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Androgen-dependent Protein Interactions within an Intron 1 Regulatory Region of the 20-kDa Protein Gene*

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The 20-kDa protein gene is androgen regulated in rat ventral prostate. Intron 1 contains a 130-base pair complex response element (D2) that binds androgen (AR) and glucocorticoid receptor (GR) but transactivates only with AR in transient cotransfection assays in CV1 cells using the reporter vector D2-tkCAT. To better understand the function of this androgen-responsive unit, nuclear protein interactions with D2 were analyzed by DNase I footprinting in ventral prostate nuclei of intact or castrated rats and *in vitro* with ventral prostate nuclear protein extracts from intact, castrated, and testosterone-treated castrated rats. Multiple androgen-dependent protected regions and hypersensitive sites were identified in the D2 region with both methods. Mobility shift assays with ^{32}P-labeled oligonucleotides spanning D2 revealed specific interactions with ventral prostate nuclear proteins. Four of the D2-protein complexes decreased in intensity within 24 h of castration. UV cross-linking of the androgen-dependent DNA binding proteins identified protein complexes of approximately 140 and 55 kDa. The results demonstrate androgen-dependent nuclear protein-DNA interactions within the complex androgen response element D2.

Steroid hormone receptors regulate gene transcription through a multistep process that requires binding to DNA sequences known as hormone response elements (HREs)[1] (1). HREs consist of a 15-bp partial palindromic sequence 5′-AGNACAnnnTGTNCT-3′ and are found in a variety of genes regulated by a glucocorticoid receptor (GR), progesterone receptor (PR), or androgen receptor (AR). Studies on the androgen-regulated prostatein C3 subunit gene identified an androgen response element (ARE) within the first intron that also functions as a glucocorticoid response element (GRE) in transient cotransfection assays (2, 3). ARE/GREs in the MMTV-LTR, tyrosine aminotransferase, and prostate-specific antigen genes mediate transcriptional responses to AR and GR (4–8). A functional ARE has also been identified in the promoter region of the androgen-regulated aldose reductase-like protein from mouse vas deferens (9). Since different classes of steroid receptors co-exist in the same cell, the commonality of shared DNA consensus sequences raises the question as to how steroid-specific gene regulation is achieved.

ARE specificity has been described in larger complex elements such as those 5′ of the mouse sex limited protein (Slp) gene (10–15), in the promoter region of the rat probasin gene (16, 17), and in intron 1 of the rat 20-kDa protein gene (18). These complex elements contain one or more simple HREs that can bind AR and GR within the context of a larger element that binds other nuclear proteins. Androgen-specific responsiveness of complex AREs appears to involve DNA binding proteins that function in cooperation with AR (10–20). Functional analyses of control regions of the genes encoding sex-limited protein (10–15), prostate-specific antigen (8, 21), and the C3 subunit gene of prostatein (2, 22, 23) indicate that regulatory sequences outside the AR binding site contribute to the androgen response. Functional cooperativity is known to occur between HREs and recognition sequences for other transcription factors (24, 25). However, little is known about the characteristics of proteins that interact with complex AREs.

The 20-kDa protein gene codes for a major androgen-regulated secretory protein in rat ventral prostate and lacrimal gland (18, 19, 26, 27). Studies in our laboratory have shown that within the first intron of this gene is a complex enhancer that responds selectively to AR (Fig. 1). This 358-bp sequence, referred to as fragment In-1c, binds both AR and GR, but in CV1 cells, it confers only AR-dependent transactivation in transient cotransfection assays (18). In PC3 and HeLa cells, transactivation was observed with both AR and GR; however, AR-induced activation was greater than that of GR. A 130-bp subfragment of In-1c, referred to as D2, retained ARE specificity in all three cell lines. D2 mediates transactivation by AR but does not contain a strong consensus 15-bp partial palindromic response element. We have identified a novel AR binding sequence in the 5′-end of this fragment.[2]

Androgen regulation of the 20-kDa protein gene requires new protein synthesis. The 20-kDa protein gene mRNA decreased to 13% of control 2d following castration and required several hours of testosterone stimulation for return to the control level. The testosterone-induced increase in 20-kDa protein mRNA was blocked by cycloheximide, suggesting that rapidly turning-over proteins are required for AR transactivation (19). Since AR interacts directly with the D2 enhancer (18), the 20-kDa protein gene would be classified as a delayed primary response gene (28). D2 contains a number of inverted and

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[1] The abbreviations used are: HRE, hormone response element; AR, androgen receptor; GR, glucocorticoid receptor; PR, progesterone receptor; bp, base pair(s); ARE, androgen response element; GRE, glucocorticoid response element; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; MMTV-LTR, mouse mammory tumor virus-long terminal repeat.

[2] C. W. Gregory, K. B. Marschke, S. G. A. Power, M. C. W. Avellar, K.-C. Ho, and F. S. French, manuscript in preparation.

direct repeats that may serve as recognition sites for cell-specific proteins and provide secondary structure that influences D2 interactions with other transcription control proteins. Thus, characterization of rat ventral prostate nuclear proteins that bind D2 is important in understanding the function of this androgen-responsive unit in the regulation of transcription.

Here we report androgen-dependent nuclear protein interactions with the complex androgen response element D2 in the 20-kDa protein gene first intron. DNase I footprinting in isolated ventral prostate nuclei revealed protected and hypersensitive regions, consistent with androgen regulation of D2 chromatin structure. D2 binding of nuclear proteins was demonstrated by DNase I footprinting *in vitro* and gel mobility shift assays. UV cross-linking of D2-bound proteins identified androgen-regulated protein complexes of 140 and 55 kDa.

EXPERIMENTAL PROCEDURES

Animals and Treatment—Sprague-Dawley rats (250–300 g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were castrated through a scrotal incision made under anesthesia with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) and acepromazine maleate (Aveco Co., Inc., Fort Dodge, IA). Castrated rats were injected with testosterone enanthate (Schein Pharmaceutical, Inc., Port Washington, NY) (5 mg/kg subcutaneously) immediately after castration. Animals were sacrificed by decapitation 24 or 48 h post-castration. Tissues were collected, stripped of connective tissues, frozen in liquid nitrogen, and kept at −80 °C until preparation of nuclear extracts as described below.

DNase I Footprinting of ARE D2 in Isolated Nuclei Using Ligation-mediated PCR—Nuclei were isolated according to Rigaud *et al.* (29) and Scarlett and Robins (12) with the following modifications. Frozen tissue (1 g) from intact and 24- or 48-h postcastration rats was pulverized under liquid nitrogen, transferred to a 50-ml tube, and allowed to thaw on ice. All buffers used to isolate ventral prostate nuclei from intact rats contained 10 nM dihydrotestosterone (Sigma). After homogenization in 15 ml TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 1.5 mM NaCl, 60 mM KCl, 0.2% Nonidet P-40, 5 mM $MgCl_2$, and 5% sucrose, using 4 strokes of a Dounce homogenizer, samples were centrifuged at 500 × *g* for 10 min at 4 °C. Pellets were resuspended in 10 ml of TE buffer containing 1.5 mM NaCl, 60 mM KCl, and 3 mM $MgCl_2$. Centrifugation was repeated, the pellets containing nuclei were resuspended in 2 ml of buffer (10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 66 mM KCl, 1 mM $MgCl_2$), and nucleic acid was quantitated by absorption at $A_{260\ nm}$. Volumes were adjusted to 10 *A*/ml. Nuclei were aliquoted (250 μl), and $MgCl_2$ and $CaCl_2$ were added to achieve 1 and 0.5 mM, respectively. Aliquots containing 0–5 units of DNase I were incubated for 10 min at 4 °C. Cleavage reactions were terminated by addition of 50 μl of stop solution (100 mM EDTA, 4 μg/ml proteinase K) and incubation for 15 s at 50 °C. After addition of 200 μl of 2.5% sodium dodecyl sulfate and incubation at 50 °C for 2 h, 50 μl of stop solution was added, and samples were kept overnight at 50 °C. DNA was extracted twice with phenol, once with phenol/chloroform, and once with chloroform. Samples were dialyzed for 48 h with 3 changes of 500 volumes of TE buffer. DNA was digested with *Bam*HI (1 unit/μg) to decrease viscosity prior to ligation-mediated PCR. Untreated DNA (50 μg) was methylated with dimethylsulfate and cleaved with piperidine according to Hornstra and Yang (30) to serve as a G ladder for D2 sequence alignment.

The following primers were used for ligation-mediated-PCR (5′ to 3′ in the 20-kDa protein gene): D1–1 (nucleotides 2852–2875), to extend complementary to D2 sequence; D1–2 (nucleotides 2779–2801) for amplification and D1–3 (nucleotides 2775–2800) for labeling; reading 5′ to 3′ on the antisense strand of the 20-kDa protein gene sequence: D1–1, GACAACCTTCTTCAGACACACAC; D1–2: GTCGGAAGTGGAACCA-AGG and D1–3, TCAGGGTCGGAAGTGGAACCCACAG. DNA (2 μg) was denatured 5 min at 95 °C, and primer D1–1 (1 μM) was annealed for 30 min at 61 °C, followed by extension for 10 min at 76 °C using Deep Vent polymerase (New England BioLabs, Beverly, MA). After PCR, an overnight ligation was performed at 16 °C using linker primer (20 μM) and T4 DNA ligase (Boehringer Mannheim). DNA was precipitated, and PCR using a ligation primer and D1–2 was performed as follows: 1) denaturation at 95 °C for 4 min, annealing at 65 °C for 3 min, and extension at 76 °C for 2 min; 2) 18 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 2 min and extension at 76 °C for 3 min adding 5 s to each subsequent cycle; 3) extension for 10 min at 76 °C. DNA was labeled in PCR reactions using 2.5 pmol [γ^{32}P] ATP-kinased primer D1–3 purified from free nucleotides on Bio-Spin 30 columns (Bio-Rad). Two rounds of PCR (4 min at 95 °C, 2 min at 66 °C, and 10 min at 76 °C) were performed, and reactions were stopped by addition of sodium acetate to 0.3 M and EDTA to 1 mM. DNA was extracted with phenol/chloroform and precipitated with ethanol, followed by resuspension in formamide loading buffer and electrophoresis on 6% sequencing gels.

Nuclear Extract Preparation—1 g of frozen ventral prostate was pulverized under liquid nitrogen, placed in 10 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 10% glycerol, 3 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 μM Pefabloc®-SC (Boehringer Mannheim), 20 μg/ml pepstatin, 20 μg/ml leupeptin, and 10 μg/ml aprotinin), homogenized with 5 strokes of a Dounce homogenizer, and centrifuged at 1500 × *g* for 10 min at 4 °C. Pellets were resuspended in 3 volumes of extraction buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM KCl, 1.1 mM EDTA, 0.5 mM Pefabloc®-SC, 20 μg/ml pepstatin, 20 μg/ml leupeptin, 10 μg/ml aprotinin), homogenized with 5 strokes in a Dounce homogenizer, and placed on ice. The suspension was frozen in dry-ice/ethanol, thawed on

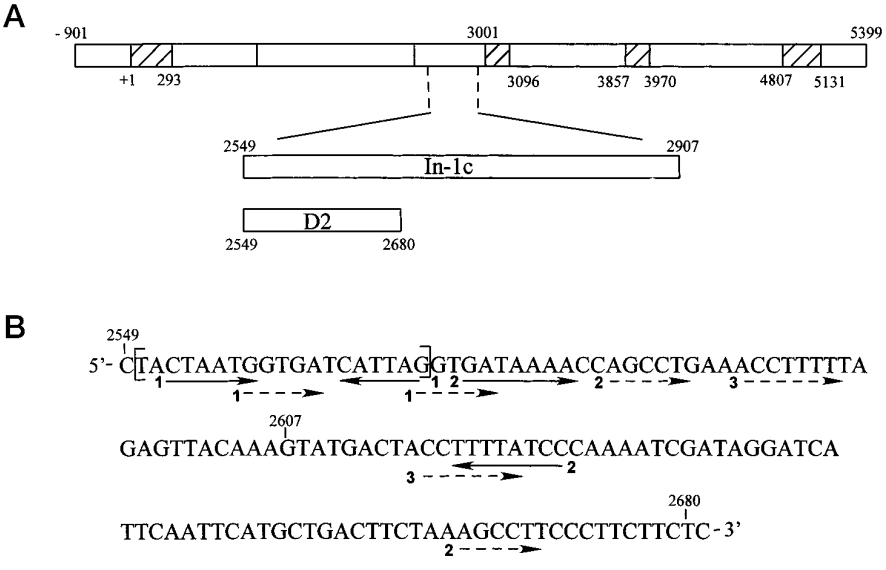

FIG. 1. **Schematic representation of 20-kDa protein gene.** Schematic diagram of the 20-kDa protein gene (*A*). *Cross-hatched bars* are exons; *open bars* are 5′-flanking, introns 1, 2, 3, and 3′-flanking regions; *residue numbers* are indicated relative to the predicted mRNA start site. The first intron fragments In-1c and D2 are shown, as described by Ho *et al.* (18). Nucleotide sequence of the D2 fragment of the 20-kDa protein gene is shown in *panel B*. The *numbers* indicate nucleotide position according to sequence. The inverted and direct repeats are indicated by *solid* and *broken lines*, respectively; *brackets* indicate the position of the AR binding site (see Footnote 2).

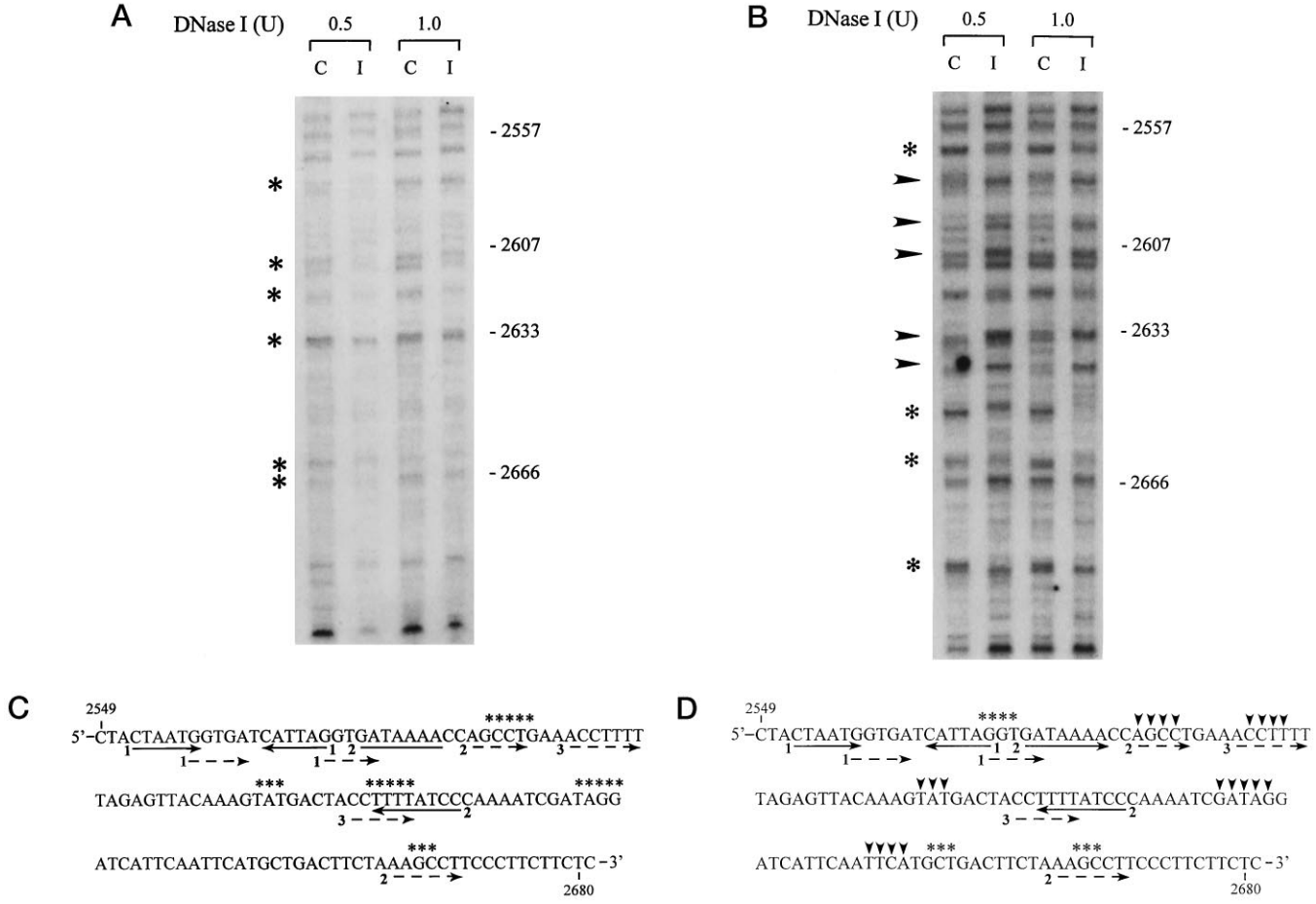

FIG. 2. **DNase I footprinting of the D2 fragment in isolated nuclei from rat ventral prostate.** Nuclei isolated from ventral prostate from intact (*I*) or 24 (*panel A*) and 48 h (*panel B*) postcastrate rats (*C*) were incubated in the presence of different concentrations of DNase I (indicated in units, *U*). DNA was isolated, and the D2 region in intron 1 of the 20-kDa protein gene was analyzed by ligation-mediated PCR, as described under "Experimental Procedures." *Numbers* on the *right* indicate the nucleotide position in the 20-kDa protein gene according to Ho *et al.* (18). Protected bases (*asterisks*) and hypersensitive sites (*arrowheads*) present in the nuclei from intact rats are indicated on the *left*. A schematic representation of the results is shown for each treatment (*panels C* and *D*). Direct and inverted repeats present in the D2 nucleotide sequence are indicated by *broken* and *solid lines*, respectively. The results shown are representative of three different experiments.

ice for 30 min, resuspended with a Dounce homogenizer, and centrifuged at 100,000 × *g* for 30 min at 4 °C. Nuclear extract pellets were resuspended in and dialyzed against DNA binding buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) for 4 h at 4 °C.

DNase I Footprinting of D2 in Vitro—D2 was obtained by digestion of the reporter vector D2-ptkCAT (18) with *XBa*I and 3′-end-labeled using [α-^{32}P]dCTP (3,000 Ci/mmol) and the Klenow fragment of DNA polymerase I (Promega Corp., Madison, WI). A second restriction digestion with *Hin*dIII yielded a labeled 130-bp fragment (D2). D2 was purified by electrophoresis on a 5% polyacrylamide gel and isolated by electroelution. DNA-binding reactions were carried out in 20-μl volumes with different amounts of nuclear extract, 2 μg of poly(dI-dC), and 30 μg of bovine serum albumin in DNA binding buffer. After 15 min on ice, ^{32}P-labeled D2 (40,000 cpm) was added and then incubated for 30 min on ice. $MgCl_2$ and $CaCl_2$ (2 mM) were added to the reaction mixture with DNase I (2 units), and the digestion was allowed to proceed for 3 min at room temperature. DNase I digestion was stopped by the addition of 4 μl of EDTA (50 mM). Samples were extracted with phenol/chloroform, and D2 was precipitated, rinsed with 70% ethanol, dried, and resuspended in 15 μl of formamide loading buffer. Samples were denatured for 5 min at 95 °C and analyzed on an 8% sequencing gel together with G and G + A chemical sequencing ladders prepared according to Maxam and Gilbert (31). The gel was transferred to Whatman 3MM paper, dried, and autoradiographed at −80 °C using an intensifying screen.

Mobility Shift Assays of Nuclear Protein Binding—Nuclear extracts were incubated with 2 μg of poly(dI-dC) and 30 μg of bovine serum albumin for 15 min on ice in DNA binding buffer with or without D2 oligonucleotide competitors (see below). The ^{32}P-labeled probe (10,000 cpm) was added, and samples were incubated for 15 min at room temperature. Protein-DNA complexes were resolved on 6% polyacrylamide gels (acryl/bisacrylamide ratio 37.5:1) buffered with 0.5 × TBE (10 mM Tris-HCl, 10 mM boric acid, 0.2 mM EDTA). Gels were transferred to Whatmann 3MM paper, dried, and autoradiographed with an intensifying screen at −80 °C.

D2 Oligonucleotides—Three oligonucleotide subfragments of D2 were chemically synthesized using the phosphoramide method and an Applied Biosystems Model 380B DNA synthesizer. Sense strand sequences are as follows: oligo A, 5′-CTACTAATGGTGATCATTAGGTGATAAAACCAGCCTGAAACCTTTT-3′, 20-kDa protein gene nucleotides 2549 to 2594; oligo B, 5′-TAGAGTTACAAAGTATGACTACCTTTTATCCCAAAATCGATAGG-3′, nucleotides 2595 to 2638; and oligo C, 5′-ATCATTCAATTCATGCTGACTCTAAAGCCTTCCCTTCTTCTC-3′, nucleotides 2639 to 2681. Oligonucleotides A, B, and C were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, annealed to the respective complementary oligonucleotide, purified on an 8% polyacrylamide nondenaturing gel, and isolated by electroelution. For competition studies, unlabeled double- and single-stranded oligonucleotides were used as competitors. A 100-bp fragment from the C3 gene (C3-C fragment) (2) was used in these studies as a nonspecific competitor.

UV Cross-linking Studies—Nuclear proteins were incubated with ^{32}P-labeled probes, and protein-DNA complexes were resolved on 6% polyacrylamide gels as described above. Gels were exposed to UV light (254 nm) for 30 min, and D2-protein complexes were visualized on overnight autoradiographic exposures. Bands were cut from the gel and homogenized in SDS sample buffer (0.25 M Tris-HCl, pH 6.8, 10% glycerol, 2.2% SDS, 1% β-mercaptoethanol). Proteins were resolved on 15% polyacrylamide denaturing gels. Controls were from lanes with ^{32}P-labeled probes either in the absence of nuclear extracts or in the

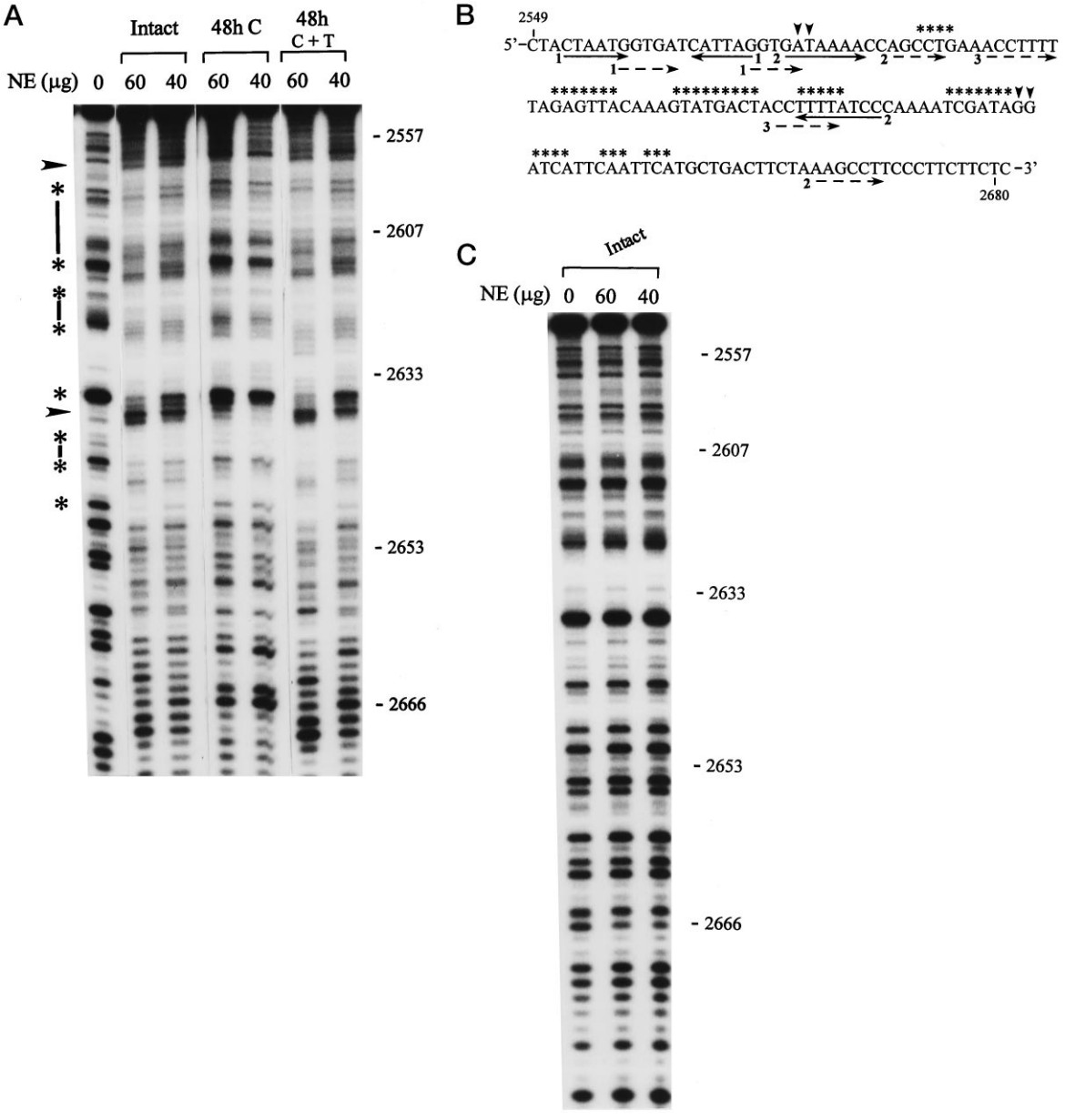

FIG. 3. **DNase I footprinting of the D2 sense strand *in vitro* with ventral prostate nuclear proteins.** D2 ^{32}P-end-labeled in the sense strand was footprinted *in vitro* using ventral prostate nuclear proteins from intact and 48-h postcastrate rats. The *lanes* contain ^{32}P-labeled D2 with no protein (0) and 60 or 40 μg of protein in ventral prostate nuclear extracts (*NE*) from intact, postcastrate (*48 h C*) or testosterone-treated castrate rats (*48 h C + T*). *A*, footprinting of D2 fragment with nuclear protein from intact and 48-h postcastrate rats. A G ladder was used for sequence alignment. Nucleotide numbers in D2 are indicated on the *right* of each panel. Protected bases (*asterisks*) and hypersensitive sites (*arrowheads*) induced by ventral prostate nuclear extracts from intact and testosterone-treated castrate rats are indicated on the *left*. *B*, schematic representation of the results. Direct and inverted repeats in the D2 nucleotide sequence are indicated by *broken* and *solid lines*, respectively. *C*, DNase I footprinting *in vitro* with kidney nuclear proteins from intact rats. The results shown are representative of three to five different experiments.

presence of nuclear extracts but with no UV light exposure. Protein size was determined using Rainbow™ high molecular weight range markers (Bio-Rad Laboratories).

RESULTS

DNase I Footprinting of the ARE D2 in Rat Ventral Prostate Nuclei Using Ligation-mediated PCR—The schematic shown in Fig. 1 demonstrates the structure of the 20-kDa protein gene. We used DNase I footprinting of D2 in isolated nuclei of rat ventral prostate and ligation-mediated PCR to detect androgen-dependent changes in chromatin structure. Nuclei were treated with DNase I, and ligation-mediated PCR was performed on isolated DNA using primer sets for amplification of the D2 region in intron 1 of the 20-kDa protein gene. DNase I cleavage sites in the sense strand of D2 indicate the presence of protected regions and hypersensitive sites in nuclei from intact rats compared with nuclei isolated 24 (Fig. 2*A*) and 48 h (Fig. 2*B*) after castration. Diminished intensities of bands in intact compared with castrate lanes 24 h after castration demonstrate several protected regions. A few weakly protected sites were more apparent at the lower concentration of DNase I. After 48 h, protected regions and several hypersensitive sites were revealed, with changes spanning the full fragment. The schematic representation in Fig. 2, *C* and *D*, illustrates the position of these changes in the nucleotide sequence of D2 for each treatment. The results indicate an androgen effect on chromatin structure in the region of D2.

DNase I Footprinting of D2 in Vitro with Ventral Prostate Nuclear Proteins—DNA-protein interactions were analyzed by

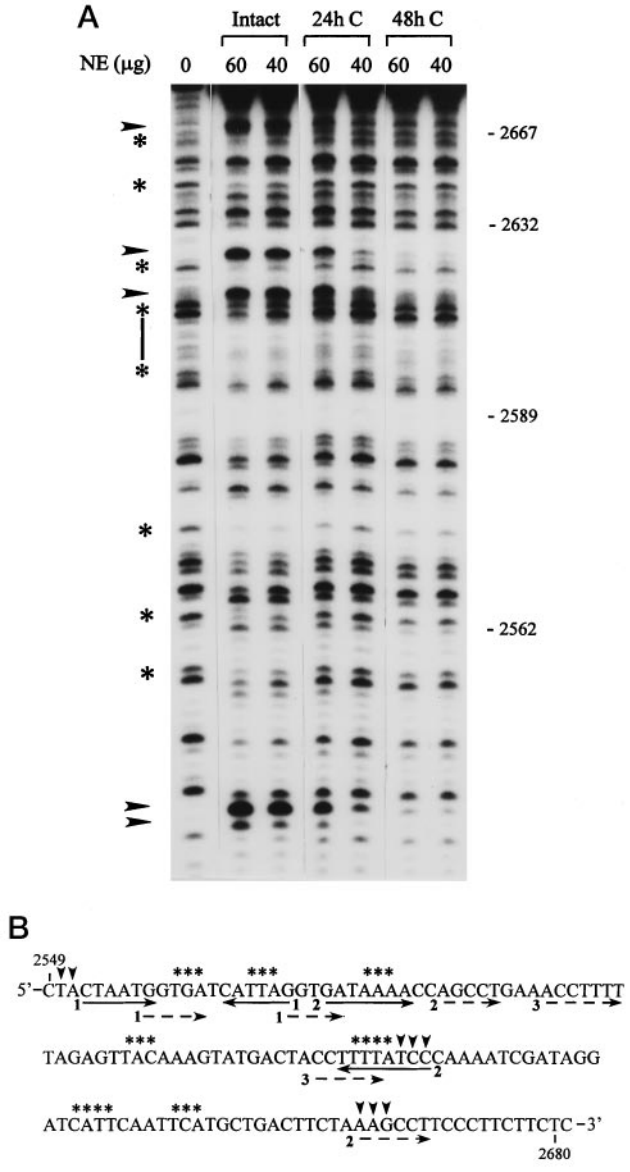

FIG. 4. **DNase I footprinting of the D2 antisense strand *in vitro* with ventral prostate nuclear proteins.** D2 ^{32}P-end-labeled in the antisense strand was footprinted *in vitro* using ventral prostate nuclear proteins from intact and 24- and 48-h postcastrate rats. *A*, *lanes* represent ^{32}P-labeled D2 with no protein (0) and 60 or 40 μg of protein in ventral prostate nuclear extracts (*NE*) from intact and castrate (*C*) rats. A G ladder was used for sequence alignment. Nucleotide numbers in D2 are indicated on the *right*. Protected bases (*asterisks*) and hypersensitive sites (*arrowheads*) induced by nuclear proteins of intact tissues are indicated on the *left*. *B*, schematic representation of the results. Direct and inverted repeats in the D2 nucleotide sequence are indicated by *broken* and *solid lines*, respectively. The results were similar in two experiments.

DNase I footprinting *in vitro* using D2 end-labeled and ventral prostate nuclear proteins (Fig. 3). Parallel binding reactions were performed without protein as a control for the nuclease digestion pattern of protein-free DNA. Fig. 3*A* shows the results obtained with D2 end-labeled in the sense strand. Protected regions (*asterisks*) and hypersensitive sites (*arrowheads*) were formed with nuclear proteins from intact rats and castrated rats treated immediately with testosterone, as compared with nuclear proteins from 48-h postcastrates, suggesting the presence of androgen-dependent D2 binding proteins in ventral prostate nuclei. A schematic representation of these androgen-dependent protected regions and hypersensitive sites in D2 is presented in Fig. 3*B*.

Footprinting *in vitro* with D2 end-labeled in the antisense strand revealed stronger androgen-dependent protected regions and hypersensitive sites than those obtained with D2 end-labeled in the sense strand (Fig. 4*A*). Comparing Figs. 3*B* and 4*B*, similar footprints in sense and antisense labeled D2 were observed in the inverted repeat 2 (GATAAA) present in the 5′ and middle region of D2. Other locations of footprints in sense and antisense strand are consistent with the complex dyad symmetry within the D2 sequence and suggest that both single- and double-stranded DNA binding proteins are involved in these footprints. These data indicate that multiple proteins bind D2, and some D2-protein interactions are androgen-dependent.

The tissue specificity of D2 footprints was examined using the same amounts of nuclear proteins from kidney of intact male rats. D2 DNase I digestion patterns were almost identical to those obtained in the absence of nuclear proteins (Fig. 3*C*).

Gel Mobility Shift Analysis of Nuclear Protein Binding to D2—To further analyze the binding of androgen-regulated nuclear proteins, three double-stranded ^{32}P-labeled oligonucleotides spanning D2 were synthesized and used in gel mobility shift assays. Oligonucleotide A spans the 5′-end (nucleotides 2549 to 2594), oligonucleotide B spans the middle region (nucleotides 2595 to 2638), and oligonucleotide C spans the 3′-end of D2 (nucleotides 2639 to 2681). The specificity of nuclear protein binding to these sequences was tested using molar excess amounts of double-stranded A, B, C, and random unlabeled oligonucleotides as competitors. Protein-DNA complexes with ^{32}P-labeled oligonucleotide A migrated at 3 different positions (A1, A2, and A3) (Fig. 5*A*). Complexes A1 and A2 were eliminated by an excess of unlabeled double-stranded oligonucleotide A but not by oligonucleotide C or a random oligonucleotide. When ^{32}P-labeled oligonucleotide B was used as a probe, four DNA-protein complexes were observed (Fig. 5*B*). Minor complexes (B1 and B2) and one major complex (B4) were competed with unlabeled double-stranded oligonucleotide B but not with oligonucleotide C or a random oligonucleotide. Bands A3 and B3 were not competed either by the specific or nonspecific unlabeled oligonucleotides, indicating nonspecific protein binding. Competition assays suggested that proteins involved in complexes A1 and A2 are related to bands B1 and B2 since unlabeled oligonucleotide B competed for complexes A1 and A2 while unlabeled oligonucleotide A competed for B1 and B2. However, unlabeled oligonucleotide A at 250 molar excess had little effect on complex B4, suggesting that this protein bound specifically to oligonucleotide B.

Since D2 contains regions of complex dyad symmetry, we tested for single-stranded D2 binding proteins in nuclear extracts by competition with unlabeled single-stranded oligonucleotides. Bands A1 and A2 observed with double-stranded ^{32}P-labeled oligonucleotide A (*^{32}P-Oligo A*) were partially competed by 250-fold excess of sense, but not by antisense, single-stranded oligonucleotide A (Fig. 5*C*). Other single-stranded oligonucleotides had no effect on these complexes. Bands B1 and B2 observed with double-stranded ^{32}P-labeled oligonucleotide B (*^{32}P-Oligo B*) were competed with unlabeled sense, but not by antisense, single-stranded oligonucleotide B or other single-stranded oligonucleotides (Fig. 5*D*). Band B3 was competed by single-stranded oligonucleotide A, B, and C, indicating it binds single-stranded DNA nonspecifically. The specific band B4 was not competed by any of the single-stranded D2 oligonucleotides. Taken together, the results suggest that related proteins with specificities for double- and single-stranded DNA are present in bands A1/A2 and B1/B2. In addition, band B4 binds duplex D2 exclusively.

Only one band was observed with ^{32}P-labeled oligonucleotide

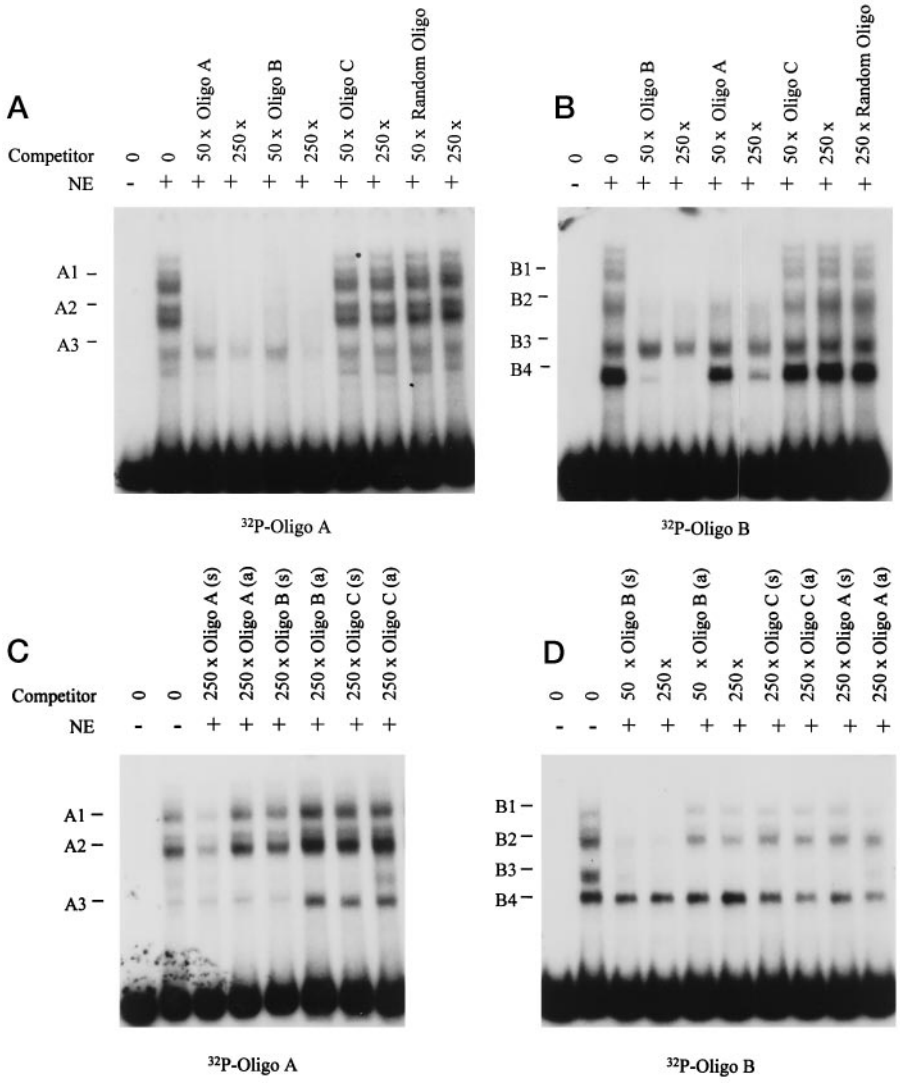

FIG. 5. **Binding of nuclear proteins to double- and single-stranded D2 subfragments.** Mobility shift assays were performed with ^{32}P-labeled synthetic oligonucleotides spanning the D2 fragment and nuclear proteins of rat ventral prostate, as described under "Experimental Procedures." 2.5 μg of protein were incubated with ^{32}P-labeled oligonucleotide in the absence (−) and presence (+) of 50- and 250-fold molar excess of unlabeled double- or single-stranded oligonucleotides used as competitors as indicated in each *lane* (*s*, sense; *a*, antisense). *A* and *C*, double-stranded ^{32}P-labeled oligonucleotide A (^{32}*P-Oligo A*; spanning nucleotides 2549 to 2594 of D2); *B* and *D*, double-stranded ^{32}P-labeled oligonucleotide B (^{32}*P-Oligo B*; spanning nucleotides 2595 to 2638 of D2). DNA-protein complexes are indicated by *numbers* on the *left*. The results are representative of four different experiments.

C, and it was competed with all unlabeled double-stranded oligonucleotides used, indicating the presence of a nonspecific DNA binding (data not shown). Although we were unable to detect specific protein binding to ^{32}P-labeled oligonucleotide C, DNase I footprinting studies both in isolated nuclei and *in vitro* indicated changes in DNase I digestion in the presence of ventral prostate nuclear proteins in the 3′ region of D2. One possibility is that binding of proteins to the 5′ and middle region of D2 induced conformational changes that altered the sensitivity of the 3′-end of D2 to DNase I digestion.

Tissue Specificity of D2-Protein Interactions—The tissue specificity of D2 complexes formed with ventral prostate nuclear proteins and oligonucleotides A and B was tested using nuclear protein extracts from male kidney and spleen, tissues that do not express the 20-kDa protein gene. Nuclear proteins from kidney and spleen bound D2 but with patterns distinct from that of ventral prostate nuclear proteins. No major bands were detected with mobility identical to A1 and A2 (Fig. 6*A*) and B1 or B2 (Fig. 6*B*). Spleen contained a less intense band corresponding to B4 (Fig. 6*B*), suggesting a protein involved in this complex is not restricted to ventral prostate or androgen target tissues.

Molecular Mass of Nuclear Proteins Bound to D2—UV cross-linking was performed to determine the size of proteins bound to ^{32}P-labeled oligonucleotides A and B. Bands A1 and A2 each contained cross-linked protein-DNA complexes of approximately 140 and 70 kDa (Fig. 7*A*). The 70-kDa complex was a minor component of A1 and a major component of A2. Band A3, a nonspecific protein complex with ^{32}P-labeled oligonucleotide A (see Fig. 5) yielded a 70-kDa band but did not contain the 140-kDa complex. B1 and B2 also contained complexes of 140 and 70 kDa, consistent with competition assays that suggested similar proteins interacted with oligonucleotides A and B (Fig. 7*B*). B3, a nonspecific complex in gel mobility shift assays (see Fig. 5*B*), also contained the 70-kDa protein. A protein of approximately 55 kDa was observed with band B4 (Fig. 7*B*). These results indicate that ^{32}P-labeled oligonucleotides A and B bind a 140-kDa protein or protein complex.

Androgen Dependence of D2 Binding of Nuclear Proteins—To identify androgen-dependent proteins binding to ^{32}P-labeled oligonucleotides A and B, mobility shift assays were performed using ventral prostate nuclear proteins from 24- and 48-h post-

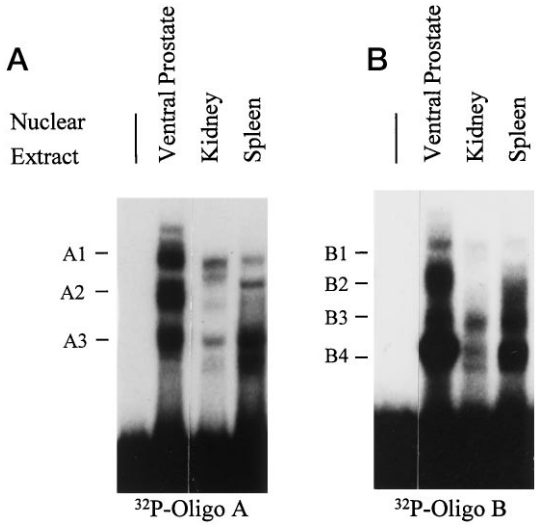

FIG. 6. **Tissue specificity of D2 binding proteins.** Mobility shift assays were performed with ^{32}P-labeled synthetic double-stranded oligonucleotides A and B and nuclear proteins from rat ventral prostate in comparison with male kidney and spleen that do not express the 20-kDa protein gene. Protein (5 μg) was incubated with respective probes as described under "Experimental Procedures." *A*, ^{32}P-oligonucleotide A (*^{32}P-Oligo A*; spanning nucleotides 2549 to 2594 of D2); *B*, ^{32}P-oligonucleotide B (*^{32}P-Oligo B*; spanning nucleotides 2595 to 2638 of D2). *Numbers* on the *left* indicate DNA-protein complexes obtained with ventral prostate nuclear extracts. Results were similar in two experiments.

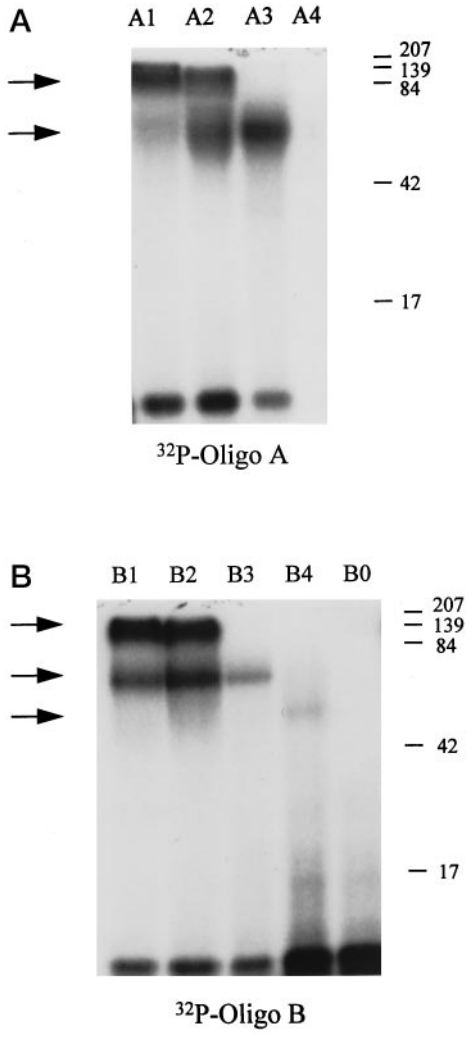

FIG. 7. **UV cross-linking of rat ventral prostate nuclear proteins bound to D2 subfragments.** Mobility shift assays were performed with ^{32}P-labeled double-stranded synthetic oligonucleotides A and B and ventral prostate nuclear protein (10 μg) from intact rats. Complexes were resolved on 6% polycrylamide gels, and UV cross-linking was performed by exposing gels to UV light (254 nm) for 30 min at 4 °C. Bands *A1*, *A2*, and *A3* for ^{32}P-labeled oligonucleotide A and bands *B1*, *B2*, *B3*, and *B4* for ^{32}P-labeled oligonucleotide B (see Fig. 5 as reference) were visualized on autoradiographs and excised from the gel. *B0* corresponds to control lanes containing probe and nuclear extracts in the absence of UV light. Cross-linked proteins were separated on 15% SDS gels, and molecular weights were determined by comparison with standards (*right*). *A*, ^{32}P-labeled oligonucleotide A (spanning nucleotides 2549 to 2594 of D2); *B*, ^{32}P-labeled oligonucleotide B (spanning nucleotides 2595 to 2638 of D2). *Arrows* indicate the size-fractionated proteins. Results are representative of three experiments.

castrated rats and from rats castrated and immediately given testosterone for 48 h (Fig. 8). DNA-protein complexes A1 and A2 with ^{32}P-labeled oligonucleotide A (Fig. 8*A*) and B1 and B2 with ^{32}P-labeled oligonucleotide B (Fig. 8*B*) decreased in intensity with castration in a time-dependent manner. Band B3 did not change while band B4 decreased only slightly. The pattern of D2 binding proteins from castrated rats treated with testosterone was identical to that of intact rats. The 55-kDa protein complex was also diminished by androgen withdrawal but only slightly reduced 24 and 48 h after castration, perhaps due to a slower turnover.

Competition for Nuclear Proteins Binding to Full-length D2—Nuclear protein from ventral prostate of intact rats formed complexes with ^{32}P-labeled D2 that migrated at three different positions (Fig. 9). Complexes 1 and 2 were eliminated by an excess of unlabeled double-stranded oligonucleotide A but not by oligonucleotide C. Complex 3 was competed only with an excess of unlabeled oligonucleotide B. These results indicate that ventral prostate nuclear proteins bind in the 5′ and middle regions of D2.

DISCUSSION

Intron 1 of the androgen-regulated 20-kDa protein gene contains a 130-bp sequence termed D2 that functions as a complex ARE in transient cotransfection assays in CV1 cells (18). Our results indicate that AR regulation of the 20-kDa protein gene involves D2 interactions with other nuclear proteins and are consistent with earlier evidence that the 20-kDa protein gene is a delayed primary response gene (19, 26–28). DNase I footprinting in isolated nuclei of ventral prostate revealed androgen-dependent protected regions and hypersensitive sites, indicative of androgen effects on chromatin structure in the D2 region. This was supported by DNase I footprinting *in vitro*, using nuclear protein extracts, that demonstrated androgen-dependent protected regions and hypersensitive sites. Mobility shift assays identified androgen-dependent nuclear protein complexes with the 5′ and middle regions of D2 (oligonucleotides A and B). Androgen-dependent complexes A1/A2 and B1/B2 interacted with double-stranded DNA of both regions but also bound to sense strand sequences, especially in oligonucleotide B, suggesting that binding might be influenced by the formation of stem-loop structures within these regions of D2. Band B4 protein bound exclusively to double-stranded DNA. Cross-linking analysis indicated that the bands A1/A2 and B1/B2 contain a protein complex of approximately 140 kDa, which was androgen-regulated, and B4, a complex of 55 kDa, which was less diminished by androgen withdrawal. One of these proteins may interact with inverted repeat 2 (GATAAA; Fig. 1) present in the 5′ and middle region of D2 since this sequence contained androgen-dependent protected and hypersensitive sites. Inverted repeats may be associated with hormone response elements or other transcription control elements (18, 32, 33). In D2, inverted repeat 2 encompasses a

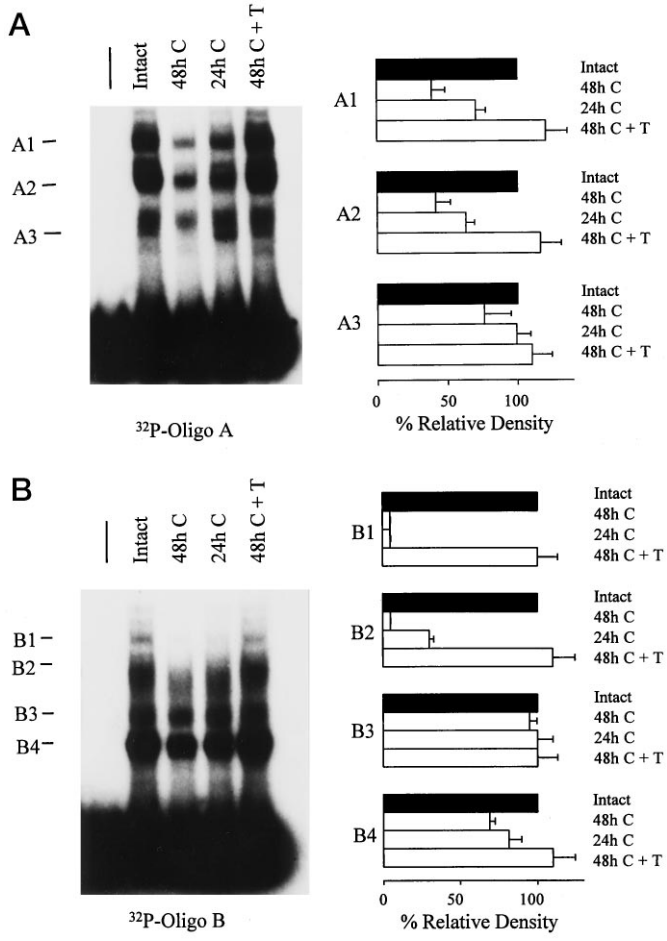

FIG. 8. **Androgen dependence of protein binding to D2 subfragments.** Mobility shift assays were performed with ^{32}P-labeled double-stranded synthetic oligonucleotides A and B and rat ventral prostate nuclear protein (5 μg) from intact and 24- and 48-h postcastrate (*C*) and testosterone-treated castrate rats (*48 h, C + T*) as described under "Experimental Procedures." *A*, ^{32}P-labeled oligonucleotide A; *B*, ^{32}P-labeled oligonucleotide B. *Numbers* on the *left* indicate DNA-protein complexes. Quantitation of the androgen dependence of respective bands was obtained by scanning autoradiographs with an Ultra Scan XL laser scanner and GelScan XL (Pharmacia LKB Uppsala, Sweden). Each band was scanned, and results are expressed in terms of percent density relative to bands of intact tissue set at 100%. Values are mean ± S.E. from four different experiments.

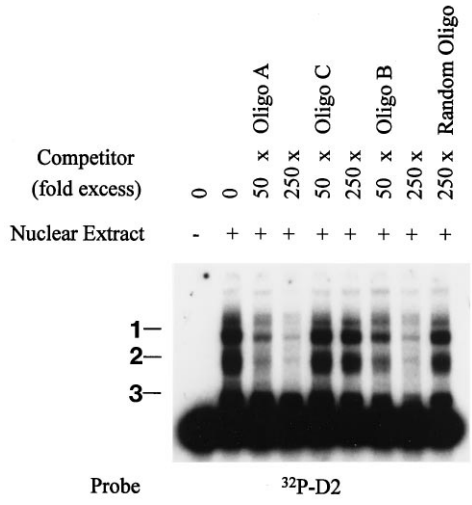

FIG. 9. **Competition of D2 subfragments for nuclear protein binding to full-length D2.** Mobility shift assays were performed with ^{32}P-labeled D2 and ventral prostate nuclear protein from intact rats as described under "Experimental Procedures." Protein (2.5 μg) was incubated with labeled ^{32}P-D2 in the absence and presence of 50- or 250-fold molar excess of unlabeled double-stranded oligonucleotides as indicated in each *lane*. Results were similar in two different experiments.

candidate ARE (GTTACAaagTATGAC), portions of which are either protected or contain hypersensitive sites.

Proteins interacting with D2 may promote selective binding or transcriptional activity of AR. De Vos *et al.* (34) reported that nuclear extracts from a variety of tissues enhance binding of AR to intron fragments of the C3 subunit gene. The high mobility group chromatin protein HMG-1 is a sequence-independent DNA-binding protein that binds preferentially in regions of secondary DNA structure (35). Recognition of DNA structure is mediated by a conserved HMG box motif (36). The HMG box motif is also present in transcription regulatory factors that bind specific DNA sequences, notably testis determining factor (37) and lymphoid enhancer factor 1 (38). Prendergast *et al.* (39) reported that purified HMG-1 enhances the binding of PR to its response element by 10-fold. Enhancement of PR DNA binding requires the HMG-1 DNA binding domain, suggesting it results from DNA bending, a known property of HMG-1. The configuration of DNA in a response element (40) may alter receptor tertiary structure (41, 42) and thereby effecting transactivation (43, 44). Interactions with nuclear receptor ligand-dependent coactivators (45) such as the AR-associated protein (ARA-70) (46) may be influenced by response element structure.

A number of reports indicate that regulation of gene expression involves a complex array of single- and double-stranded DNA binding proteins. The myogenic determination factor, Myo D1, binds to single- and double-stranded DNA containing regulatory elements from muscle-specific genes (47), and a regulatory element in the mouse adipsin gene binds multiple regulatory proteins, two of which preferentially bind to single-stranded DNA (48). Supakar *et al.* characterized a novel regulatory element associated with age-dependent expression of the AR gene in rat liver (49). Grossman and Tindall (50) identified nuclear proteins that bind single-stranded DNA in a supressor region of the AR gene promoter. Steroid induction of ovalbumin gene transcription in chicken oviduct is mediated by single- and double-stranded DNA binding protein interactions within a steroid-dependent regulatory element. At least two of these nuclear proteins are induced by estrogen (51, 52).

Functional synergism between multiple ARE-like sequences and binding sites for other transcription factors has been observed in the androgen-regulated C3 gene (22, 23). In the complex enhancer element of the Slp gene, binding of several non-receptor proteins contributes to the characteristic androgen response (10–15). Footprinting in isolated nuclei and *in vitro* indicated that these factors are influenced by androgens (12). Androgen dependence of the D2-binding proteins reported in the present study indicates that androgens may be involved both directly and indirectly in the regulation of 20-kDa protein gene transcription by way of this complex response element, directly through interactions of the AR with D2 and indirectly by controlling the expression or modification of nonreceptor D2 binding proteins. Androgen-dependent DNA binding proteins have been identified in intron 9 of the β-glucuronidase gene in mouse kidney (32) and in the promoter region of the mouse RP2 gene (20). A factor that recognizes the 5′-flanking region of the mouse RP2 gene is present in kidney nuclear extracts from both control and androgen-treated *Mus domesticus* as well as from control *Mus caroli*. However, in the latter species, a distinct androgen-induced DNA binding protein replaced the protein bound in the absence of androgen, showing that androgen can modulate the level and activity of a DNA-binding protein in

a species- and tissue-specific manner. Thus, tissue-specific proteins may have a role in determining both the magnitude and specificity of gene induction. Our results demonstrate that kidney and spleen, organs in which the 20-kDa protein gene is not expressed, did not contain the same nuclear D2 binding proteins as ventral prostate.

One hypothesis for steroid hormone modulation of gene expression suggests that receptor binding to response element DNA leads to disruption of phased nucleosomes, allowing access of other transcription factors to their binding elements (53). Another model postulates that steroid receptors exert effects on preexisting protein-DNA structure through modification of transacting factors or addition of accessory transcription factors (54). GR-induced alteration of chromatin structure in certain promoters and enhancers of hormone-responsive genes is indicated by the appearance of hormone-dependent DNase I hypersensitive sites (55–58). The general view is that these hypersensitive sites represent nucleosome-free regions of DNA (59, 60); however, the mechanism of nucleosome disruption is not fully understood (61).

Data in this report indicate that AR regulation of 20-kDa protein gene transcription by way of its intron 1 response element D2 is mediated in association with androgen dependent single- and double-stranded DNA binding proteins. These D2 binding proteins could be rapidly turning-over gene products regulated by androgens transcriptionally or post-transcriptionally. Although functions of these proteins have yet to be determined, they remain candidate modulators of the AR-specific response element.

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