Expression and Localization of Androgen Receptor in the R-3327 Dunning Rat Prostatic Adenocarcinoma[1]

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ABSTRACT

The Dunning R-3327 rat prostatic adenocarcinoma and its sublines have been developed as a model system to study prostate tumor progression. We have used this system to study the changes in androgen receptor (AR) and AR mRNA expression which occur during tumor progression from androgen dependent to androgen independent growth. Dorsal prostate and all tumor sublines contained a 10-kilobase AR mRNA on Northern blot analysis. The levels of AR mRNA in each subline compared to dorsal prostate (100%) were: H (75%) > G (48%) > HI (25%) > HI-F = AT-1 = AT-3 = MAT-Lu = MAT-Ly-Lu = <5%. Immunocytochemistry showed AR predominantly in acinar epithelial cells of dorsal prostate and the androgen sensitive H subline. In the H subline, both acinar epithelial cells and locally invasive adenocarcinoma cells within the stroma showed positive immunostaining. The androgen responsive, anaplastic G subline also showed strong positive immunostaining. The androgen resistant AT-1 and MAT-Lu sublines lacked immunostaining for the AR. Steroid autoradiography revealed a similar cellular distribution of AR. These data suggest that in the Dunning system the loss of androgen binding and responsiveness is primarily due to selective changes in gene expression and not to gene rearrangements or posttranscriptional or translational modification of the AR mRNA or protein.

INTRODUCTION

Androgens act through an intracellular receptor to regulate differentiation, growth, and maintenance of the male reproductive tract (1). The prostate gland requires androgen for growth and maintenance of differentiated function and will undergo active regression if androgen is withdrawn (2). Human prostate cancers undergo a change in androgen sensitivity during tumor progression. Most human prostatic adenocarcinomas require androgen for growth, and their growth can be arrested by androgen withdrawal. However, late-stage human prostatic tumors exposed to estrogen or androgen ablation therapy generally become androgen independent (3, 4). In a rat model for prostatic adenocarcinoma, a similar transition to androgen independence is often accompanied by a dramatic decrease in nuclear androgen receptor levels, as measured by ligand binding (5). The molecular mechanisms underlying this transition to androgen independence in cancer of the prostate are not understood.

A useful model for investigating the molecular basis of androgen sensitivity is the R-3327 Dunning rat prostatic adenocarcinoma system. The original R-3327 tumor was a papillary adenocarcinoma which arose spontaneously in the dorsal prostate of an ageing Copenhagen rat (6, 7). The G and H sublines arose from separate pieces of the parent tumor which were grown in male Copenhagen rats (7). The H tumor has given rise to several other sublines which fall into two pathways of tumor progression: H to HI to HI-F to AT-3; H to AT-1 to MAT-Lu and MAT-Ly-Lu. The Dunning sublines became more aberrant as they progressed along these pathways. These tumor sublines have now been extensively characterized and cover the full spectrum of progression from tumors which are phenotypically well differentiated, androgen sensitive, diploid, and rarely metastatic to tumors which are anaplastic, androgen independent, aneuploid, and highly metastatic (6, 8, 9). The androgen sensitive Dunning H subline contains clones of androgen dependent and independent cells but does not regress dramatically after androgen withdrawal. The G subline can grow in the absence of androgen but grows more rapidly in the presence of androgen (androgen sensitive), the HI, HI-F, MAT-Lu, MAT-Ly-Lu, AT-1, and AT-3 sublines grow in the absence of androgens (androgen independent) (5, 6, 10). The H subline continues to spontaneously progress toward less differentiated, androgen independent forms when grown in castrated male rats or nude mice (11). Ligand binding studies have suggested that AR[6] levels decrease in parallel with progression to the androgen insensitive state (Table 1). Thus, the Dunning tumors are a useful model system for studies of the relationship between androgen responsiveness, androgen receptor content, and tumor progression.

The regulatory changes which lead to decreased androgen binding activity could occur at the transcriptional, translational, or posttranslational levels. Alternatively, a change in AR gene structure could account for altered androgen binding activity. The recent cloning of cDNAs encoding the rat (12) and human (13) androgen receptors has enabled us to examine AR gene structure and steady state mRNA levels in normal prostate tissue and the Dunning tumor sublines. We have also examined AR protein distribution in selected Dunning sublines by using immunocytochemistry and steroid autoradiography.

MATERIALS AND METHODS

Tumors. All solid tumors were kindly supplied by Dr. John Isaacs (Johns Hopkins University, Baltimore, MD) and were maintained by serial passage in inbred male Copenhagen rats. The Copenhagen rats used as tumor bearers were obtained from the Charles River Breeding Laboratories (Wilmington, MA). Athymic (*nu/nu*) mice were used as host animals for all steroid autoradiography studies (14) in order to ensure uniform small tumor size which prevents artifacts due to focal necrosis. Tumor transplants (s.c.) were performed as previously described under ketamine/acepromazine anesthesia (15). All tumors were excised from anesthetized animals under sterile conditions, quick frozen in liquid nitrogen, and stored at −70°C. Characteristics of the eight sublines used in this study are summarized in Table 1.

Table 1 *Characteristics of R3327 Dunning tumor sublines*

Subline	Phenotype[a]	Androgen sensitive[a]	AR content[b]	Genotype[c]	AR mRNA[d]	Actin mRNA[d]
Dorsal prostate	Normal	Yes	1015 ± 111	Diploid	100	100
H	Well differentiated	Yes	262 ± 20	Diploid	75	255
HI	Well differentiated	No	197 ± 48	Diploid	25	159
HI-F	Poorly differentiated	No	75 ± 10[e]	Aneuploid	<5	215
G	Anaplastic	Responsive	1198 ± 395	Aneuploid	48	102
AT-1	Anaplastic	No	<50[e]	Aneuploid	<5	276
MAT-Lu	Anaplastic	No	<50[e]	Aneuploid	<5	105
MAT-Ly-Lu	Anaplastic	No	<50[e]	Aneuploid	<5	204
AT-3	Anaplastic	No	<50[e]	Aneuploid	<5	238

[a] Data from Ref. 6.
[b] Total cellular AR, expressed as fmol/mg DNA from saturation analysis (5).
[c] Data from Ref. 8.
[d] Expressed as a mean percentage of dorsal prostate values from two separate experiments.
[e] Cytosolic AR, expressed as fmol/10⁸ cells, from saturation analysis of tumors from 1 day castrated rats (34).

Isolation and Analysis of mRNA. Frozen tumors were pulverized in liquid nitrogen and homogenized in 4 M guanidine thiocyanate, pH 7.0. Total RNA was obtained by pelleting through 5.7 M cesium chloride (16). Polyadenylate containing RNA was obtained by oligodeoxythymidylate cellulose chromatography (17). RNA samples were denatured with glyoxal and dimethyl sulfoxide for 1 h at 50°C (18) and were size fractionated by electrophoresis on 1% agarose gels containing 10 mM sodium phosphate, pH 6.8. Samples were transferred to 0.2-μm nylon membranes (19) and were cross-linked by brief exposure to UV light (20). DNA hybridization probes were labeled to high specific activity with ^{32}P by nick-translation and were used in hybridization reactions as previously described (21). Autoradiographic data were quantitated with an LKB Ultrascan densitometer.

Isolation and Analysis of DNA. High molecular weight DNA was isolated from frozen Dunning tumors and normal dorsal prostate tissue (27). Aliquots of DNA were digested to completion with *EcoRI*. DNA fragments and ^{32}P-labeled molecular weight markers were size fractionated by electrophoresis in agarose gels, transferred to nylon membranes as previously described, and hybridized (21, 23).

Hybridization Probes. Probes used for hybridization were chicken β-actin cDNA (Oncor, Gaithersburg, MD) (24) and human androgen receptor cDNA, 0.7-kilobase *Hind*III to *EcoRI* fragment of ARHFL1 clone (13).

Immunocytochemistry. Antisera directed against the AR were raised in rabbits with a synthetic 15 amino acid peptide (12). IgG fractions of preimmune and immune sera used for immunostaining were obtained by protein A-agarose chromatography. The Vectastain ABC (Vector Laboratories, Burlingame, CA) method was used for immunocytochemical demonstration of the androgen receptor in tissue sections which had been fixed with 4% paraformaldehyde (12, 25). Controls included substitution of preimmune rabbit serum for the antiserum and absorption of the primary antiserum with excess immunogen.

Steroid Autoradiography. The binding of radiolabeled androgen to the androgen receptor was evaluated in samples of H, G, MAT-Ly-Lu, MAT-Lu, and AT-1 tumors growing in athymic (*nu/nu*) mice (14). Host animals were castrated 24 h prior to sacrifice to reduce circulating androgen levels. Small samples of each tumor and control dorsal prostate tissue were excised, minced into 1-mm³ mieces, rinsed, and immersed in RPMI 1640 culture medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 5 nM [³H]DHT (210 Ci/mmol; New England Nuclear, Cambridge, MA). Tissues were incubated 1 h in a shaking water bath (37°C), rinsed, and processed for autoradiography as previously described (14). The specificity of [³H]DHT binding was checked by running parallel incubations in the presence of 100-fold molar excess of unlabeled DHT.

RESULTS

The expression of androgen receptor mRNA was determined by Northern blot analysis of polyadenylate containing RNA samples isolated from the R-3327 Dunning tumor sublines and from normal dorsal prostate tissue (Fig. 1*A*). An androgen
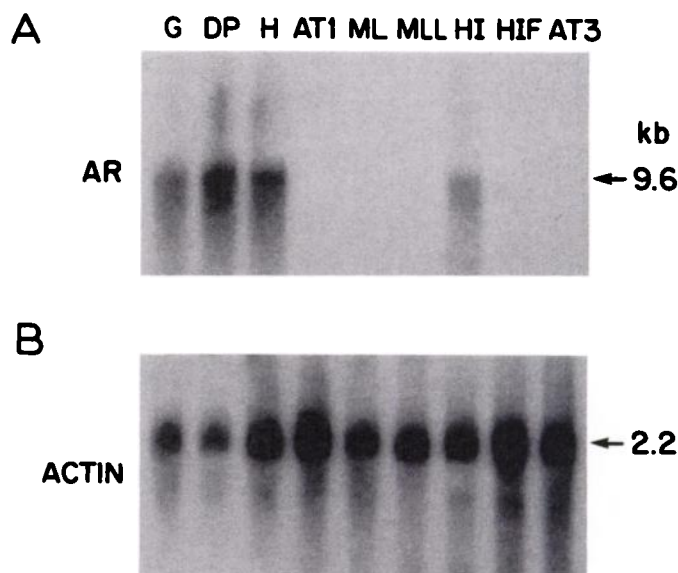

Fig. 1. *A*, Northern blot analysis of androgen receptor mRNA in dorsal prostate and Dunning tumor variants. Aliquots of polyadenylate containing RNA (15 μg) isolated from the Dunning tumor variants were analyzed by electrophoresis and blot hybridization by using a ^{32}P-labeled human androgen receptor cDNA probe. RNA samples are shown from the G tumor, normal dorsal prostate (*DP*), H, AT-1, MAT-Lu (*ML*), MAT-Ly-Lu (*MLL*), HI, HI-F, and AT-3 tumors. The size of the AR mRNA was estimated by comparison with ^{32}P-labeled molecular weight markers derived from *Hind*III cut λ-DNA. *B*, the same filter was stripped and rehybridized to an actin cDNA probe. *kb*, kilobases.

receptor mRNA of approximately 10 kilobases was detected at varying levels in all tumor sublines and in the dorsal prostate. The steady state level of AR mRNA was highest in dorsal prostate and was easily detected in the G, H, and HI tumors. Extended exposure of the filters revealed low levels of the 10-kilobase AR mRNA in the AT-1, MAT-Lu, Mat-Ly-Lu, HI-F, and AT-3 tumors (data not shown). There was no significant hybridization of the AR probe to any other RNA. To confirm the integrity of the RNA samples, filters were rehybridized to an actin probe (Fig. 1*B*). Actin mRNA (2.2 kilobases) was present in all sublines, indicating that the low levels of AR mRNA in the AT-1, MAT-Lu, MAT-Ly-Lu, HI-F, and AT-3 sublines were not due to absence or degradation of RNA. Relative levels of actin and AR mRNA in the R-3327 variants are compared with dorsal prostate in Table 1. As expected, the H and G sublines had the highest levels of AR mRNA; these sublines also contain the highest androgen receptor levels and are responsive to androgen (Table 1). The HI tumor contained a relatively high level of AR mRNA, in agreement with the presence of androgen receptors. Growth of the HI tumor is androgen independent. The remaining tumors contained low

levels of AR and its mRNA, and are categorized as androgen independent.

Immunocytochemical analysis of AR was performed with an antibody raised against a synthetic peptide whose amino acid sequence corresponded to part of the rat AR. In the dorsal prostate, most acinar epithelial cell nuclei and some stromal fibroblast nuclei stained positively for AR (Fig. 2A). The well differentiated H tumor showed a positive but heterogeneous staining pattern for AR (Fig. 2C). The nuclei of large adenocarcinoma cells found in the stromal compartment of the H tumor were stained more intensely than the acinar epithelial cell nuclei. Immunostaining was not observed in control sections using either preimmune serum (not shown) or antiserum

preabsorbed with the immunogen (Fig. 2C, inset). The intense AR immunostaining in dorsal prostate and the H tumor correlates with the high levels of AR mRNA seen by Northern analysis.

In a parallel study AR was localized in the dorsal prostate and Dunning tumor sublines by autoradiography of [³H]DHT binding. In the dorsal prostate, silver grains were primarily distributed over the acinar epithelial cells, and some stromal cells (Fig. 2B). In the well differentiated H tumor (Fig. 2D), locally invasive epithelial cells found within the stromal compartment were more heavily labeled than the acinar epithelial cells. In competition studies excess unlabeled DHT blocked binding of [³H]DHT and tissue labeling, indicating that tissue
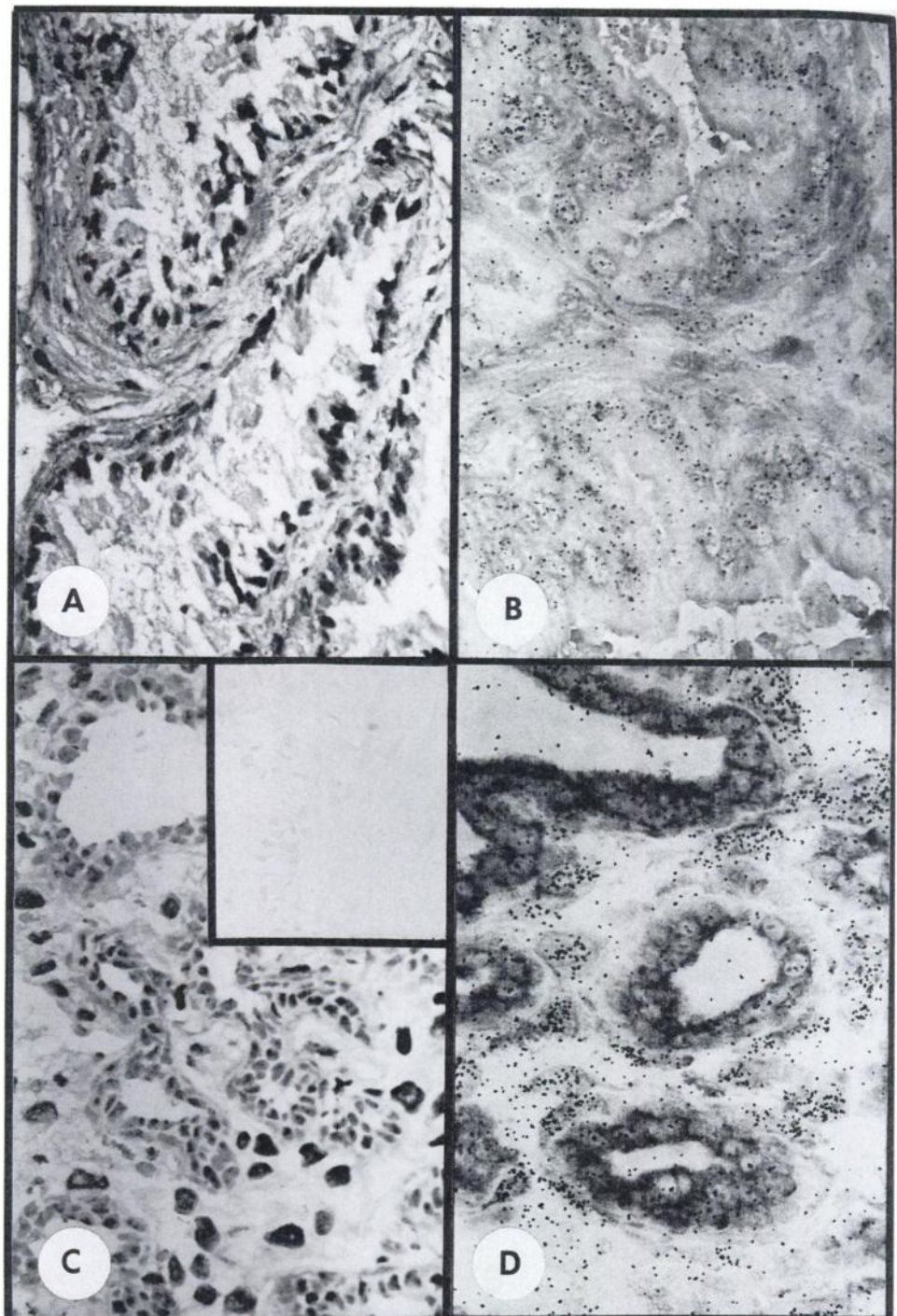

Fig. 2. Localization of AR in dorsal prostate and the Dunning R-3327 H tumor subline by immunocytochemistry and [³H]DHT autoradiography. Frozen sections of dorsal prostate and the androgen sensitive Dunning H tumor were immunostained as described previously (12) with an antibody which reacts with rat AR. All immunostained sections shown are magnified × 700 and were not counterstained. [³H]Dihydrotestosterone binding to AR was assessed by steroid autoradiography of tissue sections which were labeled in vitro with [³H] DHT (26). All autoradiographs are magnified × 1000; sections were exposed for 16 days, and were counterstained with methyl green pyronin. A, immunostained section of rat dorsal prostate; B, [³H]DHT uptake in a comparable section of dorsal prostate; C, immunostained section of R3327H tumor; inset, control section stained with antigen-absorbed antiserum; D, [³H]DHT uptake in a comparable section of R3327H tumor.

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labeling probably represented binding of the radiolabeled androgen to AR.

Three anaplastic Dunning tumor sublines were also analyzed by immunocytochemistry. The androgen responsive G subline showed strong positive AR immunostaining (Fig. 3A), whereas the androgen insensitive MAT-Lu and AT-1 sublines did not (Fig. 3, C and E). The intense AR immunostaining in the G subline correlates with the high level of AR mRNA seen by Northern analysis. Similarly, the absence of AR immunostaining in the MAT-Lu and AT-1 sublines correlates with the very low level of AR mRNA in these sublines. Steroid autoradiography showed that the G subline had a relatively uniform distribution of labeled cells (Fig. 3B). One sample of the AR negative MAT-Lu tumor was found to contain a few weakly labeled cells (Fig. 3D), although this represented a minority of cells in one tumor. The AR negative AT-1 tumor had a random silver grain distribution with no labeled cells, suggesting that there was no specific uptake of radiolabeled DHT in this tumor (Fig. 3F).

Two of the Dunning tumor sublines (HI and HI-F) contain measurable AR but are not androgen responsive. This paradox could be explained by an alteration in AR gene structure. AR
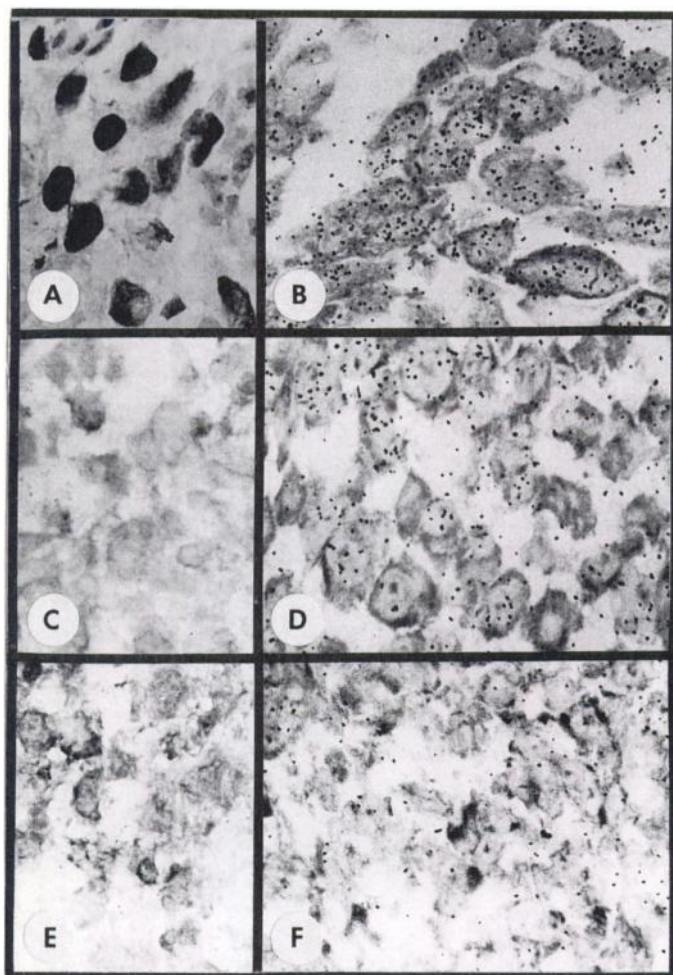

Fig. 3. Localization of AR in anaplastic Dunning sublines by immunocytochemistry and [³H]DHT autoradiography. Frozen sections of the Dunning G, MAT-Lu, and AT-1 sublines were analyzed by immunocytochemistry or autoradiography as described in Fig. 2. All immunostained sections are magnified × 600, autoradiographs are magnified × 900. A, immunostained section of the anaplastic, androgen sensitive G tumor; B, [³H]DHT uptake in a comparable section of G tumor; C, immunostained section of the anaplastic, androgen insensitive MAT-Lu tumor; D, [³H]DHT uptake in a comparable section of MAT-Lu tumor; E, immunostained section of the anaplastic, androgen insensitive AT-1 tumor; F, [³H]DHT uptake in a comparable section of AT-1 tumor.

gene structure was investigated by Southern blot analysis of genomic DNA from R-3327 tumors. Digestion with *Eco*RI yielded restriction fragments of identical sizes and signal intensities for the tumors and normal dorsal prostate (data not shown), suggesting that the AR gene is not grossly rearranged or amplified in the R-3327 tumors.

DISCUSSION

This study was designed to examine AR expression and distribution in the R-3327 Dunning tumor system by using Northern analysis, immunocytochemistry, and steroid autoradiography. The level of AR mRNA expression in the Dunning tumor sublines broadly parallels their ability to respond to androgen stimulation. While the AR mRNA was the same size (10 kilobases) in hormonally sensitive and independent Dunning tumors, the hormonally independent tumors had considerably less AR mRNA and protein by all three criteria for measurement. The similarities in AR mRNA and gene structure reported here argue against any major alterations in AR among the Dunning tumor sublines. However, we cannot exclude the presence of point mutations in the AR gene which could generate partially active or inactive mutant receptors. For example, human mammary tumor biopsy samples which contain an estrogen receptor mRNA with a point mutation have low estrogen binding activity (26). In complete androgen insensitivity syndrome, ligand binding defects in the androgen receptor have been correlated with point mutations in the steroid binding domain of the AR gene (27–29).

Immunocytochemical studies with a peptide induced anti-AR antibody have localized the AR to epithelial cell nuclei in the dorsal prostate. This antibody recognizes the native AR, and has yielded similar results in immunostaining rat ventral prostate and benign prostatic hyperplasia (12, 30). These results are also in agreement with previous steroid autoradiography data (31). Although other laboratories have isolated autoimmune antibodies directed against the AR, this is the first AR antibody to prove useful in immunocytochemical studies (32, 33).

The well differentiated androgen sensitive H tumor contains relatively high levels of AR as determined by binding assays (5). Our data suggest that both the epithelial and stromal components of the H tumor contain AR positive cells, and that within the stromal compartment it is primarily the locally invasive large neoplastic epithelial cells which contain AR mRNA and protein. These cells have been identified as epithelial in origin on the basis of positive immunostaining for laminin, intermediate filaments, and prostatic acid phosphatase content (14). Further, identical cytomorphology and autoradiographic uptake patterns have been described in Dunning R3327-H tumors carried by Copenhagen rat hosts (34). Thus, it is unlikely that the large stromal cell population observed in the Dunning H tumors represents a contaminating stromal cell population originating from the athymic mouse hosts.

The anaplastic G tumor also contains very high levels of androgen binding; steroid autoradiography and immunocytochemistry both show that the AR is relatively uniformly distributed throughout this anaplastic tumor. Immunocytochemistry failed to detect AR in the anaplastic androgen independent MAT-Lu tumor, although steroid autoradiography revealed the presence of a few labeled cells in one tumor.

In the Dunning system, tumor sublines that lack androgen binding activity from biochemical studies or steroid autoradiography appear to lack AR by immunocytochemistry and AR mRNA by Northern analysis. However, androgen sensitivity is

not determined solely by the presence of androgen receptor mRNA and protein. The HI tumor is androgen insensitive although it contains high levels of AR and AR mRNA (5, 35). The Shionogi 115 mouse mammary tumor cell line progresses to a state of androgen insensitivity while retaining functional androgen receptors (36). Thus, the presence of androgen receptors is necessary but not sufficient for androgen responsiveness.

ACKNOWLEDGMENTS

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