The Mouse Androgen Receptor Gene Contains a Second Functional Promoter Which Is Regulated by Dihydrotestosterone†

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Received September 8, 1994®

ABSTRACT: The androgen receptor (AR) is a developmental and tissue-specific transcription factor which is activated by binding testosterone or dihydrotestosterone. Several different methods of transcriptional regulation of the AR have been shown, including regulation by androgens, follicle-stimulating hormone, epidermal growth factor, and the cAMP pathway. In order to further characterize the transcriptional regulation of the AR, portions of the mouse androgen receptor (mAR) promoter were cloned into the promoterless pBLCAT3 vector and assayed for chloramphenicol acetyltransferase activity. The results indicate that in addition to the previously characterized promoter (+1) there is a second distinct promoter located 3′ to the first promoter. Amplification of the 5′-end of the AR gene indicates that RNA originating from the second promoter is initiated from 162 and 170 bases downstream from the 5′-most previously characterized site. Northern blot analysis indicated that RNA initiated from the two promoters is differentially expressed in several cell lines and multiple tissues. Androgen ablation by castration showed that both promoters are controlled by androgens in the kidney. Sequence analysis revealed that the second promoter does not contain a TATA or CAAT box. Further characterization of this promoter may provide important insights into the transcriptional regulation of the androgen receptor since previous studies have often included only the first promoter.

The androgen receptor (AR)[1] is a nuclear phosphoprotein that mediates the action of testosterone and dihydrotestosterone in target cells (Lindzey et al., 1994). The mechanism of AR-mediated action is thought to be similar to that of other steroid receptors which bind ligand, interact with specific sequences of DNA, and regulate transcription in either a positive or a negative manner (Simental et al., 1991). Steroid receptors appear to have many common regulatory features. The AR, estrogen receptor (ER), progesterone receptor (PR), and glucocorticoid receptor (GR) all possess promoters which are GC-rich and lack TATA boxes (Faber et al., 1991; Keaveney et al., 1991; Huckaby et al., 1987; Zong et al., 1990). In addition, the PR, GR, and ER have previously been shown to contain multiple promoters (Kastner et al., 1990; Strahle et al., 1992; Keaveney et al., 1991). Finally, the AR, ER, PR, and GR are autoregulated (Quarmby et al., 1990; Maxwell et al., 1987; Mullick & Katzenellenbogen, 1986; Rosewicz et al., 1988). It is likely that mechanisms of AR transcriptional regulation exist in addition to autoregulation. For instance, it has been shown that follicle-stimulating hormone (FSH), epidermal growth factor (EGF), dibutyryl-cyclic AMP (dbcAMP), and forskolin (FSK) can all regulate transcription of AR (Blok et al., 1989; Henttu & Vihko, 1993; Lindzey et al., 1993). Further characterization of the mouse androgen receptor (mAR) promoter will help to fully define factors involving in regulation of the AR.

Previously, a 1.5 kb *Eco*RI fragment located 5′ to the start of translation had been isolated from an EMBL3 mouse genomic library (Clonetch Laboratories) using the 5′-most *Eco*RI−*Afl*II fragment of the mouse AR cDNA as a probe (He et al., 1990). The clone was sequenced from both directions and found to extend 1568 bp upstream from the ATG translation start site and contains the previously characterized promoter (Faber et al., 1991). We have designated this region promoter 1. Sequence analysis of this region revealed no TATA or CAAT boxes. The mAR promoter does contain an initiator (CTTTCCACCTCCA) preceding the +1 site of transcription and a perfect GC box (consensus GGCGGG) in a GC-rich region approximately 45 bp upstream. The initiator and GC box are both required for transcription from some TATA-less genes (Smale & Baltimore, 1989; Courey et al., 1989). In addition, the mAR promoter has a homopurine region that is 54 bp long and contains six GGGGA repeats. Promoter 1 contains two main sites of transcription initiation that are 13 bp apart. One of these sites has been shown to be regulated by the GC box when assayed by S1-nuclease protection (Faber et al., 1993). In addition, promoter 1 is utilized by diverse human tissues and cell lines (Tilley et al., 1990). The 5′-most nucleotide that serves as a transcription start site has been designated +1 (Faber et al., 1991). The current study identifies a second

† This work was supported by NIH Grants DK47592, HD09140, and CA58225.

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® Abstract published in *Advance ACS Abstracts,* November 1, 1994.

[1] Abbreviations: AR, androgen receptor; mAR, mouse androgen receptor; DHT, dihydrotestosterone; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; AIS, androgen insensitivity syndrome; ER, estrogen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; FSH, folicle-stimulating hormone; EGF, epidermal growth factor; dcbAMP, dibutyryl-cyclic AMP; FSK, forskolin; mARS, mouse androgen suppressor; SFCS, charcoal-stripped fetal calf serum; SHS, charcoal-stripped horse serum.

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distinct promoter and further characterizes the regulation of the mAR through the use of this site.

MATERIALS AND METHODS

Construction of CAT Expression Plasmids. In order to characterize the mouse AR (mAR) promoter, a 1.5 kb fragment of the 5′-flanking region of the mAR gene was used as a template to generate PCR products. The 5′-most nucleotide that serves as a transcription start site has been designated +1 (Faber et al., 1991). The 5′ primers contain *Bam*HI enzyme restriction sites, and the 3′ primers contain *Xho*I enzyme restriction sites. Following amplification, the fragments were cut with *Bam*HI and *Xho*I and then ligated into pBLCAT3. Conditions were as described in the GeneAmp PCR reagent kit (Perkin Elmer Cetus) with the following modifications: the total volume of each reaction was 100 μL, the polymerase enzyme was Pfu (Strategene), and 10× buffer 2 contained 200 mM Tris-HCl, pH 8.8, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 15 mM $MgCl_2$, and 1% Triton X-100. The DNA was purified using a Gene Clean kit (BIO 101 Inc.), digested with *Bam*HI and *Xho*I, Gene Cleaned again, and ligated into the promoter-less pBLCAT3 vector. Clones were sequenced using the fmol sequencing system (Promega).

Cell Culture and Transfection Procedure. GT1-7 and αT3-1 cells (kindly provided by Dr. P. Mellon) express AR transcripts as shown by analysis of steady-state levels of RNA on a Northern blot. The cells were grown to confluency in T-175 flasks using DMEM (with pen/strep) with 5% charcoal-stripped fetal calf serum (SFCS)/5% charcoal-stripped horse serum (SHS). The cells were harvested using trypsin/EDTA, washed (2×), and counted. Cells (20×10^6) were pelleted, resuspended in 400 μL of 0.1% glucose/PBS containing 15 mg of CAT vector and 5 mg of RSV-β-GAL vector, and electroporated at 960 mF and 0.35 kV. The cells were incubated on ice for 7 min, resuspended in 1 mL of PBS with 4% SFCS, incubated at room temperature for 7 min, and resuspended in 42 mL of DMEM with 5% SFCS/5% SHS. Ten milliliters of cell suspension was plated into each of four 100 mM culture dishes and harvested 48 h later with PBS 2 mM EDTA. Cells were lysed in 100 mM Tris (pH 7.8)/0.1% Triton X-100, and the resulting supernatant was assayed for CAT, RSV-β-GAL activity, and protein. In brief, the CAT assay consisted of 2−10 μg of protein in Tris/Triton buffer placed in a scintillation vial and incubated at 70 °C for 10 min. The mixture was cooled to room temperature, 100 μL of AcCoA cocktail [2.5 mM chloramphenicol, 0.1 M Tris (pH 7.8), and 2 μL of [^{3}H]AcCoA (5 Ci/mM)] was added, and 2 mL of Ecoscint O scintillation fluid was overlaid. After acetylation, the ^{3}H entered the organic phase and was counted a minimum of 5 times over 2−6 h. CAT activity was calculated by linear regression and normalized to RSV-β-GAL values in the GT1-7 cells or protein in the αT3-1 cells.

RNA Preparations. Total RNA was isolated from tissues or cells by lysing the cells with 3 M LiCl/6 M urea. The samples were then homogenized on ice for 1 min with a polytron and incubated on ice for 3−18 h. The samples were then centrifuged 20 min at 25 000 rpm. The pellet was resuspended in ES (0.1% SDS/0.2 mM EDTA), extracted with phenol once and chloroform/isoamyl alcohol twice, and precipitated by adding 0.1 volume of 3 M NaOAc/HOAc (pH 5.2) and 2.5 volumes of EtOH. The RNA pellet was resuspended in water and quantitated.

Determination of Transcription Initiation Start Sites. The 5′-end of the mAR gene was obtained using the 5′-AmpliFINDER RACE method (CLONTECH) with the following modifications. In brief, 8 μg of total mRNA was used as a template for AMV reverse transcriptase to synthesize a single strand of cDNA which was primed by an mAR-specific primer (+803 to +819). The RNA was then hydrolyzed and excess primer removed. The AmpliFINDER anchor (3′-GGAGACTTCCAAGGTCTTAGCTATCACTTA AGCAC-5′) was then ligated to the 3′-ends using T4 RNA ligase. The ligation mixtures were diluted 1:20, and 1 μL was used for a PCR reaction as described above using the anchor primer (5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′) and the mAR-specific primer (+657 to +672). A second PCR reaction was then performed using 0.5 μL of the first reaction with the same anchor primer and a nested mAR primer (+512 to +529). The bands were excised, Gene Cleaned, and ligated into the TA cloning vector (Invitrogen). DH5α-competent cells (Gibco-BRL) were transformed. Isolated colonies were grown, and plasmid DNA was purified using an RPM kit (BIO 101). Sequencing was performed as previously described.

RNA Separation and Blotting. RNA was separated by electrophoresis on a 1% agarose gel which contained 2% formaldehyde using 1× MOPS buffer [0.02 M 3-(*N*-morpholino)propanesulfonic acid, 0.005 M NaOAc, and 0.001 M EDTA]. The RNA was then transferred to a Hybond-N membrane (Amersham) and UV-cross-linked to the membrane using a Stratalinker (Stratagene). The probes were labeled by denaturing 125 ng of DNA in 12 μL of water by heating to 100 °C for 3 min. Next, 4.5 μL of solution olb [0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP, 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol], 0.5 μL of Klenow (1 unit), and 3 μL of [α-^{32}P]-dATP were added. The probes were then incubated 45 min at 25 °C and purified over a Sephadex G-50 column. Blots were prehybridized for 1 h at 42 °C with Hybmix (45% formamide, 0.5% SDS, 10% Denhardt's solution, 10 mM phosphate buffer, 15% dextran sulfate, and 150 μg/mL salmon sperm DNA). Probes ($1 \times 10^{6-7}$ cpm/mL) were then denatured by heating at 100 °C for 3 min, added to the hybmix, and incubated at 42 °C for 18 h. The blots were then washed and exposed to X-ray film. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe used in Figure 5 was added concurrently with the AR probes but at a concentration of 1×10^5 cpm/mL. Densitometry analysis was performed using the AMBIS Optical Imaging System.

Evaluation of Dihydrotestosterone in Vivo. Adult male mice approximately 12 weeks old which averaged 28 g in weight were divided into three groups of three or more. The animals were either sham-operated (group 1) or castrated (groups 2 and 3). Beginning on day 2 and continuing every other day until day 14, the animals received sesame oil (groups 1 and 2) or sesame oil with 200 μg of dihydrotesterone (DHT) subcutaneously (group 3). On day 14, the animals were sacrificed, and tissues were snap-frozen using liquid nitrogen. Animal experiments were conducted in accordance with the principles and procedures as outlined by the NIH Guide for the Care and Use of Laboratory Animals.

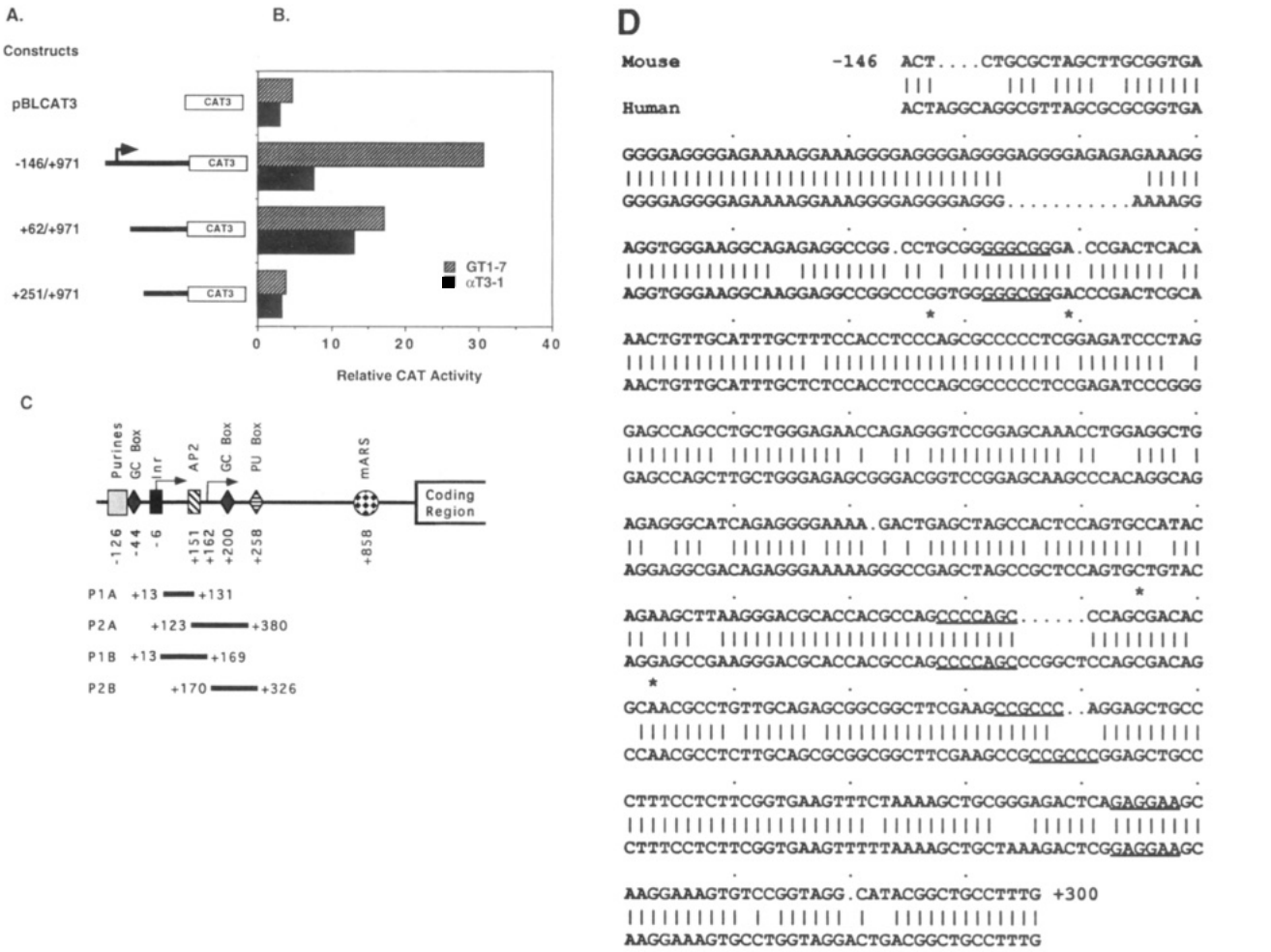

FIGURE 1: Promoter activity of the mAR 5′-region in αT3-1 and GT1-7 cells. (A) Scale diagram of deletion mutants of the mAR 5′-region transiently transfected into αT3-1 and GT1-7 cells. Bold lines represent regions of the 5′-flanking region inserted into the promoter-less pBLCAT3 vector. The exact bases inserted from the mAR 5′-flanking region are shown to the left of the schematics. The arrow represents the 5′-most site of transcription initiation (+1). (B) Bar graph of CAT activity of mAR promoter deletion mutants illustrated in (A). Bars represent the cpm per minute per microgram of protein for the αT3-1 cells or the cpm per minute per milliunit of RSV-β-GAL for the GT1-7 cells. A minimum of two separate electroporations were performed in both cell lines for each construct. (C) Diagram of the 5′-flanking region used to assay transcriptional regulation of the mAR is drawn approximately to scale with putative transcriptional elements listed above the schematic and the first nucleotide of each element below. Purines is a homopurine region, GC box is a consensus SP1 binding site, Inr is a possible initiator sequence, AP2 is a potential AP2 binding site, PU box is a PU.1/Spi-1 binding site, and mARS is the mouse androgen receptor suppressor binding site. Arrows show the sites of transcription initiation. The +1 site represents the 5′-most site of transcription initiation. The probes used for Northern blots are shown below the diagram. The coding region is shown as an open box. (D) The sequence of a portion of the mouse AR 5′-flanking region (top) is compared to the human AR 5′-flanking region (bottom). Numbers correspond to the mouse numbering where +1 is the 5′-most site of transcription initiation. Regions of homology are connected by bars. The sites of transcription initiation have asterisks over them. Putative regulatory elements are underlined and include an AP2 binding site (CCCCAGC), two GC boxes (GGGCGG, CCGCCC), and a PU box (GAGGAA).

RESULTS

CAT Assays Reveal the Presence of Two Promoters. In order to define the mAR promoter, we inserted several fragments of the 5′-untranslated region into the promoterless pBLCAT3 vector (Figure 1A). These constructs were transiently transfected into the mouse αT3-1 and GT1-7 cell lines, and CAT activity was assessed. Figure 1B illustrates the CAT activity of the constructs expressed as a fold increase over the pBLCAT3 vector alone. As expected, there was promoter activity in the −146/+971 construct which contains the previously described mAR promoter. The low level of CAT activity can be attributable to the mouse androgen receptor suppressor (mARS) which is located in the 5′-untranslated region of the construct (Grossmann et al., 1994). The +62/+971 construct contains none of the previously characterized transcription initiation start sites (Faber et al., 1991, 1993; Tilley et al., 1990) and was not expected to contain measurable promoter activity. Unexpectedly, the promoter activities of −146/+971 and +62/+971 constructs were similar. Removal of the region from +62 to +251 to create the +251/+971 construct removed all promoter activity. These results indicate that a second promoter may exist between +62 and +251.

In order to confirm the presence of a second promoter and define the relative basal levels of activity of the two potential promoters, three additional constructs were created using the pBLCAT3 vector (Figure 2A). Figure 2B shows that both the −146/+131 and the +123/+380 constructs contain promoter activity, providing additional verification of the presence of two promoters. Furthermore, the +123/+380 construct had a much higher level of basal activity than the −146/+131 construct. We have defined the region from −146 to +131 as mAR promoter 1 and +123 to +380 as mAR promoter 2. The −146/+380 construct shows that

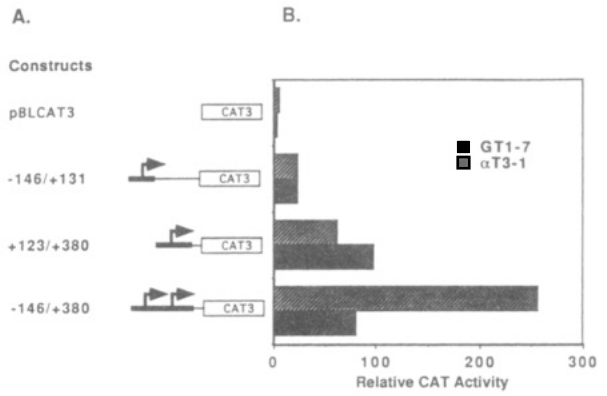

FIGURE 2: CAT assays showing two mAR promoters. (A) Schematic of mAR deletion mutants used to assay potential promoter activity of the mAR. Bold lines are a nonscale representation of the sequences inserted into pBLCAT3 vectors. Arrows indicate the two sites of transcription initiation. The 5′- and 3′-ends of each insert are shown to the left of the schematics. (B) Bar graph showing CAT activity of promoter deletion mutants illustrated in (A) using αT3-1 and GT1-7 cell lines. GT1-7 cell extracts were normalized for RSV-β-GAL and are shown as cpm per minute per milliunit of β-GAL. The αT3-1 cell extracts were normalized for protein and are shown as cpm per minute per microgram of protein. A minimum of two separate eletroporations were performed for each construct in each cell line.

the two basal promoters do not work in a synergistic manner in the αT3-1 cells but may in the GT1-7 cells, illustrating the possibility that different tissues may utilize the promoters in varying fashions.

Characterization of Start Sites of Transcription Initiation. In order to characterize the start sites for transcription initiation, the 5′-AmpliFINDER RACE method was performed using total RNA from mouse kidney tissue and αT3-1 cells. S1-nuclease protection assays were also attempted but were unsuccessful in showing either promoter 1 or promoter 2. The 5′-AmpliFINDER RACE is a modification of the SLIC (*s*ingle-strand *li*gation to single-stranded *c*DNA) and RACE (*r*apid *a*mplification of *c*DNA *e*nds) methods (Frohman et al., 1988; Edwards et al., 1991). One primer specific for the single-stranded anchor oligonucleotide which was ligated to the 3′-end of the cDNA corresponding to the noncoding strand and a second primer specific for the AR 5′-untranslated region from +514 to +529 were used in the final amplification. Figure 3 illustrates that both kidney and αT3-1 RNA could be used to generate two distinct PCR bands. The upper band is approximately 600 bp, and the lower band is approximately 430 bp. The length of these two PCR products supports the concept of two AR promoters located within the −146/+131 and +123/+380 regions described above.

In order to define the extract sites of transcription initiation, the two bands generated using the kidney RNA and the lower band from the αT3-1 RNA were excised and cloned into the TA Cloning vector. The DNA from isolated colonies was then sequenced to determine the exact start sites. A total of 11 clones from the upper band were sequenced. All clones contained the sequence ACTTAAGCAC which is specific for the anchor oligonucleotide and is not contained in the PCR primer. All 11 clones end at +13 which is 1 of the 2 main sites of transcription initiation that has been described previously. The lack of detection of the +1 site may be due to the differences between S1 protection assays and the 5′-AmpliFINDER RACE method or differential use

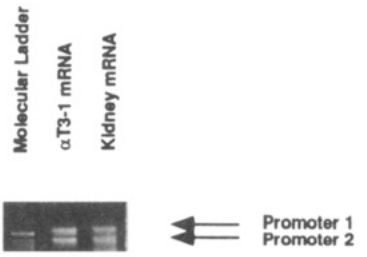

FIGURE 3: PCR amplification of the 5′-end of the mAR. PCR products generated using the 5′-AmpliFINDER RACE kit (CLONTECH) were loaded on a 1.5% agarose gel and visualized by ethidium bromide staining. A molecular weight marker was run to the right of the PCR products to estimate the sizes of the bands. Sizes of the molecular weight marker from top to bottom in base pairs (bp) are 1000, 517, 396, 344, and 298 bp. The mRNA used in each reaction is shown above the gel. The upper arrow on the right highlights a 600 bp band which corresponds to products originating from promoter 1. The lower arrow on the right highlights a 430 bp band which corresponds to products originating from promoter 2.

of the two sites in kidney tissue versus prostate tissue. The latter possibility seems especially likely since the two sites have been shown to be differentially regulated by the SP1 site (Faber et al., 1993).

To identify the transcription start sites for the second promoter, 21 clones from the lower band of the kidney material and 3 clones from the αT3-1 were isolated and sequenced. All clones contained the sequence specific for the anchor oligonucleotide. In addition, all of the clones contained AR gene sequences and ended at +170 or 162 for the kidney and αT3-1 material, respectively. These sites are in close agreement with the size estimated from the PCR bands in Figure 3 and indicate that the second promoter utilizes multiple sites for transcription initiation.

Differential Levels of Total RNA Are Initiated from the Two AR Promoters. In order to study whether various tissues or cell lines utilize the two promoters in different manners, two probes (P1A +13/+131 and P2A +123/+380; see Figure 1C) were labeled with ^{32}P and hybridized to a Northern blot which contained total RNA from several tissues and cell lines. Figure 4A shows that using the P1A probe and a low-stringency wash, AR message was detected only in the GT1-7 and αT3-1 cell lines and in Dunning tumor. The lack of a detectable message in the tissues does not indicate the total lack of AR mRNA initiated from the first promoter, but is an indication that mRNA initiated from this promoter may be only part of the total AR mRNA found in cells. Additionally, the AR mRNA observed in the GT1-7 cells is larger than that in the αT3-1 cells. The 2.2 and 2.45 kb bands in the GT1-7 and αT3-1 lanes appear to be due to cross-hybridization of the P1A probe with mRNA from the SV40 T-antigen gene. The SV40 T-antigen mRNA is present in the GT1-7 and αT3-1 cells because it was used to transform the cells (Windle et al., 1990; Mellon et al., 1990). Identical 2.2 and 2.45 kb bands were observed when the blot was stripped and hybridized to an SV40 T-antigen probe (data not shown). In addition, comparison of the DNA sequences of the P1A probe and SV40 T-antigen revealed a homologous region which is probably sufficient for hybridization to the SV40 T-antigen mRNA at the low wash stringency used for this experiment.

Figure 4B is the same blot as seen in Figure 4A after it had been stripped and rehybridized with the P2A probe.

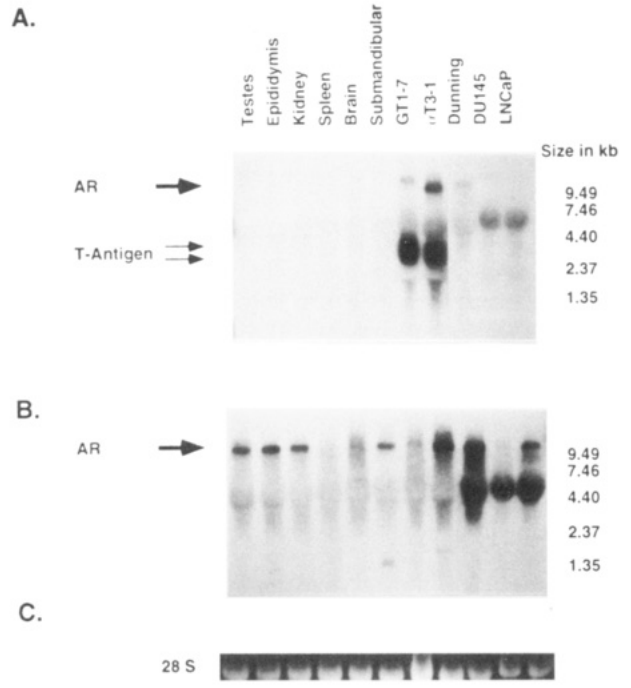

FIGURE 4: Northern blot hybridized with probes from the first and second mAR promoter regions. Total RNA was isolated from the tissues of adult male mice or from cell lines, and 20 μg was loaded in each lane. The RNA was separated on a 1% agarose gel containing 2% formaldehyde and transferred to a nylon membrane. The tissues and cell lines represented are illustrated above the lanes. The length in kilobases (kb) is shown to the right of the gel, and the bands which hybridized are highlighted to the left. (A) Northern blot which has been probed with the P1A probe. (B) Identical Northern blot which had been stripped and hybridized with the P2A probe. (C) Ethidium bromide staining of the 28S ribosomal band of the Northern gel used to produce the blot probed in (A) and (B).

Using the P2A probe, more AR message is detected in all tissues and cell lines except spleen and DU145 cells, neither of which expresses AR mRNA. Moreover, a comparison of Dunning tumor and αT3-1 cells indicates that the increase in the AR mRNA detected with the P2A probe is not equal for these cells. In addition, the AR mRNA detected in the GT1-7 cells with the P2A probe does not appear to increase when compared with the P1A probe and appears to be slightly different in size from the GT1-7 AR message detected with the P1A probe. Finally, a band can be seen between the 4.40 and 7.46 kb markers in the Dunning, DU145, and LNCaP lanes. This band corresponds to the site of the 28S ribosomal band and does not represent a specific band. All lanes in this figure are from the same blot and were treated in exactly the same manner, but the species differences created a cross-hybridization between the P2A probe and the 28S RNA. Comparison of Figure 4A and Figure 4B suggests that the two promoters are used differently by various cell lines. They also indicate that mRNA initiated from the first promoter is less abundant as compared to mRNA initiated from the second promoter region.

Effects of Androgen on Promoter Activities in Vivo. Male mice were used to determined the effects of androgen ablation and DHT treatment on the two promoters *in vivo*. One group of mice was sham-operated, a second was castrated, and a third was castrated and treated with DHT. After 14 days, the animals were sacrificed, and total RNA was isolated from the tissues.

In order to determine whether the animals were responding to castration and DHT substitution in a normal physiological manner, the seminal vesicles was collected, seminal fluid was extruded, and the weights of the seminal vesicles were normalized for the weights of the animals. The average weight of the seminal vesicles in the sham-operated animals was set at 100%. The average weight of the seminal vesicles from the castrated animals was 42%, and the average weight of the seminal vesicles from the castrated animals which received DHT was 94% as compared to the sham-operated animals. These results indicate that the animals were responding to the loss of androgens and treatment with DHT in a normal physiological manner.

In order to better assess androgenic effects on the two mAR promoters, probes (P1B +13/+169 and P2B +170/+326) of identical length were constructed (see Figure 1C). Total kidney and spleen RNA from the three groups of animals described above was probed using the P1B probe (Figure 5A) and a probe for rat G3PDH. An identical blot was probed using the P2B probe and the G3PDH probe (Figure 5B). These data show that the spleen does not express AR. However, the kidney does express AR RNA, and the level of expression is regulated by androgens (Figure 5A,B). Figure 5C shows mAR kidney RNA quantified by densitometry and expressed as counts × 100 in each lane after it has been normalized for the G3PDH in that lane. The data suggest that both promoters are down-regulated by DHT in the kidney. Moreover, a higher level of expression is associated with the second promoter even though hybridization conditions were equal or favored P1B; i.e., both probes were of equal length, equal counts were hybridized to the blots, the washing and exposure conditions were identical, and the GC content of the P1B probe was higher than for the P2B probe (62% and 59%, respectively). Due to the extremely small size of the mouse prostate, this tissue was not tested in these experiments. The results using the kidney tissues support the hypothesis that the mAR utilizes two promoters *in vivo* and that both promoters appear to be regulated by DHT.

While AR is regulated by androgens *in vivo*, it has not been shown to regulate expression using *in vitro* CAT assays. Since the original CAT assays were performed in media lacking androgens, additional experiments were performed using the GT1-7 cells to determine if the two promoters were androgen-responsive *in vitro*. The experiments (data not shown) indicated that addition of DHT for 48 h following transfection resulted in neither an increase nor a decrease in the level of CAT activity using any of the previously described constructs. Cotransfection of the AR promoter constructs with an AR expression plasmid to ensure that functional AR was present in the cell also created no response to DHT (data not shown). To confirm that the AR expression vector was functional, it was cotransfected with a MMTV CAT vector. Addition of DHT did cause activation of the MMTV CAT construct (data not shown). Therefore, it is likely either that additional sequences of the 5′-flanking region are necessary for DHT regulation or that transcription factors specific for DHT regulation of the mAR are not available in these cell lines.

DISCUSSION

These experiments demonstrate that the mouse androgen receptor contains two promoters. Both promoters lack

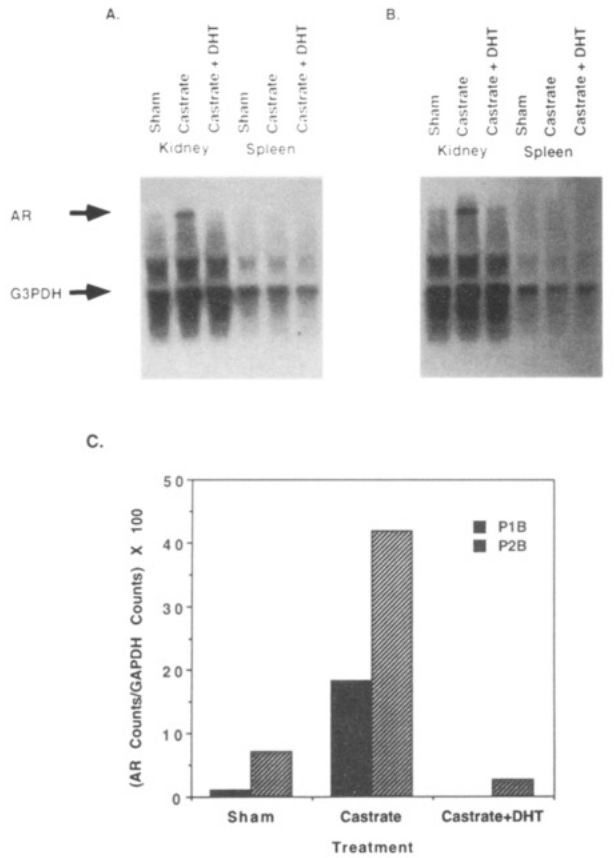

FIGURE 5: Northern blots and densitometry measurements elevating the effects of androgen ablation. Total RNA was isolated from the tissues of adult male mice which had undergone sham castration, castration, or castration with dihydrotesterone (DHT) replacement. Twenty micrograms of total RNA was loaded in each lane and separated on a 1% agarose gel containing 2% formaldehyde. (A) The RNA was then transferred to a nylon membrane and hybridized with the P1B probe. (B) An identical blot was also hybridized with the P2B probe. Exact bases of each probe are shown in Figure 4A. Each tissue with the treatments above is labeled over the lanes in which their RNA was run. The rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was probed at the same time and used for normalization. (C) Bar graph of the AR mRNA from the three groups of kidney tissues. An AMBIS optical imaging system was used to quantitate by densitometry the AR and G3PDH bands for each lane and express the density of each band as counts. The G3PDH was then used to normalize each AR band. Bands obtained using the P1B probe are illustrated as solid bars. Bands obtained using the P2B probe are illustrated as hatched bars.

consensus TATA and CAAT boxes. The second promoter contains multiple sites of transcription initiation that are located 162 and 170 bp downstream from the 5′-most site of transcription initiation in the first promoter. These promoters have been shown to be functional using *in vitro* and *in vivo* assays. In addition, both promoters appear to be regulated by androgen *in vivo*.

The androgen receptor has been shown previously to contain a promoter located approximately 1000 bp upstream from the site of translation which lacks both TATA and CAAT boxes (Faber et al., 1991, 1993; Tilley et al., 1990). Functional *in vitro* analysis using CAT assays to examine the mouse androgen receptor 5′ region in more detail revealed that a second promoter exists that had not been characterized previously. This promoter is located downstream from the first promoter. Using the 5′-AmpliFINDER RACE method, two sites of transcription iitiation were observed. This method is more sensitive than the primer extension technique since the extension step is followed by PCR amplification (Frohman et al., 1988). Previous work on the AR promoter 1 has often utilized S1-nuclease protection assays of mRNA obtained from cell lines or transiently transfected cell lines (Faber et al., 1991, 1993; Tilley et al., 1990). The data presented in this paper lend support to the idea that two AR promoters are present not only in cell lines but also in normal tissue. In addition, Northern blots using several tissues and cell lines showed that the two promoters appeared to be differentially expressed in some tissues and cell lines. Finally, Northern blot analysis showed that mRNA originating from both promoters appeared to be down-regulated by DHT *in vivo* in the kidney. Although it has been shown that prostate tissue is regulated by androgens, the extremely small size of the mouse prostate made it impractical for these experiments.

Sequence analysis of the mAR 5′ region revealed that the second promoter lacks both TATA and CAAT boxes. However, the second promoter does contain several potential transcription factor binding sites including an AP2 binding site, a GC box, and a PU box (Figure 1D). The GC box has been shown to be involved in the regulation of TATA-less promoters (Courey et al., 1989) and is also present in the first promoter. The PU box (GAGGAA) binds to the PU.1/Spi-1 protein which is a tissue-specific transcription factor that is related to the *ets* oncogene (Klemsz et al., 1990). It is possible that a member of this family can bind to this region. All of these elements are conserved between the mouse and human androgen receptors, and there is a 90% sequence homology overall, indicating that the human AR may also contain a second promoter.

Regulation of the androgen receptor by androgens *in vivo* has been observed by several investigators. It is thought that this autoregulation is a critical element in the overall regulation of the AR. Inclusion of the second promoter in future studies should enable researchers to better define the mechanism of androgen receptor regulation at the molecular level.

ACKNOWLEDGMENT

We thank Marceil Blexrud for her excellent technical assistance in conducting the experiments for this paper.

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