's *t* test (unpaired group), significant difference from the levels of the controls is shown by *($p<0.05$) and **($p<0.01$).


A
control
+EGF
+DHT
+EGF & DHT
CAT ACTIVITY
(dpm/min/mg protein)
500
400
300
200
100
0
*
*
- rAR

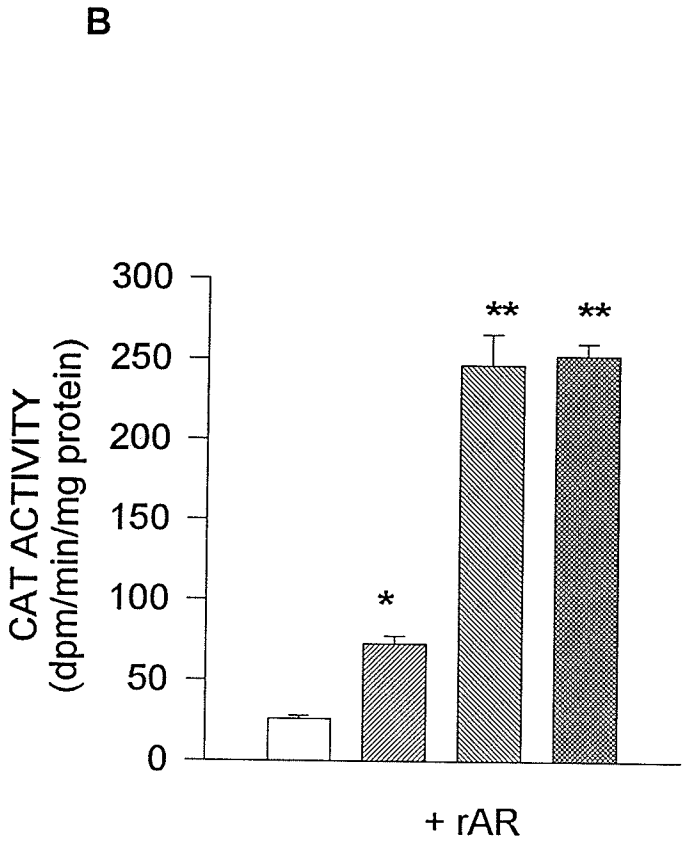
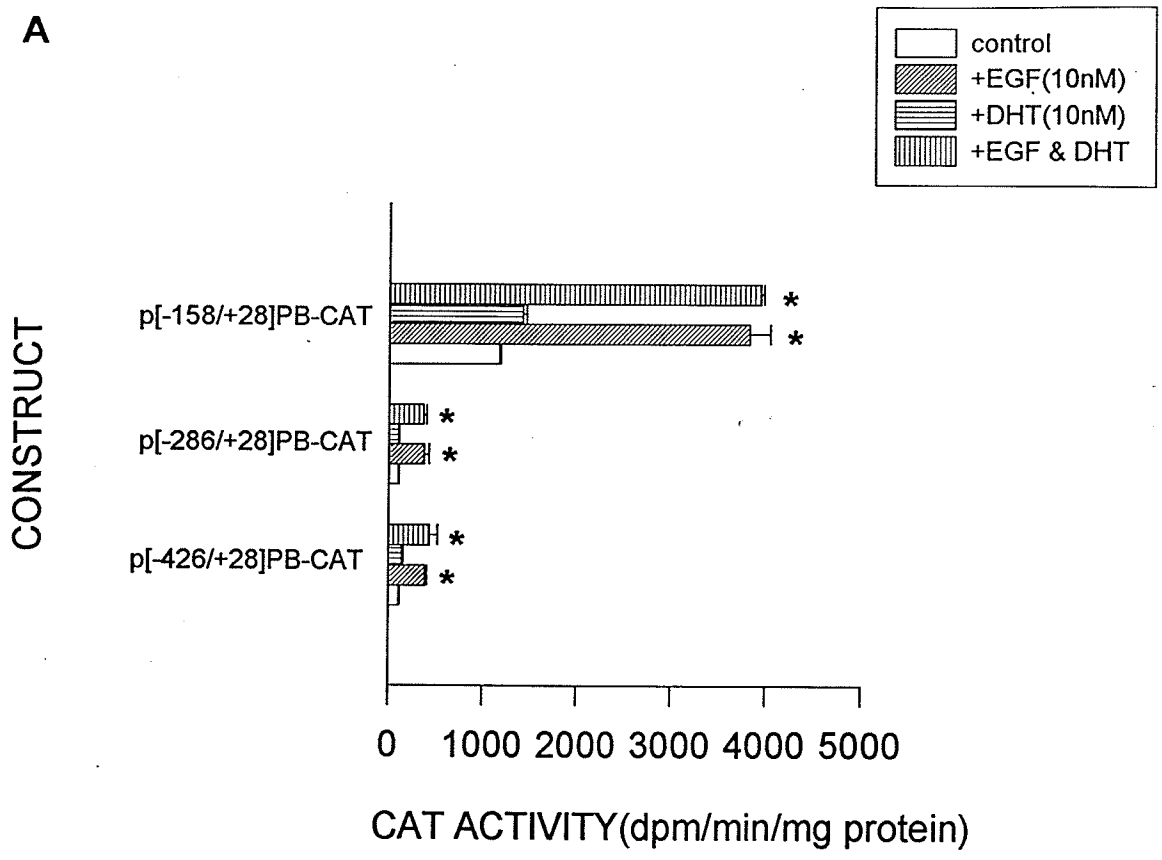

B
CAT ACTIVITY
(dpm/min/mg protein)
300
250
200
150
100
50
0
*
**
**
+ rAR

Figure 13. Effects of EGF and DHT on the p(-426/+28) PB-CAT construct in DU145 cells. The construct of p(-426/+28) PB-CAT was transfected into DU145 cells without (A) or with (B) the rAR expression vector and treated with EGF, DHT or both as indicated in the legends, respectively. The CAT activity without addition of EGF or DHT served as a base line (control). All CAT assays were performed by scintillant two phase fluor diffusion method and activity was expressed as the mean ± S.E. of triplicate determinations after being normalized for transfection efficiency. Based on SigmaPlot Student's *t* test (unpaired group), significant difference from the levels of the controls is shown by * ($p<0.05$) and ** ($p<0.01$), respectively.

A


control
+EGF(10nM)
+DHT(10nM)
+EGF & DHT
CONSTRUCT
p[-158/+28]PB-CAT
p[-286/+28]PB-CAT
p[-426/+28]PB-CAT
0 1000 2000 3000 4000 5000
CAT ACTIVITY(dpm/min/mg protein)

B

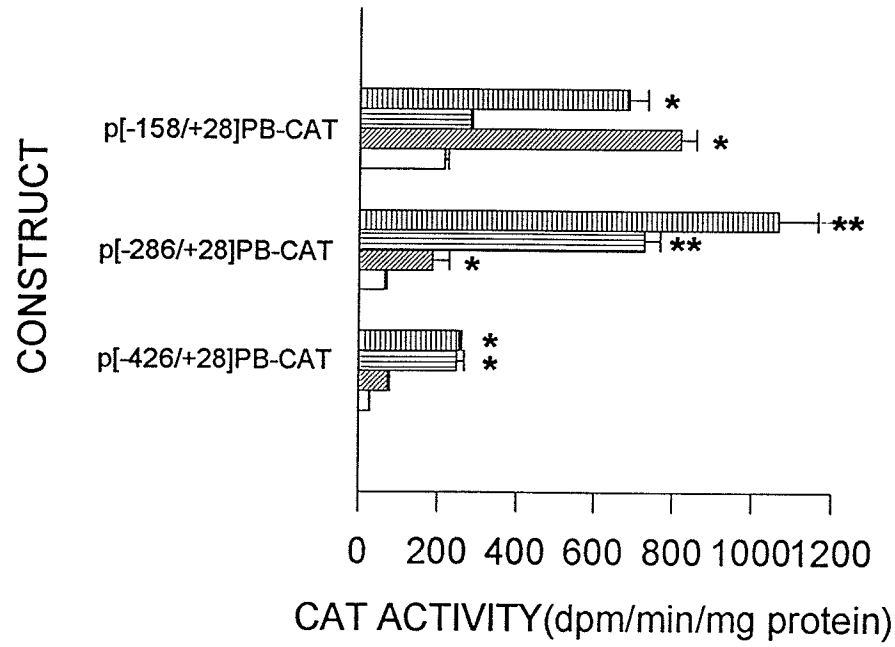
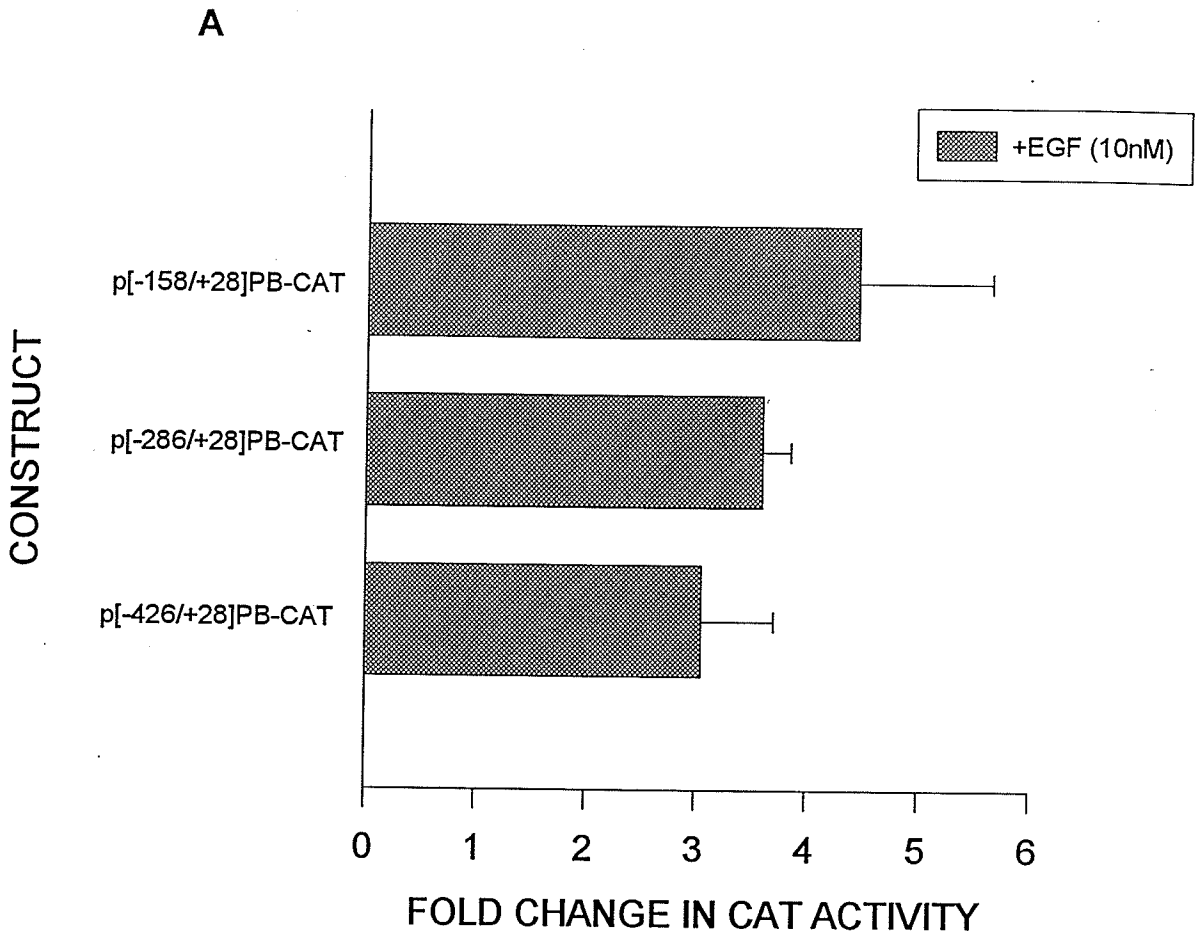

CONSTRUCT
p[-158/+28]PB-CAT
p[-286/+28]PB-CAT
p[-426/+28]PB-CAT
0 200 400 600 800 10001200
CAT ACTIVITY(dpm/min/mg protein)

Figure 14. Effects of EGF and DHT on 5'-Deletion of PB-CAT Constructs in DU145 Cells. Deletions of 5'-flanking sequences (-426, -286 and -158 bp) in PB-CAT were transfected into DU145 cells without (A) or with (B) the rAR expression vector and treated with EGF, DHT or both EGF and DHT as indicated in the legends, respectively. The CAT activity without addition of EGF or DHT served as control. All CAT assays were performed by scintillant two phase fluor diffusion method and activity was expressed as the mean ± S.E. of triplicate determinations after being normalized for transfection efficiency. Based on SigmaPlot Student's *t* test (unpaired group), significant difference from the levels of the controls is shown by * ($p<0.05$) and ** ($p<0.01$), respectively.


A
+EGF (10nM)
p[-158/+28]PB-CAT
p[-286/+28]PB-CAT
p[-426/+28]PB-CAT
CONSTRUCT
0
1
2
3
4
5
6
FOLD CHANGE IN CAT ACTIVITY

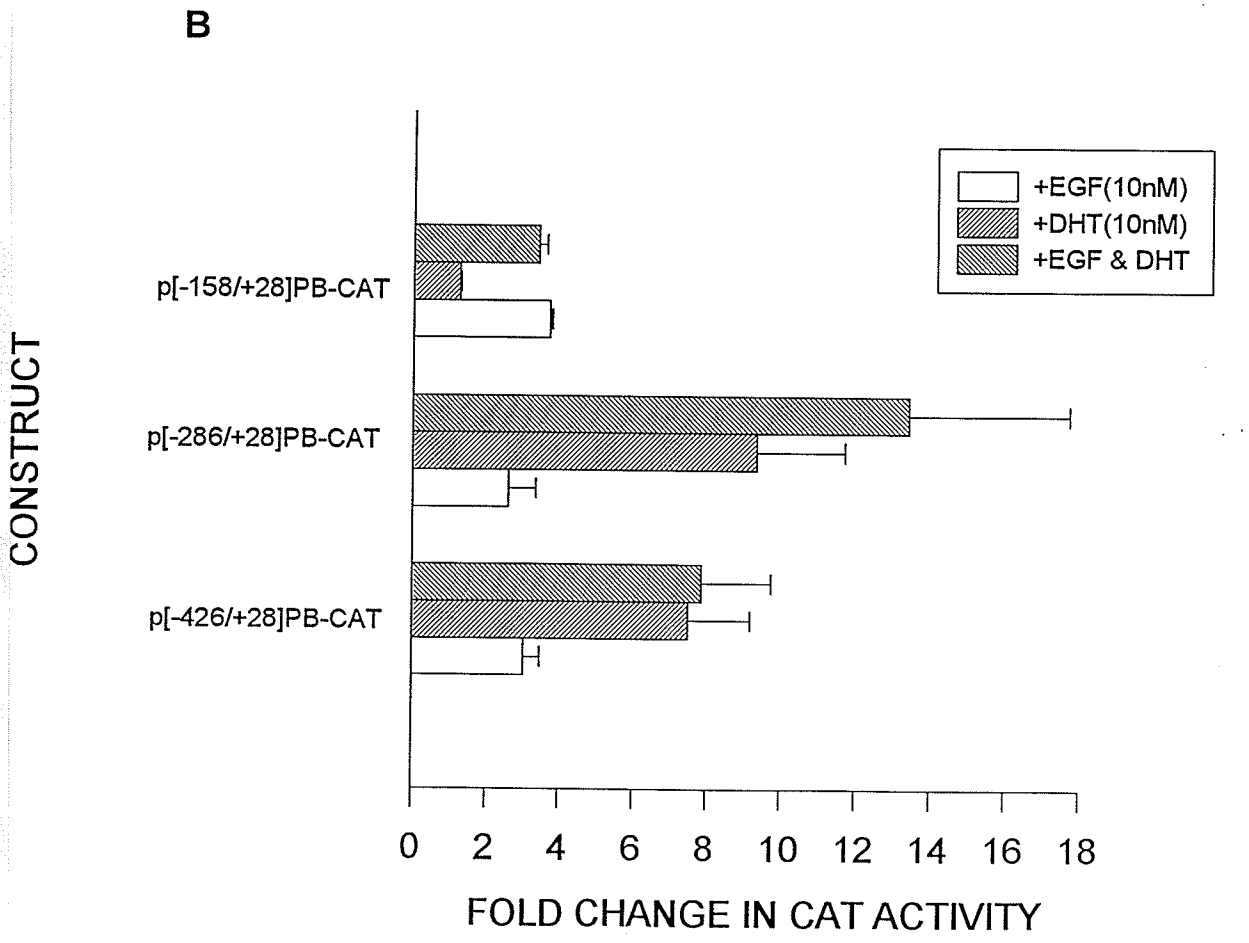
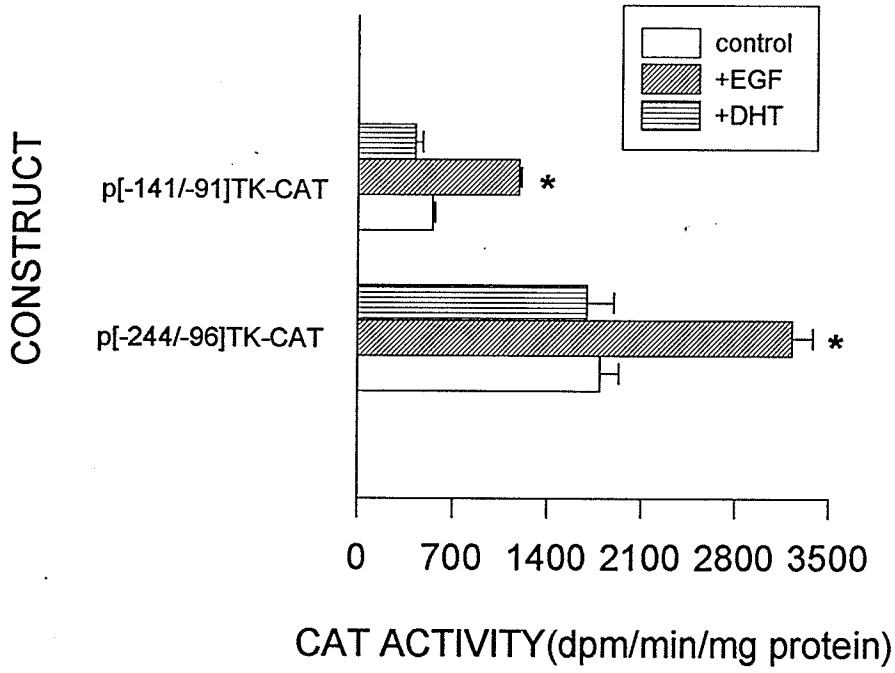

B
+EGF(10nM)
+DHT(10nM)
+EGF & DHT
p[-158/+28]PB-CAT
p[-286/+28]PB-CAT
p[-426/+28]PB-CAT
CONSTRUCT
0
2
4
6
8
10
12
14
16
18
FOLD CHANGE IN CAT ACTIVITY

Figure 15. Fold Change in CAT Activity of 5' -PB Deletion Constructs. The DU145 cells were transfected with the 5'-deletion constructs of Figure 14, co-transfected without (A) or with (B) the rAR expression vector and treated with EGF and/or DHT. CAT activity was measured in triplicate determinations after being normalized for transfection efficiency and expressed in fold induction (i.e., the ratio of EGF and/or DHT-treated over the controls). The CAT activity from the cells untreated with EGF or DHT served as controls and is represented as 1. The bar graph shows mean ± S.E. from at least two independent experiments.

A


control
+EGF
+DHT
CONSTRUCT
p[-141/-91]TK-CAT
p[-244/-96]TK-CAT
*
*
0
700
1400
2100
2800
3500
CAT ACTIVITY(dpm/min/mg protein)

B

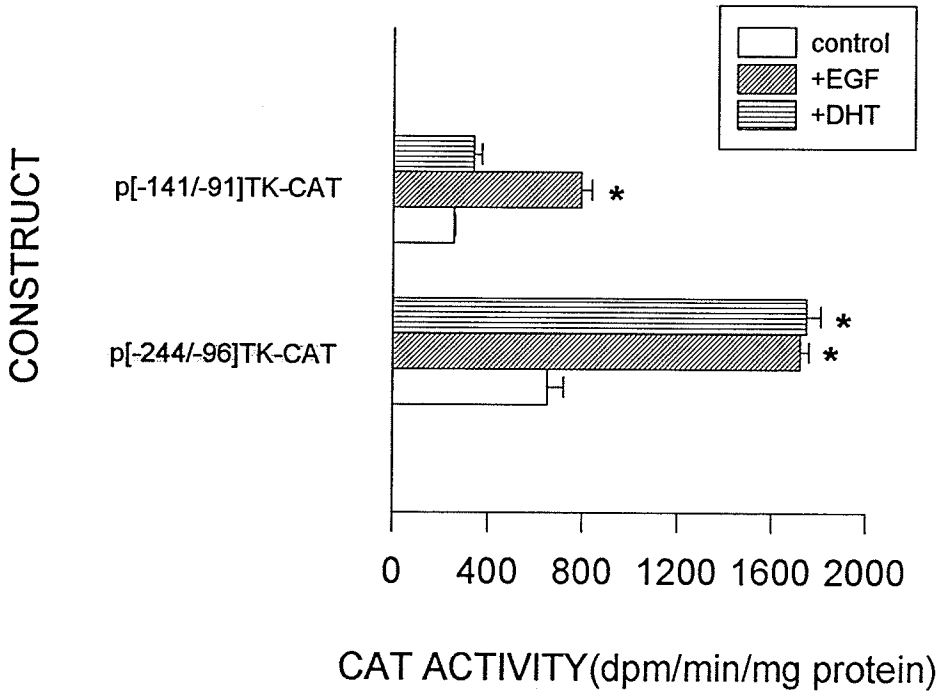
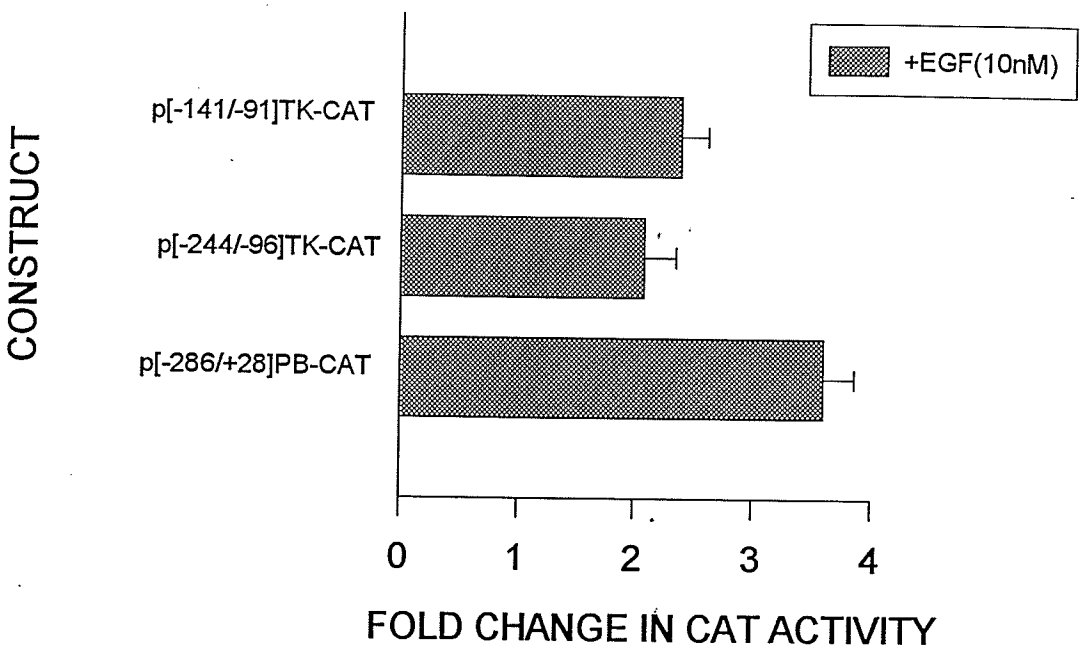

control
+EGF
+DHT
CONSTRUCT
p[-141/-91]TK-CAT
p[-244/-96]TK-CAT
*
*
*
0
400
800
1200
1600
2000
CAT ACTIVITY(dpm/min/mg protein)

Figure 16. Effects of EGF and DHT on -244/-96 and -141/-91 TK-CAT Constructs. The constructs (-244/-96 and -141/-91 TK-CAT) were co-transftected without (A) or with (B) the AR expression vector into DU145 cells which were treated with EGF or DHT (hatched bars). The CAT activity from the cells without addition of EGF or DHT served as controls (open bars). The CAT activity was expressed as the mean ± S. E. of triplicate determinations after being normalized for transfection efficiency and compared with -286/+28 PB-CAT construct. Based on SigmaPlot Student's *t* test (unpaired group), significant difference from the levels of the controls is shown by * ($p<0.05$).

A


+EGF(10nM)
p[-141/-91]TK-CAT
p[-244/-96]TK-CAT
p[-286/+28]PB-CAT
CONSTRUCT
0
1
2
3
4
FOLD CHANGE IN CAT ACTIVITY

B

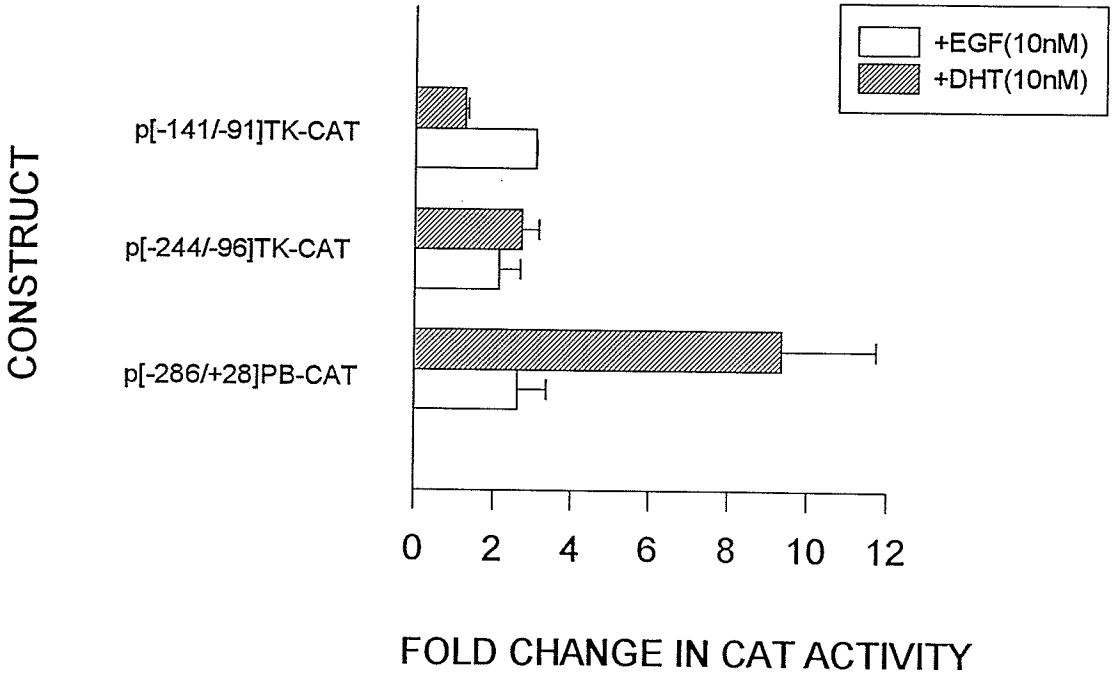
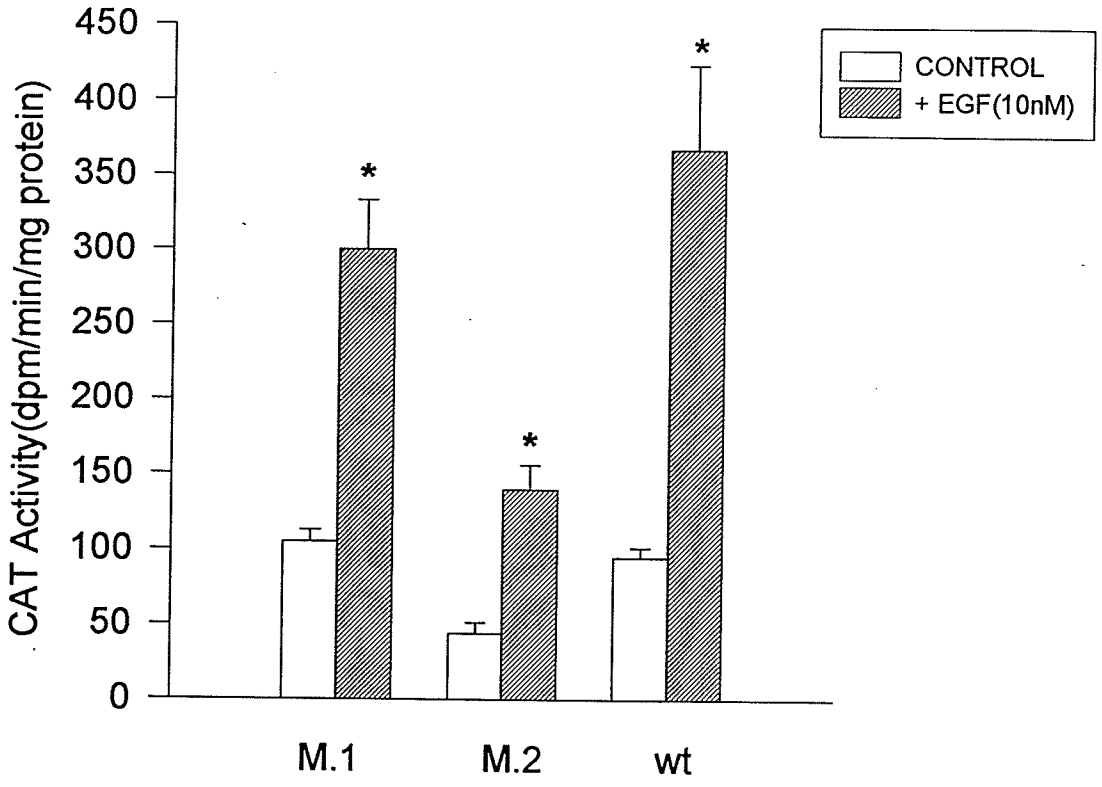

+EGF(10nM)
+DHT(10nM)
p[-141/-91]TK-CAT
p[-244/-96]TK-CAT
p[-286/+28]PB-CAT
CONSTRUCT
0
2
4
6
8
10
12
FOLD CHANGE IN CAT ACTIVITY

Figure 17. Fold Change in CAT Activity of -244/-96 and -141/-91 TK-CAT Constructs. The DU145 cells were transfected with the constructs of Fig. 16, co-transfected without (A) or with (B) the rAR expression vector and treated with EGF or DHT as indicated in the legends. The CAT activity without treatment of EGF or DHT served as controls and represented as 1. The CAT activity was compared with -286/+28 PB-CAT construct, which contains the endogenous PB promoter, after being normalized for transfection efficiency and expressed in fold induction (i.e., the ratio of EGF- or DHT-treated over the controls). The bar graph shows means and standard errors for at least three independent experiments, each containing triplicate plates of transfected cells.

A


450
400
350
300
250
200
150
100
50
0
CAT Activity(dpm/min/mg protein)
CONTROL
+ EGF(10nM)
*
*
*
M.1
M.2
wt
CONSTRUCT

B

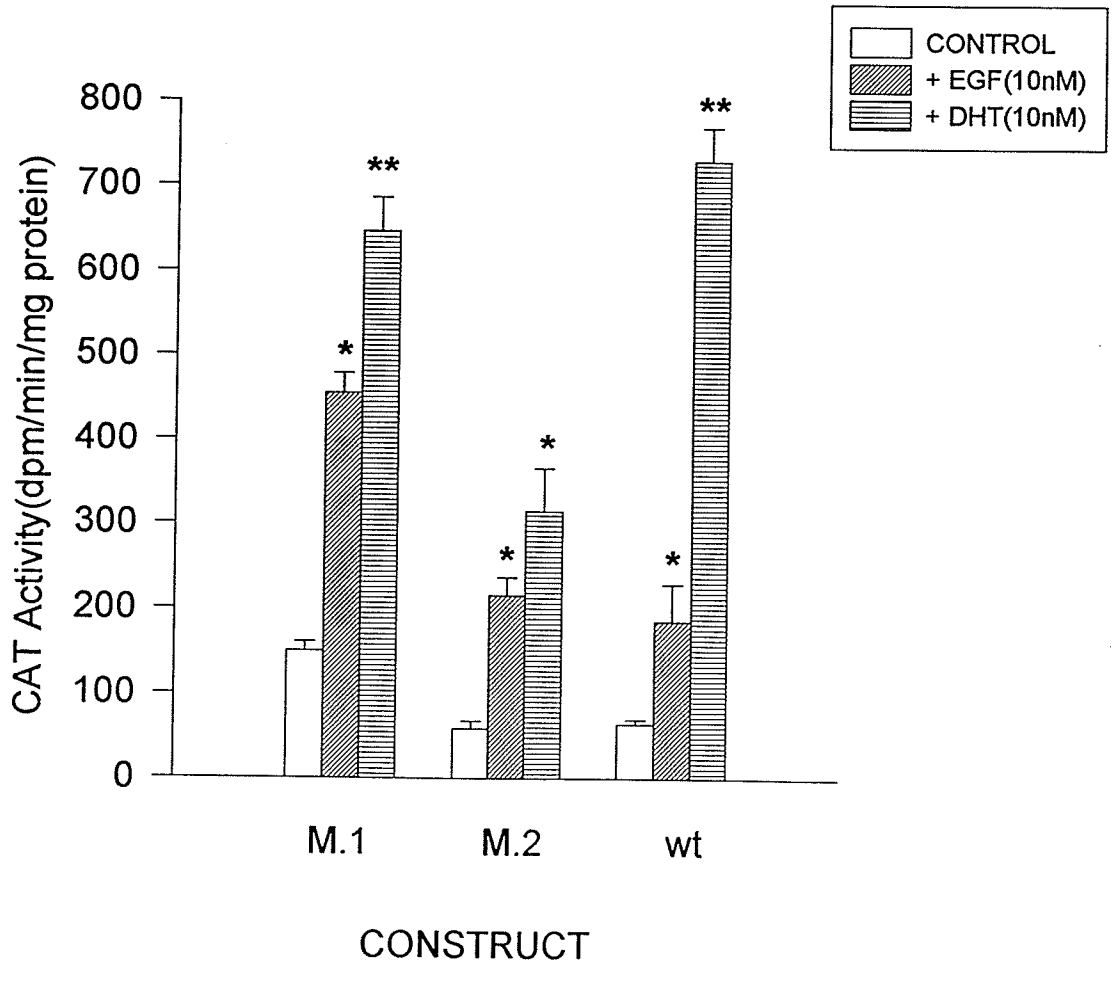
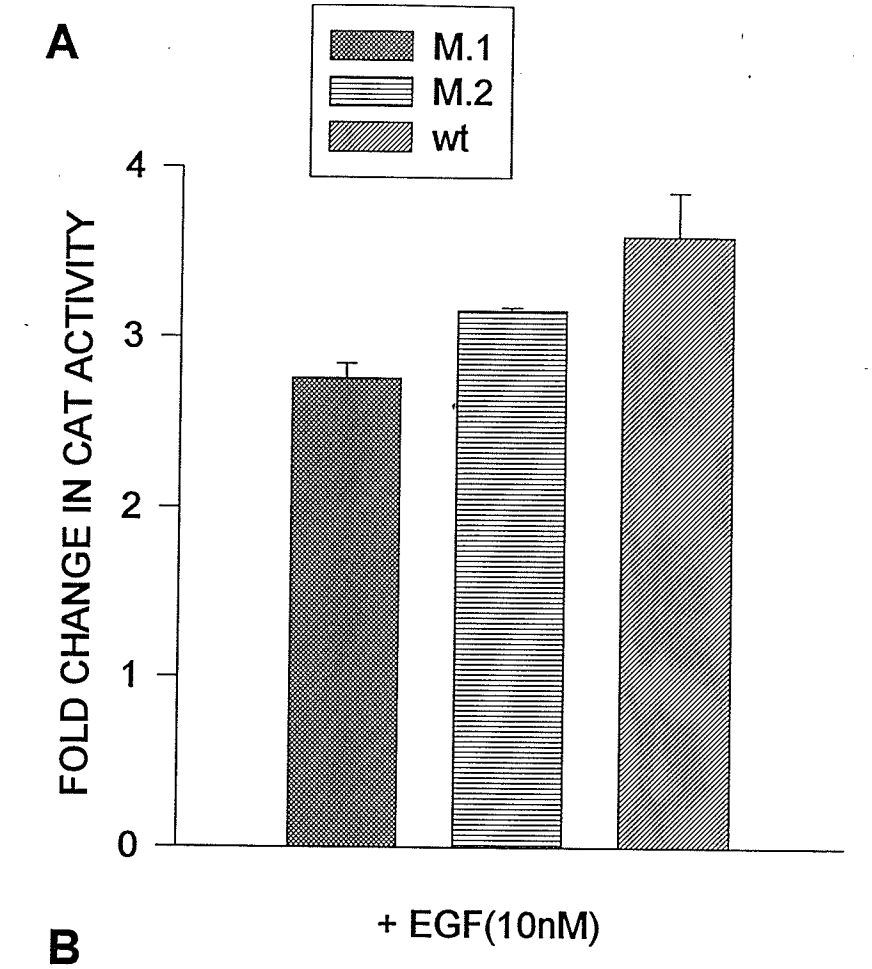

800
700
600
500
400
300
200
100
0
CAT Activity(dpm/min/mg protein)
CONTROL
+ EGF(10nM)
+ DHT(10nM)
*
**
*
*
*
**
M.1
M.2
wt
CONSTRUCT

Figure 18. Effects of EGF and DHT on Point mutation PB-CAT Constructs. Within ARE-1, base -231 G was changed to an A [p(-286/+28)M.1PB-CAT (M.1)] or within ARE-2, base -123 C was changed to an A [p(-286/+28)PB-CAT (M.2)]. These constructs were co-transfected without (A) or with (B) the rAR expression vector into DU145 cells, which were incubated with EGF or DHT (hatched bars). The CAT activity from the cells without addition of EGF or DHT (open bars), served as controls. CAT activity was compared to the wild type (wt) PB-CAT gene [p(-286/+28)PB-CAT]. All CAT assays were performed by scintillant two phase fluor diffusion method and activity was expressed as the mean ± S.E. of triplicate determinations after being normalized for transfection efficiency.


A
M.1
M.2
wt
FOLD CHANGE IN CAT ACTIVITY
0
1
2
3
4
+ EGF(10nM)

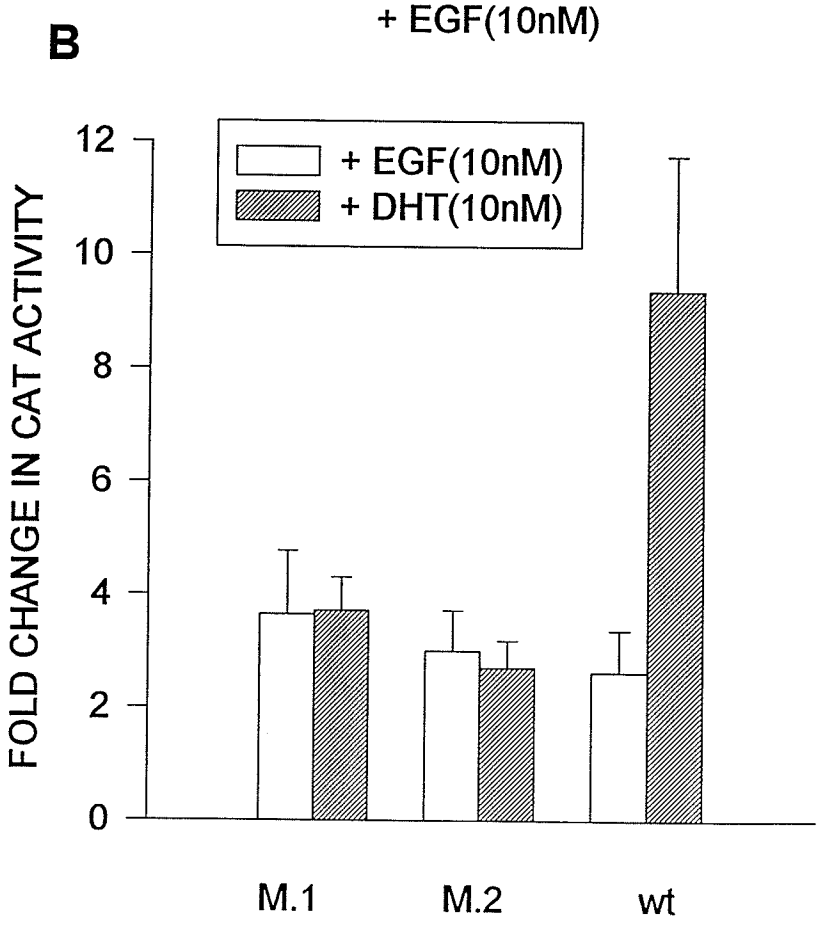

B
+ EGF(10nM)
+ DHT(10nM)
FOLD CHANGE IN CAT ACTIVITY
0
2
4
6
8
10
12
M.1
M.2
wt

Figure 19. Fold Change in CAT Activity of Point Mutation PB-CAT Constructs. The DU145 cells were transfected with the point mutations constructs of Fig. 15, co-transfected without (A) or with (B) the AR expression vector and treated with EGF or DHT. CAT activity was measured in triplicate determinations after being normalized for transfection efficiency and expressed in fold induction (i.e., the ratio of EGF or DHT-treated over the controls). The CAT activity from the cells without addition of EGF or DHT served as controls and is represented as 1. CAT activity was compared with the wild type (wt) PB-CAT gene [p(-286/+28)PB-CAT].The bar graph shows mean ± S.E. from at least two independent experiments.

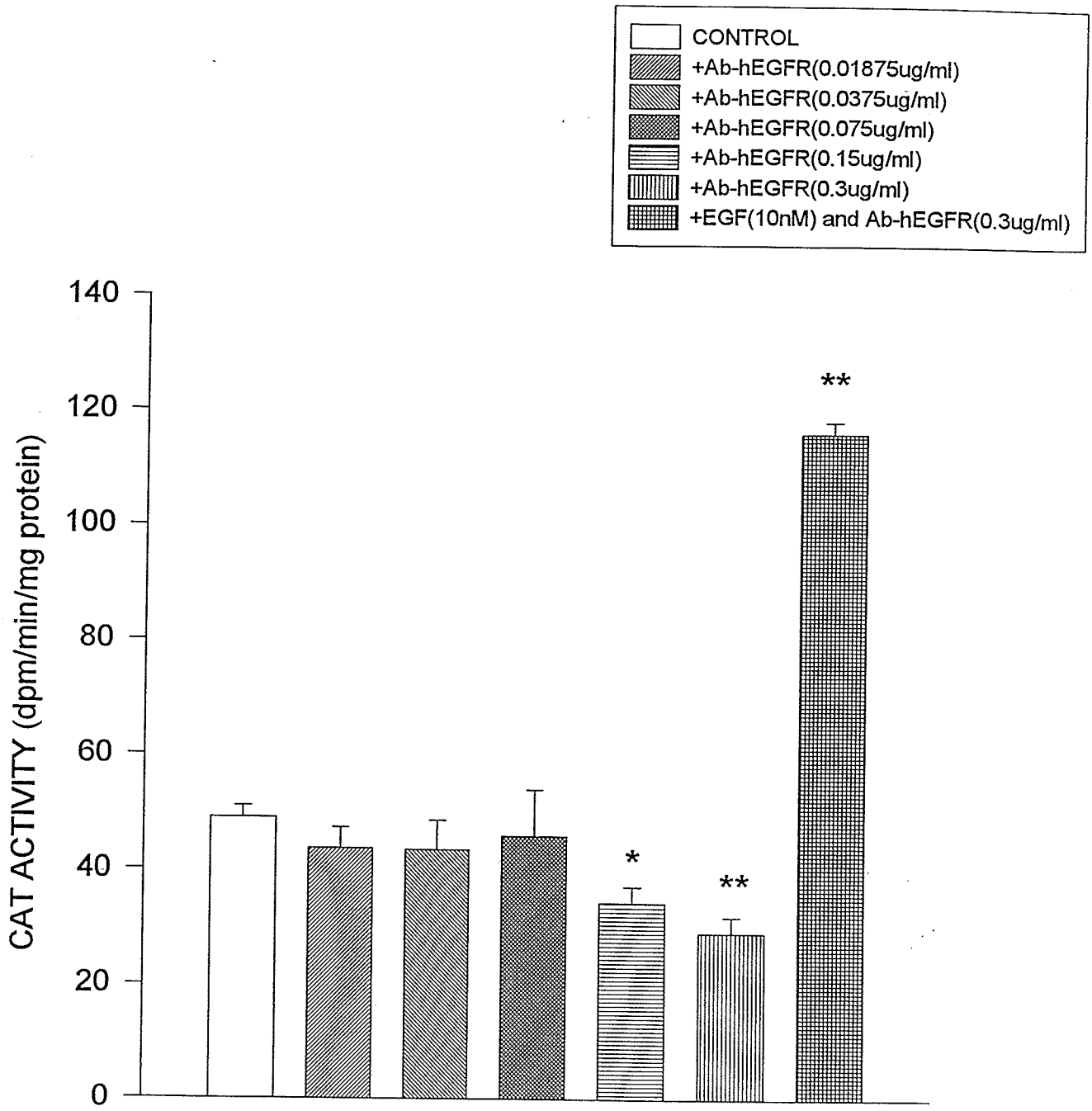

CONTROL
+Ab-hEGFR(0.01875ug/ml)
+Ab-hEGFR(0.0375ug/ml)
+Ab-hEGFR(0.075ug/ml)
+Ab-hEGFR(0.15ug/ml)
+Ab-hEGFR(0.3ug/ml)
+EGF(10nM) and Ab-hEGFR(0.3ug/ml)
CAT ACTIVITY (dpm/min/mg protein)
140
120
100
80
60
40
20
0
*
**
**

Figure 20. Effect of Ab-hEGFR on the PB-CAT Expression in DU145 Cells. Cell culture and DNA transfection were performed as described. After the glycerol shock, cells were cultured in serum free MEM plus 1% antibiotics (v/v), 2μg/ml of insulin and transferin with various amounts of Ab-hEGFR (from 0.01875 to 0.3μg/ml) or both of EGF (10nM) and Ab-hEGFR (0.3μg/ml). For control, only the media but no EGF or Ab-hEGFR was added. Cells were incubated at 37^0C with 5% CO_2 for about 40-48 hours before harvested. CAT assays were performed by scintillant two phase fluor diffusion method and the activity was expressed as the mean ± S.E. of triplicate determinations after being normalized for transfection efficiency. Based on SigmaPlot Student's *t* test (unpaired group), significant difference from level of control is shown by *($p<0.01$) and **($p<0.001$).

DISCUSSION

The focus of this project was to determine whether the effects of EGF on gene regulation are mediated through the androgen receptors and the possible interactions between EGF and androgens in gene regulation in the prostate. To reach the goals, we have done both *in vivo* and *in vitro* studies.

We used the transgenic mouse model generated by the PB-CAT fusion gene to test the effects of EGF or androgen withdrawal on the expression of a prostate-specific gene. Our data indicated that in the prostate tissues of the transgenic mice, both androgen withdrawal induced by Cx and EGF withdrawal induced by Sx affected the PB-CAT transgene expression (see Fig. 6 and 7). However, EGF only affected the PB-CAT expression in DLP but not in VP. In addition, Sx only affected the PB-CAT expression in the early stage (14 day post-Sx) post-Sx. The PB-CAT expression had returned to the control levels by 28 day post-Sx without further EGF treatment (Fig. 7). These observations led us to question: (1) Are EGF, TGF-α and EGF-R differentially regulated in the different lobes of the prostate? (2) Does EGF withdrawal affect the expression of EGF, TGF-α and EGF-R ? and (3) Is the rebound of PB-CAT expression due to the local expression of EGF, TGF-α and EGF-R?

To answer the questions dressed above, we measured relative mRNA levels of EGF, TGF-α, and EGF-R in different lobes of normal sexually mature mouse prostate using RT-PCR. Our data indicated that indeed, there is deference for the expression of EGF, TGF-α and EGF-R, in different lobes of the prostate. Compared to dorsal and lateral lobes, the

ventral lobes possess the lowest mRNA levels for EGF and EGF-R (Fig. 10). Specifically, the amount of EGF-R mRNA in the ventral lobes is only half and one third as much in the dorsal and lateral lobes, respectively. As mentioned before, the rodent prostate gland is composed of four anatomically distinct lobes. The ventral lobe has been demonstrated to differ the most from the other lobes biochemically (Ho et al., 1987; Matusik et al., 1986). Our observation that the higher EGF and EGF-R mRNA levels in dorsolateral lobes suggests that EGF might play a more important role in dorsolateral than in ventral lobes in the prostate. The dorsolateral prostate more responsive to EGF than the ventral prostate which may explain why EGF withdrawal did not affect the PB-CAT expression in the ventral prostate of the transgenic mice. The changes of the prostate weight after Sx also support the idea. As shown in Table 2 (Sx 1), 14 days after Sx, the weight of DLP dropped to 85% compared to that of the controls. In contrast, the weight of VP increase to 140% compared to that of the controls. Because EGF has been reported to inhibit the testicular androgen secretion in mouse (Liu et al., 1994), the increase of VP weight could be due to the increase of circulating androgens.

In addition, the dorsolateral prostate has been reported to be the site of development of prostatic carcinoma in the rat (Issacs, 1987). Very recently, Matusik and co-workers have developed an transgenic mouse model for prostate cancer. Several lines of transgenic mice have been generated by using up to ~ 12 kb of the PB promoter to drive expression of the simian virus 40 large tumor antigen-coding region. The highest levels of transgene expression were detected in the DLP and VP in sexually mature males. However, all tumors were generated from the DLP; none of the tumors were generated from the VP even though the transgene expression is present in the VP (personal communication). This is consistent

with our data that the VP is more stable and less responsive to changes in the hormonal or growth factor signals than the rest of the prostate.

We observed that the PB-CAT expression had returned to the control levels without further EGF replacement by 28 days post-Sx. To determine whether the rebound was due to EGF, TGF-α and EGF-R produced locally, we tested the EGF, TGF-α and EGF-R mRNA levels in the prostate at 28 day post-Sx. As shown in Fig. 12, EGF-R mRNA level returned to the pre-Sx level in DP and VP. Even though the level of EGF was lower than that of the controls, TGF-α mRNA was increased in the DP. However, all mRNA levels were decreased in the LP. In the mouse, a large amount of EGF is produced by salivary glands (Barka, 1980; Gospodarowicz, 1981). Tsutsumi and co-workers have reported that after sialoadenectomy in mouse, plasma EGF decreased rapidly and was undetectable by 3 weeks, but this did not affect the circulating levels of testosterone (Tsutsumi et al., 1986). Liu et al. have also reported that Sx completely depleted the circulating levels of EGF after 28 days Sx in mouse, however, they observed that intratesticular and serum levels testosterone increased 2- and 6- fold, respectively, by 28 days post-Sx (Liu et al., 1994). According to these findings, our observation that the PB-CAT expression rebounded to the pre-Sx levels by 28 days post-Sx in DLP is unlikely to be due to any increase of circulating EGF; however, it could be due to the increase of circulating androgens (Liu et al., 1994) and to the local expression of EGF-R and its ligands.

EGF, TGF-α and EGF-R have been identified in the prostate and in cultured cells derived from the prostate (Sherwood and Lee, 1995). The presence of EGF in human, mouse, and rat prostate has been well established by radioimmunoassay, Western blotting and competitive binding studies (Nishi et al., 1991; Liu et al., 1992; Myers et al., 1993; Wu

et al., 1993). It has been demonstrated that the prostate is the primary source of EGF in seminal plasma (Fuse et al., 1992) and that EGF is an important factor in the maintenance of the structural integrity of the adult mouse prostate (Liu et al., 1992). TGF-α has been identified in both developing and adult rat prostate (Taylor and Ramsdell, 1993; Sherwood and Lee, 1995). EGF-R has also been identified in normal rodent and human prostate (Maygarden et al., 1992; Ibrahim et al., 1993; Taylor and Ramsdell, 1993). Studies have shown that castration results in an increase of the EGF-R expression in rat prostate (St Arnaud et al., 1988). Based on these evidence we proposed that the rebound of PB-CAT expression is at lest partially due to an autocrine-stimulated mechanism involving EGF.

In vitro studies have provided evidence that not only EGF/TGFα and its receptor but other growth factors, such as TGF-β and IGF-I, also affect prostatic epithelial growth (Chaproniere and McKeehan, 1986; Schuurmans et al., 1988b; Sitaras et al., 1988; Wilding et al., 1989b; Fiorelli et al., 1991). There is the possibility that other growth factors may be involved in the PB gene regulation in the prostate tissues.

The results obtained from our experiments indicated that EGF treatment alone could block the decrease of PB-CAT expression induced by Sx but not by Cx. The effects of EGF on the PB-CAT regulation *in vivo* showed an androgen-dependent pattern. This led to the following questions: (1) What are the effects of androgen withdrawal on EGF, EGF-R and AR expression in the prostate tissues? (2) Are the effects of EGF on the PB-CAT regulation mediated through the AR? (3) Does EGF need the presence of androgens to elicit its function in PB gene regulation?

Androgens have been reported to down-regulate AR expression in rat ventral prostate (Lubahn et al., 1988a; Quarmby, 1990; Blok et al., 1992). Our data also indicated

that the AR mRNA level was much higher in the DP and VP after Cx than that of pre-Cx (Fig. 11 and 12). In contrast, the AR mRNA level was decreased in the LP after Cx. This evidence indicated that androgen-receptor is differentially regulated by androgens among the different lobes of the prostate. Prins and Woodham also observed that androgens differentially regulate AR mRNA expression in the different lobes of rat prostate (Prins and Woodham, 1995). They They demonstrated that AR mRNA levels are down-regulated by androgens in all prostate lobes. Using *in situ* hybridization, they further confirmed that the increase in AR mRNA levels immediately following androgen withdrawal is due to increased transcripts per cell. However, they found that the AR mRNA elevation upon androgen withdrawal was transient and the value returned to control levels in the ventral and dorsal lobes within three days while the elevetion of AR message in the lateral lobe was prolonged. Besides AR mRNA, androgens also differentially regulate nuclear androgen receptor (nAR) levels in the different lobes of rat prostate (Prins and Reiher, 1987). Prins and Reiher have reported that early after castration the nAR levels decreased in all prostatic lobes. However, LP nAR levels began to increase on day 3 post-Cx and reaching intact values by day 7 and 125% intact levels by day 14, whereas, levels of nAR continued to decline in the VP and DP to undetectable levels on day 10 post-Cx (Prins and Reiher, 1987).

There is now extensive data to indicate important androgen-EGF interactions. Jacobs et al. reported that EGF synthesis in the rat ventral prostate requires androgens (Jacobs et al., 1987). Androgens also affect production of EGF in salivary glands. It is reported that castration causes more than 10-fold reduction in EGF production in male mouse salivary glands (Byyny et al., 1974). Traish and co-worker observed that androgens down-regulate prostate cell membrane EGF-R and proposed that androgen deprivation

might allow EGF-R induction, thereby, promoting cell growth and preventing complete prostatic regression with castration (Traish and Wotiz, 1987). Fiorelli and co-workers also found that EGF receptor binding was significantly higher in prostate tissue from men treated for 3 months with endocrine therapy, which reduce plasma androgens, than in BPH tissue from untreated men (Fiorelli et al., 1989). These observations further suggest that androgens negatively affect EGF receptor expression in both rat and human prostate. Our data also revealed a decreased EGF mRNA level in all lobes of the prostate and an increased EGF-R mRNA level in the DP and VP after Cx (Fig. 11 and 12).

Because androgen withdrawal would increase EGF-R expression (Fiorelli et al., 1989), we propose that EGF might play an enhanced role in regulation of the prostate under conditions without androgens. If so, then why did treatment with EGF alone not restore the PB-CAT expression in the transgenic mice after Cx? Before answering this question, it is necessary to look at the structure and components of the prostate. The rodent prostate gland is composed of four anatomically distinct regions and the four different lobes are defined: ventral, dorsal, lateral and anterior (coagulating gland). The relative numbers of epithelial and stromal cells within the prostate differ between species (Cunha et al., 1987). DeKlerk and co-workers have reported that the epithelial/stromal cell ratio remains relatively constant once sexual maturity is attained. In the adult human male , the ratio of epithelial/stromal cells is equal, whereas in the adult male rat, the ratio is 5:1 (DeKlerk et al., 1978). Androgen withdrawal affects the epithelial cell population much more than that of the stromal cells. It has been found that epithelial cells undergo a 93% reduction in population upon androgen withdrawal, however, the stromal cells only regress by 22% (DeKlerk et al., 1986).

The PB-CAT expression has been identified to be restricted to prostate epithelium (Greenberg et al., 1994). However, castration alters the ratio of epithelial to stromal cells in favor of the stromal component according to the evidence that DeKlerk and co-workers reported. So under this condition, there is possibility that we might not be able to detect the CAT activity from the whole protein contents of which only a very small portion was from the epithelium. That means that EGF potentially might affect the PB-CAT expression after Cx, but we were unable to measure it by the CAT assay because of the small content of epithelium. It would be necessary to repeat these experiments using *in situ* hybridization - using a radioactive cDNA probe and counting the exposed grains of the emulsion which overlay individual cells to resolve this issue.

All these arguments indicate that there are two ways to explain why EGF replacement alone could not restore the PB-CAT expression in the transgenic mouse prostate after Cx: (1) EGF regulates the PB-CAT expression through the AR, and EGF treatment alone could not restore the PB-CAT expression after Cx because of the absence of nAR in the DP and VP. (2) EGF effects the PB-CAT expression through an androgen receptor-independent pathway, and the failure of EGF replacement alone to block the decrease of PB-CAT expression after Cx is due to the loss of the epithelium. To resolve whether or not EGF effects the PB-CAT expression through the AR, we turned to *in vitro* studies.

We transfected (-426/+28)PB-CAT construct (which is the same construct as the one used to generate the transgenic mice) and several other constructs with either mutation or deletions of the known androgen response element from PB 5'-flanking region into human prostate cancer cell line- DU145. As mentioned earlier, the DU145 cell line was

derived from brain of metastasized prostate cancer and has been demonstrated to express EGF-R (Connolly et al., 1990; Morris and Dodd, 1990; Ching et al., 1993), but lacks AR (Stone et al. 1978; Connolly et al., 1990). That makes this cell line EGF-sensitive but androgen-insensitive. The effects of EGF on the PB gene regulation could be identified by measuring the gene product (CAT activity). Also, the effects of androgen on the CAT expression could be identified by co-transfecting an AR expression vector. Using this system, we measured transient expression of the PB-CAT constructs. Our data indicated that EGF induced the CAT expression for all constructs regardless of the presence or absence of androgen or the AR (Fig. 13 and 16-19). These observations demonstrated that EGF regulated the PB gene through an androgen- and androgen receptor-independent pathway.

Generally, the signaling pathways for steroid and peptide growth factors are separate and distinct. Steroid receptors belong to a family of transcription factors that contain a highly conserved "zinc finger" motif responsible for DNA binding (Beato, 1989). Once the steroid binds to the nuclear receptor, the hormone-receptor complex interacts with regulatory sequences called Hormone Responsive Elements (HRE), contained within the non-coding regions of specific target genes and stimulates target genes to be expressed (Ponta et al., 1985). Peptide growth factors interact with membrane receptors and elicit a cellular response mediated by an intracellular second messenger signaling pathway. However, it has been reported that peptide growth factors may induce effects similar to those of steroid hormones in gene regulation by an interaction between the growth factor signaling pathway and the classical steroid receptors. For example, Culig and co-workers have constructed a vector in which the reporter gene (CAT) was driven either by an artificial

promoter consisting of one or two androgen-responsive elements in front of a TATA box or by the promoter of the prostate-specific antigen (PSA) gene, a naturally occurring androgen-inducible promoter, which contains one ARE, (Culig et al., 1994). They co-transfected the vector and an AR expression vector into DU145 cells and found that growth factors including IGF-I, KGF and EGF could directly activate CAT expression in the absence of androgens. However, EGF could only activate the vectors which contained two AREs. Since the CAT expression was completely inhibited by the pure AR antagonist casodex, they proposed that the effects of the growth factors are AR mediated (Culig et al., 1994). Estrogen regulation of chick oviduct genes is the classic example for steroid regulation of gene expression (Dean et al., 1984, Gaub et al., 1987). However, Ignar-Trowbridge and co-workers have reported that EGF may induce effects similar to those of estrogen in the mouse uterus by an interaction between the EGF signaling pathway and the estrogen receptor (Ignar-Trowbridge et al., 1992). They observed that EGF effected phosphatidylinositol (PI) lipid metabolism in the mouse uterus. Activation of uterine PI lipid turnover is one of the earliest and most persistent effects of estrogen administration and this effect is mediated by the ER (Grove and Korach, 1987, Ignar-Trowbridge et al., 1991). They also showed that EGF induced uterine DNA synthesis and PI lipid metabolism were significantly attenuated by an ER antagonist. Nelson et al. also have reported that an EGF-specific antibody administered prior to estradiol partially blocked estrogen-induced uterine epithelial cell proliferation (Nelson et al., 1991). This observation suggested that production of EGF might be necessary for some estrogen-induced responses. Nevertheless, the presence of cross-talk between growth factor signaling pathways and steroid receptors

suggests that complex may modulate hormone action and influence normal and aberrant function in mammalian cells.

At first glance, our observations appear to be in contrast to the results obtained by Culig et al (Culig et al., 1994); however, upon examination, we find some agreement. First, they found that EGF needed the presence of two AREs to elicit its function through the AR. The promoter they used consisted of two artificial ARE sequences in front of the TATA box. And compared to IGF-I, EGF only showed very minor stimulatory effects. Secondly, EGF showed no induction of the reporter gene when it was under the control of PSA promoter. In addition, EGF also failed to stimulate the CAT expression in LNCaP cells which produced endogenous AR. PSA secretion in the LNCaP cells has been previously shown to be AR mediated (Montgomery et al., 1992), however, in Culig's experiments EGF treatment could not stimulate the PSA secretion in this cell line even though the AR was present. These observations do not provide strong support for the idea that EGF regulates gene expression through the AR in the prostate.

Furthermore, Henttu and Vihko have also investigated the effects of EGF/TGF-α on the PSA gene regulation using LNCaP cell line (Henttu and Vihko, 1993). They found that EGF/TGF-α reduced the secretion of PSA by the cells. This was in contrast to the effects of androgens on the PSA gene expression in this cell line. This evidence suggested that androgen and EGF activate different molecular mechanisms in PSA gene regulation. Because the promoter of PSA gene contains a putative AP1 recognition element and EGF-R activation has been demonstrated to activate expression of the genes (*c-fos* and *c-jun*), from which AP1 is the product, they proposed that the direct participation of AP1 in the regulation of the PSA gene expression could take place. They concluded that the effects of

EGF/TGF-α on PSA gene regulation were most likely through the EGF-R and AP1. Based on these observations, it is not surprising that Culig et al. failed to see an increase in the level of the PSA protein in LNCaP cells after EGF treatment. Taken together with our data on PB-CAT expression, the weight of evidence does not support AR-activation by EGF-dependent pathways as a major mechanism of gene regulation in the prostate.

Previous work has demonstrated the presence of a silencing activity located between -426 and -286 of PB gene upstream elements, the silencer affects androgen induction of the PB-CAT (Rennie et al., 1993). We obtained the same result in androgen induction experiments (Figure 14 B). However, this sequence did not affect EGF induction of the PB under the condition in which there was no rAR expression vector (Fig. 14 A). Comparing -426 with -286, without the rAR, we obtained similar CAT activity from -426 and -286 constructs (Figure 14 A). Whereas with the rAR, we obtained higher CAT activity from the -286 construct than the -426 construct. This evidence supports the presence of a silencer located between -286 and -426 of the PB promoter, which affects both basal and EGF induction of the PB-CAT. Interestingly, when cotransfected with the AR expression vector, addition of both EGF and DHT revealed different results between -426 and -286 constructs (Figure 14 B). For the -286 construct, treatment with both EGF and DHT showed an additive effect. However, for the -426 construct, the CAT activity was the same with EGF and DHT as that treated with DHT alone. This suggests that the silencer located between -426 and -286 bp of PB gene could also affect EGF inducibility but only at the condition in which both AR and DHT were present. We may further propose that the entire gene of PB is regulated mainly by androgens under the conditions

in which both AR and androgens are present. Once the cells lost functional AR (such as DU145 cells), growth factors, such as EGF could regulate the gene predominantly instead.

Henttu and Vihko have used LNCaP cells to investigate the interactions between EGF and androgens (Henttu and Vihko, 1993). They chose the LNCaP cell line as a model because these cells are androgen responsive and produce prostatic epithelial proteins (Horoszewicz et al., 1980; Horoszewicz et al., 1983). This cell line also possesses EGF receptors (Schuurmans et al., 1988a; Schuurmans et al., 1988b; Wilding et al., 1989), secretes immunoreactive EGF and TGF-α (Wilding et al., 1989; Connolly and Rose, 1990), and responds to EGF with increased growth (Wilding et al., 1989, Ching et al., 1993). They tested the effects of EGF and androgen on the regulation of expression of the genes coding for prostatic differentiation markers, prostatic acid phosphatase and PSA. They observed that the secretion of the PSA was down-regulated by EGF. However, the up-regulatory effect of androgen was dominant in the regulation of PSA gene expression when both EGF and androgen were added to the system.

The expression of the human androgen receptor (hAR) is down-regulated by androgen and EGF in the LNCaP cell line (Henttu et al., 1992). They found that hAR mRNA level in the cells treated with both EGF and androgen was significantly lower than that in cells treated only with androgen. They proposed that in the presence of androgens, the effect of EGF on the other androgen-regulated genes may be partly mediated by the reduction in the expression of the AR gene.

In our system, the cells do not possess the AR, so that the effect of EGF on the PB-CAT regulation is unlikely through this mechanism. Nevertheless, the presence of rAR does effect EGF-induced CAT expression. Comparing -426, -286 and -158 PB-CAT

expression under the conditions without and with the rAR (Fig.14 A, B), the CAT activity was higher from all constructs in the absence of the rAR group. It appears as if the presence of high levels of rAR could inhibit the signal mediated through the ERE. Competition for binding to the PB promoter may occur among transcription factors including the AR and AP1 proteins. This might explain why the CAT activity was lower with the presence of AR even though without androgens in our system (Fig. 14B). It remains to be elucidated; however, why the additive effects of EGF and DHT were obtained from the -286 construct.

We have identified that EGF could induce the PB-CAT expression both *in vivo* and *in vitro*, and that expression was not mediated through the AR. Our next aim was to identify the putative EGF regulatory element (ERE) in the PB promoter. To reach the goal, we transfected a series of constructs with either 5' or 3' deletions of the PB 5'-flanking region into DU145 cells. Our data indicated that EGF induced the CAT expression for all constructs tested (Fig. 14-17). This indicated that the putative ERE exists in the smallest construct that still gives an EGF-response. This construct is p-(-141/-91)TK-CAT which contains 51 nucleotides located at -141 to -91 bp of the PB 5'-flanking region.

After very carefully comparing this sequence with the ERE sequences previously described in literature, we found that this sequence differs from previously described EGF response elements found in prolactin (Elsholtz et al., 1986), gastrin (Merchant et al., 1991), the tyrosine hydroxylase (Lewis and Chikaraish, 1987), the transferrin receptor (Ouyang et al., 1993), and the mouse lactoferrin (Shi and Teng, 1994) promoters. The common feature of these EGF-response elements are their GC-rich nature. However, a

sequence within the -141/-91 PB 5' flanking region resembles the EGF-response elements found in transin (Kerr et al., 1988) and *c-fos* (Fisch et al., 1989) promoters. These EREs contain a similar structure which is highly homologous to the consensus AP1 motif. The region between -106 and -100 contains an 7-bp nucleotide sequence: 5'-TGACACA-3', which differs from the consensus AP1 motif (TGACTCA) by only one nucleotide. The details about the sequence from -141 to -91 bp of the PB 5'-flanking region and the region of the AP1 homology as well as the region of dyad symmetry within the -141/-91 PB promoter are shown in Fig. 21.

The nuclear-acting group of proto-oncogenes includes *c-fos, c-myc, c-myb, c-erb*A, p53, and *c-jun* (Bishop, 1987, Vogt et al., 1987). Because of their nuclear location, the products of these genes have been suggested to function as transcriptional regulators (Kingston et al., 1985). It has been identified that the AP1 is one of the transcriptional regulators coded by *c-jun* gene (Bohmann et al., 1987; Angel et al., 1988). However Chiu et al. have reported that the *c-fos* gene product, Fos, stimulated gene expression not by direct binding to DNA but by interaction with the sequence-specific transcription factor AP1. Therefore, recognition of specific *cis*-elements by AP1 is a prerequisite for Fos-mediated stimulation of gene expression. Combining Fos with *c-jun* protein forms AP1 heterodimer, which they termed AP1:Fos complex (Chiu et al., 1988).

It has been confirmed that binding of EGF to its membrane tyrosine kinase receptor activates many second messenger pathways and modulates the activity of several intracellular kinases by phosphorylation as well as stimulates the expression of a number of early response genes, including *c-fos* and *c-jun* (Carpenter and Cohen, 1990; Fisher and Lakshmanan, 1990). The EGF-R tyrosin kinase directly phosphorylates phospholipase C,

the enzyme which activates diacylglycerol, the activator of protein kinase C (Margolis et al., 1989; Meisenhelder et al., 1989; Nishibe et al., 1989). PKC stimulation is followed by activation of the transcription factor AP1 (Merchant et al., 1991). The consensus AP1 motif TGACTCA has been found in many phorbol ester-responsive promoters (Merchant et al., 1991). However, that the AP1 motif could mediate EGF responsiveness in some promoters has also been reported. For example, Kerr et al. have reported that EGF effects on transin transcription involved factors recognizing the AP1 motif in the transin promoter (Kerr et al, 1988). They observed that when the *c-fos* protein level in the experimental system increased, the stimulation effect of EGF in the transin gene expression was also elevated. However, abolished *c-fos* expression could not completely block the function of EGF induction of the transin gene expression. Thereby, they concluded that there are two pathways in EGF regulation of transin gene expression: *c-fos*-dependent and *c-fos*-independent pathways. Since an oligonucleotide containing the AP1 motif competed for factor binding with the transin promoter and inhibited EGF stimulation by both pathways, they proposed that the effects may be mediated through the same *cis*-acting transcription element-AP1 motif.

Furthermore, Fisch et al. have also reported that EGF could regulate the *c-fos* gene mediated through a sequence element highly homologous to the consensus AP1 binding site in the promoter (Fisch et al., 1989). They have proved that EGF up-regulated *c-fos* through the AP1 sequence. As mentioned earlier, *c-fos* protein can form AP1:Fos complex (or AP1 heterodimer) by binding with *c-jun*/AP1. The AP1:Fos complex also regulates gene expression through the AP1 motif (TGACTCA). A model for EGF regulated gene

expression should include: EGF → EGF-R → *c-fos* → AP1 → transcription of AP1 responsive genes through the AP1 motif.

Our evidence very strongly supports the model that EGF regulates the PB gene through the AP1 motif. However, to confirm our hypothesis, it would be necessary either to do competition binding assay by using an oligonucleotide containing the AP1 motif or to generate another PB-CAT construct with deletion of the AP1 sequence in the PB promoter to test the EGF induction in DU145 cells. Furthermore, DNase I footprinting also could verify the hypothesis.

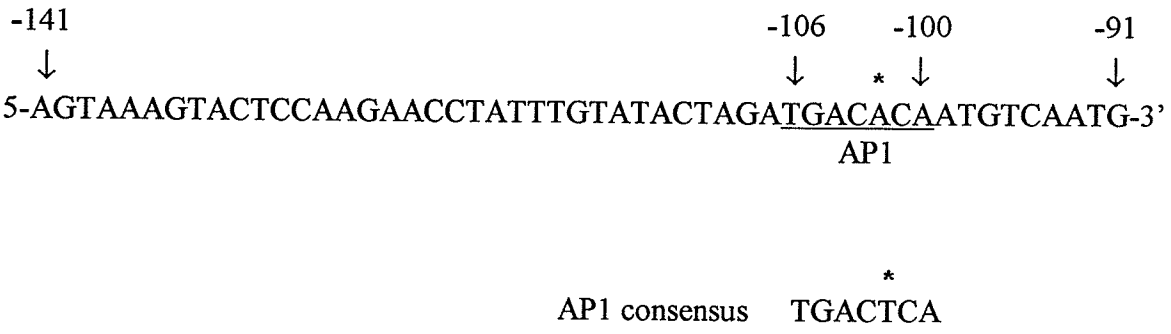


Figure 21. Nucleotide sequence from -141 to -91 bp of PB 5'-flanking region. The AP1 homology is underlined. The AP1 consensus sequence is shown below. The base pair in the -106/-100 sequence which is divergent from the AP1 consensus is marked by *.

CONCLUSION

Having investigated the effects of EGF and DHT on the PB promoter both in prostate tissues of the transgenic mice and in DU145 cells and, on EGF, TGF-α, EGF-R and AR mRNA expression in different lobes of the mouse prostate, we obtained the following conclusions:

(1) The PB promoter is regulated by both EGF and androgens in the prostate tissues of the transgenic mice. However, androgens regulate the PB in the whole prostate whereas EGF only affects the gene in dorsolateral prostate.

(2) In different lobes of the normal mouse prostate, EGF, EGF-R, TGF-α and AR mRNA expression are differentially regulated.

(3) Both castration (Cx) and sialoadenectomy (Sx) affect EGF, EGF-R, TGF-α and AR mRNA expression in the mouse prostate. However, the effects are different among different lobes and different periods post- Cx and Sx.

(4) Both EGF and DHT induced PB-CAT or PB-TK-CAT expression in DU145 cells. However, EGF is able to induce the CAT activity either in presence or absence of the AR expression vector, which suggests that EGF induction is androgen receptor-independent.

(5) A series deletions from 5' and/or 3' direction of the PB 5'-flanking DNA fragment from -426 to +28 bp did not significantly affect EGF induction of the CAT activity. Because the smallest construct tested in the experiments contains PB 5'-flanking fragment from -141 to -91, it is suggested that a putative ERE could exist in this region of

the PB promoter. Since a sequence nearly identical to the AP1 consensus sequence is present in this region (-141/-91), we now postulate that EGF regulates the PB gene through the AP1 motif.

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