

LOCALIZATION AND CHARACTERIZATION OF THE
ANDROGEN RESPONSIVE ELEMENTS IN THE
RAT PROBASIN GENE

BY

SHELLEY ANN MCQUEEN

38

A Thesis
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

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LOCALIZATION AND CHARACTERIZATION OF THE ANDROGEN
RESPONSIVE ELEMENTS IN THE RAT PROBASIN GENE

BY

SHELLEY ANN MCQUEEN

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

MASTER OF SCIENCE

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BY

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A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the
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MASTER OF SCIENCE

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List of Abbreviations

| | |
|------------------------------------|---------------------------------------------------------------|
| A ₂₆₀ | absorbance at 260 nm, 1 A ₂₆₀ unit=40 μg/mL DNA |
| α-2-u globulin | alpha 2 urinary globulin |
| AR | androgen receptor |
| ARE | androgen response element |
| bp | base pairs |
| BPH | benign prostatic hyperplasia |
| CAT | chloramphenical acetyltransferase |
| cDNA | complementary DNA |
| CH ₃ COOH | acetic acid |
| CH ₃ COOK | potassium acetate |
| CH ₃ COONa | sodium acetate |
| CH ₃ COONH ₄ | ammonium acetate |
| CHEF | primary chicken embryo fibroblast cells |
| cpm | counts per minute |
| cPR | chicken progesterone receptor |
| CsCl ₂ | cesium chloride |
| CV-1 | mouse kidney cells |
| dATP | deoxyadenine triphosphate |
| dCTP | deoxycytosine triphosphate |
| ddh ₂ O | double distilled water |
| DEX | dexamethasone |
| dGTP | deoxyguanine triphosphate |
| DHT | dihydrotestosterone |

| | |
|-------------|------------------------------------------------------------------------|
| DMEM | Dulbecco's modified Eagles' medium |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| dpm | degradations per minute |
| DTT | dithiothreitol |
| DU-145 | human prostatic adenocarcinoma cells |
| DU-145-A | human prostatic adenocarcinoma cells obtained from ATCC |
| DU-145-D | human prostatic adenocarcinoma cells obtained from Dr. J. G. Dodd |
| DU-145-R | human prostatic adenocarcinoma cells obtained from Dr. P. S. Rennie |
| EDTA | ethylenediaminetetraacetic acid |
| Eagles' MEM | Eagles' minimal essential medium |
| ER | estrogen receptor |
| ERE | estrogen response element |
| EtBr | ethidium bromide |
| FCS | fetal calf serum |
| GR | glucocorticoid receptor |
| GRE | glucocorticoid response element |
| GUS | murine β -glucuronidase |
| HeLa | human cervical epithelial carcinoma cells |
| hPR | human progesterone receptor |
| HRE | hormone response element |
| hsp | heat shock protein |

| | |
|-------------------|----------------------------------------------------------------------|
| kbp | kilobase pair |
| kb | kilobase |
| LNCaP | human prostatic adenocarcinoma cells derived from the lymph nodes |
| LTR | long terminal repeat |
| μ g | microgram |
| μ L | microliter |
| mg | milligram |
| mL | milliliter |
| MgCl ₂ | magnesium chloride |
| mm | millimeter |
| MMTV | mouse mammary tumour virus |
| MMTV-CAT, | mouse mammary tumour virus chimaeric |
| MTV-CAT | CAT construct |
| MMTV-LTR | mouse mammary tumour virus long terminal repeat |
| mRNA | messenger ribonucleic acid |
| MOPS | N-morpholinol propane-sulfonic acid |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| nls | nuclear localization signal |
| NMR | nuclear magnetic resonance |
| % | percent |
| P | progesterone |
| PB | probasin |
| PB-CAT | probasin CAT chimaeric construct |

| | |
|---------|-------------------------------------------------------------------------|
| PBP | prostatic binding protein |
| PBS | phosphate buffered saline |
| PC-3 | human prostatic adenocarcinoma cells |
| pCMVrAR | cytomegalovirus rat androgen receptor |
| PR | progesterone receptor |
| PSA | prostate specific antigen |
| rAR | rat androgen receptor |
| rGR | rat glucocorticoid receptor |
| RSB | 15 mM Tris-Cl pH 7.5, 15 mM sodium chloride and 3 mM magnesium chloride |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of the mean |
| SEN | sodium sarcosyl |
| slp | sex-limited protein |
| SSC | 3M sodium chloride and 0.3M sodium citrate |
| SV40 | simian virus 40 antigen |
| TAT | tyrosine aminotransferase |
| TAT-CAT | tyrosine aminotransferase chimaeric CAT construct |
| TBE | Tris base, boric acid and sodium EDTA |
| TE | tris(hydroxymethyl)aminomethane hydrochloride and EDTA |
| Tfm | testicular feminized male |
| TK | thymidine kinase |

| | |
|---------|---------------------------------------------|
| TK-CAT | thymidine kinase chimaeric CAT construct |
| TR | thyroid receptor |
| TRE | thyroid response element |
| tRNA | transfer ribonucleic acid |
| 2XFCS | twice stripped fetal calf serum |
| UGE | urogenital sinus epithelium |
| UGM | urogenital sinus mesenchyme |
| UGS | urogenital sinus |
| ZR-75-1 | human mammary carcinoma cells |

Abstract

Probasin (PB) is an androgen and zinc regulated rat dorsolateral prostatic gene. Initial localization of the androgen responsive element(s) (ARE) was achieved by transfection of chimaeric Chloramphenicol Acetyltransferase (CAT) constructs prepared by sequential 5'-end deletions of -426 bp of the 5'-flanking PB DNA linked to the CAT gene, cotransfection with the expression vector for either the rat androgen receptor (rAR) or the rat glucocorticoid receptor (rGR) and treatment with dihydrotestosterone (DHT) or dexamethasone (DEX), respectively, in human prostatic adenocarcinoma (PC-3) cells. The results indicated the following information: (1) that cis-elements in the first 140 bp (-426 to -286) were not required for androgen or glucocorticoid regulation; (2) that the ARE(s) existed, at least in part, between -286 bp and -157 bp since maximal CAT activity was observed at deletion -286, decreasing until minimum at -157 for both androgen and glucocorticoid induced CAT expression; and (3) that for each chimaeric PB-CAT construct tested, preferential androgen over glucocorticoid induction of CAT expression was observed.

In order to determine the specific sequences necessary and sufficient to confer hormonal regulation, two 3'-end PB deletion CAT constructs were prepared, -244/-96 and -244/-165 linked to the heterologous thymidine kinase (TK) promoter. Subsequent transfections with these constructs in PC-3 cells

revealed the following points: (1) neither DHT nor DEX were able to regulate the -244/-165 PB-TK-CAT chimaeric CAT gene; and (2) preferential androgen induction was lost when the PB DNA sequences containing the endogenous promoter (-96 to +28 bp) were replaced with the heterologous TK promoter as the CAT induced expression by DHT and DEX of -244/-96 PB-TK-CAT were essentially equal.

Confirmation of the location of the AREs was obtained from DNase I footprinting performed by Rennie et al., 1993, revealing two putative AREs, ARE-1 between -236 and -223 bp and ARE-2, between -140 and -117 bp.

To address the question of cell line specificity, transfections of -244/+28 PB-CAT or -244/-96 PB-TK-CAT were performed in another prostatic adenocarcinoma (DU-145) cell line. Cotransfection with the rAR or rGR expression vector and treatment with DHT or DEX respectively, disclosed the subsequent results: (1) preferential androgen induced expression of the -244/+28 PB-CAT gene was observed, paralleling data obtained in the PC-3 cells; (2) glucocorticoid induced CAT expression of the -244/-96 PB-TK-CAT gene was greater than androgen, not equal as in the PC-3 cells; and (3) CAT activity of -244/-96 PB-TK-CAT was larger than -244/+28 PB-CAT whether induction was by DHT or DEX.

Comparison of PB with two known androgen regulated genes, Mouse Mammary Tumour Virus (MMTV) and Tyrosine Aminotransferase (TAT), demonstrated PB as the most potent

androgen responsive gene.

In summary, the PB gene is the most powerful androgen responsive gene reported to date which requires the presence of two androgen responsive elements.

Introduction

A. Developmental Biology of the Prostate

The prostate is an androgen dependent exocrine gland located at the base of the urethra in mammalian males. In the human the prostatic ducts originate from the urethra and extend to the periphery in a radial fashion (McNeal, 1983; Lewis et al., 1981). However, in the rat the prostate is organized into four separate and encapsulated lobes, the dorsal, lateral, ventral and anterior (or coagulating gland) which surround but do not encompass the urethra (Cunha et al., 1987). Although these defined lobes are not distinct in the human prostate, McNeal (1983) has distinguished four morphologically discrete zones: the anterior fibromuscular stroma, the peripheral zone, the central zone and the transition zone. The importance of these zones will be addressed later.

Studies on the histology of the prostate indicate that it is a complex arrangement of ductal-acinar structures (Cunha et al., 1987). In the human, compound tubuloalveolar glands comprise the prostate (McNeal, 1983; McNeal, 1984; Franks, 1976). The epithelial cells of the acini vary from pseudostratified columnar to cuboidal (McNeal, 1983; Bloom and Fawcett, 1975). The distal ducts are layered with simple cuboidal or columnar epithelium, whereas the proximal ducts are lined with transitional epithelium. In the rat, the compound ductal arrangement of the prostate is apparent,

however true acinar termini are found only in lateral type 1 and type 2 epithelium and dorsal prostate (Hayashi et al., 1991). Furthermore, each lobe has a distinctive ductal branching pattern (Cunha et al., 1987; Sugimura et al., 1986). The individual ducts are lined with pseudostratified columnar secretory epithelium, the height of which varies depending on the state of the gland. Non-secretory basal epithelial cells are also present along the basement membrane (Mao and Angrist, 1966; Dahl et al., 1973; Timms et al., 1976). The functional significance of these cells in the prostate is unknown. Suggestions for the role of these basal cells in the prostate range from the capacity to serve as a reserve or stem cell population capable of differentiating into columnar secretory cells (Mao and Angrist, 1966; Ichihara and Pellinieni, 1975; Timms et al., 1976) to transportation of materials between the secretory epithelial cells and the extracellular matrix (Ichihara and Pellinieni, 1975; Ichihara et al., 1985) and maintaining regulation of the columnar epithelium (Brandes, 1966; Heatfield et al., 1979).

The mesenchyme or stromal component of the prostate is composed of smooth muscle cells, fibroblasts, blood vessels, wandering connective tissue cells, nerve terminals and lymphatics all enmeshed in a loose collagenous extracellular matrix (Amuller, 1983). The ratio of epithelial to stromal cells within the prostate vary both developmentally and between species, a point which will be addressed in detail

later.

The prostate is derived from the epithelial prostatic buds which extend from the endodermal urogenital sinus (UGS) into the urogenital sinus mesenchyme (UGM) immediately below the developing bladder (Cunha et al., 1987). In the human these epithelial buds or outgrowths appear around the 10th week of gestation (Lowsley, 1912; Kellokumpu-Lehtinen, 1985; Kellokumpu-Lehtinen et al., 1980). By week 13, these outgrowths have developed into approximately 70 primary ducts which have lengthened, branched and canalized (Lowsley, 1912; Kellokumpu-Lehtinen et al., 1980). In the rat, these prostatic buds appear on the 18th or 19th day of gestation (Price and Ortiz, 1965). Unlike the human though, these outgrowths give rise to a specified number of ducts which in turn give rise to the 4 separate lobes of the rat prostate. Each lobe has a distinctive branching or arborization pattern which develops largely post-natally. The epithelial buds that give rise to the ventral prostate lengthen, infiltrate and branch dichotomously into the mesenchyme of the UGS, giving an "elm tree configuration" (Cunha et al., 1987; Sugimura et al., 1986). In contrast, the ducts comprising the developing dorsal prostate initially elongate without branching. Further growth of the dorsal ducts result in 3 to 6 terminal branches per main duct. The lateral lobe branching configuration can be subdivided into two distinct patterns. The lateral type 1 prostate is formed from 5-7 main ducts originating from the

urethra just beneath the bladder neck. The main ducts extend quite a distance before terminating in 20-30 branches per duct. The lateral type 2 prostate is formed from 5-7 ducts per side which originate just below the lateral type 1 prostate and branch extensively near to the urethra resulting in 40 to 60 terminal acini per duct (Hayashi et al., 1991). The anterior prostate is derived from a solid epithelial bud originating from the top portion of the urogenital sinus. As the solid outgrowth extends into the mesenchyme it begins to canalize and subsequently develop a high degree of intraductal infolding, much like that seen in the seminal vesicles (Hayashi et al., 1990).

Recent data presented by Hayashi et al., (1990) have demonstrated by ¹⁴C-thymidine wholemount autoradiography that the thymidine incorporation into DNA was much higher in the ductal tips versus the more proximal portions of the prostate in mice, confirming the observation that new ductal formations are added distally to the pre-existing more proximal ductal complexes. However, as this author and others stress, the ductal branching pattern which is in part a result of the increased DNA synthesis at the distal tips is ultimately induced and defined by the stroma or mesenchyme (Sugimura et al., 1986; Bernfield et al., 1973; Alescio and Cassini, 1962; Kratochwil, 1969; Sakakura and Nishizuka, 1976; Shannon and Cunha, 1984). Accordingly, the differences in ductal branching present in the four separate lobes are probably a result of

the regional differences in the regulatory properties of the mesenchyme in the developing rat prostate. Furthermore, different proteins are secreted from the epithelial cells of the different lobes presumably the result of differences in mesenchymal induction, a point which will be expanded upon later.

Prostatic growth and ductal morphogenesis begin prenatally and continue essentially uninterrupted until sexual maturity in the rat. However, in the human male, normal prostatic growth and ductal morphogenesis occur in two separate periods, prenatally and at puberty. Further prostatic growth and branching can occur in pathological situations such as benign prostatic hyperplasia (BPH) and adenocarcinoma although the onset is not usually seen until the 4th decade of life (Zonek and Zonek, 1975; Berry et al., 1985).

It is well known that the development and growth of the prostate is androgen dependent. The testes begin production of androgen prenatally and continue this role during periods of prostatic morphogenesis (Weniger and Zeis, 1972; Resko, 1978; Pointis et al., 1980; Siiteri and Wilson, 1974; Winter et al., 1981). If the testes are inhibited from producing androgen either by surgical removal or chemical obliteration during the ambiguous period of sexual differentiation, the prostate is restrained from normal development and growth (Jost, 1953; Raynaud and Frilley, 1974; Neumann et al., 1970; Elger et al., 1974; Neumann et al., 1974; Burns, 1961; Greene et al., 1939,

Greene, 1940). In fact, castration of the neonate or the adult produces the same response -- greatly inhibited growth of the prostate (Price and Ortiz, 1944; Berry and Isaacs, 1984; DeKlerk and Coffey, 1978). In the adult, androgen withdrawal results in prostatic atrophasia, cell death and ultimately atrophy (Bruchovsky et al., 1978; Bruchovsky et al., 1987). The effects of castration can be countered by exogenous application of androgen (Kyprianow and Isaacs, 1987). However, in normal and castrated males, androgen will not induce growth beyond reaching normal size. According to Bruchovsky et al., (1978 and 1987) homeostatic constraint mechanisms such as negative feedback, come into play once the prostate has attained its normal size.

The majority of androgen produced by the fetal testes is testosterone. Nonetheless, dihydrotestosterone (DHT) appears to be the intracellular active androgen responsible for prostatic morphogenesis (Cuhna et al., 1987). Testosterone is enzymatically reduced in the UGS to DHT by the action of 5 α -reductase in rats and human males (Lasnitzki and Franklin, 1972; Wilson and Lasnitzki, 1971 and Wilson et al., 1981). Inhibition of this enzyme in the fetus results in restricted prostatic morphogenesis and the appearance of feminized external genitalia in both rats and humans (Imperato-McGinley et al., 1985).

Androgen action is transmitted via specific intracellular androgen receptors (Shannon et al., 1981; Shannon and Cuhna,

1983 and Takeda et al., 1985). In the rat fetus, autoradiographic studies have demonstrated the presence of androgen receptors in the mesenchyme but not in the epithelium (Shannon et al., 1981; Shannon and Cunha, 1983 and Takeda et al., 1985). However, in the adult, androgen receptor is present in both the mesenchyme and the epithelium.

Tissue recombination experiments using explants under the kidney capsule of UGS and urogenital sinus epithelium (UGE) from either wild type or testicular feminized male (Tfm) mice demonstrated that only wild type UGM combined with either wild type or Tfm UGS resulted in the prostate developing. When Tfm UGM was recombined with either wild type or Tfm UGS these tissues formed the equivalent of the vagina. This data confirmed that, in the fetus, it is the mesenchyme which ultimately directs and determines what type of epithelial cell population will develop (Cunha et al., 1980). This is also true for the adult male, a point which will be discussed later.

Another role played by the mesenchyme is controlling the final size of the prostate. Specifically, it is the amount of mesenchyme present which determines the final organ size (Chung and Cunha, 1983). These investigators demonstrated by increasing either the amount of UGE while keeping the UGM constant or vice versa, only those recombinant tissues when the UGM varied (ie: increased) but the UGE was kept constant showed a corresponding increase in tissue mass. There appeared

to be a direct correlation between the amount of UGM present and the final tissue mass such that when the amount of recombined UGM was increased, a parallel increase in tissue mass was observed (Cunha et al., 1987).

However, some size constraints are in effect since exogenous application of androgen elicits no further increase in the size of the prostate once it has reached its normal size. Furthermore, the prostate of the castrated males which have received androgen replacement therapy grow only to the normal size of the intact rat. Additional applications of androgen evoke no further growth in prostate size in the normal or androgen treated castrated male (Berry and Isaacs, 1984).

Bruchovsky and colleagues (1975) have outlined the following three homeostatic constraints which regulate the growth of cells in the normal prostate: (1) initiation; (2) negative feedback; (3) autophagia/self-destruction. According to Bruchovsky, undifferentiated or involuted cells resulting from castration will initiate new cycles of DNA synthesis and cell proliferation in the presence of androgen. Once the prostate has attained its standard size, a negative feedback system is triggered, shutting down DNA synthesis and cell proliferation. As long as androgen is present the organ size and functional integrity is maintained. However, should androgen be removed, autophagia will result decreasing the size of the prostate, arresting prostatic secretions, and

reducing, but not obliterating, the number and the size of epithelial cells (Higgins and Scott, 1945). In fact, epithelial cells undergo a 93% reduction in population upon withdrawal of androgen, whereas the stromal cells only regress by 22% (DeKlerk and Coffey, 1984). Furthermore, the reduction in the prostate is not uniform throughout the organ. The distal tips undergo the greatest regression while the more proximal segments are maintained but in an atrophic state (Sugimura et al., 1986). Although the majority of epithelial cells succumb to cell death with the withdrawal of androgen, a small population of cells survive castration and are propagated in the absence of hormone (Lesser, 1974; Bruchovsky et al., 1975). Apparently, these "stem cells" are not under the same hormonal control as the differentiated epithelial cells. In actuality, the primary function of the stem cells appears to be their involvement in the growth response of regenerating tissue (Bruchovsky et al., 1987).

Consequently, withdrawal of androgen alters the ratio of epithelial to stromal cells in favour of the stromal component. It was thought that a ratio of epithelial/stromal which favoured the stromal components determined cell growth and proliferation. However, as the data presented later demonstrates, this is not the case.

In the immature male the development of the mesenchymal component initially far exceeds the epithelial cell count (5:1). However, as prostatic growth and ductal morphogenesis

proceeds, the mesenchyme is invaded by proliferating epithelium which shifts the ratio in favour of the epithelial cell component. Once sexual maturity is attained the epithelial/stromal cell ratio remains relatively constant. In the adult human male the ratio is 1:1, while in the adult male rat it is 5:1, respectively (DeKlerk and Coffey, 1978).

Biomorphogenic experiments using portions of the adult prostatic ducts containing epithelial cells were grafted alone or in combination with either UGM or bladder mesenchyme. Although individual ducts were maintained when grafted alone or with bladder mesenchyme, only those recombinant grafts containing UGM (UGM + prostatic ducts) demonstrated an increase in prostatic ducts and weight (Norman *et al.*, 1986). In contrast to the idea presented above by Bruchovsky, these results imply that epithelial cell loss or atrophy induced castration is not a necessary requirement for the regenerative growth response. In other words, the stromal element need not be in excess of the epithelial element, just present, in order for tissue regeneration to occur.

B. Prostatic Cancer

As stated above, further prostatic growth is observed in pathological conditions such as Benign Prostatic Hyperplasia (BPH) and prostatic adenocarcinoma. Investigations have shown that these cells grow in the absence of one or more of the three homeostatic constraints that are initiation, negative feedback and autophagia. Should the negative feedback

mechanism be absent or aberrant, then abnormal prostatic cells will grow unlimited in response to androgens. With the removal of androgen, the normal autophagic constraint will be triggered, ultimately resulting in death and lysis of these irregular prostatic cells as well as a decrease in the proliferative rate. This variety of tumour is referred to as androgen-dependent.

An androgen-sensitive tumour has lost both the negative feedback and autophasic mechanisms. Its growth, like the androgen-dependent tumour, is unlimited in the presence of androgen. However, with the autophasic constraint lacking, the withdrawal of androgen results in the inhibition of further tumour cell growth or proliferation. Consequently, the tumour will either remain in a stationary state of growth or decline its growth to the pre-treatment rate (Bruchovsky et al., 1987).

In the normal developing prostate, androgen application results in the initiation of cell growth and proliferation. The loss of the homeostatic initiation constraint allows tumourgenesis to proceed unrestricted in the presence or absence of hormone. Specifically, there is no visible correlation between the growth of the tumour cells and the presence or absence of androgen. Therefore, these tumours are autonomous and are thus referred to as androgen-independent.

C. Models for Prostatic Cancer

In Canada and the United States, prostatic adenocarcinoma

is the second leading cause of death by cancer (Silverberg et al., 1990). Furthermore, the incidence of prostatic cancer is predicted to increase markedly by the year 2000 as the "baby boomer" population reaches their fourth and fifth decade of life. Nevertheless, the lack of appropriate animal models and tissue/cell culture systems which display the various stages of cancerous growth in man have left prostatic cancer research sorely lagging.

The animal models available are in the form of transplantable tumour cell lines, derived originally from the rat dorsal prostate. Nevertheless, these in vivo systems do not accurately reflect the human condition. In man, prostatic cancer cells are initially androgen-dependent. As the disease progresses the cells transform, becoming androgen-sensitive and ultimately androgen-independent with extensive metastases to the bone.

Dunning tumours are a series of tumour sublines derived from the original R-3327 rat prostatic adenocarcinoma (Dunning, 1963). The tumour sublines are either androgen responsive but non-metastatic (R-3327H) or androgen-independent and metastatic, but not to the bone (MAT-lylu) (Smolev et al., 1977; Isaac and Coffey, 1981). The PA-III subline is one of the tumour sublines developed by Pollard (Pollard, 1973; Pollard and Luckert, 1975). It does metastasize to the bone but it is androgen-independent and thus limits developing information on the etiology of

prostatic cancer progressing to the independent state. The Noble sublines (Noble and Hoover, 1975; Noble, 1977) are androgen-responsive and metastasize to the lung, but not to the bone.

Therefore none of the aforementioned animal models correctly mirrors the onset of human prostatic carcinogenesis. What does remain are the immortalized cell lines derived from xenografts of human prostatic adenocarcinoma in nude mice. These in vitro tissue/cell culture systems offer a different approach to uncovering the mechanisms of prostatic carcinogenesis.

Most recently, data derived from transgenic mice experiments performed by Greenberg et al., 1994, provide a more promising in vivo model of the transformation of the differentiating prostate. Armed with 426 base pairs (bp) of the rat probasin (PB) gene promoter and 28 bp of the 5'-untranslated region fused to the bacterial chloramphenicol acetyl transferase (CAT) gene, these researchers were able to specifically target CAT gene expression to the prostate in transgenic mice. Furthermore, the PB-CAT gene expression was male specific and confined to the epithelial cells of the dorsal, lateral and ventral prostatic lobes, mirroring the expression of PB in vivo in normal rats. Consequently, it is feasibly to design experiments which can unravel the molecular and cellular events regulating the transformation of the differentiating prostate in vivo.

D. Steroid Hormone Regulation of Specific Gene Expression

The classical model of steroid hormone regulation of gene expression defines two specific events. The first event occurs in the cytosol where the hormone molecule binds to its cognisant hormone receptor protein. This binding results in a conformational change in the hormone receptor whereby the previously occluded nuclear localization signal sequence becomes exposed. The second events occurs once the hormone receptor complex has translocated to the nucleus. There, the deoxyribonucleic acid (DNA) binding domain of the receptor protein recognizes and interacts with regulatory sequences called Hormone Responsve Elements (HRE), contained within the non-coding regions of specific target genes (Ponta et al., 1985). The progesterone receptor (PR), glucocorticoid receptor (GR) and androgen receptor (AR) proteins all contain a high degree of sequence homology within their respective DNA binding domains which is reflected in their common interactions with target genes possessing one or more 15 bp imperfect palindromic sequences (Beato, 1989, Cato et al., 1987, Ham et al., 1988, Denison et al., 1989, DeVos et al., 1991, Riegman et al., 1991 and Rennie et al., 1993).

Two criteria are necessary for proof that a specific gene of interest is hormonally responsive: (1) the steroid-receptor complex must interact specifically with the HRE located within the gene in question. This may be demonstrated by in vitro methods such as gel retardation, DNase I protection assays and

DNA footprinting; (2) the association of the hormone-receptor complex with its corresponding putative HRE confers hormone responsiveness to the gene alone or to a reporter gene situated near the native gene when transferred into a suitable cell line. This criterion may be illustrated by in vivo methods such as gene transfer experiments, specifically, transfections.

The following represents a brief summary of genes demonstrated to be regulated by androgens.

1. Alpha 2 urinary (α_{2u})-globulins

The α_{2u} -globulins are a group of closely related proteins encoded by a gene family of approximately twenty. The primary site of secretion of these proteins occurs in the liver of the male rat, however, secretion of small quantities have been shown in other tissues (Roy et al., 1979; Kurtz, 1981; Laperche et al., 1983; Vandoren et al., 1983; Gutitz et al., 1984; MacInnes et al., 1986). Multihormonal control of these proteins in the liver have been demonstrated although androgen appears to be the principal physiological regulator (Van Dijck et al., 1989).

Two genes within this family, RAP 1 and RAO 1, initially showed promise in terms of their potential to act as mediators of androgen regulation. The RAP 1 gene was demonstrated to interact specifically and bind with relative high affinity to the Androgen-Receptor complex. The responsive element within this gene is localized to a region between -642 and -584 bp

upstream of the start site of transcription and bears a strong similarity to the consensus glucocorticoid response element (GRE) (Van Dijck et al., 1989). The RAO 1 gene has undergone a mutation within this putative androgen response element (ARE) sequence which has rendered it unable to bind both AR and GR (Van Dijck et al., 1989; Van Dijck et al., 1987). A second sequence located between -252 and -118 bp is shared by both RAP1 and RAO 1 is able to bind GR but fails to bind AR (Van Dijck et al., 1989). In any case, these demonstrations have only addressed one of the two criteria for proving a sequence is an HRE. They have presented no biological data or information on the action of these sequences in vivo.

2. Murine β -Glucuronidase Gene

The murine β -glucuronidase gene (GUS) is expressed constitutively in many tissues cell types, however, there is a marked but slow rise in GUS messenger ribonucleic acid (mRNA) and enzyme levels in kidney epithelium cells when treated with androgens (Paigen, 1989). Furthermore, GUS responds to androgenic steroid in a specific manner (Dofuku et al., 1971; Dofuku et al., 1971) but is unaffected by either glucocorticoids or progestins (Fishman, 1951).

The most recent information on the androgenic response of the GUS gene is derived from inbred strains of mice. Lund et al. (1991) describes three phenotypes: (1) a strong response in mice strains of the Gus^a haplotype, (2) a reduced response in strains of the Gus^b and Gus^h haplotypes, and (3) no response

in Gus^{or} haplotype. Further investigations revealed an androgen-inducible nuclear hypersensitivity site in intron 9 of the Gus^a but not Gus^{or} mice. Gel shift analysis using this Gus^a hypersensitivity site with kidney nuclear extracts of Gus^a mice treated or not treated with androgens showed two shifted bands whose intensity was much enhanced for the treated mice compared with the untreated. Sequence analysis uncovered two regions of importance: a 57 bp region of complex dyad symmetry reflecting binding of a kidney-specific factor present of Gus^a and Gus^{or} but partially deleted in Gus^h and Gus^b; and a GRE sequence motif 130 bp upstream of the 57 bp region which is present in all phenotypes but Gus^{or}. The authors suggest that the results indicate that both elements in the proximal end of intron 9 of the GUS structural gene are responsible for androgen responsiveness of GUS (Lund et al., 1991).

3. Prostate-Specific Antigen

Prostate-specific antigen (PSA) is a member of the kallikrein gene family. It is expressed solely in human prostatic epithelial cells as a 240 amino acid single chain glycoprotein (Watt et al., 1986). It is found in high concentrations in the seminal fluid and its proteolytic activity has been implicated in semen liquifaction and male fertility (Liljia, 1985; Liljia et al., 1987).

Interest in PSA has grown since the discovery that it is more superior and reliable as a tumour marker for prostate cancer previously employed prostatic acid phosphatase and

alkaline phosphatase (Killian et al., 1986; Stamey et al., 1987; Ercole et al., 1987; Nadji et al., 1981; Allhoff et al., 1983).

Two important pieces of data have subsequently emerged which lead to the suggestion that PSA may be under androgenic regulation. First, the expression of PSA protein in prostatic tissues of the developing male has been demonstrated to be related to the corresponding levels of androgen (Goldfarb et al., 1986). Secondly, the loss of androgen responsiveness of LNCaP cells, a cell line derived from lymph nodes of humans with prostatic adenocarcinoma, corresponds to the loss of PSA expression (Hasenson et al., 1989). It is well established that PSA protein is found in abundance in the epithelium of normal prostate and benign prostatic hyperplasia. However, what still remained unresolved was the potential link between the heterogeneity of expression of PSA and androgen responsiveness in high grade prostatic carcinomas. Therefore, further investigations were performed to elucidate the mechanism of PSA regulation and expression and its potential value as a model of androgen action.

Employing a PSA-specific probe, Young et al., (1991), have localized the mRNA in the epithelium of BPH prostate using in situ hybridization. In addition, androgen regulation of PSA mRNA was demonstrated using LNCaP cells treated with mibolerone or DHT at various concentrations over a nine hour time period. The RNA was extracted at various time periods and

Northern blots performed on the fractions. These investigators found that PSA mRNA increased within 1 hour of hormone treatment reaching a maximum level after 9 hours. Furthermore, when 200 fold excess of hydroxyflutamide, an antiandrogen, was added in the presence of DHT, PSA mRNA was reduced, lending support to the idea that androgen induction of PSA mRNA might be via the androgen receptor.

4. C3(1) Gene of the Prostatic Binding Protein

Prostatic Binding Protein (PBP) or Prostatein (Viskochil et al., 1983) is the major secretory protein of the rat ventral prostate (Hurst and Parker, 1983; Parker et al., 1983; Peeters et al., 1982; Peeters et al., 1983). It consists of three subunits, C1, C2 and C3 arranged in two heterodimers (C1C3) and (C2C3). Isolation of the genomic sequences and restriction enzyme mapping of the three subunits indicated that each subunit contains 3 exons separated by 2 introns. The coding sequences are contained within 2.8 kilo base pairs (kbp) of the genomic DNA (Viskochil et al., 1983; Hurst and Parker, 1983; Parker et al., 1983). Of particular interest is the C3 subunit which is encoded by 2 genes only one of which is transcribed (Viskoshil et al., 1983, Hurst and Parker, 1983; Parker et al., 1983; White and Parker, 1983). According to Parker and others (Peeter et al., 1980; Parker et al., 1980; Page and Parker, 1982; Zhand and Parker, 1985; Bossyns et al., 1986; Quarmby et al., 1987), androgen regulation of C3 mRNA has been demonstrated both at the level of transcription

and mRNA turnover.

Protein binding experiments performed by other labs (Perry et al., 1984) showed that partially purified AR binds preferentially to two fragments within the C3 subunit: a 0.3 kbp fragment 5' to the initiation start site, including the promoter and part of the first exon and a 0.5 kbp fragment within the first intron. Although both these fragments contained the imperfect 15 bp palindromic sequence similar to the consensus GRE, the specific sequence to which the AR bound was not identified.

Subsequent experiments revealed the following data: (1) upon transient transfection of the 0.5 kbp intronic fragment into T47D cells (having endogenous AR, GR and PR) and treatment with androgens, androgen regulation was observed, (2) mutations within this sequence containing a GRE-like motif, abolished its androgen responsiveness, thus suggesting that this sequence can function as an ARE (Tan et al., 1992).

Sequence analysis of the 0.3 kbp and the 0.5 kbp fragments as well as the intervening 0.9 kbp fragment uncovered 12 GRE-like sequences. However, when binding assays were performed on these putative AREs only 3 sequences bound AR with higher affinity than the controls. Furthermore, sequence, A, localized to the 0.3 kbp fragment of the 5'-flanking DNA, exhibited much less binding affinity than the two other sequences, B and C, are contained within the 0.5 kbp fragment of the first intron (Perry et al., 1984; Rushmere et

al., 1987). Transient transfections of these fragments with Herpes simplex thymidine kinase (tk)CAT as promoter in to CV-1 cells and cotransfection of the cytomegalo virus rat androgen receptor (pCMVrAR) expression vector demonstrated corresponding data, namely the 0.5 kbp fragment encompassing sequences B and C showed stronger transcriptional action than did the 0.3 kbp fragment containing sequence A. Furthermore, sequence C alone was able to transactivate androgen regulation equally as well as sequences B and C together (Tan et al., 1992).

5. Mouse Mammary Tumour Virus Long Terminal Repeat

The Mouse Mammary Tumour Virus (MMTV or MTV) is a retrovirus which contains genes encoding the viral coat proteins and reverse transcriptase. Flanking these genes are repetitive DNA sequences called Long Terminal Repeat (LTR). It is the sequences within the LTR of MMTV which have proven to exhibit hormonal enhancement capabilities. Originally, these sequences were demonstrated to enhance glucocorticoid and progesterone responsiveness (Yamamoto, 1985; Cato et al., 1986; Strahle et al., 1987; Beato, 1989). Subsequently, the LTR of MMTV was demonstrated to mediate androgen-dependent activation of gene transcription using Shionogi S115 mouse mammary tumour cells (Darbre et al., 1986).

Following these initial investigations, a series of transient transfection employing different cell lines were performed to further characterize androgen responsiveness of

the LTR. Notably, human breast cancer cell lines T47D and ZR-75-1, containing endogenous AR, GR and PR, were transiently transfected with chimaeric MMTV-LTR-CAT constructs and treated with androgens. Indeed, both cell lines were demonstrated to be androgen responsive (Cato et al., 1987; Cato et al., 1988; Harris et al., 1988; Claessens et al., 1989; Ham et al., 1988).

Denison and colleagues (1989) further characterized the ability of MMTV-LTR to mediate androgen-dependent activation of transcription using the human prostate cancer cell line LNCaP. Most recently, addressing the potential of receptor interference (discussed later), the monkey kidney cell line, CV-1, was transiently transfected with the MMTV-LTR-CAT, cotransfected with the AR expression vector and treated with androgens. Again, the LTR was demonstrated to enhance androgen-dependent transcription (Govindan 1990; Rundlett et al., 1990; Simental et al., 1991).

6. Mouse Sex-Limited Protein Gene

The mouse sex-limited protein (Slp) gene is a duplicated complement of the C4 gene (Shreffler, 1982; Chaplin et al., 1983). Its expression in several tissues is androgen-dependent due to the influence of an inserted LTR provirus upstream of the structural Slp gene (Stavenhagen and Robbins, 1988; Cox and Robbins, 1988). The LTR functions as a hormone-dependent enhancer and shows multiple nuclear protein binding sites (Adler et al., 1991). One element within this fragment has

been demonstrated to be necessary but not sufficient for strong induction is a consensus HRE (HRE-3). This is the only element which shows AR binding and some hormonal responsiveness itself (Adler et al., 1991). Specifically, 3 chimaeric CAT constructs, C' 9, C' 2 and 2XHRE-3, were assayed for their potential as hormone enhancers in CV-1 cells. The C' 9 construct, a 120 bp fragment containing the HRE-3 enhancer, exhibited a 20 fold increase in CAT activity when cotransfected with the rat androgen receptor (rAR) expression vector and treated with androgen. When the same construct was assayed for glucocorticoid or progesterone responsiveness, that is cotransfected with rat glucocorticoid receptor (rGR) expression vector and treated with dexamethazone (DEX) or with human progesterone receptor (hPR) expression vector and treated with progestins, respectively, no response was observed. These data indicates that the C' 9 fragment possessed sequences which conferred androgen-specific regulations.

The C' 2 construct consisting of 160 bp, 40 bp upstream of and contiguous with the 120 bp of the C' 9 construct, was assayed in the same manner as C' 9 construct. The data revealed no difference in the level of induction between glucocorticoid and androgen responsiveness and only a slight decrease in the progesterone response (Adler et al., 1992). The authors argue that nonspecific factors may be involved through binding sites not present in the AR-specific DNA

sequences, in effect overriding androgen responsive specificity.

Evidence supporting this idea is presented in the final set of transient transfections. A construct containing only the HRE-3 in duplicate was tested for its ability to mediate androgen, glucocorticoid and progesterone induced transcription via their appropriate cognisant receptors. The results showed that both glucocorticoid and progesterone responses were more than double that of androgen (Alder et al., 1992).

7. Probasin Gene.

The probasin (PB) gene is an androgen (Dodd et al., 1983) and zinc (Matusik et al., 1986) regulated rat prostatic gene. It has been localized within the ducts and the nucleus of epithelial cells of the dorsal, lateral and ventral lobes of the prostate (Spence et al., 1989). Although no ligand has been found for PB, amino acid sequence predicted by sequencing of the PB complementary deoxyribonucleic acid (cDNA) reveals homology to the rat α 2-urinary glubulin, rat odorant binding protein and the bovine β -lactoglobulin, all members of a ligand carrier family of proteins (Spence et al., 1989).

The expression of PB in the rat prostate is developmentally regulated at least in part, by androgens. The immature rat has low levels of PB mRNA in the ventral prostate. At the beginning of sexual maturation, these levels of PB mRNA decrease further in the ventral prostate but increase substantially in the dorsal and lateral lobes.

E. Steroid Receptor Structure

I have outlined the known influences of androgen on the development and morphogenesis of the prostate at the cellular and organ level. Although a great deal of research has been focused on androgen regulation of growth and function of the prostate, little is known about the actual molecular mechanisms involved in transcription. The question of how androgen regulates the expression of androgen-responsive genes still remains. The following is a summary of what is known about steroid hormone regulation of specific gene expression.

Until the early 1970's very little information was available addressing the question of how hormones exert their control at the molecular level. With the use of radioactively labelled ligands, steroid and thyroid receptors were identified (Jensen and DeSombre, 1972; Tompkins, 1974; Tata et al., 1978). According to Ivarie and O'Farrell (1978), interaction of the hormone with the receptor altered the receptor in a way that caused it to associate with specific high affinity binding sites in the chromatin. This association resulted in the induction or repression of a number of genes, estimated at that time to be 50 to 100 per cell. What determines which genes will be induced depends, at least in part, on the cell type and tissue. Since chromatin structure is arranged differently in each cell type and tissue, it follows that only certain clusters of genes will be accessible to the receptor-hormone complex.

After initial identification of steroid binding proteins, intense effort was directed towards isolating and purifying these steroid receptors. However, their trace presence in the cell (10^3 to 10^4 molecules per cell) made the task insurmountable until the development of high-affinity synthetic analogues of the steroid hormones (Krieger, 1981). By 1988, the last of the major steroid receptors, the androgen receptor, was cloned and sequenced (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988). What emerged from these studies on AR was similarity in function and structure to different steroid receptors. For example, purification and biochemical characterization of the androgen receptor uncovered specific regions in the protein intimately involved in DNA and hormone binding. In addition, these regions on receptors could be separated biochemically through limited proteolysis thus providing the first evidence for domain structures within the steroid hormone receptors (Wrange and Gustafson, 1978; Wrange et al., 1979; Okret et al., 1982; Carlstedt-Duke et al., 1982; Pellwege et al., 1982 and Wrange et al., 1984).

Subsequently, a generalized structure for steroid hormone receptors was described. The steroid receptors can be divided into various functional protein domains. The A/B domain is located at the amino-terminus and consists of hypervariable region in both size and amino acid composition. It has been termed the "transcriptional modulation domain" consistent with

the observations that this region contains information which enhances transcription and sequences which permit enhanced activation of particular genes (Tora et al., 1988; Conneely et al., 1989). Support for this modulatory function comes from deletion and mutational analysis of the glucocorticoid receptor. Specifically, deletions in the A/B domain decreases activity by 10 to 20 fold (Hollenberg et al., 1987; Danielson et al., 1987). Furthermore, analysis of the NTⁱ (nuclear transfer increased) mutant glucocorticoid receptor revealed that although these receptors can bind hormone, they are biologically not functional. Apparently, this mutant receptor contain an altered amino terminus (Yamamoto et al., 1976). Further support for this modulatory function in the A/B domain comes from the observation and analysis of the progesterone receptor. Two versions of this receptor exist, A and B forms, differing only in the first 128 amino acids (at the amino terminus). Initial studies of the progesterone receptor forms indicate that it is entirely possible that these two forms have remarkably different abilities to modulate gene expression. Specifically, Chambon and colleagues reported that the two forms of chicken progesterone receptor (cPR), in the presence of hormone, could activate the HRE in the MMTV-LTR in both HeLa and primary chicken embryo fibroblast (CHEF) cell cultures (Tora et al., 1988). In contrast, only the A form of cPR, in the presence of hormone, could transactivate transcription of the progesterone response element (PRE) in

the chicken ovalbumin gene when assayed in HeLa cells. These investigators suggest that transcriptional activation of the chicken ovalbumin gene PRE requires not only the progesterone receptor and hormone, but also a cell specific factor absent in HeLa cells (Tora et al., 1988). How this region functions to modulate or "transactivate" gene expression has not been precisely defined, however, current thought indicates that this function is transmitted through charge aggregates or higher order structures and not by specific stretches of amino acids (O'Malley, 1990). For the glucocorticoid receptor, two transactivating domains have been identified, one in the A/B domain and the other in the E domain (the hormone binding domain). Both sequences are strongly acidic in character and are functionally, position independent. Nonetheless, they are structurally unrelated (Hollenberg and Evans, 1988).

The C domain is a small, centrally located region, consisting of approximately 66 amino acids. By far, it is the best characterized and most highly conserved region (40 to 95% homology between the different steroid hormone receptors), in terms of structure and function (Danielson et al., 1989). This region comprises the DNA binding domain and is distinguished by 9 conserved cysteine residues, eight of which are thought to form 2 tetrahedrally coordinated zinc (ion) fingers, comparable to the zinc finger motifs first described by Miller et al., (1985) for *Xenopus* protein TFIIIA (Hollenberg and Evans, 1988). More recent evidence presented by Luisi and

colleagues (1990) demonstrated that the similarity between these two structures was primarily superficial. Nuclear Magnetic Resonance (NMR) crystallography was performed on two protein crystals comprised of the rGR DNA binding domain (amino acids 440-525) and the GRE (target DNA) with either 3 (GRE_{3s}) or 4 (GRE_{4s}) base pairs between the two half-sites. These investigators showed that although the TFIIIA and the rGR DNA binding domain both tetrahedrally coordinate two zinc ions by out-folding or looping of the protein resulting in finger-like structures, this is where the similarity ends. The two zinc ions in TFIIIA are coordinated by two cysteines and 2 histidine residues whereas the two zinc ions of rGR are coordinated by 4 cysteines. Furthermore, the chirality of the amino zinc-sulphur centre assumes an 'S' configuration, as found in *Xenopus* Xfin (Lee et al., 1989) and other metal-binding proteins (Berg, 1988), whereas the carboxyl zinc-sulphur centre assumes an 'R' configuration (Luisi et al., 1990). According to Luisi and colleagues (1990), not only do the zinc nucleated motifs of rGR differ from those found in other metal-binding proteins, but they also differ structurally and functionally from each other. In the *Xenopus* TFIIIA, the zinc finger motifs are stabilized individually by a hydrophobic core. Each finger functions as an autonomous conformationally stable structural unit, each assisting in DNA binding (Pavletich and Pabo, 1991). However, the zinc nucleated modules of the rGR protein are stabilized primarily

by the interaction of the aromatic side chains contained within their amphipathic helices flanking each finger and are components of a larger compact globular folded structure (Luisi et al., 1990).

Of the approximately 66 amino acids embracing the D domain of rGR, thirteen amino acids form the intervening loop between the two pairs of cysteines in the first or amino-terminal zinc finger and twelve amino acids form the loop in the second or carboxyl zinc finger. The finger motifs are linked by sequence of 15 to 17 amino acids (Umesono and Evans, 1989). Site-directed mutagenesis experiments performed by Green and Chambon (1987) clearly demonstrated the requirement for the conserved cysteine residues in DNA binding. Furthermore, according to Berg (1989), the first 8 of the 9 cysteines are necessary for proper DNA binding domain function, since mutation of any one of these cysteines leads to the abolishment of activity.

The amino terminal loop consists of a small anti-parallel β structure which aids in orienting the residues to make contact with the phosphates in the DNA. The two distal cysteines (of the amino zinc module) and the nine intervening amino acid residues comprise an α -helix of which amino acid residues 460, 461 and 466 supply the only base contacts with the major groove of the DNA. Furthermore, there are no proteins contacts with the minor groove of the DNA (Luisi et al., 1990).

Genomic analysis demonstrated that each zinc motif was encoded by a separate exon (Arriza et al., 1987; Huckaby et al., 1987) and appeared to be structurally discrete (Hollenberg and Evans, 1988). Finger swap experiments performed by Green and Chambon (1987) demonstrated that the amino terminal zinc finger region was involved in the recognition of specific DNA sequences in the target gene. Specifically, these investigators replaced the carboxyl terminal zinc finger of the estrogen receptor (ER) with that of the glucocorticoid receptor and vice versa. Regardless of the origin of the carboxyl zinc finger, it was the amino zinc finger which appeared to be responsible for the functional discrimination between the GRE and an estrogen response element (ERE) (Green et al. 1988).

Early structural analysis of the different steroid hormone receptors indicated that they may be subdivided into 2 groups based upon primary amino acid sequence of the two residues located between the two distal cysteines of the first zinc finger (residues 458 and 459, GR numbering for clarity; Beato, 1988; Danielson et al., 1989). Group 1 comprise the glucocorticoid, progesterone, mineralocorticoid and androgen receptors. All four of these receptors have glycine and serine at residues 458 and 459, and are able to work through a glucocorticoid response element (Sträle et al., 1987; Cato et al., 1987; Arriza et al., 1987; Beato, 1989). Group 2 have glutamate and glycine at residues 458 and 459. These

characteristic amino acid residues are found in the estrogen thyroid hormone, retinoic acid and vitamin D₃ receptors (Danielson et al., 1987; Beato, 1989). The two conserved distal cysteines of the first zinc finger, residues 457 and 460, the two intervening variant amino acids, residues 458 and 459 are referred to as the P-box and are suggested to be involved in recognizing the variant nucleotides in the steroid responsive element. Support for this concept came from substituting the glycine at position 458 of GR with a glutamic acid characteristic of the ER. As a result, the receptor not only recognized a GRE but now also recognized an ERE. More recent mutagenesis experiments have revealed amino acid residue 462 in addition to residues 458 and 459 as determining receptor sequence-specific DNA recognition (Danielson et al., 1989; Mader et al., 1989). Whereas GR has a valine at residue 462, ER has an alanine. Furthermore, exchanging loop sequences between receptors does not alter target DNA specificity (Umesono and Evans, 1989). In other words, it is the knuckle and not the finger, as originally proposed by Hughes and colleagues (1988) that makes contact with the target DNA. Indeed recent investigations by Luisi et al. (1990) have shown that only the side chains of the amino acid residues make base contact with the DNA. Furthermore, these investigators demonstrated that the amino terminal zinc module lies within the large groove of the DNA and the carboxyl module is positioned over the minor groove but makes no nucleotide

contact with the DNA (Luisi et al., 1990)

The carboxyl zinc nucleated motif consists of two α -helices. The first α -helix consisting of amino acid residues 486-491 is distorted whereas the second α helix, amino acids 492-503 assumes a regular structure, similar to the α helix found in the amino zinc motif. The amino acid residues 477, 479, 481, 483, 487, and 491 together with cysteine residues 476 and 482 comprise the "D box", or the dimerization interface. Phosphate contacts are made with amino acids 475, 489, 490 and 496. The conformation of the major loop is a reverse β turn which is maintained by the R configuration of the zinc-sulphur centre. The uniqueness of the chirality of zinc centre together with the exposed hydrophobic isoleucine residues 483 and 489 resulting from the reverse β turn of the major loop all suggest a potential function for this domain in contacting other domains of the receptor or possibly with other proteins (Scheda et al., 1989). Indeed, Luisi et al. (1990) suggest the reason for uniqueness of the chirality of the zinc centre lies in the fact that the carboxyl terminal zinc module provides all the known protein contacts (Nauber et al., 1988).

The carboxyl zinc motif provides the phosphate contacts and the dimerization interface as well as a site implicated in the positive control of transcription (Scheda et al., 1989). Until recently, the specific manner in which the carboxyl zinc motif assisted transcriptional control remained elusive. It

was known that steroid hormone receptors with mutations in their carboxyl terminals that extend into the second zinc finger rendered them transcriptionally inactive due to their inability to bind DNA (Danielson et al., 1986; Hollenberg et al., 1987; Miesfield et al., 1987). Furthermore, point mutations as well as amino acid insertions in the carboxyl zinc finger of glucocorticoid receptor leads to its inactivity (Danielson et al., 1986; Hollenberg and Evans, 1988).

Previous reports by Wrange et al., (1986) suggested that one glucocorticoid receptor molecule might bind to one GRE sequence in the MMTV promoter. However, recent evidence based on gel retardation and methylation interference experiments presented by Tsai and colleagues (1988) suggest the glucocorticoid receptor binds to the hormone response element as a dimer and that the binding of each monomer is not simultaneous nor of equal affinity. Specifically, methylation interference experiments using a truncated form of glucocorticoid receptor demonstrated sequential binding: first binding to the upstream half-site and then to the downstream half-site of the GRE. In fact, if the truncated GR concentration is low, it will bind to the left half-site preferentially. Apparently, binding to the upstream half-site fosters binding to the downstream half-site.

More recent evidence presented by Umesono and Evans (1989) suggest that the proximal pair of cysteines and the 3 intervening nucleotides of the second zinc finger, the D box,

is involved in recognition of half-site spacing of the palindromic HRE. Both GREs and EREs have 3 bp between their two half sites however their DNA sequences vary. The consensus thyroid response element (TRE) has no bp spacing between the two half sites but the DNA sequence is identical to that of the consensus ERE (5'...AGCTCAnnntGACCT... 3'). In addition, both GR and ER proteins dimerize upon DNA binding whereas thyroid receptor (TR) proteins do not, suggesting that the spacing between the half sites is a critical prerequisite for receptor dimerization.

Recent NMR crystallography analysis of GR protein complexed with the consensus GRE having 4 bp between the two half sites (GRE_{s4}) has revealed a clearer picture of the DNA-protein and protein-protein interactions. The fragment 440-525 (of GR protein) is monomeric in solution. Binding of the GR monomer to the upstream half sites produces a conformational change of the entire structure resulting in the DNA acting as a fluid scaffold now able to accommodate and coordinate protein-protein interactions, primarily dimerization as well as DNA-protein interaction, namely receptor-target DNA binding. Based upon the S4 crystal analysis, these investigators found that the spacing between the two half sites did not affect dimerization but did influence binding of the second monomer to the downstream half site. Previous investigations by Dahlman-Wright and colleagues (1991) demonstrated that increasing the spacing between the half

sites by one bp resulted in the two monomers binding non-cooperatively to the target DNA. This information suggests that the loss of cooperativity results from the second monomer facing sequences in the DNA which it does not specifically recognize and therefore can not bind with the same affinity. Taken together, these observations imply that the stability of the dimerization interface must exceed the stability furnished by the specific base interactions of the half site otherwise the monomers would not dimerize but would bind independently to the DNA. Whereas the amino acid sequences of the P box is highly conserved within the steroid hormone receptor family, the D box sequences are markedly divergent (Umesono and Evans, 1989). These authors propose that the D box of the receptor recognizes the spacing in the HREs in the target genes through interaction with another protein and not with the target DNA.

Although the actual regions in the receptor responsible for dimerization are still unclear, there are leucine-rich regions in the glucocorticoid and progesterone receptors. These regions have been implicated in the formation of "leucine zippers", a motif which apparently stabilizes the dimer formation of certain transcription factors (Landschulz et al., 1988).

Immediately carboxyl to the DNA binding domain is a small region (approximately 45 amino acids) defined as the D domain (Beato, 1989). Although characterization of this region has

not been well defined, there appears to be short run of basic amino acids similar to those found in simian virus (SV40) Large T antigen, which in the latter, is responsible for nuclear translocation (Kalderon et al., 1984). These sequences are highly conserved within the steroid receptors and appear to be, at least in part, responsible for their nuclear targeting (Jenster et al., 1991). Specifically, for the PR, the SV40 Large T antigen-like sequence, within the D domain appears to be sufficient for its nuclear localization (Guiochon-Mantel et al., 1989).

However, for the GR, this nuclear localization signal (NLS)-like sequence is not sufficient to effect its nuclear translocation. Evidently, there appears to be another hormone-dependent NLS present extending into the steroid binding domain (E domain, see below). In the absence of hormone, this second putative NLS is obscured by the heat shock protein 90 (hsp90) which binds to the GR, providing a cytoplasmic anchor (Sanchez et al., 1985; Catelli et al., 1985; Kost et al., 1989). One line of reasoning proposes that upon hormone binding, the heat shock protein (hsp) 90 is released from the GR protein, exposing the NLSs and allowing for nuclear relocation of the GR.

Recent investigations by Jenster and colleagues (1991) indicate that mode of AR nuclear localization may be very similar to that described for the GR protein: the AR contains two nuclear localization signals, one of which is hormone-

dependent. Nevertheless, the majority of the AR resides in the nucleus in the absence of hormone whereas the majority of the GR (in the absence of hormone) is found in the cytoplasm (Picard and Yamamoto, 1987; Wikström et al., 1987; Husmann et al., 1990; Sar et al., 1990).

F. Hypothesis and Objectives

Our hypothesis is that PB is preferentially regulated by androgens (over glucocorticoids and progestins) and that the putative ARE(s) reside, at least in part, between -286 bp and -158bp of the 5'-flanking DNA of PB.

Our objectives are to first demonstrate preferential androgen regulation. Transfection experiments of PB-CAT chimaeric constructs containing PB 5'-flanking sequences (-426, -346, -307, -286, -244, -235, and -157 bp) cotransfected with rAR, rGR or cPR and treated with the appropriate steroid in PC-3 cells would be performed. PB-CAT induced expression by each steroid would be compared for each PB-CAT chimaeric construct.

Our second objective is to localize the putative ARE(s). This would be addressed by comparison of the level of androgen induced CAT expression of PB-CAT for all PB-CAT chimaeric constructs tested.

MATERIAL AND METHODS

A. Cell Culture

Both PC-3 and DU-145 cell lines were obtained from different sources, as outlined below. However, only the DU-145

cells from different sources appeared to have different characteristics and therefore were specified by a letter following the identity of the cell line, DU-145.

Human prostatic adenocarcinoma (PC-3) cells were obtained from P. Rennie (Cancer Control Agency, Vancouver) and American Type Culture Collection (ATCC, Rockville, Maryland). The cells were counted (Coulter counter, Coulter Electronics Inc., Hialeah, Florida) plated at an initial density of 1×10^6 /100 millimeter (mm) dish in Eagle's Minimum Essential Medium (MEM, Gibco, Grand Island, N.Y.) supplemented with 10 percent (%) Fetal Calf Serum (FCS) obtained from Hyclone (Logan, Mich.) or Gibco (Grand Island, N.Y.) and 5 milliliter (ml) Bonus (200 mM L-glutamine and 10×10^3 units Penicillin G; 1×10^5 microgram (μg)/ml streptomycin sulphate in 0.85% saline) per 500 ml bottle. After transfection, the PC-3 cells were supplemented with 5% twice (2X) charcoal stripped FCS (2X SFCS), MEM and Bonus.

Human prostatic adenocarcinoma (DU-145) cells were acquired from 3 different sources and appeared to have different characteristics thus they will be subsequently referred to as: DU-145-R, from P. Rennie (Cancer Control Agency, Vancouver); DU-145-D, from J. Dodd (Department of Physiology, University of Manitoba, Winnipeg); and DU-145-A, from ATCC (Rockville, Maryland). These cells were plated at an initial density of 2×10^6 /100 mm dish in MEM supplemented with 10% FCS and Bonus. Following transfection, the DU-145

cells were supplemented with 3% 2X SFCS, MEM and Bonus.

Human mammary carcinoma (ZR-75-1) cells were obtained from Leigh Murphy (Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg). The ZR-75-1 cells were plated at an initial density of 4×10^6 /100 mm dish in Dulbecco's Modified Eagle's Medium (DMEM-low glucose) supplemented with 7.5% FCS, 30% glucose (5 ml in 500 ml media) and Bonus. After transfection, the ZR-75-1 cells were supplemented with 3% 2X SFCS, DMEM and Bonus.

Human cervical carcinoma (HeLa) cells were obtained from ATCC (Rockville, Marland). The HeLa cells were plated at an initial density of 2×10^6 /100 mm dish Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and Bonus. After transfection, the HeLa cells were supplemented with 1% 2X SFCS, DMEM and Bonus.

B. Transfections

Transient transfections of PC-3, DU-145, ZR-75-1 and HeLa cells with plasmid DNA were performed using a modified version of calcium phosphate DNA precipitation method (Cattini *et al.*, 1986). Briefly, a solution containing 5 μ g of plasmid DNA per 100 mm dish, 2 Molar (M) calcium chloride and double distilled water (ddH₂O) was bubbled with air into 1 ml 28 millimolar (mM) sodium chloride (NaCl); 50 mM HEPES free acid; and 1.5 mM Na₂HPO₄ (HEBS) pH 7.1 for 1 minute. This mixture was left for 30 minutes, at room temperature, in order to allow time for

the plasmid DNA to form calcium precipitates. The media from the cells to be transfected was removed and replaced with fresh media. The precipitated plasmid DNA was added drop by drop (450 microliter (μ l) in total) to the dish of cells. The cells were then incubated for 6 hours at 37°C. The media was then removed and the cells were treated with 2 ml of 20% glycerol in either MEM or DMEM for 2 minutes. The cells were rinsed twice with 5 ml Phosphate Buffered Saline (PBS), pH 7.1. Media containing 2X SFCS (concentrations indicated) and Bonus was added to each transfected plate. The appropriate steroid (1×10^{-8} M) was then added to the transfected cells. After 24 hours, the cells were harvested in 1 mM ethylenediaminetetraacetic acid (EDTA)/PBS and subjected to centrifugation (Model HN-S, International Clinical Centrifuge, Needham Heights, Mass.) at 1000 rpm for 4 minutes at room temperature. Cell pellets were lysed with 0.1 M Tris-HCl/ 0.1% Triton X-100, pH 7.8, and left on ice for 15 minutes. Centrifugation was carried out at 14,000 rpm for 15 minutes at 4°C subsequent to lysing to separate the soluble protein containing fraction from the insoluble fraction.

C. CAT Assays

All CAT assays were performed using the two phase flour diffusion assay (Nactigal et al., 1989). After the soluble protein concentration of each plate of transfected cells was determined by colorimetric analysis using the BioRad Protein

Assay (Richmond, Ca.), 200 μ g of protein from each plate was diluted to a volume of 200 μ l with 0.1 M Tris-HCl, pH 7.8 and 0.1% Triton X-100 and placed in a scintillation vial. The vials were heated for 10 minutes at 65°C to inactivate any interfering acetylases. After cooling, 75 μ l of a solution composed of 0.5 M chloramphenicol (Sigma, St. Louis, Mo.), 0.1 M Tris-HCl pH 7.8, ³H-Acetyl Coenzyme A (Amersham Canada Lt., Oakville, Ont.) and ddH₂O was added to each vial. This solution was overlaid with 4 ml of Scintillene (Fisher, Fairlaom, N.J.) and left to incubate, in the dark, for 30 minutes at room temperature. The samples were then loaded into the Liquid Scintillation Counter (model 1216 Rackbeta, LKB Wallac, Helsinki, Finland) and cycled for at least 5 times of counting. Each cycle was timed and the CAT activity was calculated at each time point.

D. Transfection Efficiency

All transfection experiments were corrected for transfection efficiency by probing the DNA from transfected cells with radiolabelled CAT DNA insert. The DNA from each transfected plate was extracted from the insoluble fraction of harvested cells using the following method:

1. DNA Isolation

The insoluble fraction remaining after isolation of protein for CAT assay contained the DNA. It was resuspended in 420 μ l of 15 mM Tris-HCl pH 7.5, 15 mM NaCl and 3 mM magnesium

chloride (MgCl_2) (RSB). To this solution, 80 μl of 10 mM EDTA, 0.16 M NaCl and 2.2% sodium sarcosyl (SEN) was added. Twenty-five microlitres (25 μl) of pronase (10 mg/ml, Boehringer, Mannheim, Montreal, Quebec) was then added and the sample was incubated overnight at 37°C. The following day, the samples were incubated for 2 hours at 37°C with 10 μl RNase (10 mg/ml-DNase free, (Pharmacia, Quebec) and then with 25 μl pronase (10 mg/ml) at the same temperature overnight. The next day 1/20th volume 3 M sodium acetate (CH_3COONa), pH 5.2 was added to each sample and the DNA was isolated by phenol/iso-amyl chloroform (1:1:1) extraction performed twice, followed by a single extraction using iso-amyl alcohol: chloroform (24:1). The DNA was then precipitated by the addition of one volume isopropanol. The samples were centrifuged (Eppendorf Centrifuge model 5415, Brinkmann Instruments, Westbury, N.Y.) at 14,000 rpm for 5 minutes at room temperature, pellets washed with 70% ethanol, vacuum dried and then resuspended in 50 to 100 μl (depending on the size of the DNA sample) in 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA (TE). The DNA samples were left to rehydrate at 4°C for 2-3 days. A 1/100 dilution of each DNA sample was prepared (using TE) and then the absorbance read on the Gilford spectrophotometer at A_{260} . To determine the concentration of DNA, the value of the absorbance reading was multiplied by a factor of 40 and expressed as $\mu\text{g/ml}$.

2. Slot Blots

After determining the amount of DNA isolated per cultured plate of transfected cells, 5 μg of DNA was diluted to 140 μl with TE. The DNA was then denatured by the addition of 60 μl of 1 N sodium hydroxide (NaOH) and incubated at 37°C for 30 minutes. Subsequently, the samples were put immediately on ice to prevent renaturation of the DNA. An equal volume (200 μl) of cold 2M ammonium acetate ($\text{CH}_3\text{COONH}_4$) was added to each tube. The resulting solution was mixed and left on ice. The nitrocellulose (Bio-Rad TransBlot Transfer Medium, Richmond, Ca.) was rinsed with TE and assembled into the Minifold II slot blotter apparatus (Schleicher and Schuell, Keene, N.H.). Each well is then washed with 200 μl of 1 M $\text{CH}_3\text{COONH}_4$, loaded with 2 μg denatured DNA (160 μl) and washed again with 200 μl of 1M $\text{CH}_3\text{COONH}_4$. After the addition of each solution, the liquid is left to be suctioned through until the well is dry before the addition of the next liquid. The nitrocellulose is then removed from the assembly, rinsed in 6X 3 M sodium chloride, 0.3 M sodium citrate, SSC, (Mallinckrodt, Pointe Claire, Quebec) and blotted on 3M filter paper (Whattmann, Mainstone, England) to remove excess liquid. The blots are then baked at 80°C for 2.5 hours. As a control for non-specific hybridization, untransfected cells were subjected to the same protocol as described above.

3. CAT Probe

In order to determine whether equal amounts of

transfected DNA were actually taken up by the cells, the protein coding DNA region of the CAT gene was isolated, purified and used to probe the isolated DNA from the transfected cells. The CAT insert was obtained in the following manner: 35 μg of pTKC-VI (TkCAT) was digested overnight at 37°C with Eco RI. The following day the restriction cut plasmid was run on a 0.6% agarose gel for 1.5 hours. Afterward, the expected band of the CAT insert of 1.3 kilobases (kb) was localized on the gel. A well was cut in the gel just downfield of the 1.3 kb band, filled with 5 times (5X) 0.25 M Tris Base; 0.25 M boric acid and 0.05 M Na_2EDTA (TBE). The band was electroeluted at 200 volts until it migrated into the cut out well. The solution within the well was manually removed and placed in an Eppendorf tube. Two volumes of 100% ethanol and a final concentration of 0.3 M CH_3COONa were added and the CAT DNA was allowed to precipitate out of solution over 3 days. Subsequently, the DNA was centrifuged (JA 20 rotor, Beckmann, Model J2-21, Palo Alto, Ca.) at 14,000 rpm for 30 minutes at room temperature, pellets were washed with 70% ethanol, vacuum dried and resuspended in 50 μl TE. To estimate the concentration of CAT insert, a 1.5% agarose gel was run with 200 ng of ϕX -DNA as marker and 1 μl of isolated CAT insert. It was estimated from the intensity of the 1383 bp band (ϕX) that the concentration of CAT DNA was 102 ng/ μl , and the total amount extracted was 5 μg .

4. Nick Translation

In order to prepare the CAT DNA as a probe for the slot blots, nick translation was performed in a reaction mix of 150 ng of CAT DNA diluted with 6 μ l of ddH₂O, followed by the addition of 4 μ l cold 100 mM deoxynucleotide triphosphate (dNTP) solution, containing a 1:1:1 ratio of deoxycytosine (dCTP), deoxyguanine (dGTP), and deoxythymine (dTTP) nucleotide triphosphates, 7 μ l of ³²P- α -deoxyadenosine triphosphate (dATP) and 2 μ l of enzyme mixture (100 pg DNase and 5 units DNA polymerase I in a buffer solution, Amersham, Oakville, Ontario) and then incubated at 15°C for 1.5 hours. The reaction was stopped by adding 1.5 μ l 0.5 M EDTA and 4 μ l 10 mg/ml yeast transfer ribonucleic acid (tRNA) (Sigma, St. Louis, Mo.) and heating at 65°C for 15 minutes. After cooling, the solution was applied to a commercially prepared Nick column (Sephadex G-50, DNA grade, Pharmacia, Quebec) and eluted in 400 μ l 1 mM Tris-HCl, pH 7.5, and 0.5 M EDTA. To determine the specific activity of the CAT probe, 2 μ l of the nick translation purified reaction was added to 4 ml of Aquasol (Dupont, Boston, Mass.) and counted on the liquid scintillation counter.

5. Prehybridization and Hybridization

The dried blots were rehydrated with water and placed into Micro-Seal (#6011, Dazey Ind., Industrial Airport, Kansas) bags (2 blots per bag). The prehybridization solution [50%

formamide, 25% 20x SSC, 5% 100x Denhardt's, 5% 1 M Na_2PO_4 , 0.1% sodium dodecyl sulphate (SDS) and ddH_2O] was heated to 42°C. Each bag containing 2 blots received 10 ml of prehybridization solution. The bags were sealed and placed in a 42°C shaking water bath overnight.

The CAT probe was boiled in water for 5 minutes with 1 mg/ml of both yeast tRNA and salmon sperm DNA and then placed on ice. A volume of CAT probe containing a specific activity of 10×10^6 cpm was added to each bag. The bags were sealed and placed in a shaking water bath at 42°C overnight.

6. Washing

The following day the hybridization solution was carefully removed and the blots were rinsed with 2x SSC/0.1% sodium dodecylsulphate (SDS) at room temperature, for 10 minutes, then at 65°C for 30 minutes. The blots were removed from this solution and placed in 0.2x SSC/0.1% SDS, at 65°C for 20 minutes. After a final wash in 0.2x SSC/0.1 %SDS at 65°C for 15 minutes, the blots were then placed in clean Decosonic Polythrene plastic (St. Laurent, Qué.), sealed (Decosonic Vacuum Sealer #828, St. Laurent, Qué.) and put on Kodak X-OMat RP, XRP-5 film (Sigma, St. Louis, Mo.). Following the appropriate amount of exposure time, the films were developed and the exposed slots analyzed by densitometry.

7. Densitometry

Each blot was scanned using the Transmittance Reflectance Scanning Densitometer (Model 1650, Bio Rad Labs, Richmond, Ca.). The highest peak per blot was given the value of 100 and each of the other peaks (on that blot) were expressed as a percentage of the highest peak. 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 | D | E |
|--------|-------|--------|-------|--------|-------------------|
| 1 0 | 11.3 | 79.3 | 84.2 | 94.2 | |
| 2 \$ | 13.6 | 95.4 | 82.7 | 115.4 | 103.6 ±10.8 |
| 3 x | 13.9 | 97.2 | 96.1 | 101.2 | |
| 4 ▲ | 234.9 | 1644.4 | 74.3 | 2213.2 | |
| 5 * | 218.9 | 1532.6 | 100.0 | 1532.6 | 2004.4 ± 410.0 |
| 6 z | 297.7 | 2083.9 | 91.9 | 2267.6 | |

A: SLOPE OF THE LINE (cpm/minute)

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. Graph I and II represent the average slope of the line

(cpm/min) of triplicate transfections of the same construct (-286/+28 PB CAT) cotransfected with the rat AR expression vector and treated with (graph II, samples 4-6) and without (graph I, samples 1-3) DHT.

| Sample # | Slope of the Line (cpm/min) |
|----------|-----------------------------|
| 1 | 11.3 |
| 2 | 13.6 |
| 3 | 13.9 |
| 4 | 234.9 |
| 5 | 218.9 |
| 6 | 297.7 |

B: CAT Activity (dpm/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) and divided by the amount of protein assayed. For the example given above, 200 μ g of protein from one transfected plate of cells was assayed. Therefore, the formula becomes:

$$\frac{(1.4)(\text{slope of the line [cpm/min] determined in \{A\}) (1000\mu\text{g/mg})}{200 \mu\text{g protein}}$$

| Sample # | CAT Activity (dpm/min/mg protein) |
|----------|-----------------------------------|
| 1 | 79.3 |
| 2 | 95.4 |
| 3 | 97.2 |

| | |
|---|--------|
| | 50 |
| 4 | 1644.4 |
| 5 | 1532.6 |
| 6 | 2083.9 |

C: PERCENTAGE INCORPORATION of CAT DNA

As indicated above, the DNA from transfected and harvested cells was isolated, quantified, slotted onto nitrocellulose, probed with labelled CAT DNA insert and exposed to film. Each probed blot was scanned with a densitometer and the highest peak (on that blot) was given a value of 100%. Each of the other peaks were expressed as a percentage of the highest peak. Specifically for the example given above:

| Sample # | Percentage of CAT DNA Incorporated/Blot |
|----------|-----------------------------------------|
| 1 | 84.2% |
| 2 | 82.7% |
| 3 | 96.1% |
| 4 | 74.3% |
| 5 | 100.0% |
| 6 | 91.9% |

D: CORRECTED CAT ACTIVITY (dpm/min/mg protein)

To correct for transfection efficiency, the CAT activity calculated in {B} (dpm/min/mg protein) for one sample was divided by the percentage of CAT DNA incorporated into that same sample calculated in {C}:

CAT Activity (dpm/min/mg protein)

CAT DNA incorporated (%)

| Sample # | Corrected CAT Activity (dpm/min/mg protein) |
|----------|---------------------------------------------|
| 1 | 94.2 |
| 2 | 115.4 |
| 3 | 101.2 |
| 4 | 2213.2 |
| 5 | 1532.6 |
| 6 | 2267.6 |

E: CORRECTED AVERAGE SLOPE of the LINE (dpm/min/mg protein) ± SEM

The final calculation was to determine the average CAT activity of triplicate determinations of the same transfection and the standard error of the mean. For the example above

| Sample # | CAT Activity (dpm/min/mg protein ± SEM) |
|------------|-----------------------------------------|
| 1-3 (-DHT) | 103.6 ± 10.8 |
| 4-6 (+DHT) | 2004.4 ± 410.0 |

E. Plasmid Preparation**1. Bacterial Cultures**

Bacterial cultures containing 5 ml of 10 g Tryptone, 5 g NaCl, 5 g Yeast extract and ddH₂O to 1 litre (L-Broth) and Ampicillin (320 µg/ml, Sigma, St. Louis, Mo.) were inoculated

with the glycerol stock of the appropriate plasmid and placed overnight in a shaking incubator at 37°C. The following day 400 ml cultures of L-Broth containing Ampicillin were inoculated with 400 μ l of overnight culture and put into the shaking incubator at 37°C overnight. The inoculated flasks are poured into Beckmann 500 ml plastic bottles and centrifuged at 4000 rpm (Beckmann, model J2-21, JA 10 rotor, Paulo Alto, Ca.) at 4°C for 10 minutes.

Plasmid preparations were performed using either cesium chloride (CsCl_2) gradients or commercially prepared Qiagen Plasmid Maxi Preparation kits (Diagen Inc., Chatsworth, Ca.).

I. Cesium Chloride Gradient

The supernatant is discarded, cell pellets were lysed in a freshly pre-made solution of 25 mg lysozyme (Sigma, St. Louis, Mo.) and 10 ml TE/400 ml inoculated culture, divided equally into 2 Oakridge tubes and left for 5 minutes at room temperature. The solution was treated with the addition of freshly made 0.2 M sodium hydroxide (NaOH)/1% SDS (10 ml per Oakridge tube). The tubes were inverted sharply and placed on ice for 10 minutes. Subsequently, 7.5 ml of 0.3 M potassium acetate (CH_3COOK)/0.115 M acetic acid (CH_3COOH) was added to each tube, again inverting sharply and placing them on ice for 10 minutes. The tubes are then centrifuged at 15,000 rpm for 20 minutes at 4°C. The cultures which had been divided into 2 Oakridge tubes are now combined: the supernatants from both

Oakridge tubes are poured into 150 ml Corex bottle containing 27 ml isopropanol and left at room temperature, for 30 minutes, in order to precipitate the DNA out of solution. The precipitated DNA is pelleted by centrifugation at 5000 rpm at room temperature for 30 minutes. The resulting pellets are rinsed with 70% ethanol, vacuum dried, and resuspended in 10 ml/400 ml culture. To each tube, 9.5 g CsCl₂ was added and the tubes were then incubated at 37°C for 5 minutes to allow the CsCl₂ to dissolve. After dissolving, 0.9 ml of 5 mg/ml ethidium bromide (EtBr) was added to each tube, the tubes were inverted a few times and covered with aluminum foil to prevent nicking of the DNA in the presence of light, and left for 5 minutes at room temperature. The resulting sedimentation was removed by centrifugation (model HN-S, International Clinical Centrifuge, Needham Heights, Mass.) at 2000 rpm for 15 minutes. With a Pasteur pipette, the clear supernatant was drawn up and placed in Beckmann Ti-75 Quick Seal tubes. To ensure the tubes did not collapse while centrifuging, mineral oil was applied to the top of the solution. The tubes were then balanced to ± 0.05 g, sealed and placed in Beckmann ultracentrifuge (model 28-70M), at room temperature for 18 hours at 55,000 rpm and subsequently for 1 hour at 45,000 rpm. The following day the tubes are carefully removed from the centrifuge and the DNA bands were localized with hand held ultra violet light (UV Products Ltd., St. Gabriel, Ca.). To eliminate the vacuum created in the tubes, a 16 gauge needle

(without syringe) was inserted into the top of the Quick Seal tubes. To extract the bands, a 21 gauge needle (with syringe attached) was inserted into the tube just below the plasmid band, bevel side up. The plasmid DNA band is slowly collected in the 21 gauge syringe (approximately 1.5 ml) and the collected sample is placed in a 15 ml polypropylene tube containing 3 ml of water. An equal volume of ddH₂O-saturated butanol was added and the tubes were handheld on a Vortex for several minutes, then left to allow liquid phases to clear. The top butanol layer is carefully removed and discarded. This procedure was repeated 4-5 times until all the visible EtBr (pink color) was in the butanol layer and not in the aqueous layer. In order to precipitate the DNA out of solution, 2.5 volumes of ethanol and 1/10th volume of 3 M CH₃COONa was added to each tube, inverted several times and left at -20°C overnight. The next day, the tubes were centrifuged at 9.5 K (Beckmann, model J2-21, JS-13 rotor) for 30 minutes at 4°C. The supernatants were aspirated off and the pellets were washed with 70% ethanol, vacuum dried and resuspended in 500 μl of ddH₂O and transferred to Eppendorf tubes. To eliminate RNA and protein contamination, the resuspended DNA solution was treated first with RNase, DNase free (10mg/ml, Sigma, St. Louis, Mo.) at 37°C for 2 hours and then with Proteinase K (1-5 mg/ml) at 65°C for 1 hour. The plasmid DNA was then twice extracted to remove the remaining protein by adding an equal volume phenol/isoamyl/chloroform (1:1:1) and once with 2x

volume isoamyl/chloroform (1:1), and the DNA was precipitated out of solution with the addition of 2x volume of 100% ethanol and 1/10th volume of 3 M CH_3COONa left at -20°C overnight. The following day, the tubes were centrifuged (JS-13, Beckmann, Paulo Alto, Ca.) at 10,000 rpm for 15 minutes at 4°C . The supernatant was aspirated off and the pellets were washed with 70% ethanol, vacuum dried and resuspended in 200-400 μl TE. The absorbance at A_{260} was read to determine DNA concentration.

II. Qiagen Plasmid Maxi Preparation

After centrifugation of the 400 ml inoculated culture at 4000 rpm (Beckmann, model J2-21, JA 10 rotor) at 4°C for 10 minutes, the supernatant was discarded and each cell pellet was resuspended, on ice, in 10 ml of buffer P1 containing 100 $\mu\text{g}/\text{ml}$ RNase in 50 mM Tris/HCl, pH 8.0 and 10 mM EDTA. Each tube was mixed using a hand held vortex prior to the addition of the following solution. Degradation of the RNA in the bacterial cells was achieved by the addition of 10 ml of 200 mM NaOH and 1% SDS (Buffer P2) per tube. The resulting solution was then mixed gently and incubated on ice for 5 minutes. Subsequently, 10 ml of 2.55 M CH_3COOK , pH 8.0, (Buffer P3) was applied to each tube, followed by immediate but gentle mixing. The tubes were then centrifuged at 15,000 rpm for 30 minutes at 4°C (JA-21, Beckmann, Paulo Alto, Ca.). The supernatants were carefully removed and placed in 50 ml polystyrene tubes, ensuring that no particulate matter was

resuspended in the lysate. For each 400 ml bacterial culture, one Qiagen-tip 500 column was equilibrated with 10 ml of 750 mM NaCl, 50 mM 3-[N-Morpholino] propane-sulfonic acid (MOPS, Sigma, ST. Louis, Mo.), 15% ethanol and 0.15% Triton X-100, pH 7.0 (Buffer QBT). After this solution had entirely drained from the column, the supernatant was applied and allowed to completely enter the resin bed by gravity flow. The column was then washed four times with 10 ml of 1.0 M NaCl, 50 mM MOPS and 15% ethanol, pH 7.0 (Buffer QC). The DNA was eluted with the addition of 15 ml of 1.25 M NaCl, 50 mM MOPS and 15% ethanol, pH 8.2 (Buffer QF), precipitated with 0.7 volumes (10.5 ml) of isopropanol at room temperature and subsequently centrifuged at 9,000 rpm (Beckmann, J2-21 model, JS 13 rotor, Paulo Alto, Ca.) at 4 °C for 30 minutes. The DNA pellets were then washed with 70% ethanol, air dried for 5 minutes, redissolved in 200 to 400 μ l of TE, depending on the size of the pellet. The absorbance at A_{260} was read to determine the DNA concentration.

2. Restriction Endonuclease Analysis

In order to confirm the identity and the relative purity of the plasmid DNA prepared, 100 ng of each plasmid preparation was digested for at least 2 hours at 37°C with 1 μ l (Pharmacia, Quebec) of the appropriate restriction endonuclease, 1 μ l of 10x corresponding buffer (Low salt (L): 100 mM Tris/HCl, pH 7.5, 100 mM MgCl₂ and 10 mM DTT (Sigma,

St. Louis, Mo.); Medium salt (M): 100 mM Tris/HCl, pH 7.5, 100 mM MgCl₂, 10 mM DTT and 500 mM NaCl; or High salt (H): 500 mM Tris/HCl, pH 7.5, 100 mM MgCl₂, 10 mM DTT and 1.0 M NaCl) and 7 μ l ddH₂O. The following day, 2 μ l of gel loading buffer (50% glycerol, 100 mM EDTA, 0.15% xylene cyanol and 0.15% bromophenol blue) was added to each digestion. A BioRad Mini Sub DNA Cell containing a 1.0% agarose gel with 1 μ l of 5 mg/ml EtBr was then filled with 1x TBE. The resulting solutions 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 1 μ l of commercially prepared DNA marker (λ /Hind I, ϕ X or Bst E; Pharmacia, Quebec). Electrophoresis was performed at 70 volts and 2 mamps (Bio-Rad, model 200/2.0 Power Supply, Richmond, Ca.) for approximately 1.5 hours, until the DNA fragments had migrated sufficiently as visualized with a hand held ultraviolet light (UV Products Ltd., St. Gabriel, Ca.). The gel was removed from the DNA cell apparatus (gel box). A photograph was taken of the gel using the DNA Transilluminator (Fotodyne In., New Berlin, Wisc.). This procedure was followed for each of the plasmids prepared. Specifically, digestion of the expression vectors for the steroid receptors yielded the following results: rAR was digested with Eco RI giving 3 bands of 3.3, 2.5 and 0.5 kb; rGR was digested with Eco RI yielding 2 bands of 3.1 and 2.5 kb; and cPR (chicken progesterone receptor B) was digested with Sac I resulting in 4 bands of

3.7, 2.44, 2.4 and 1.6 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 -165 bp and -244 bp to -96 bp in front of Tk gene promoter, were double digested with Hind III/Xba I and Hind III/Acc I respectively, and resulted in bands of 3200 and 79 bp for -244/-165 TK CAT and 3200 and 148 bp for -244/-96 Tk CAT. Mouse Mammary Tumour Virus-Long Terminal Repeat (MMTV-CAT, Ham *et al.*, 1988) and Tyrosine Amino Transferase (TAT-CAT, Denison *et al.*, 1989) were digested with Pvu II and Eco RI, respectively, generating bands of 800, 1800 and 2300 bp for MMTV-CAT and 2.8 and 0.9 kb for TAT-CAT.

RESULTS

A. Localization of the Androgen Responsive Element(s)

A series of deletions of PB 5'-flanking DNA sequence coupled to CAT (PB-CAT) were constructed to localize the hormone response element(s). Figure 1 shows the PB 5'-flanking DNA sequence commencing at nucleotide -426. The stars above the nucleotides indicate the 5' end of that particular chimaeric PB-CAT construct: -426, -346, -306, -286, -244, -235 and -158 PB-CAT. Transient transfection of these constructs and co-transfection with the expression vector for the rat androgen receptor (rAR) in PC-3 cells treated with

dihydrotestosterone (DHT) resulted in an increase in androgen induced CAT activity with deletion of -426/+28 to a maximum at -286/+28 (figure 2, panel A). Decreasing activity was seen with further 5'-end deletions of PB, -244/+28 and -235/+28, until -158/+28 at which point DHT induced CAT activity was minimal. A similar profile was observed when the same constructs were co-transfected with the expression vector for the rat glucocorticoid receptor (rGR) and treated with Dexamethasone (DEX) in PC-3 cells (solid diamonds, panel A, figure 2). The net CAT activity induced by DEX was approximately a tenth and the fold change a fifth when compared to that with DHT (Figure 3). This indicated three points: (1) cis-element(s) in the first 140 bp of 5'-flanking PB (-426 to -286) are not required for DHT and DEX regulation; (2) the loss of DHT and DEX regulation following deletion to nucleotide -158 implied that an androgen responsive element (ARE) resided, at least in part, between nucleotides -286 and -158 of the PB gene; (3) these sequences are preferentially regulated by DHT.

Concurrently, a collaboration with P. Rennie's laboratory in Vancouver, B.C., produced some vital information. The DNase I footprinting with the DNA binding and steroid binding domains of the rAR synthetically produced in bacteria and the PB 5'-flanking DNA generated two footprints: one between positions -241 and -223 (ARE-1); and the other between -140 and -117 (ARE-2). This data demonstrated that the rAR bound to

two sequences within the PB 5'-flanking (Rennie *et al.*, 1993). With this knowledge, a chimaeric construct containing PB 5'-flanking sequences from -244 to -165 adjacent to heterologous TK promoter linked to CAT (-244/-165 TK-CAT) was created to determine whether ARE-1 sequence was sufficient to confer hormonal regulation via a heterologous promoter. Figure 4 indicates, this was not the case: transient transfections of -244/-165 PB-TK-CAT and co-transfection with either rAR or rGR and treatment with DHT or DEX, respectively, showed minimal steroid induced CAT activity. Thus, even though the -244/-165 PB-TK-CAT construct contained the first ARE (ARE-1) binding site for the synthetic AR, these sequences were NOT sufficient to confer hormonal regulation via the heterologous TK promoter.

Another chimaeric CAT construct was generated containing both ARE-1 (-241 to -223) and ARE-2 (-140 to -117) in the PB 5'-flanking DNA fragment, -244 to -96 adjacent to the heterologous promoter TK (-244/-96 PB-TK-CAT). Transfection of this construct into PC-3 cells co-transfection with rAR or rGR and treatment with DHT or DEX, respectively, showed almost equal induction of CAT activity by each steroid (1332 dpm/min/mg protein \pm 284 and 1486 dpm/min/mg protein \pm 185, figure 5).

To ensure that we were working at the correct concentration of steroid, dose response curves for both androgens and glucocorticoids were generated. Transient

transfections were performed in PC-3 cells using the -244/+28 PB-CAT construct, co-transfecting with pAR or pGR and treating with DHT or DEX, respectively, at concentrations ranging from 0.01 nM to 10 nM (figure 6). This experiment was then repeated using the -244/-96 PB-TK-CAT construct and concentrations of steroid ranging from 0.1 nM to 10 nM (figure 7). As the data indicates, the greatest androgen and glucocorticoid induced CAT activity of both constructs was achieved using a concentration of 10 nM per transfected plate of cells. A composite of these results is given in figure 8. Thus, comparing the induction by AR and GR of -244/+28 PB-CAT and -244/-96 PB-TK-CAT the following conclusions were formed: (1) both AREs are necessary and sufficient for regulation by AR and GR of -244/+28 PB-CAT and -244/-96 PB-TK-CAT; (2) preferential induction by AR was lost when PB DNA sequences between -96 and +28 (containing the PB promoter) are removed and replaced with the heterologous TK promoter.

B. Comparison of Activities of HREs in PB-, MMTV-, and TAT-CAT.

After having shown that the HREs in the 5'-flanking region of PB gene were preferentially induced by androgens and then by glucocorticoids, the next question asked was how did HREs in PB compare with other known androgen responsive elements found in MMTV and TAT? Therefore, the activities of the HREs in -286/+28 PB-CAT, MMTV-CAT and TAT-CAT were

compared:

(1) Androgen Induced CAT Activity

The -286 PB-CAT, MMTV-CAT and TAT-CAT constructs were transiently transfected into PC-3 cells, co-transfected with rAR and treated with and without DHT. Figure 9 demonstrates graphically that the fold change in androgen induced CAT activity was greatest for PB-CAT (74 fold), smaller for MMTV-CAT (36 fold) and least for TAT-CAT (8 fold).

(2) Glucocorticoid Induced CAT Activity

When transient transfections in PC-3 cells of the same three chimaeric CAT constructs (co-transfected with rGR) were compared for DEX induced CAT activity (figure 10), the fold change in CAT activity was greatest for MMTV-CAT (57 fold) followed by TAT-CAT (36 fold) and the smallest for PB-CAT (29 fold).

(3) Progesterone Induced CAT Activity

Finally, to compare PR induced CAT activity, -286 PB-CAT, MMTV-CAT and TAT-CAT were transiently transfected into PC-3 cells, cotransfected with the expression vector for the chicken progesterone receptor (cPR) and treated with progesterone (figure 11). From this data, maximum progesterone induced fold change in CAT activity (figure 12) was observed with MMTV-CAT (5 fold), then PB-CAT (4 fold) and minimally with TAT-CAT (2 fold). To confirm this data, a second cell line which could have different cell specific factors needed for PR activity was tested. HeLa cells were chosen for two

reasons: (1) optimal conditions for transient transfections had originally been worked out in HeLa cells using MMTV-CAT as a standard; (2) all 5'-flanking PB-CAT deletion constructs had been previously shown to be regulated by both GR and AR in HeLa cells.

C. Different Concentrations of Progesterone Receptor Transfected into HeLa and PC-3 Cells

In order to address the question of whether 5 μ g of chicken progesterone receptor expression vector (cPR) transfected per plate of cells was appropriate, different concentrations of cPR expression vector DNA were cotransfected into HeLa cells using MMTV-CAT as standard. Transfection of MMTV-CAT, co-transfection of 0, 2 or 5 μ g of pPR and treatment of 10 nM progesterone in HeLa cells yielded the results summarized in figure 13. Maximum progesterone induced CAT activity was greatest when only 2 μ g of cPR was co-transfected (8030 dpm/min/mg protein \pm 686). In comparison, when 5 μ g cPR/plate of cells was cotransfected, progesterone induced CAT activity was remarkably small (163 dpm/min/mg protein \pm 22). Therefore, we chose to perform a subsequent dose response curve of cPR in PC-3 cells again utilizing MMTV-CAT as the standard. Figure 14 represents the progesterone induced CAT activity of MMTV-CAT when transfected into PC-3 cells, cotransfected with 250, 500, 1000 or 2000 ng of cPR and treated with progesterone. Clearly, maximum CAT activity

(1837.8 ± 68.1 dpm/min/mg protein) and fold change in CAT activity (3 fold) was seen when 2000 ng of cPR was co-transfected into PC-3 cells. Nevertheless, when -286/+28 PB-CAT was transfected under the same conditions (figure 15) even though a 3.2 fold change in CAT activity was observed the maximum progesterone induced CAT activity was again, notably small (336.9 ± 69.8 dpm/min/mg protein). To ensure that the -286/+28 PB-CAT construct was functioning, a positive control was included in this transfection: PC-3 cells were transfected with -286/+28 PB-CAT, cotransfected with rAR expression vector and treated with DHT. The data indicated that the -286/+28 PB-CAT was induced by AR in the presence of DHT (figure 15).

We had observed a preferential androgen > glucocorticoid induction of -286/+28 PB-CAT and -244/+28 PB-CAT, but an **equal** androgen and glucocorticoid induction of -244/-96 PB-TK-CAT (in PC-3 cells). This information led to the question of whether removing the endogenous PB promoter and replacing it with the heterologous TK promoter (-244/-96 PB-TK-CAT) might render the construct progesterone inducible. Thus, PC-3 cells were transfected with -244/-96 PB-TK-CAT, cotransfected with the expression vector for rAR, rGR or cPR and treated with DHT, DEX or PG, respectively. The greatest CAT induced activity was observed with GR (57 fold), then with AR (45 fold) and the least induction with PR (5 fold, figure 16; table I). Regardless, the fold change in CAT activity by cPR plus steroid was greater for -244/-96 PB-TK-CAT (5 fold) than

for -286/+28 PB-CAT (3.2 fold).

D. Progesterone Induced Response of -286/+28 PB-CAT, MMTV-CAT and TAT-CAT in ZR-75-1 Cells.

Concerned that the chicken progesterone receptor expression vector (cPR) was, in some manner, incompatible with both PC-3 and HeLa cell lines, we chose a cell line that possessed endogenous PR, the ZR-75-1 cell line. Since this cell line has endogenous AR, GR and PR, no co-transfection with the expression vectors was necessary. Transient transfections of -286/+28 PB-CAT, MMTV-CAT and TAT-CAT into ZR-75-1 cells and treatment with progesterone gave the results summarized in figure 17: -286/+28 PB-CAT exhibited minimal progesterone induced response; MMTV-CAT showed a 24.4 fold change in CAT activity and progesterone induction of TAT-CAT gave a 1.5 fold change in CAT activity. Nonetheless, the standard error of the mean (SEM) of both MMTV-CAT and TAT-CAT after progesterone application, was remarkably high (SEM= \pm 5761 dpm/min/mg protein for MMTV-CAT and SEM= \pm 2700 dpm/min/mg protein for TAT-CAT). In addition, the uninduced (basal) CAT activity of TAT-CAT was large (10,547 \pm 1550 dpm/min/mg protein). This result is reflected in the small fold change in CAT activity of TAT-CAT even though the maximum progesterone induced CAT activity was the very large (15,556.9 \pm 2700 dpm/ min/mg protein).

E. Prostatic Cell Specificity

When PC-3 cells were transfected with -244/-96 PB-TK-CAT, the data showed an **equal** induction in CAT activity by rAR and rGR when steroid was added (Figure 8). However, when -286/+28 PB-CAT or -244/+28 PB-CAT was transfected into PC-3 cells, androgen induction was **greater** than glucocorticoid (Figure 8). One of the questions asked was if this induction profile by androgen, glucocorticoid and progesterone of PB-CAT was true in other prostatic cells lines? There are two other recognized prostatic cell lines, the LNCaP and the DU-145. Recent data had shown that the endogenous AR in the LNCaP cell line was mutated (Veldscholte, 1992). Thus, the DU-145 cell line was chosen. As stated in Materials and Methods, the DU-145 cells came from three different sources. Initial experiments were performed in the DU-145 cells obtained from J. Dodd (University of Manitoba): the DU-145-D cells were transfected with -244/-96 PB-TK-CAT alone, and with the expression vector for rAR and treated with and without DHT (figure 18). As the data indicated, even without cotransfecting with the rAR expression vector, but treating the cells with DHT, there was a small increase in CAT activity (1.5 fold). When the expression vector for rAR was cotransfected and the cells treated with DHT, the CAT activity increased 4.6 fold above DU-145-D cells treated with DHT but not cotransfected with rAR expression vector, and 7.6 fold above the DU-145-D cells simply transfected with -244/-96 PB-TK-CAT. Hence, the -244/-

96 PB-TK-CAT construct was induced by androgens in DU-145-D cells. However, there were concerns that the small change might reflect changes that occur due to passage of a cell line. Therefore, DU-145 cells were obtained from another source, P. Rennie's (B.C, Cancer Agency, Vancouver) lab (DU-145-R) and later from American Type Culture Collection (DU-145-A).

Initial experiments in the DU-145-R cell line demonstrated a preferential GR over AR induction when the appropriate expression vector was cotransfected with -244/-96 PB-TK-CAT and treated with the corresponding steroid (Table II, column A, lines 2 and 4). However, when the PB-CAT construct containing the endogenous promoter (-244/+28 PB-CAT) was transfected into DU-145-R cells, cotransfected with either the expression vector for the rAR or rGR and treated with DHT or DEX, respectively, the DHT induced CAT activity was greater than the CAT activity observed with DEX (Table II, column A, lines 1 and 3). In summary, the data shows (1) the net DEX induced CAT activity of -244/-96 PB-TK-CAT was not equal, but **greater** than DHT in DU-145-R cells; (2) DHT induced CAT activity of -244/+28 PB-CAT was greater than DEX in DU-145-R cells; (3) the DHT and DEX induced CAT activity was always greater using the construct with the heterologous TK promoter (-244/-96 PB-TK-CAT). This last point was also confirmed for the DU-145-A cell line (figure 19). A comparison of the DHT induced fold change in CAT activity of -244/-96 PB-TK-CAT in

all three DU-145 sublines is given in figure 20. The greatest response was seen in DU-145-D cells (7.4 fold), followed by DU-145-R cells (3.8 fold) and the least responsive were the DU-145-A cells (1.3 fold). As the data indicated, there was a difference in the degree of DHT induced fold change in CAT activity of the different DU-145 sublines even though they were supposedly all the same type of cell line. The following questions arose from this information: (1) was there a real difference in the response of these DU-145 cells which came from different sources? (2) were there discrepancies in CAT induced activity observed between DU-145-R and DU-145-A cells when other CAT constructs (such as -286/+28 PB-CAT and MMTV-CAT) were used? Therefore, transfections of -286/+28 PB-CAT, cotransfection of the expression vector for either the rAR or rGR and treatment with DHT or DEX, respectively, in both DU-145-R and DU-145-A cells were performed (figure 21). As the data indicate, both DU-145-R and DU-145-A cells were preferentially DEX induced whether expressed as CAT activity (dpm/min/mg protein \pm SEM) or fold change in CAT activity (figure 22). Furthermore, the CAT induced activity seen in DU-145-R cells was greater than the activity seen with DU-145-A cells (figure 21).

When MMTV-CAT was transfected into DU-145-R cells, cotransfected with the expression vector for either the rAR or rGR, and treated with DHT or DEX, respectively, a similar result was obtained. The MMTV-CAT was preferentially induced

by DEX (figure 23). The MMTV-CAT activity of the DU-145-R cells transfected with the expression vector for rAR but not treated with DHT was high (3420 ± 293 dpm/min/mg protein) but the MMTV-CAT activity of DHT treated cells was still lower than those cells cotransfected with rGR and treated with DEX.

When the same transfection was performed in DU-145-A cells, a similar response was seen (figure 24) with one exception. The basal CAT activity of DU-145-A cells transfected with MMTV-CAT, cotransfected with rAR but not treated with DHT was lower than the basal CAT activity of those cells cotransfected with the rGR expression vector. Nonetheless, MMTV-CAT was preferentially induced by DEX. Again, the data indicated that the overall steroid induced CAT activity in the DU-145-A cells was markedly lower than the CAT activity observed with DU-145-R cells (figure 21, 23 and 24). Regardless of which subline of DU-145 cells were used (either DU-145-R or DU-145-A), or which form the results were expressed as (dpm/min/mg protein \pm SEM or fold change in CAT activity), MMTV-CAT was preferentially induced by DEX over DHT (figures 23, 24 and 25). Further, when the CAT activity was represented as fold change in CAT activity (figure 25), the greatest fold change in CAT activity of MMTV-CAT induced by DEX was seen when this transfection was performed in DU-145-A cells (48 fold), even though the overall CAT activity ($12,974$ dpm/min/mg protein \pm 1170, figure 24) was much lower than the CAT activity observed when DU-145-R cells transfected with

MMTV-CAT, cotransfected with rGR and treated with DEX (53,273 dpm/min/mg protein \pm 6213, figure 23).

Figure 1: Sequence of Probasin 5'-Flanking DNA. Genomic clones were isolated from a rat genomic library, subcloned into pUC119, and sequenced. The start of transcription is shown with the number starting immediately after as +1. All negative numbering is relative to the start site of transcription. The ARE-1 and ARE-2 sequences are boxed and the CAAT box, and TATAA box are underlined.

-426

*

AAGCTTCCACAAGTGCATTTAGCCTCTCCAGTATTGCTGATGAATCCACAGTTCAGGTTC

-346

*

-307

*

AATGGCGTTCAAACTTGATCAAAAATGACCAGACTTTATATTCTTACACCAACATCTAT

-286

*

-251

*

CTGATTGGAGGAATGGATAATAGTCATCATGTTTAAACATCTACCATTCCAGTTAAGAAA

-244

*

-235

*

ATATGATAGCATCTTGTCTTAGTCTTTTTCTTAATAGGGACATAAAGCCCACAAATAAA

-158

*

AATATGCCTGAAGAATGGGACAGGCATTGGGCATTGTCCATGCCTAGTAAAGTACTCCAA

-101

*

-95

*

-82

*

-76

*

GAACCTATTTGTATACTAGATGACACAATGTCAATGTCTGTGTACAACCTGCCAACTGGGA

-48

*

-27

*

TGCAAGACACTGCCCATGCCAATCATCCTGAAAAGCAGCTATAAAAAGCAGGAAGCTACT

+1

*

+28

*

CTGCACCTTGTCAGTGAGGTCCAGATACCTACAGAGCTCACACACG ATG AGG GTC
Met Arg ValATC 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 SerATG ATG ACA GAC AAG AAT CTC AAA AAG AAG CTAGCAGAC
Met Met Thr Asp Lys Asn Leu Lys Lys Lys

Figure 2: The 5'-Deletion Mapping of HRE in 5'-Flanking PB DNA. Deletions of 5'-flanking sequences (-426, -346, -307, -286, -244, -235, and -158 bp) in PB-CAT were co-transfected in PC-3 cells with rAR or rGR expression vectors and treated with DHT [●] or DEX [▲] respectively, in the presence of 5% STR-FCS (panel A). The activity of CAT with rAR [○] or rGR [△] expression vector without the addition of hormone, served as a baseline (panel B). All CAT assays were performed by scintillant two phase fluor diffusion method and activity was expressed as the mean (dpm/min/mg protein ± Standard Error of the Mean) of at least triplicate determinations after being normalized for transfection efficiency.

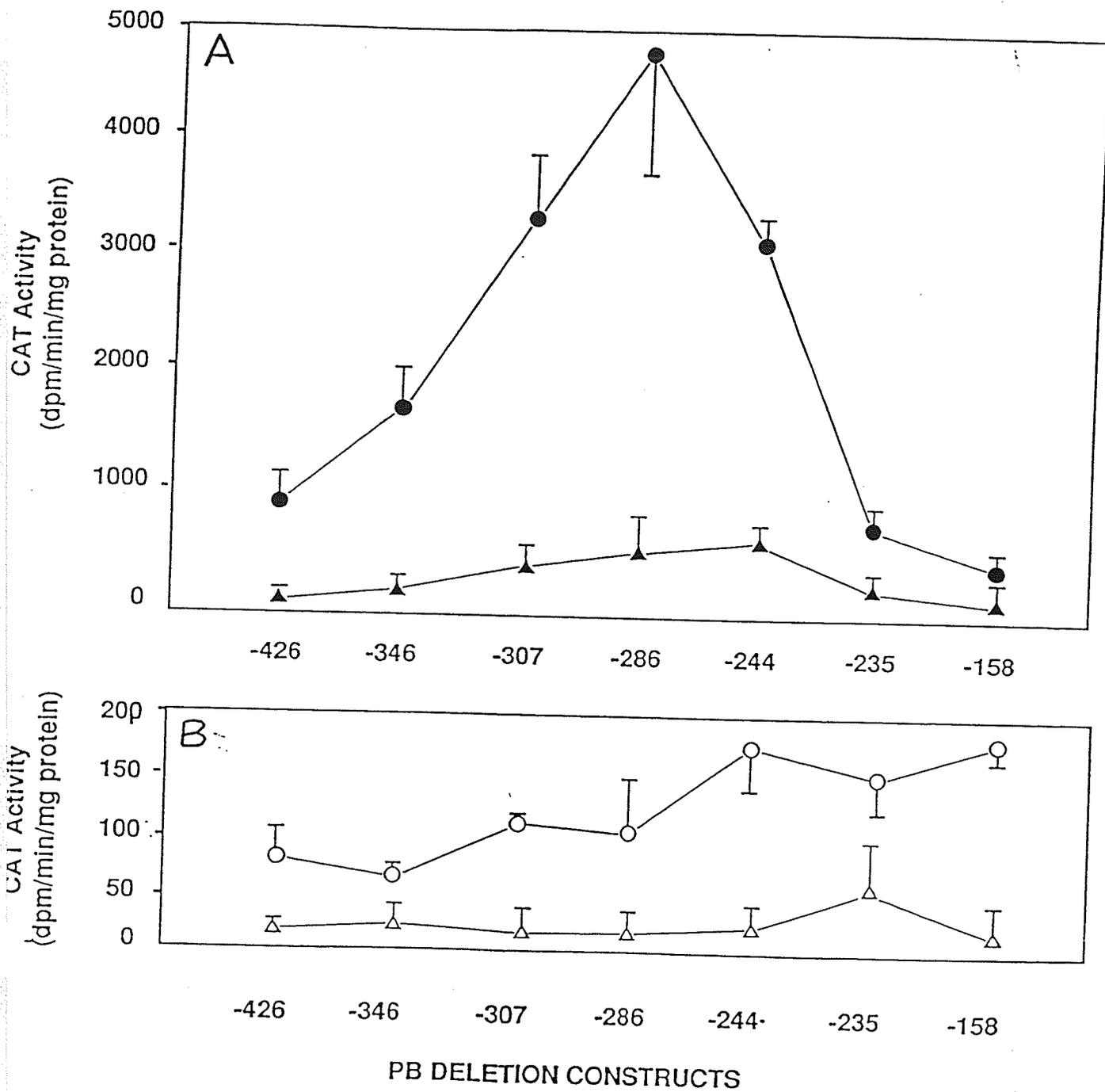


Figure 3: Fold Change in CAT Activity of 5'-PB Deletion Constructs. The PC-3 cells were transfected with the 5'deletion constructs of figure 2, cotransfected with rGR and rAR and treated with DEX (open bars) or DHT (solid bars). The CAT activity was expressed as fold change in CAT activity relative to controls where PC-3 cells were cotransfected with the appropriate receptor expression vector (rAR or rGR) but not treated with steroid.

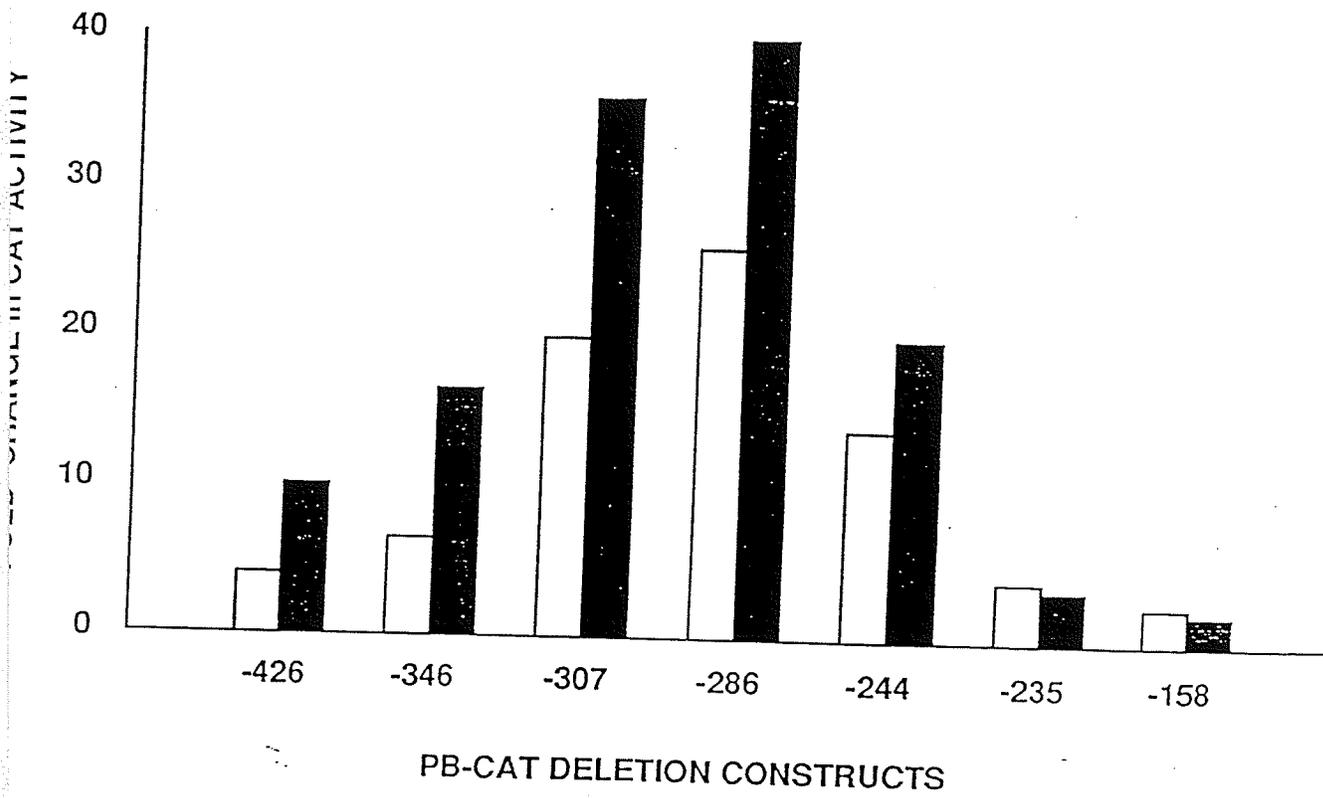


Figure 4: Androgen and Glucocorticoid Induction of -244/-165 PB-TK-CAT. The PC-3 cells were transfected with -244/-165 PB-TK-CAT, cotransfected with the expression vector for either rAR or rGR and treated without (open bars) and with (solid bars) DHT or DEX, respectively. The CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after being corrected for transfection efficiency.

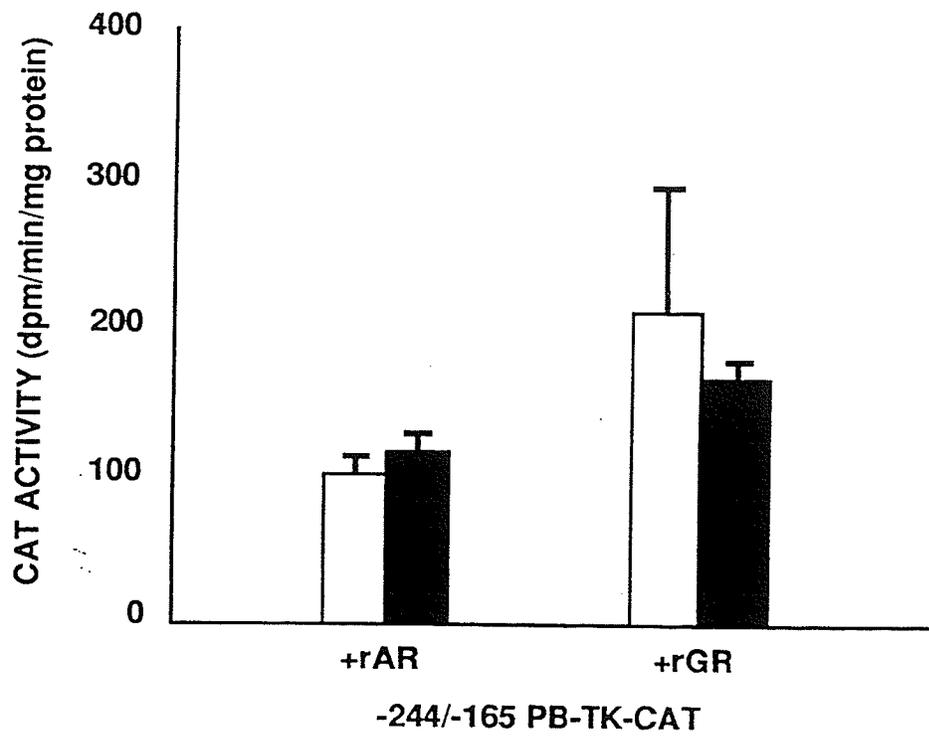


Figure 5: Androgen and Glucocorticoid Induction of -244/-96 PB-TK-CAT. The PC-3 cells were transfected with -244/-96 PB-TK-CAT, cotransfected with the expression vector for either rAR or rGR and treated without (open bars) and with (solid bars) DHT or DEX, respectively. The CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after being corrected for transfection efficiency.

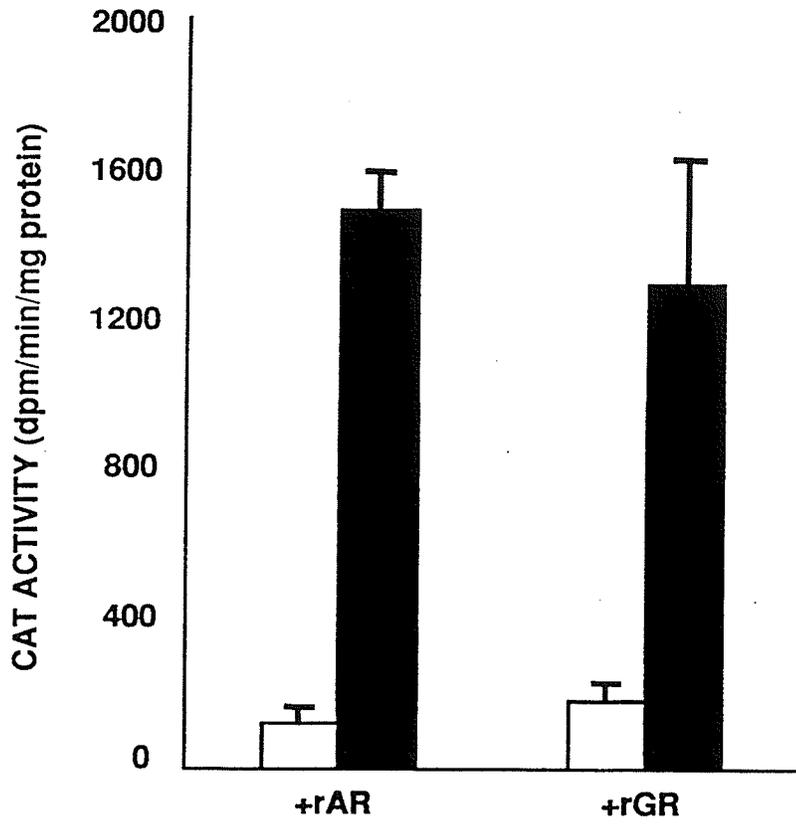


Figure 6: Dose Response Curve for -244/+28 PB-CAT. The PC-3 cells were transfected with -244/+28 PB-CAT and cotransfected with either rAR or rGR expression vectors and treated with DHT (solid bars) or DEX (open bars), respectively. The CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after being corrected for transfection efficiency.

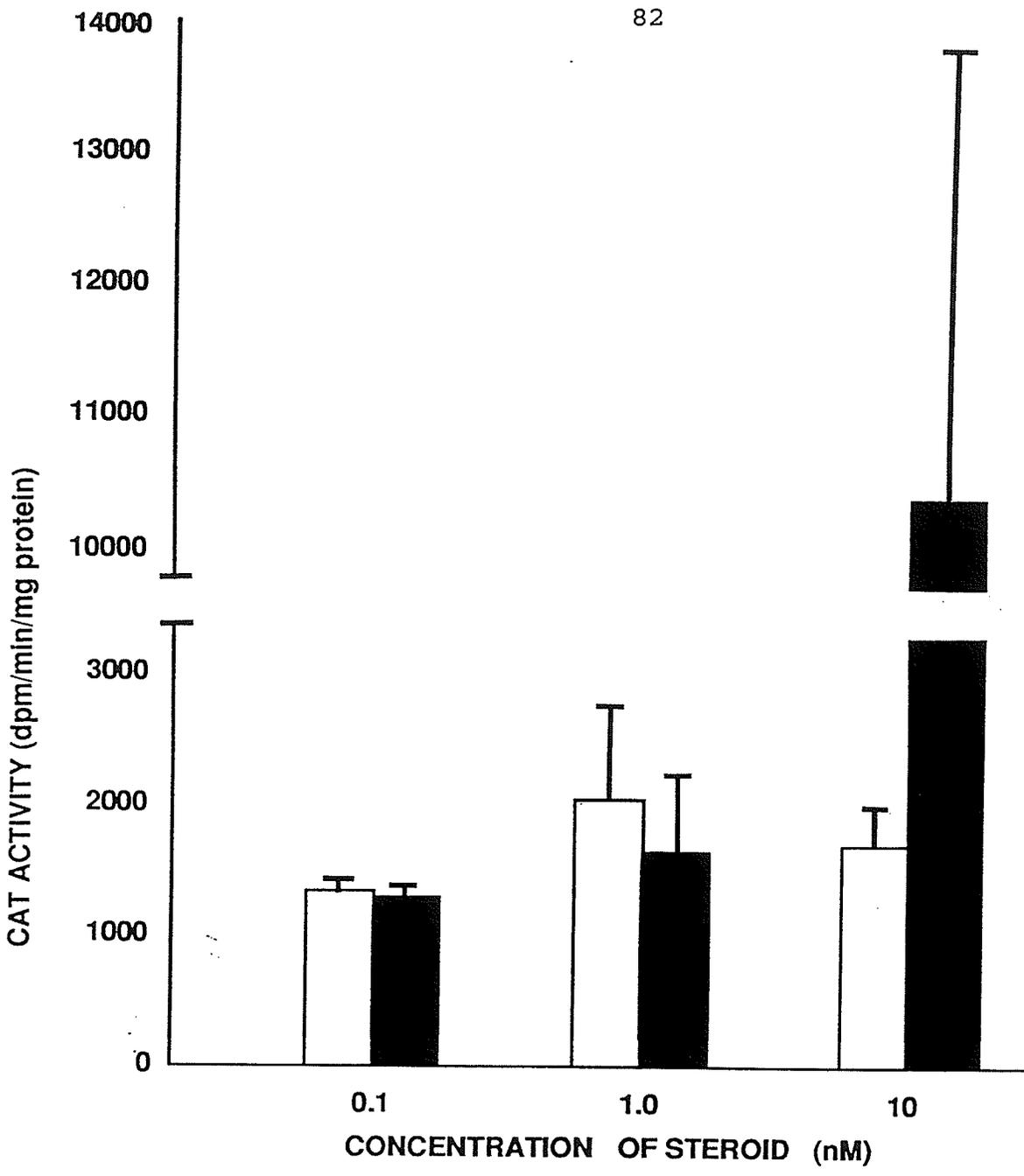


Figure 7: Dose Response Curve for -244/-96 PB-TK-CAT. The PC-3 cells were transfected with -244/-96 PB-TK-CAT and cotransfected with either rAR or rGR expression vectors and treated with 10 nM DHT (solid bars) or DEX (open bars), respectively. The CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after being normalized for transfection efficiency.

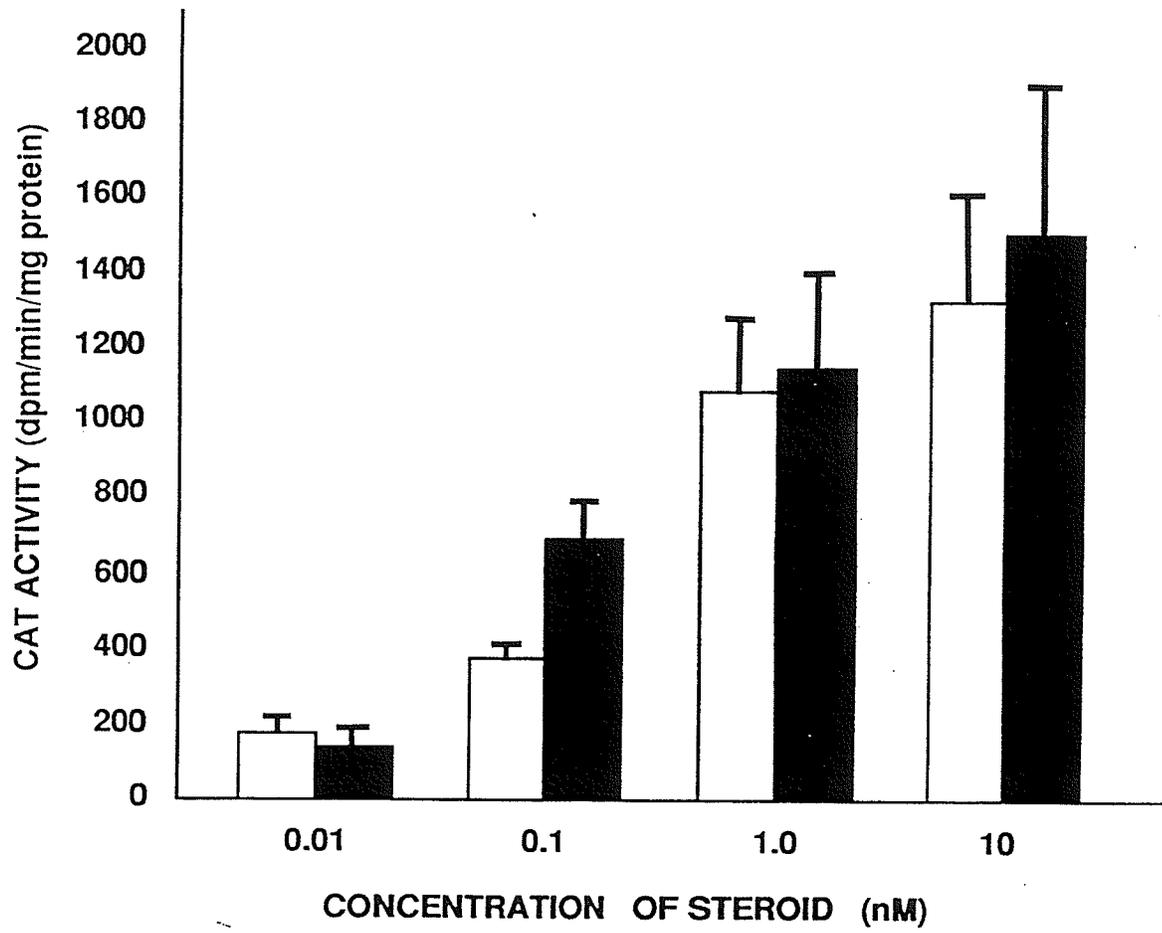


Figure 8: A Composite of Androgen and Glucocorticoid Induction of -244/+28 PB-CAT and -244/-96 PB-TK-CAT. The CAT activity of PC-3 cells transfected with -244/+28 PB-CAT (figure 6) and -244/-96 PB-TK-CAT (figure 7), cotransfected with rAR or rGR and treated with either DHT or DEX, respectively, at 10 nM were compared. The CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after being corrected for transfection efficiency.

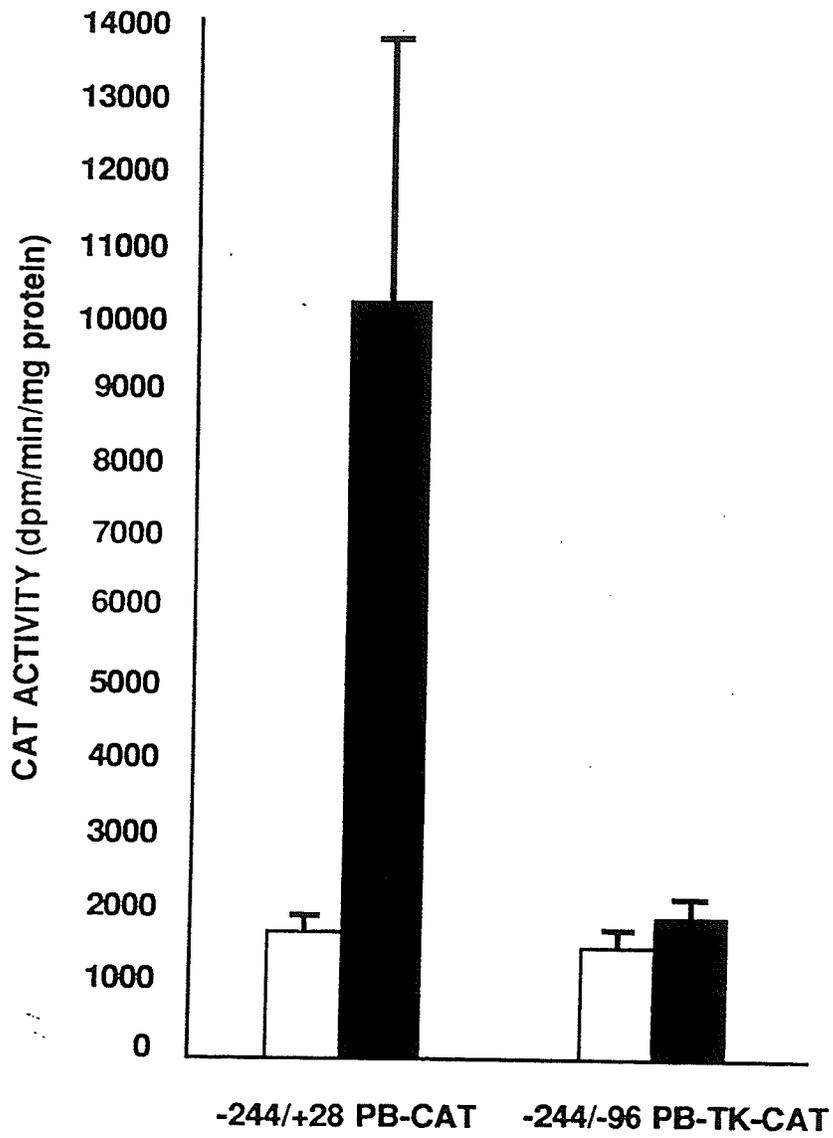


Figure 9: Androgen Induced Fold Change in CAT Activity of Three Chimaeric CAT constructs. The PC-3 cells were transfected with -286/+28 PB-CAT, MMTV-CAT and TAT-CAT and cotransfected with the expression vector for rAR and treated with DHT. The induced CAT activity was expressed as fold change in CAT activity relative to controls where PC-3 cells were cotransfected with rAR expression vector, but not treated with DHT.

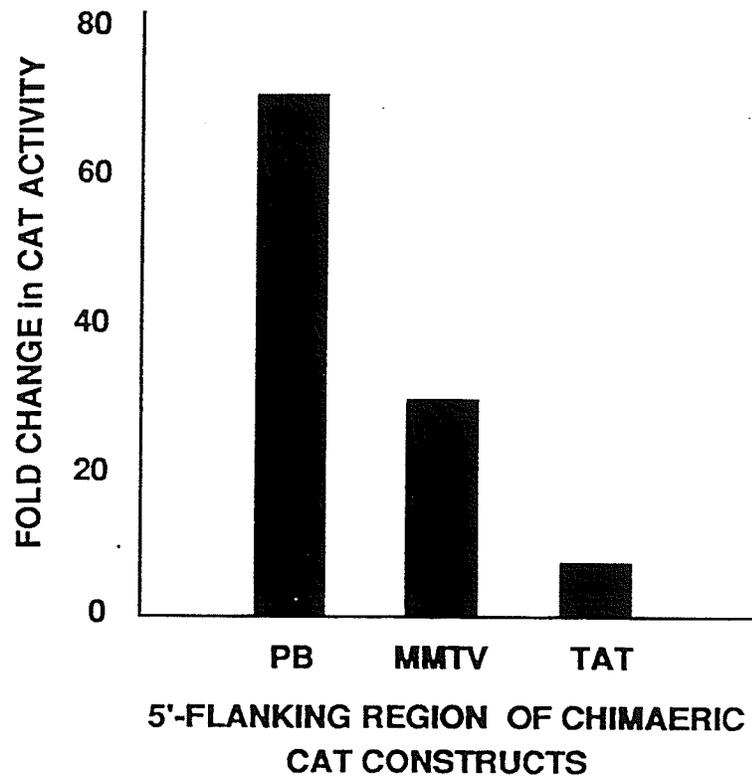


Figure 10: Glucocorticoid Induced Fold Change in CAT Activity of Three Chimaeric CAT Constructs. The PC-3 cells were transfected with -286/+28 PB-CAT, MMTV-CAT and TAT-CAT, cotransfected with the expression vector for rGR and treated with DEX. The CAT induced activity was expressed as fold change in CAT activity relative to control where PC-3 cells were cotransfected with rGR but not treated with DEX.

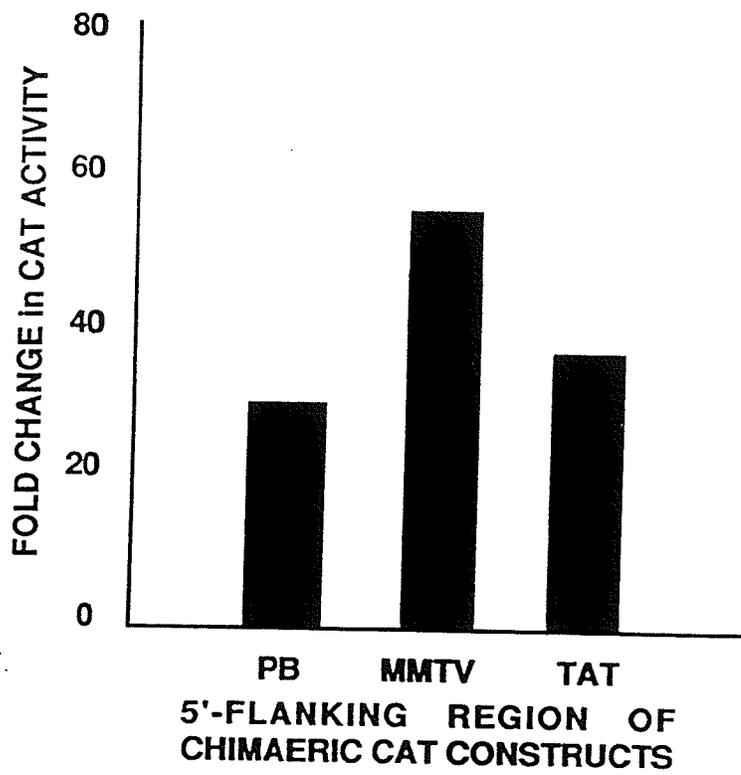


Figure 11: Progesterone Induced Change in CAT Activity of Three Chimaeric CAT Constructs. The PC-3 cells were transfected with -286/+28 PB-CAT, MMTV-CAT and TAT-CAT, cotransfected with 5 μ g of plasmid per plate of cells with the chicken PR expression vector and treated with (solid bars) and without (open bars) progesterone. The CAT activity was measured was the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after correction for transfection efficiency. Note that the PB-CAT activity of both the untreated and DHT treated cells exhibited very low CAT activity and thus the difference between the two is not clear graphically: the basal CAT activity of PC-3 cells transfected with -286/+28 PB-CAT and cotransfected with rAR but not treated with DHT was 29 \pm 10 dpm/min/mg protein whereas the induced PC-3 cells (ie: treated with DHT) demonstrated a CAT activity of 116 \pm 9 dpm/min/mg protein.

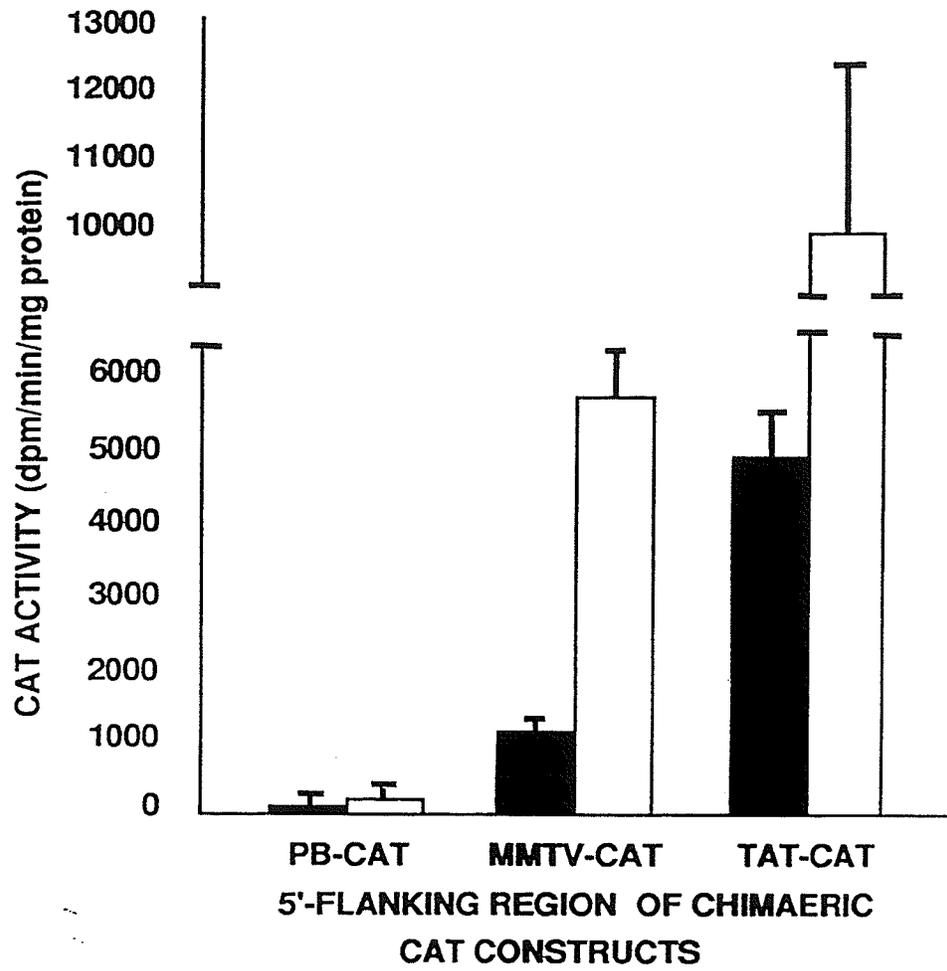


Figure 12: Progesterone Induced Fold Change in CAT Activity of -286/+28 PB-CAT, MMTV-CAT and TAT-CAT. The PC-3 cells were transfected with -286/+28 PB-CAT, MMTV-CAT and TAT-CAT, cotransfected with 5 μ g of plasmid per plate of cells of chicken PR expression vector and treated with progesterone. The CAT activity was expressed as the fold change in CAT activity relative to controls where PC-3 cells were cotransfected with chicken PR but not treated with progesterone.

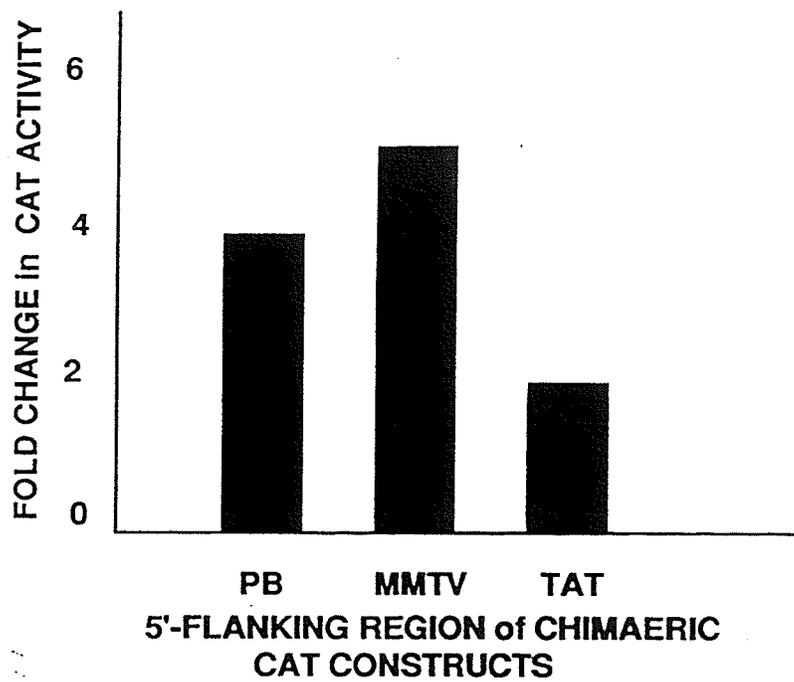


Figure 13: Progesterone Response of -286/+28 PB-CAT, MMTV-CAT and TAT-CAT in HeLa Cells. The HeLa cells were transfected with -286/+28 PB-CAT and cotransfected with the expression vector for chicken PR, at 2 μg ; the MMTV-CAT was cotransfected with cPR at 0, 2, 5 μg ; the TAT-CAT was cotransfected with cPR at 2 μg , and treated with (solid bars) and without (open bars) progesterone. The CAT induced activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after normalizing for transfection efficiency.

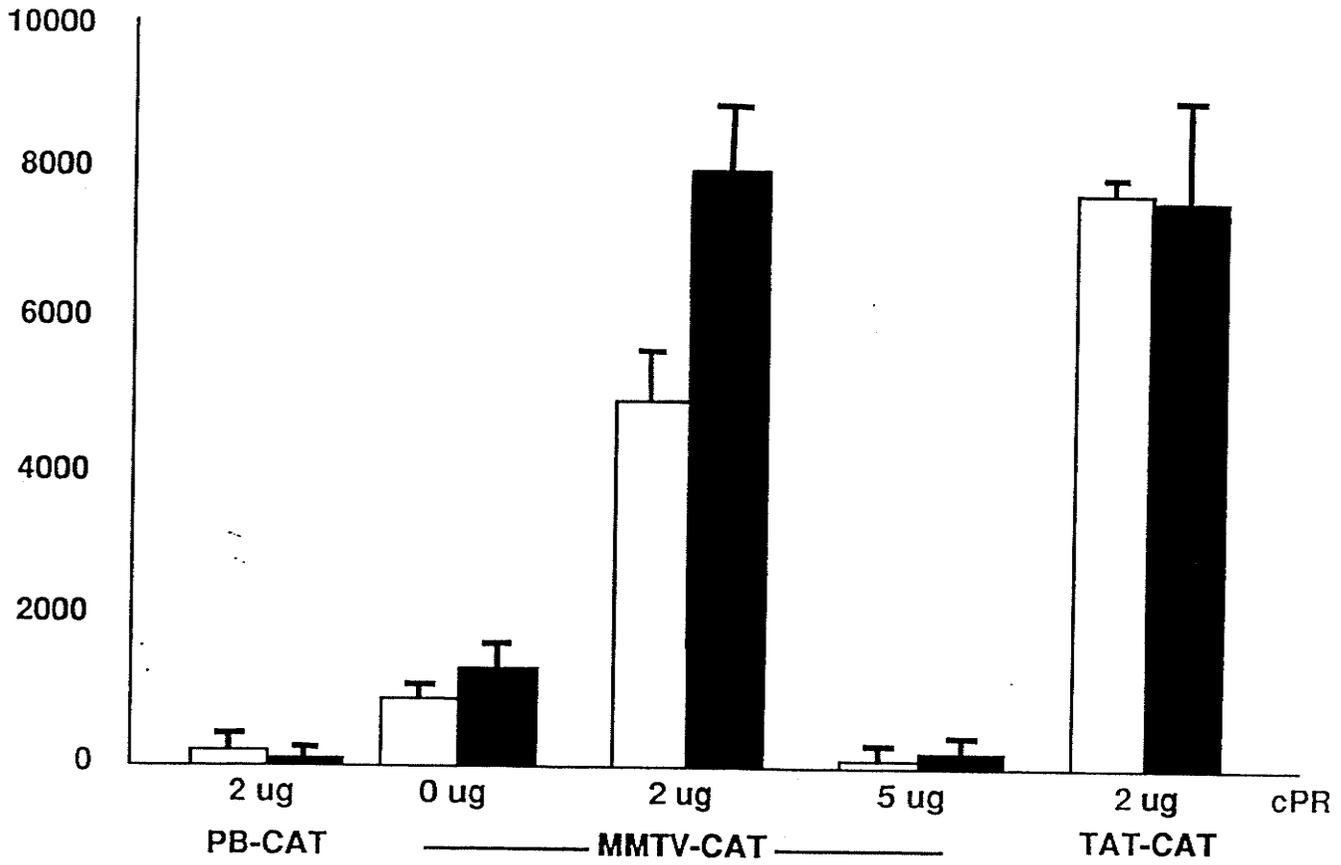


Figure 14: Cotransfections at Different Concentrations of cPR with MMTV-CAT in PC-3 Cells. The PC-3 cells were transfected with MMTV-CAT and cotransfected with 250, 500, 1000 or 2000 ng of the expression vector for chicken PR, and treated with (solid bars) and without (open bars) progesterone. The induced CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after correction for transfection efficiency.

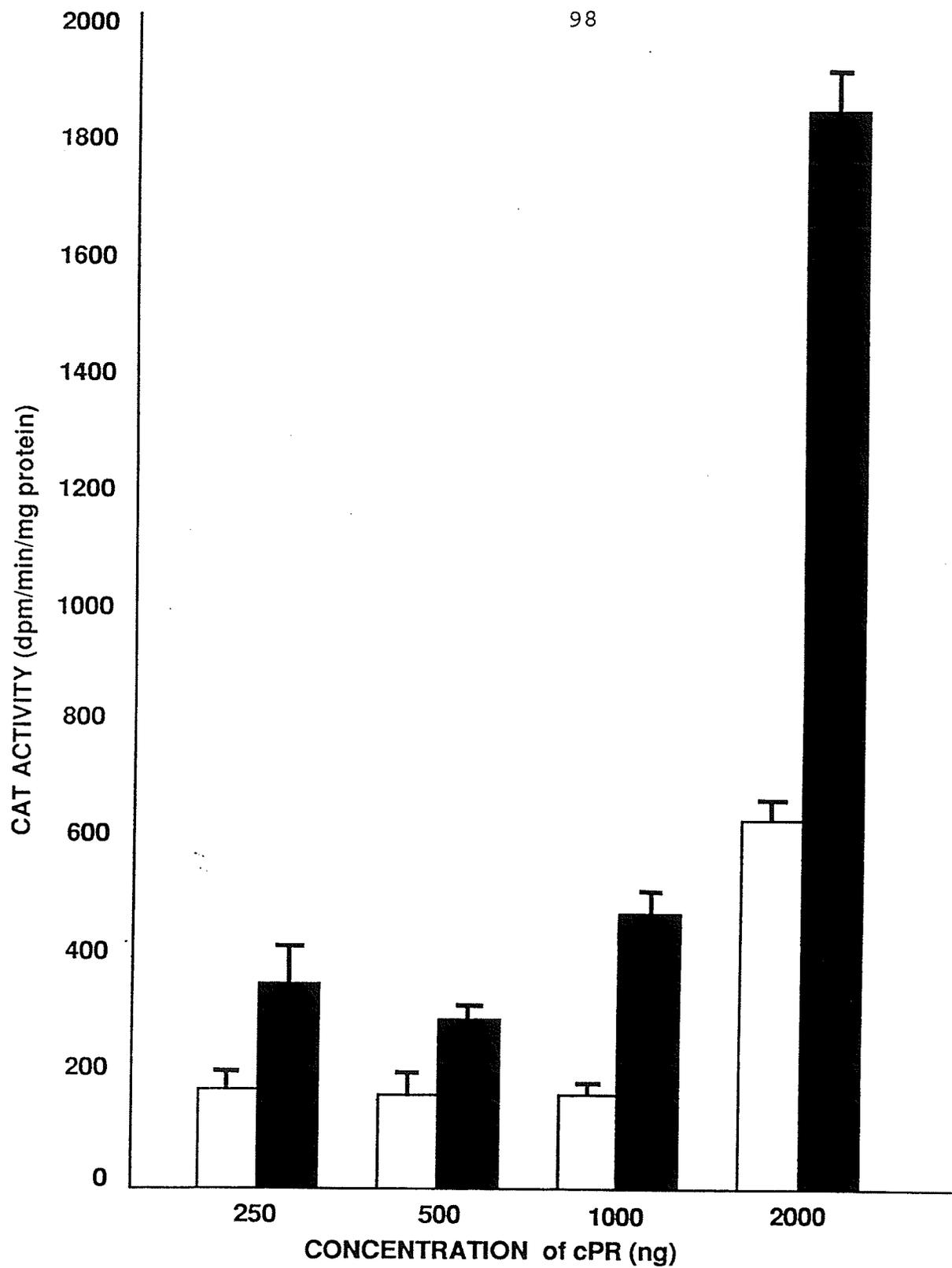


Figure 15: Progesterone Response of -286/+28 PB-CAT and MMTV-CAT. The PC-3 cells were transfected with -286/+28 PB-CAT and MMTV-CAT, cotransfected with cPR at 2 μ g and treated with (solid bars) and without (open bars) progesterone. As a positive control, PC-3 cells were transfected with -286/+28 PB-CAT, cotransfected with rAR at 5 μ g and treated with (solid bar) and without (open bar) DHT. The induced CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after being normalized for transfection efficiency.

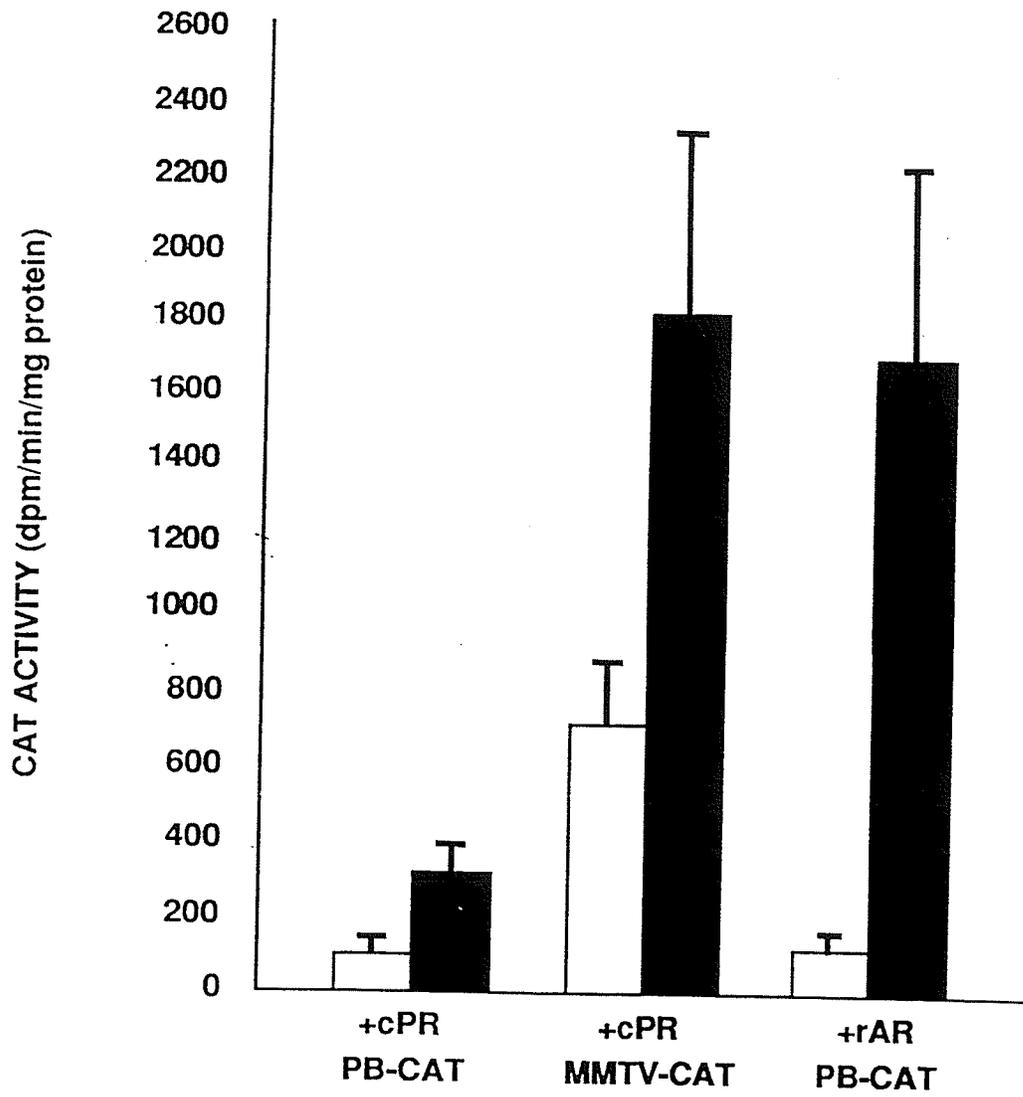


Figure 16: Androgen, Glucocorticoid and Progesterone Induction of -244/-96 PB-TK-CAT. The PC-3 cells were transfected with -244/-96 PB-TK-CAT, cotransfected with rAR, rGR or cPR and treated with (solid bars) and without (open bars) DHT, DEX or progesterone, respectively. The induced CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after being corrected for transfection efficiency.

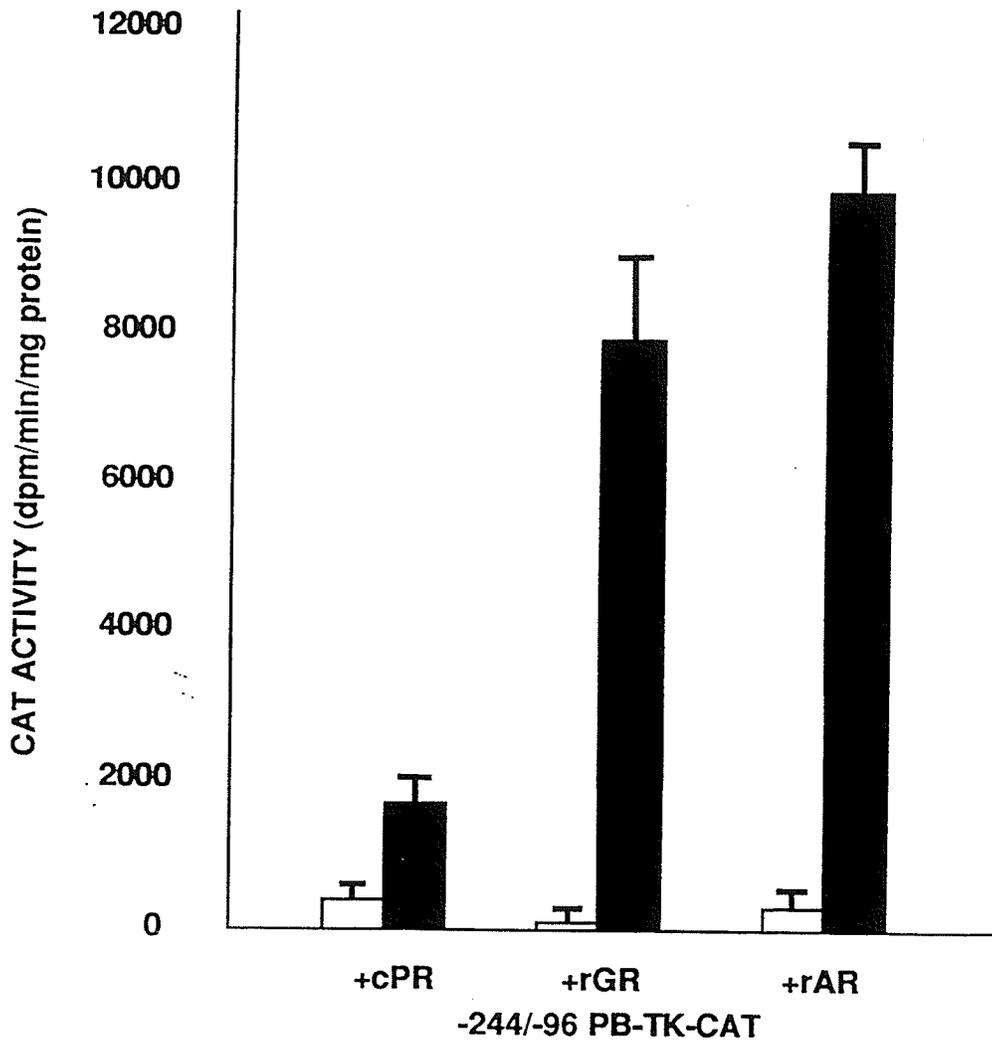


TABLE I: Fold Change in CAT Activity Induced by PR, GR and AR of -244/-96 PB-TK-CAT in PC-3 Cells. The CAT activity (dpm/min/mg protein \pm SEM) of figure 16 was expressed as the fold change in CAT activity relative to controls where PC-3 cells were transfected with the appropriate receptor expression vector, but not treated with hormone.

| | FOLD CHANGE in CAT ACTIVITY | | |
|---------------------------|----------------------------------------|-------------|-------------|
| | +P | +DEX | +DHT |
| -244/-96 PB-TK-CAT | 5 | 59 | 45 |

Figure 17: Progesterone Induced CAT Activity in ZR-75-1 Cells.
The ZR-75-1 cells were transfected with -286/+28 PB-CAT, MMTV-CAT and TAT-CAT and treated with (solid bars) and without (open bars) progesterone. The CAT induced activity was measured as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after normalizing for transfection efficiency.

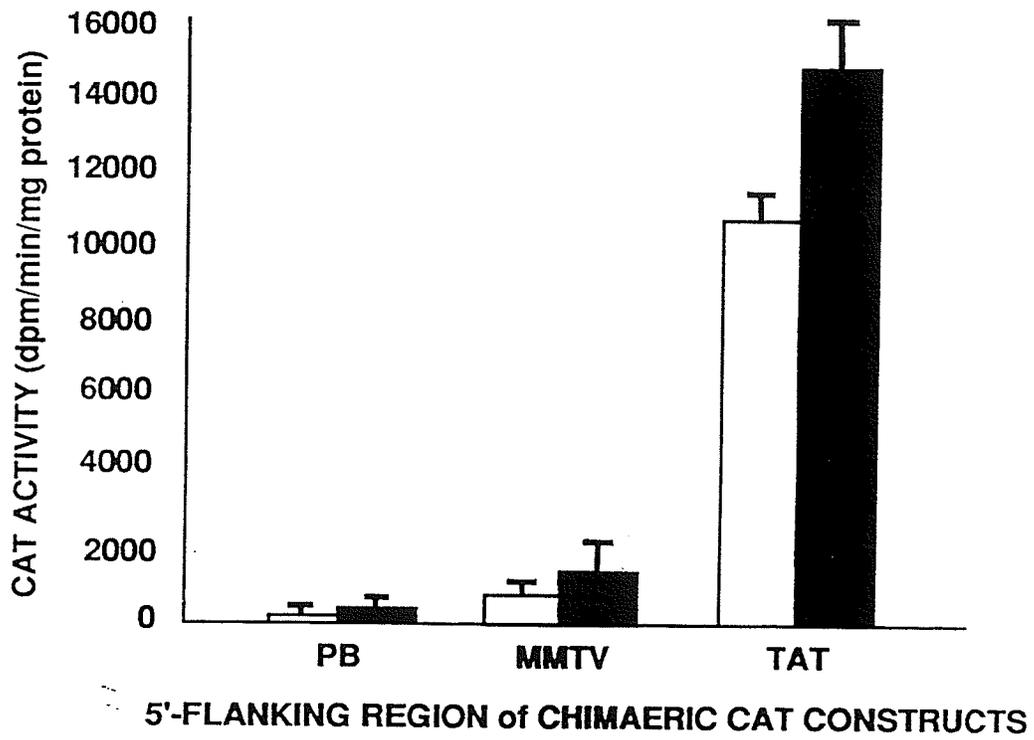


Figure 18: Androgen Induction of -244/-96 PB-TK-CAT in DU-145-D Cells. As a control, the DU-145-D cells were transfected with -244/-96 PB-TK-CAT and treated with (hatched bar) and without (open bar) DHT. The -244/-96 PB-TK-CAT was transfected into DU-145-D cells, cotransfected with the expression vector for rAR and treated with DHT (solid bar). The CAT induced activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after correcting for transfection efficiency.

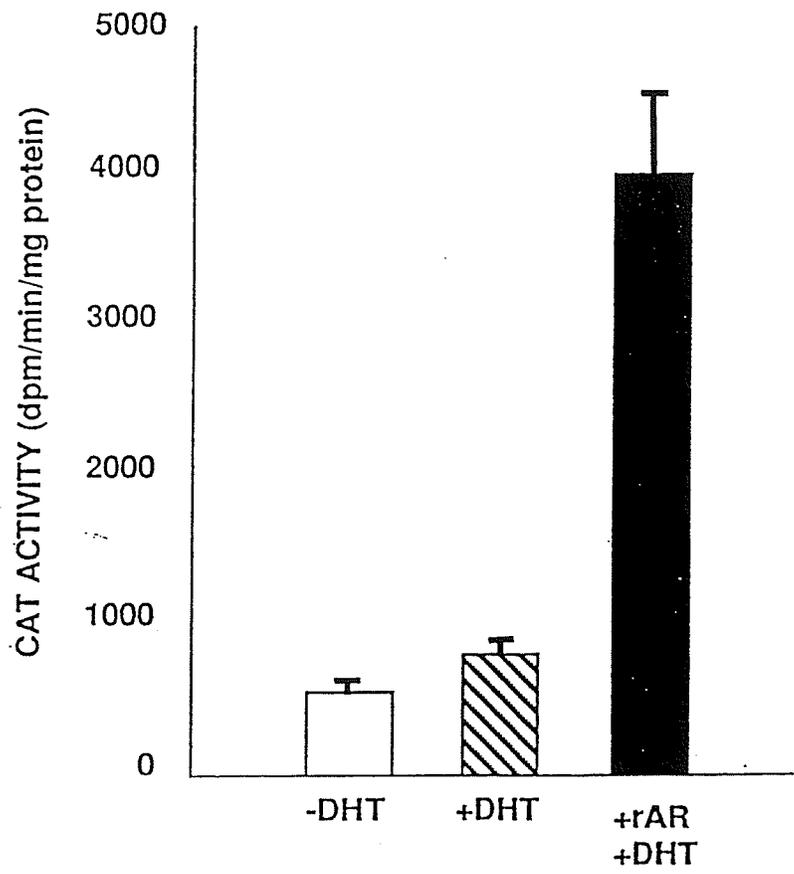


Figure 19: Comparison of Androgen Induction of -244/-96 PB-TK-CAT and -244/+28 PB-CAT in DU-145-A Cells. The DU-145-A cells were transfected with -244/-96 PB-TK-CAT (open bar) and -244/+28 PB-CAT (solid bar), cotransfected with the expression vector for rAR and treated with DHT. The induced CAT activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after normalizing for transfection efficiency.

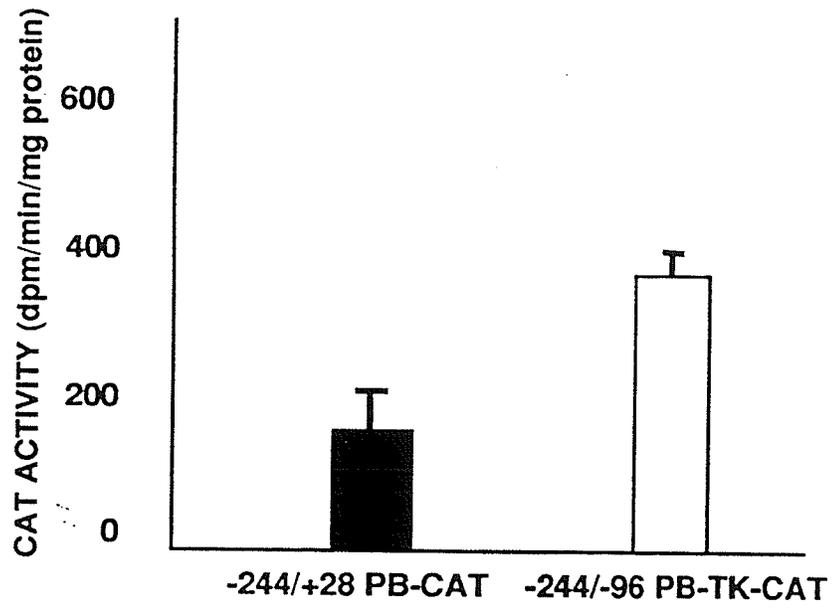


Figure 20: Comparison of Androgen Induction of -244/-96 PB-TK-CAT in Different Sublines of DU-145 Cells. Different sublines of DU-145 cells were transfected with -244/-96 PB-TK-CAT, cotransfected with the expression vector for rAR and treated with DHT: (D) DU-145-D; (R) DU-145-R; (A) DU-145-A cells. The CAT induced activity was expressed as the fold change in CAT activity relative to controls where DU-145 cells were cotransfected with rAR but not treated with DHT.

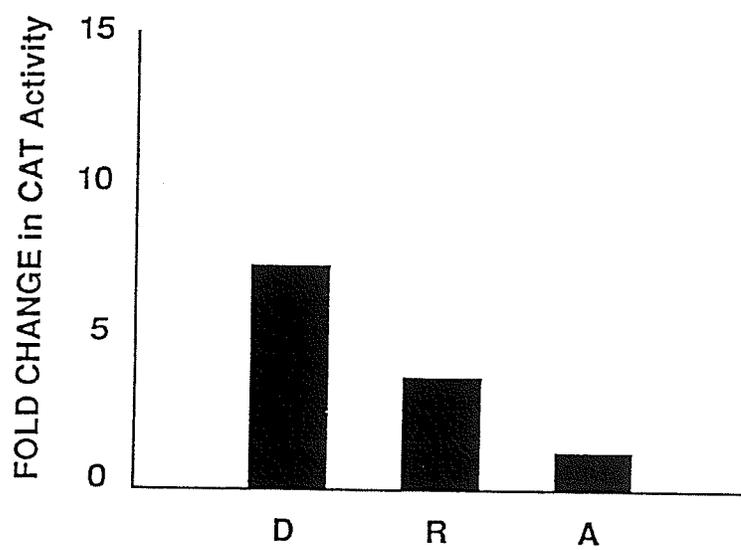


Figure 21: Androgen and Glucocorticoid Induction of -286/+28 PB-CAT in DU-145-R and DU-145-A Cells. Both the DU-145-R (open bars) and DU-145-A (solid bars) cells were transfected with -286/+28 PB-CAT, cotransfected with either the expression vector for rGR or rAR and treated with DEX or DHT respectively. The CAT induced activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after correcting for transfection efficiency.

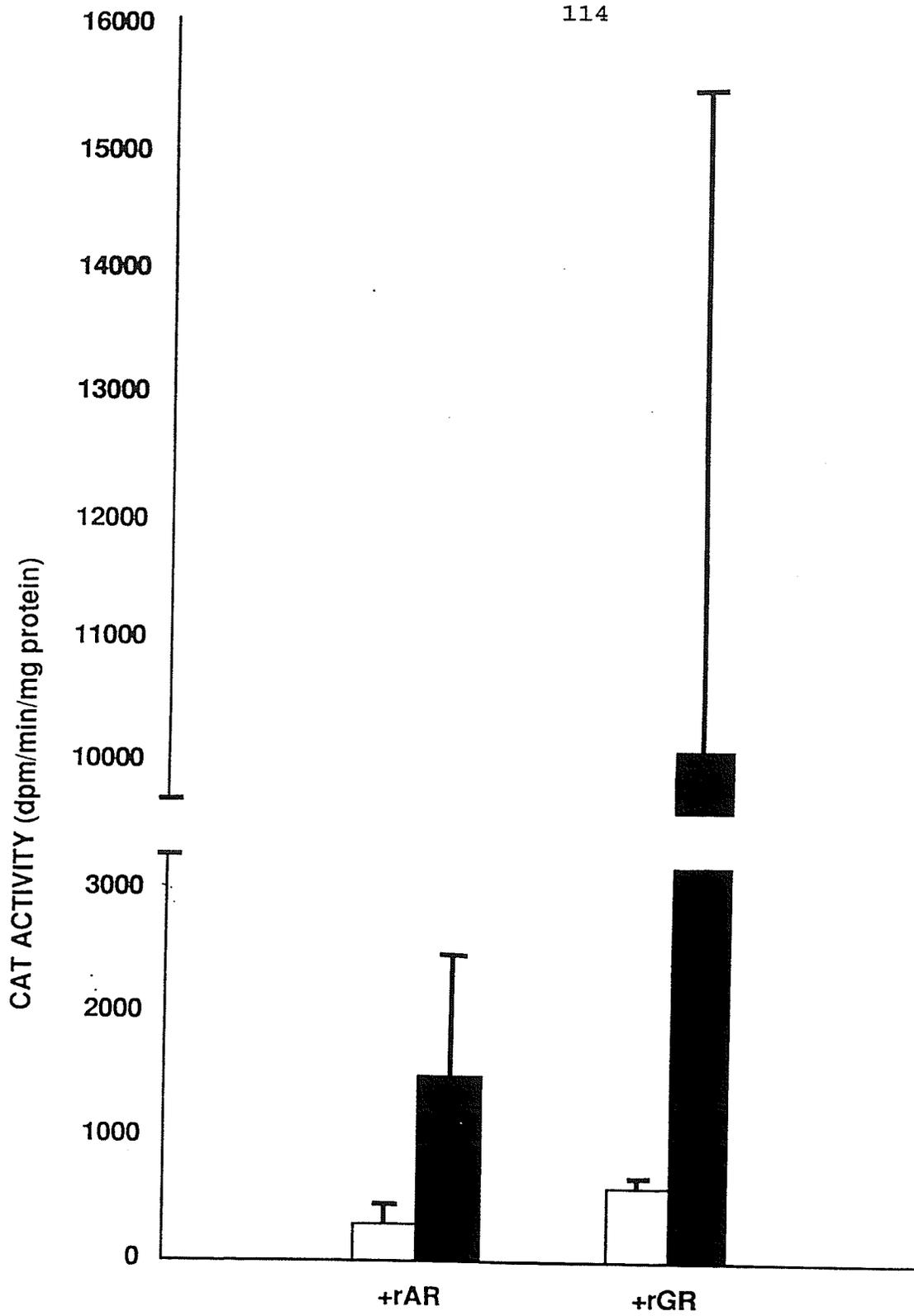


Figure 22: Androgen and Glucocorticoid Induced Fold Change in CAT Activity of -286/+28 PB-CAT in DU-145-R and DU-145-A Cells. The DU-145-R (open bars) and DU-145-A (solid bars) cells were transfected with -286/+28 PB-CAT, cotransfected with either the expression vector for rAR or rGR and treated with DHT or DEX, respectively. The CAT induced activity was expressed as the fold change in CAT activity relative to controls where DU-145-R and DU-145-A cells were cotransfected with the appropriate receptor expression vector but not treated with hormone.

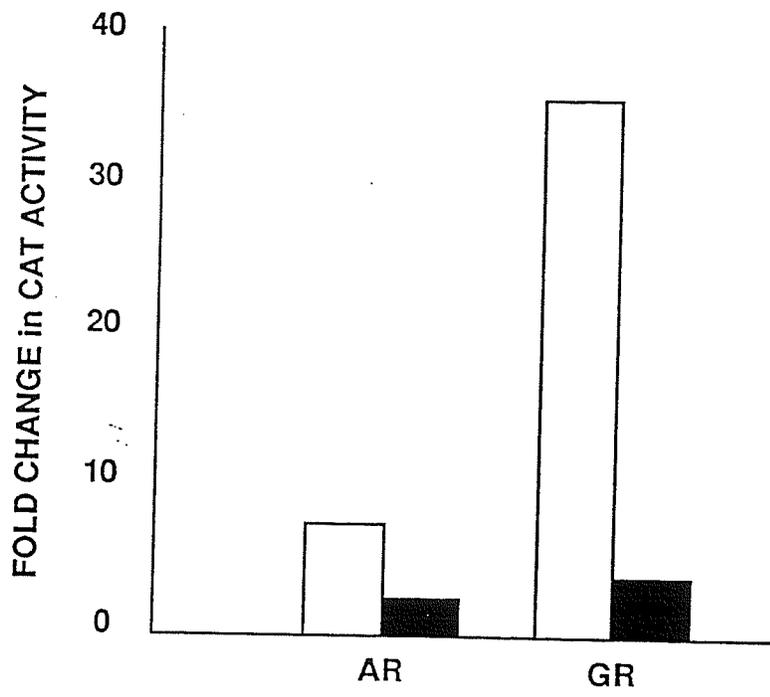


Figure 23: The Induction of MMTV-CAT by AR and GR in DU-145-R Cells. The DU-145-R cells were transfected with MMTV-CAT and cotransfected with the expression vector for rAR or rGR and treated with (solid bars) and without (open bars) the appropriate steroid. The CAT induced activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after normalizing for transfection efficiency.

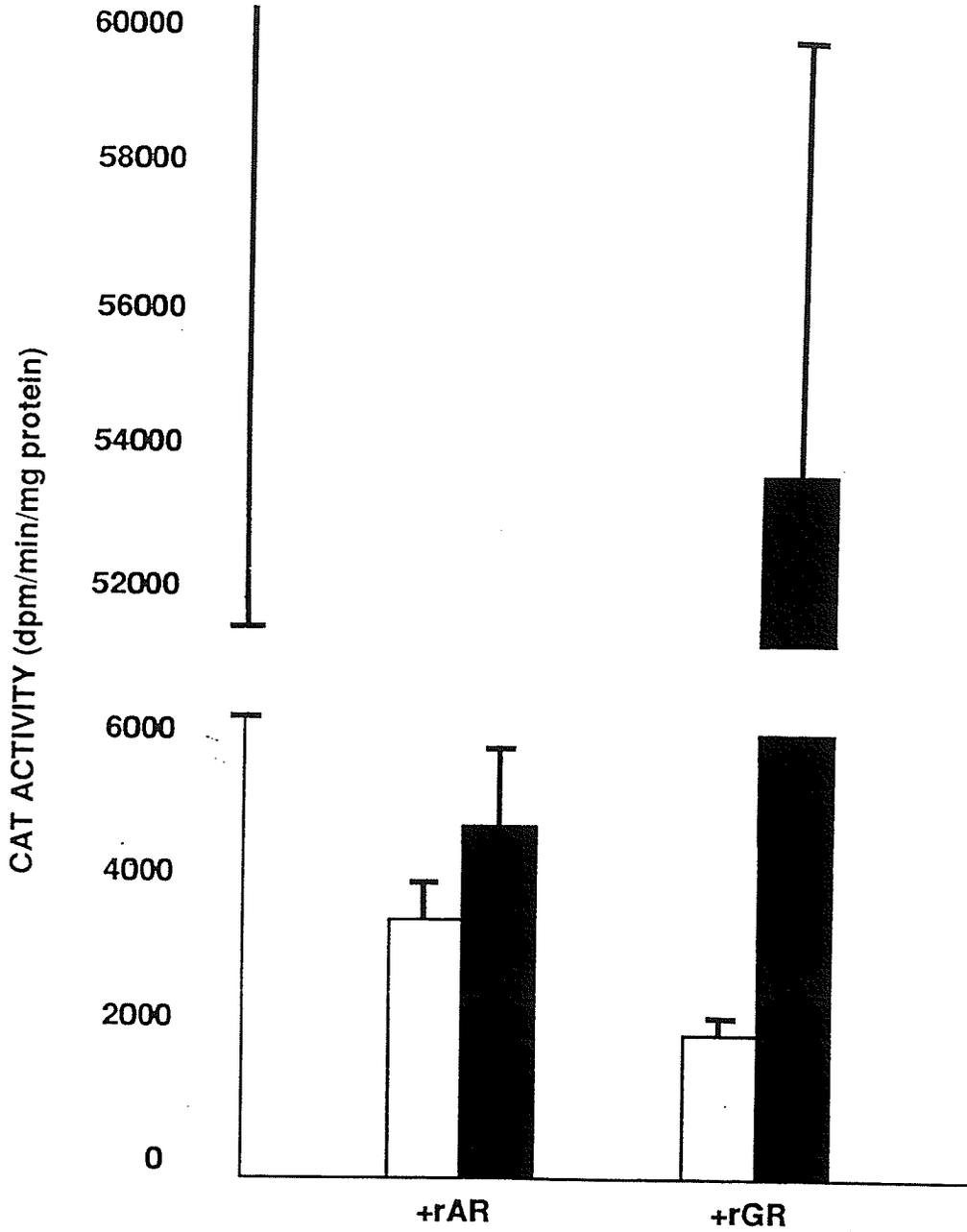


Figure 24: The Induction of MMTV-CAT by AR and GR in DU-145-A Cells. The DU-145-A cells were transfected with MMTV-CAT and cotransfected with the expression vector for rAR or rGR and treated with (solid bars) and without (open bars) the appropriate steroid. The CAT induced activity was expressed as the mean (dpm/min/mg protein \pm SEM) of triplicate determinations after normalizing for transfection efficiency.

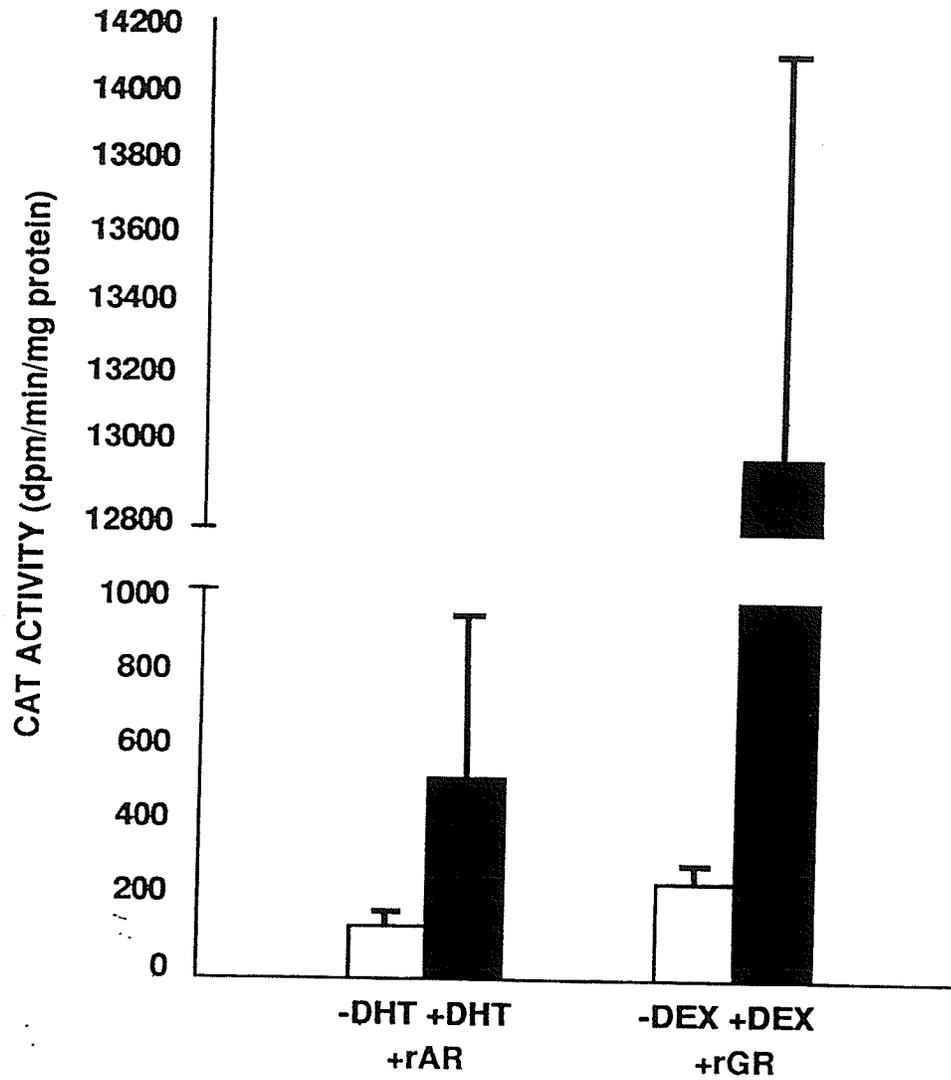


Figure 25: Comparison of Induction of MMTV-CAT by rGR and rAR in DU-145-R and DU-145-A Cells. Transfection of MMTV-CAT, cotransfection of rAR (solid bars) or rGR (open bars) and treatment with appropriate hormone was carried out in both DU-145-R and DU-145-A cells. The CAT induced activity was expressed as Fold change in CAT activity relative to controls where DU-145-R and DU-145-A cells were both cotransfected with the appropriate receptor expression vector, but not treated with hormone.

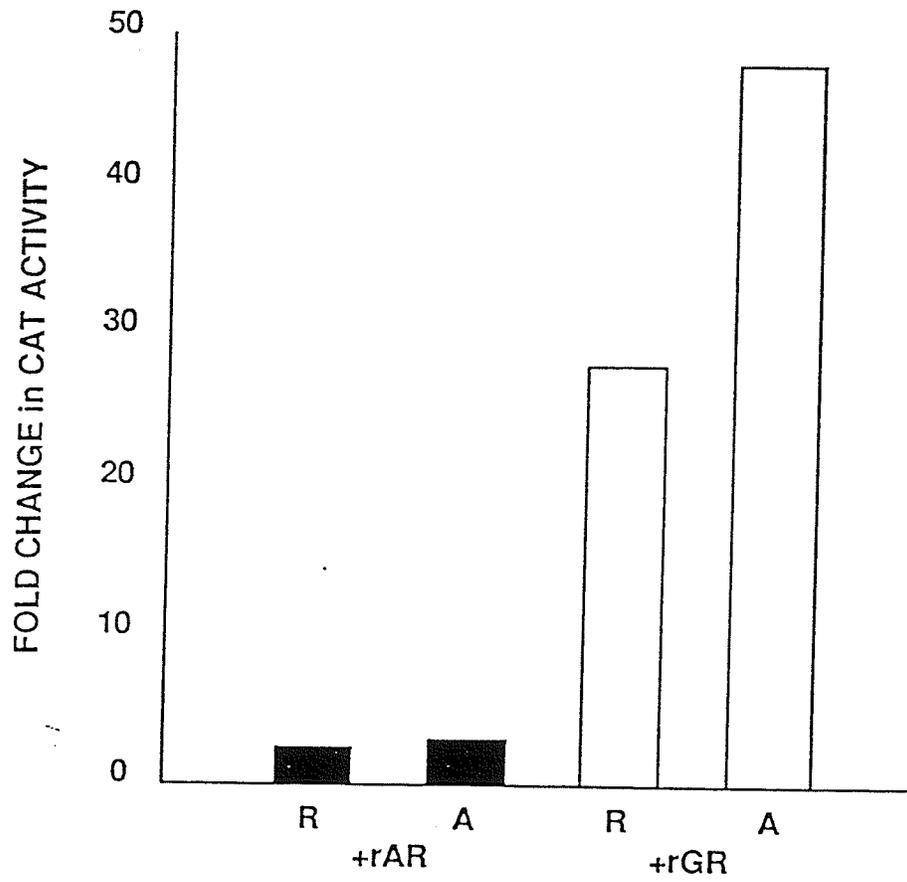


TABLE 2: Androgen and Glucocorticoid Induction of -244/+28 PB-CAT and -244/-96 PB-TK-CAT in DU-145-R cells. The DU-145-R cells were transfected with -244/+28 PB-CAT and -244/-96 PB-TK-CAT, cotransfected with the expression vector for either rAR or rGR and treated with DHT or DEX, respectively. Column (A) represents the mean (dpm/min/mg protein \pm range) of duplicate determinations with steroid; Column (B) represents the fold change in CAT activity or -244/-96 PB-TK-CAT relative to -244/+28 PB-CAT; in column (C), the induction of -244/-96 PB-TK-CAT by DHT or DEX is given an arbitrary value of 100%; the induction of -244/+28 PB-CAT is then a percentage of -244/-96 PB-TK-CAT.

| <u>Androgen Induction</u> | (A) | (B) | (C) |
|---------------------------------|-------|-------|------|
| -244/+28 PB-CAT | 1612 | 1404 | 21% |
| -244/-96 PB-TK-CAT | 7697 | 120 | 100% |
| <u>Glucocorticoid Induction</u> | | | |
| -244/+28 PB-CAT | 1175 | 213 | 10% |
| -244/-96 PB-TK-CAT | 11203 | 10000 | 100% |

TABLE III. Sequence Homology of the HREs. The asterisk indicates point of 5'-flanking deletion (-235 bp) that results in the major loss in androgen regulation. Sequence in lower case letters of ARE-1 does not footprint with the androgen receptor (Rennie et al., 1993). Nucleotides in bold show the homology between ARE-1 and ARE-2. Numbers indicate the location in the PB 5'-flanking DNA.

Consensus GRE

GGTACAnnnTGGTCT

*

ARE-1

-241

atagcATCTT**GTTCT**TAGT

-223

tatcgTAGAACAAGAATCA

ARE-2

-140

GTAAAGTACTCCAAGAACCTATTT -117

CATTTCATGAGG**TCTT**GGATAAA

DISCUSSION

The focus of this investigation was to determine the location and characteristics of the Hormone Responsive Elements (HRE) within the 5'-flanking DNA of Probasin, a rat prostatic gene.

Previous work had shown that the PB-CAT deletion constructs were regulated by rGR and rAR, in the presence of steroid, in human cervical carcinoma (HeLa) cells. However, it was questioned (1) whether there were any transcription factors (TF) in the HeLa cell line which inhibited the full potential induction by rAR and DHT; (2) whether a prostatic (adenocarcinoma) cell line might better reflect the 'natural' setting for regulation of a prostatic gene in prostatic cells. Thus, transient transfections of the PB-CAT deletion constructs were performed in PC-3 cells, a human prostatic adenocarcinoma cell line. Indeed, preferential DHT (over DEX) induction of each PB-CAT construct was observed (figure 2), not only of those cells treated with steroid (panel A, figure 2), but also in the basal expression of the PB-CAT construct in the presence of AR without steroid (panel B, figure 2). Furthermore, with successive 5'-end deletions of the -426/+28 PB-CAT, an increase in CAT induced activity was seen up to deletion -286/+28 PB-CAT (panel A, figure 2). This inferred that there were PB DNA sequences from -426 to -286 bp that functioned to repress the maximum potential DHT and DEX induction of PB-CAT. In addition, because the maximum CAT

induced activity was exhibited by the -286/+28 PB-CAT construct, it could be argued that the PB DNA sequences from -426 to -286 bp were not necessary for androgen/glucocorticoid regulation.

With further 5'-end deletions, we observed a decrease in CAT induced activity of -244/+28 PB-CAT, but a significant drop in DHT and DEX induced CAT activity at -235/+28 PB-CAT (panel A, figure 2). This data implies the loss of hormonal regulation was due to the loss of the hormonally responsive DNA sequences (HREs). Therefore, it was reasoned that the HRE (in PB-CAT) resided, at least in part, between -286 and -235 bp. Examining the 5'-flanking DNA sequence of PB (figure 1), a 15 bp imperfect palindrome was identified (-241 to -227 bp) which was found to have 70% homology to the consensus Glucocorticoid Response Element (GRE) (Beato, 1989). Only the first four bp were different (Table III). Therefore, it was not surprising that only minimal androgen and glucocorticoid regulation resulted with the -235/+28 PB-CAT since this construct deleted the first 6 bp of the HRE, the element necessary to confer steroid regulation by hormone receptor complex. Apart from the PB-CAT constructs which included only part or none of this first HRE (-235, -158 bp), all other constructs were preferentially induced by DHT (over DEX) whether expressed as CAT activity (dpm/min/mg protein \pm SEM, figure 2) or fold change in CAT activity (figure 3). However, it was unclear whether DNA sequences 3' to this HRE were

necessary for hormonal induction of PB-CAT.

In an attempt to specifically identify the PB-CAT DNA sequences necessary and sufficient for hormonal regulation a 3'-end deletion, the -244/-165 PB-TK-CAT construct, was generated. As the data indicated (figure 4), neither DHT or DEX were able to regulate this construct. This information was rather surprising since this construct contained the HRE as well as DNA sequences 3' to this element. However, it is possible that sequences within this fragment of PB DNA (-165 to +28) specified binding sites for transcription factors which assist DHT and/or DEX in conferring hormonal response via their cognitive receptors. Indeed, it is well established that GR cooperates with other transcription factors in binding to the DNA and when treated with DEX in transfection experiments, elicits an enhanced glucocorticoid response (Schule et al., 1988). Furthermore, eliminating the 193 bp (-165 to +28) of the PB gene promoter may have affected the conformation of the DNA in such a manner as to occlude or eliminate necessary binding sites for potential transcription factors assisting in the hormonal response.

By far, the most valuable information was obtained from the DNase I footprinting of PB with the synthetic AR. Dr. Rennie's laboratory (Rennie et al., 1993) observed two footprints the first from -241 to -222 bp, called ARE-1 and the second from -140 to -117 bp, called ARE-2. The footprint over ARE-1 was anticipated since it contained the 15 bp

palindrome of a GRE-like sequence. However, because the DNA sequences of the ARE-2 on the coding strand (5' to 3') shared no homology to any known HREs, this second footprint was not expected. Upon analysis of the PB DNA sequences protected by DNase I footprinting, it was discovered that the ARE-2 had sequence homology to ARE-1 on the non-coding strand (Table III). It now became apparent why the rAR and rGR, in the presence of steroid, were unable to regulate the -244/-165 PB-TK-CAT construct (figure 4) since it only contained the ARE-1. It also became apparent that each ARE alone was insufficient to confer hormonal regulation via promoter: the -158/+28 PB-CAT construct, containing the ARE-2, but not the ARE-1, was only minimally regulated by both DHT and DEX (figures 2 and 3).

Thus, it was evident that both AREs (ARE-1 and ARE-2) in PB-CAT were necessary for hormonal regulation. In fact, this conclusion was strongly supported when the construct containing both ARE-1 and ARE-2 (and the PB DNA sequences between the AREs) adjacent to the heterologous TK promoter linked to CAT was transfected into PC-3 cells and cotransfected with either the expression vector for rAR or rGR (figure 5) and treated with steroid. Both DHT and DEX were able to regulate this construct. This was confirmed by making individual point mutations in ARE-1 or ARE-2 in the presence of the PB promoter. These mutations demonstrated that both AREs were required (Rennie et al., 1993). The necessity for

more than one HRE for strong androgen induction is not unique. Adler et al., 1993, report that the two HREs contained within the 5'-flanking sequences of the slp gene are both necessary and sufficient for strong androgen response. Furthermore, neither HRE alone was able to confer androgen responsiveness via its cognisant receptor complex. In parallel, are the PSA gene (Riegman et al., 1992) and the β -glucuronidase gene (Lund et al., 1991). Both of these genes require DNA sequences flanking their respective HREs for strong androgen induction.

What was more remarkable though, was the apparent loss of preferential androgen induction with -244/-96 PB-TK-CAT. The DHT and DEX induced CAT activity (dpm/min/mg protein \pm SEM) were equivalent (figure 5). This suggested that the PB sequences between -96 and +28 bp (containing the endogenous promoter), in some manner, conferred preferential androgen regulation. Alternatively, binding sites for transcription factors which can cooperate with GR to enhance glucocorticoid mediated response, overriding preferential androgen receptor response, may have been occluded or unavailable due to the spatial conformation of the AR-ARE interaction. When these DNA sequences are removed, it is possible that the spatial conformation of the DNA no longer favors specific interaction with AR, but now enables either AR and/or GR to interact with the PB DNA in a non-preferential manner. Support for this interpretation is derived from investigations by Adler et al., 1992 who demonstrated that preferential AR over GR mediated

response was lost when a fragment 40 bp longer, upstream and contiguous with the 120 bp fragment upstream of the slp gene was transiently transfected into CV-1 cells. These investigators suggest that non-specific factors may aid in a general steroid response via binding sites not present in the AR-specific DNA sequence thereby overriding androgen-mediated specificity of response.

To ensure the correct concentration of steroid was being employed, a dose response curve for both DHT and DEX using both -244/+28 PB-CAT (figure 6) and -244/-96 PB-TK-CAT (figure 7) were generated. At concentrations of 0.1 nM and 1 nM of DHT or DEX, the CAT induced activity of -244/+28 PB-CAT were equivalent (figure 6). However, at 10 nM of DHT or DEX, there was a significant difference: the DHT induction of -244/+28 PB-CAT far exceeded the CAT induced activity observed with DEX.

The DHT and DEX dose response curves for -244/-96 PB-TK-CAT (figure 7) showed that at low concentrations (0.01, 0.1 and 1.0 nM) of DHT or DEX, CAT induced activity was equivalent. Unlike the androgen and glucocorticoid induction of -244/+28 PB-CAT, the CAT induced activity by DHT or DEX at the 10 nM concentration of -244/-96 PB-TK-CAT construct was equal.

In addition, the profiles of the dose response curves for -244/+28 PB-CAT (figure 6) and the -244/-96 PB-TK-CAT (figure 7) differ. As the concentration of steroid tested was

increased from 0.1 to 10 nM, the CAT induced activity of -244/-96 PB-TK-CAT increased. This was not true for the DHT and DEX induced CAT activity of -244/+28 PB-CAT (figure 6). As the concentration of DEX was increased from 0.1 nM to 10 nM, the CAT induced activity did **not** increase but remained the same (within the margin of error). As the concentration of DHT was increased from 0.1 nM to 1.0 nM, no apparent increase in CAT activity was observed. The only significant change in CAT activity for -244/+28 PB-CAT was seen when 10 nM DHT was used. In any case, the greatest induction of CAT activity for both -244/+28 PB-CAT and -244/-96 PB-TK-CAT was observed when 10 nM of DHT or DEX was used (figure 5, 6 and 7).

A few points should be further emphasized. In comparing DEX induction of -244/+28 PB-CAT and -244/-96 PB-TK-CAT, the CAT activities (dpm/min/mg protein \pm SEM) were equivalent (figure 6 and 7). This implied that removing the endogenous PB promoter (contained within -96 to +28 bp) and replacing it with the exogenous TK promoter had no visible effect on the glucocorticoid induction of -244/-96 PB-TK-CAT. In contrast, androgen induction of -244/-96 PB-TK-CAT compared with -244/+28 PB-CAT, had been substantially reduced (figure 8), inferring that the DNA sequences from -96 to +28 bp, including the endogenous PB promoter contained in at least part androgen specific elements which imparted preferential androgen over glucocorticoid induction.

Having localized the AREs in PB-CAT, the focus of this

investigation shifted to characterizing these AREs. The question then became how well did these AREs in PB-CAT compare to other recognized androgen responsive genes? It is a well established fact that the GREs in MMTV-CAT (Ham et al., 1988) and TAT-CAT (Denison et al., 1989) responded to androgens. Therefore, transfections of -286/+28 PB-CAT, MMTV-CAT and TAT-CAT, cotransfection with rAR expression vector and treatment with DHT were performed (figure 9). Clearly, the -286/+28 PB-CAT gene construct exhibited the greatest fold change in CAT activity (74 fold), followed by MMTV-CAT (36 fold) and the smallest fold change in CAT activity was seen with TAT-CAT (8 fold). Indeed, this was the most encouraging result. Until recently, MMTV-CAT had been the "benchmark" androgen responsive gene, even though the four GREs in MMTV-CAT responded better to glucocorticoids than androgens (Ham et al., 1988). Our data reflected the potent nature of these GREs in MMTV, when DEX induction of -286/+28 PB-CAT, MMTV-CAT and TAT-CAT was examined (figure 10). MMTV-CAT showed the largest fold change in CAT activity (57 fold) followed by TAT-CAT (36 fold) and finally -286/+28 PB-CAT (29 fold).

Optimum conditions for cotransfections with the cPR expression vector in PC-3 cells were not established previously. However, since the appropriate concentrations of rAR and rGR expression vector cotransfected per plate of PC-3 cells had been previously established and were equal (5 μ g), preliminary cotransfections of cPR at 5 μ g with -286/+28 PB-

CAT, MMTV-CAT and TAT-CAT were performed. While the basal (uninduced) and progesterone induced CAT activity of -286/+28 PB-CAT were both small (figure 11), the fold change in CAT activity (4 fold, figure 12) was considerable in comparison to MMTV-CAT (5 fold) and TAT-CAT (2 fold). However, both MMTV-CAT and TAT-CAT exhibited high basal CAT activity (figure 11), thus appearing as a relatively small fold change in CAT activity.

Therefore, using MMTV-CAT as standard, PC-3 cells were cotransfected with different concentrations of cPR expression vector and treated with progesterone (figure 13). Both the CAT activity and the fold change in CAT activity of MMTV-CAT were greatest when 2000 ng of cPR expression vector was cotransfected (figure 13). Also evident was that at low concentrations of cPR (250, 500 and 1000 ng, figure 13), the basal CAT activity was small. This result was in agreement with previous transfections of MMTV-CAT in PC-3 cells. However, when MMTV-CAT was transfected into PC-3 cells and cotransfected with 2000 ng cPR expression vector, the basal CAT activity increased more than 3 fold above the CAT activity observed when the other three concentrations of cPR expression vector were cotransfected. Nonetheless, the best progesterone induced response of MMTV-CAT was achieved when 2000 ng cPR expression vector was cotransfected into PC-3 cells.

When PC-3 cells were transfected with -286/+28 PB-CAT cotransfected with 2 μ g of cPR expression vector and treated

with progesterone, a 3.2 fold change in CAT activity was observed in spite of the recurrent low progesterone induced CAT activity (337 ± 70 dpm/min/mg protein, figure 14).

Although many explanations are possible for this result although, the most compelling explanation is that the presence or absence of certain cell specific factors in the human PC-3 cell line may be incompatible with the expression vector for the cPR. Indeed, the PR expression vector was derived from the chicken unlike both the GR and AR which were derived from the rat. Therefore, the HeLa cell line was chosen to assess the progesterone response of -286/+28 PB-CAT, MMTV-CAT and TAT-CAT. The HeLa cell line was chosen for the following two reasons: (1) optimal conditions for transfections had originally been established using the HeLa cell line with MMTV-CAT as standard; (2) all 5'-PB-CAT deletion constructs had been previously demonstrated to be regulated by both glucocorticoids and androgens in HeLa cells.

Transfections were performed in HeLa cells cotransfected with 2000 ng cPR expression vector with -286/+28 PB-CAT and TAT-CAT and 0, 2000 and 5000 ng cPR with MMTV-CAT (figure 15). Examining these results, the following points emerge: (1) there was no change in CAT activity of -286/+28 PB-CAT when 2000 ng cPR was cotransfected into HeLa cells and treated with and without progesterone. This result differed from the result obtained when the same transfection was carried out in PC-3 cells (figures 11 and 12); (2) In the absence of steroid but

in the presence of cPR, the CAT activity of TAT-CAT was elevated (figure 13). Addition of progesterone elicited no further increase in CAT activity of TAT-CAT but basal activity was high when the cPR was present. This implied that the HeLa cells cotransfected with cPR expression vector produced transcriptionally active PR without the presence of ligand, in this case progesterone.

Analyzing the CAT activity of MMTV-CAT cotransfected with different concentrations of cPR the following observations were made: (1) the largest increase in CAT activity of MMTV-CAT was observed when 2 μ g of cPR expression vector was cotransfected into HeLa cells (8030 ± 686 dpm/min/mg protein); (2) however, the greatest fold change in CAT activity was observed when 5 μ g of cPR expression vector was cotransfected into HeLa cells and treated with progesterone (1.7 fold); (3) the HeLa cells cotransfected with 2 μ g cPR expression vector/per plate of cells exhibited higher CAT activity than those cells cotransfected with 5 μ g cPR. This data indicated that cotransfecting HeLa cells with 5 μ g of cPR was inhibitory (at least for MMTV-CAT).

As stated above, we had observed a preferential DHT over DEX induction of those PB-CAT constructs containing both the ARE-1 and the ARE-2 and the endogenous PB promoter (figures 1 and 2). When the PB DNA sequences from -96 to +28 bp (of the -244/+28 PB-CAT construct) were substituted with the heterologous TK promoter (-244/-96 PB-TK-CAT), the androgen

and glucocorticoid induction became equivalent (figure 8). Conceivably, the replacement of the PB promoter with the TK promoter might enable a more substantial progesterone induced response of PB-CAT.

Consequently, androgen, glucocorticoid and progesterone induction of -244/-96 PB-TK-CAT in PC-3 cells were compared (figure 16). Even with the heterologous TK promoter, the progesterone induced CAT activity (figure 16) and the fold change in CAT activity was small (5 fold, Table 1). Nonetheless, the progesterone induction of -244/-96 PB-TK-CAT was comparable to that of MMTV-CAT (figure 13). Furthermore, the progesterone induced fold change in CAT activity of -244/-96 PB-TK-CAT (5 fold, Table 1) exceeded that observed with -286/+28 PB-CAT (3.2 fold, figure 14). However, it should be noted that there are small differences in the fold change.

All of this data generated in order to characterize the progesterone response of PB-CAT in comparison to the standards, MMTV-CAT and TAT-CAT support that the cPR expression vector, was incompatible with the PB-CAT gene in both PC-3 and HeLa cell lines. Therefore, it was decided that a cell line possessing endogenous PR would alleviate the necessity of cotransfection with cPR expression vector and might better demonstrate the progesterone induced response of PB-CAT, MMTV-CAT and TAT-CAT. Since other investigators had used the mammary tumour ZR-75-1 cell line (which possesses endogenous AR, GR and PR) to characterize steroid responses of

MMTV-CAT (Ham et al., 1988) and TAT-CAT (Luckow and Schutz, 1987) this cell line was chosen. Nevertheless, no change in progesterone induced CAT activity of -286/+28 PB-CAT was exhibited (relative to the control, figure 17). The progesterone induced fold change in CAT activity of MMTV-CAT in ZR-75-1 cells was markedly increased (24 fold, figure 17) in comparison to the progesterone induction of MMTV-CAT in both PC-3 and HeLa cell lines (figures 13-15). Further, the recurrent high basal CAT activity observed in both PC-3 and HeLa cells when the cPR expression vector was cotransfected with TAT-CAT was not eliminated when the progesterone response of TAT-CAT was tested in ZR-75-1 cells (figure 17).

In summary, whether PC-3, HeLa or ZR-75-1 cells are used, the progesterone induction of -286/+28 PB-CAT was minimal, in contrast to androgen and glucocorticoid induction (in PC-3 cells, figures 1 and 2). Given this data, it appears that PB-CAT is preferentially induced by androgen > glucocorticoid > progestin, in the presence of steroid. Although MMTV-CAT has been demonstrated to be androgen responsive, it is reported to be preferentially induced by progestin > glucocorticoid > androgen (Ham et al., 1988). The data presented here indicate preferential glucocorticoid > androgen > progestin of MMTV-CAT, but the basal expression of MMTV-CAT always increased dramatically when PR was present and steroid was absent. This very high basal activity accounted for the small fold change when progestins were added. Clearly, MMTV-CAT was not

preferentially induced by DHT. Based upon the data presented, the best androgen responsive gene is PB-CAT.

The focus of this investigation then shifted. Was the induction profile of PB (ie: DHT > DEX > Progestin) true for other human prostatic carcinoma cell lines? Two other human prostatic carcinoma cell lines have been characterized and are available. The LnCaP cell line had recently been reported to have a single point mutation (A to G in codon 868) in the steroid binding domain of the androgen receptor gene and cDNA. According to Veldscholte et al (1992), this mutation affects the binding specificity for and transcriptional activation by several steroids. The DU-145 cell line lacks androgen receptor (Tilley et al., 1990).

The preliminary transfections were performed in DU-145-D cells obtained from Dr. J. Dodd (Department of Physiology, University of Manitoba). Transfections of -244/-96 PB-TK-CAT alone and with DHT showed a small increase in CAT activity (531 ± 67 to 833 ± 62 dpm/min/mg protein, Figure 18) suggesting some transcriptional activation without the presence of AR. However, when -244/-96 PB-TK-CAT was transfected into DU-145-D cells, cotransfected with the rAR expression vector and treated with DHT, an increase in CAT activity was observed (3869 ± 550 dpm/min/mg protein, figure 18). This resulted in a 7.6 fold change in CAT activity.

However, the DU-145-D cells were cultured differently than standard culture conditions in our lab. Normally, we

supplement cells with the same concentration of L-glutamine and Penicillin G. The DU-145-D cell line had been previously cultured in media supplemented with antimycotic/antibiotic (Gibco/BRL). Previous work in this lab had shown that PC-3 cells which had formerly been cultured in media containing antimycotic/antibiotic and then switched to media supplemented with L-glutamine and Penicillin G failed to survive. This suggests that different cell populations could be selected depending upon the type of antibiotic used. At the time we acquired DU-145 cells from Dr. P.S. Rennie (Cancer Center, Vancouver, British Columbia) (DU-145-R). Additionally, we ordered the DU-145 cells from the original source, ATCC, and called these cells DU-145-A.

Thus DU-145-R cells were transfected with -244/+28 PB-CAT and -244/-96 PB-TK-CAT, cotransfected with either the expression vector for rAR or rGR and treated with DHT or DEX, respectively (Table II). Three conclusions resulted from this data: (1) the -244/-96 PB-TK-CAT was preferentially induced by DEX (over DHT) by a factor of approximately 2 (compare lines 2 and 4, table 2). This result differs from the data obtained from the same transfections in PC-3 cells where the induction of -244/-96 PB-TK-CAT by rAR and rGR (in the presence of steroid) was equivalent (figures 5 and 8); (2) Androgen induced CAT activity of -244/+28 PB-CAT was greater than the CAT activity induced by glucocorticoids (table 2). This result was in agreement with data obtained from PC-3 cells (figures

7 and 8); (3) The -244/-96 PB-TK-CAT construct always exhibited greater CAT induced activity in DU-145-R cells compared to the -244/+28 PB-CAT construct (compare lines 1 and 3 with 2 and 4, table 2). This is in opposition to the results obtained in PC-3 cells (figure 8). However, before drawing conclusions from the data presented in table 2, it is important to focus on a limitation of these results. Only duplicate (**not** triplicate) assays of each transfections were performed (lines 1-4, table 2). Unfortunately, there was a substantial range in the CAT induced activity of two transfections. The DHT induced CAT activity of -244/+28 PB-CAT ranged from 619 to 2605 dpm/min/mg protein while the DEX induced CAT activity of -244/+28 PB-CAT ranged from 4067 to 11,203 dpm/min/mg protein. Consequently, this large range diminished the confidence in this data. Given this information, it is possible that the induction of -244/-96 by DHT and DEX could be equivalent.

In an attempt to validate or refute the results summarized in table 2, DU-145-A cells were transfected with -244/+28 PB-CAT and -244/-96 PB-TK-CAT, cotransfected with the expression vector for rAR and treated with DHT (figure 19). The DHT induced CAT activity of -244/-96 PB-TK-CAT (386 ± 8 dpm/min/mg protein, figure 19), exceeded that of -244/+28 PB-CAT (174 ± 74 dpm/min/mg protein, figure 19) by just more than two fold.

The fold change in CAT activity of -244/-96 PB-TK-CAT

induced by rAR for all three sublines of DU-145 cells are summarized in figure 20. Clearly, there is not a great difference between the fold change in CAT activity of DU-145-D (7.6 fold), DU-145-R (3.8 fold) and DU-145-A (1.3 fold). However, a pattern is suggested. As the number of cell passages increase and the culture conditions are changed, the DU-145 cells exhibit increased androgen responsiveness (figure 20). The DU-145-D cells were maintained under different culture conditions than DU-145-R and DU-145-A cells, and show the greatest DHT induced fold change in CAT activity (figure 20). It is likely that the DU-145-D and the DU-145-R cell population had been selected for lines that are more androgen responsive.

Unlike the PC-3 cells, the DU-145-R and DU-145-A cell lines exhibited preferential DEX over DHT induction of -286/+28 PB-CAT, whether expressed as CAT activity (figure 21) or fold change in CAT activity (figure 22). However, the CAT activity and the fold change in CAT activity exhibited by DU-145-A cells was diminished in comparison to DU-145-R cells (figures 21 and 22). Apparently, even with the presence of the endogenous PB promoter in a (human) prostatic carcinoma cell line, the -286/+28 PB-CAT was preferentially induced by DEX. Comparing the results (figure 21, 22 and Table II) suggest the DU-145 cells and PC-3 cells contain different transcription factors. The DNase I footprinting using nuclear extracts for each cell line may provide valuable clues as to the important

factors/DNA sequences responsible for preferential regulation by androgens in PC-3 cells.

Not surprisingly, in both DU-145-R (figure 24) and DU-145-A (figure 25) cells, MMTV-CAT was preferentially induced by DEX. However, DEX induced fold change in CAT activity (figure 25) of MMTV-CAT in DU-145-A cells was **almost double** that observed in DU-145-R cells. This was yet another difference between DU-145-R and DU-145-A cells. The CAT activity of the DU-145-R cells was always greater than the CAT activity exhibited by DU-145-A cells (figures 20-22, 24 and 25).

In summary, the data presented here show AR function preferentially over GR and PR on the PB promoter and that the PB promoter contains 2 AREs necessary, sufficient and responsible for transcriptional activation.

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