Steroid Requirement for Androgen Receptor Dimerization and DNA Binding

MODULATION BY INTRAMOLECULAR INTERACTIONS BETWEEN THE NH$_2$-TERMINAL AND STEROID-BINDING DOMAINS

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Choi-iok Wong†, Zhong-xun Zhou‡, Madhabananda Sarfi, and Elizabeth M. Wilson†‡

From the Laboratories for Reproductive Biology and the Departments of †Biology, ‡Pediatrics, *Cell Biology and Anatomy, and *Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

Infection of Spodoptera frugiperda Sf9 insect cells with recombinant human androgen receptor (AR) baculovirus results in expression of a 118-kDa phosphoprotein that displays high affinity androgen binding and androgen-dependent targeting to the nucleus. Using the DNA mobility shift assay, specific in vitro binding of full-length AR to androgen response element DNA (ARE) requires intracellular hormone exposure. The ability of a variety of steroids to induce ARE binding paralleled their transcriptional potential. Certain antihormones, cyproterone acetate and RU486, promote ARE binding, but a pure antiandrogen, hydroxyflutamide, inhibits AR binding to ARE DNA. AR dimerization requires incubation of recombinant baculovirus-infected insect cells with androgen, but only when one or both components of the dimer contain the NH$_2$-terminal domain. Based on the intensities of ARE binding and lack of binding to an ARE half-site, it appears that, unlike the glucocorticoid receptor, AR binds DNA primarily as a dimer. Thus, full-length baculovirus-expressed AR requires intracellular hormone exposure for dimerization and ARE binding to overcome inhibition imposed by the AR NH$_2$-terminal domain. Antihormones with agonist activity promote dimerization and ARE binding, while a pure antiandrogen blocks AR DNA binding. It is concluded that intramolecular interactions between the NH$_2$-terminal and steroid-binding domains are regulated by the specificity of hormone binding and modulate receptor dimerization and DNA binding.

The requirement for sufficient amounts of steroid receptors for structure/function studies and in vitro transcriptional analysis led to the application of the baculovirus system for overexpression of recombinant receptors. Overexpression of the glucocorticoid (1, 2), mineralocorticoid (3), vitamin D (4), progestrone (5, 6), and androgen (7, 8) receptors was achieved using baculovirus. Expression in baculovirus is advantageous because of the potential for protein processing that occurs in eukaryotic cells. For example, phosphorylation of several steroid receptors was shown to occur in Spodoptera frugiperda (Sf9) cells infected with recombinant baculoviral vectors (2, 3, 5), and the expressed receptors were functional in transcriptional activation studies in vitro (6). Since transient transfection in monkey kidney COS cells yields androgen receptor (AR) in a predominantly insoluble form in limited amounts, baculovirus was used for overexpression of human AR.

An important question that remains in the functional analysis of AR is whether dimer formation requires hormone binding and occurs in association with DNA binding. Many transcription factors, including members of the steroid receptor family, undergo dimer formation in acquiring high affinity DNA binding (9). Dimerization of steroid receptors was demonstrated in the mobility shift assay using wild type and truncated forms of the progesterone, glucocorticoid (10), and estrogen (11) receptors. Heterodimers were reported for the thyroid hormone, retinoic acid (12-14), and vitamin D receptors. The vitamin D receptor forms heterodimers with a ubiquitous 55-kDa protein that enhances DNA binding (15, 16). Heterodimer formation with coregulatory proteins stabilizes specific DNA interactions of this group of transcriptional regulatory proteins (14).

Studies outlined in this report demonstrate that AR expressed in baculovirus displays high affinity androgen binding, androgen-dependent nuclear translocation, and phosphorylation. Mobility shift assays indicate that full-length baculovirus expressed AR requires intracellular androgen exposure to effect dimer formation and specific DNA binding. A surprising finding was that AR dimerization required androgen exposure in cells only when the NH$_2$-terminal domain was present. The results indicate that the NH$_2$-terminal domain acts to inhibit AR dimerization and DNA binding in the unliganded receptor. Furthermore, a pure antiandrogen, hydroxyflutamide, lacks agonist activity because it inhibits AR

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**To whom correspondence should be addressed: Laboratories for Reproductive Biology, CB#7500 MacNider Bldg., University of North Carolina, Chapel Hill, NC 27599.

1 The abbreviations used are: Sf9, Spodoptera frugiperda; AR, androgen receptor; AcMNPV, Autographa californica nuclear polyhedrosis virus; R1881, methyltestosterone; PCR, polymerase chain reaction; ARE, androgen response element; flutamide, α-α-α-trifluoro-2-methyl-4′-nitro-m-propionotoluamide, or 4′-nitro-3′-trifluoromethoxyisobutyranilide or SCh 13521; hydroxyflutamide, α-α-α-trifluoro-2-methyl-4′-nitro-m-lactotoluamide or SCh 18423; cyproterone acetate, 6α-chloro-17α-hydroxy-1α,2α-methylene-4,6-pregnadiene-3,20-dione-17-aceate; RU486, Roussel Uclaf list no. 36,486, mifepristone, 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(prop-1-ynyl)-estr-4,9-diene-3-one; DHT, 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; AR1-660 or N, expressed fragment of human AR containing amino acids 1-660; AR507-919 or C, expressed fragment of human AR containing amino acids 507-919; m.o.i., multiplicity of infection; kb, kilobase(s); rpm, counts/min; PBS, phosphate-buffered saline; bp, base pair(s).
DNA binding, and thus, represents a new class of antihormones.

**EXPERIMENTAL PROCEDURES**

**Materials**—Spodoptera frugiperda SF9 cells derived from the Fall Army worm ovary, Trichoplusia ni HighFive® insect cells from calf blood, Human embryonic kidney 293 cells, High Five insect cells from Sf9 cells, and SF9 cells were plated in 100 cm dish, infected with recombinant virus for 40–48 h at m.o.i. 1000, and analyzed on an 8% SDS-polyacrylamide gel as previously described (20).

**Immunoblot Analysis**—In a control experiment, AR expressed from SF9 cells was analyzed on immunoblots. SF9 cells were plated at 105 cells/10 cm dish, infected with recombinant virus for 40–48 h at m.o.i. 1000, and analyzed on an 8% SDS-polyacrylamide gel as previously described (20).

**Immunocytochemistry**—SF9 or HighFive® insect cells were infected with recombinant baculovirus at m.o.i. 4 in serum free Excell 400 medium at 27 °C for 20 h. Cells were washed in PBS, pH 6.4, and harvested in the same buffer. Cells were smeared on glass slides, air dried, and fixed in 95% alcohol at ~20 °C for 10 min. After washing with PBS, pH 7.4, cells were incubated with primary antibody AR52 overnight as previously described (22–24). After incubation, cells were incubated with biotinylated goat anti-rabbit IgG (1:400; Organon Teknika, Cochraville, PA) for 1 h at room temperature. Slides were washed with PBS, pH 7.4, and mounted using 90% glycerol, 1 mM Tris, pH 7.6, and a cover glass placed on the cells. Slides were viewed using a Nikon-UXF-DA fluorescent microscope with a B-2A filter.

**Steroid Binding Activity**—Steroid binding was performed on intact SF9 cells infected with human AR recombinant baculovirus using concentrations of [3H]R1881 ranging from 0.1 to 20 nM in the presence and absence of a 100-fold excess of unlabeled hormone. Cells were infected for 48 h at m.o.i. 2, incubated in Excell 400 media for 2 h at 27 °C, and then as described above. Cells were solubilized in SDS sample buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 7.4, and 1 mM EDTA) for 1 h on ice. Solubilized cell extracts were subjected to electrophoresis on 10% SDS-PAGE gels. After electrophoresis, protein bands were visualized by autoradiography and quantified by phosphorimaging. Band intensities were determined by the method of Schaefer (25). For mobility shift assays to demonstrate AR dimerization, the amounts of cell extracts ranging from 20–50 μg were subjected to electrophoresis on 10% polyacrylamide gels and analyzed on an 8% acrylamide minigel. Immunooblots were performed using antipeptide antibody AR52 as previously described (21).

**Gel Mobility Shift DNA Binding Assay**—DNA binding was assessed in vitro using recombinant full-length or deletion mutant human AR baculovirus expressed in SF9 cells at m.o.i. 4 in the presence and absence of androgen or various hormones and antihormones. After 48 h of viral infection, cells were incubated an additional 3 h or throughout the entire 48-h infection period at 27 °C with various hormones. Cells were infected in ice-cold PBS, pH 6.4, and resuspended in 0.33% yeastolate, 0.33% lactalbumin hydrolysate, 1% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 70 μg/ml gentamicin. Cells were exposed to three cycles of freeze/thaw, incubated on ice for 1 h, microfuged for 30 min, and the supernatant dialyzed against the above resuspension buffer containing 50 mM KCl. The dialyzed supernatant and the pellet were solubilized in SDS sample buffer (2% SDS, 10% glycerol, 1 mM Tris, pH 6.8) and analyzed on an 8% acrylamide minigel. Immunooblots were performed using antipeptide antibody AR52 as previously described (21).

**Cell Culture and Transfection**—Sf9 cells were maintained in 60–100-mL spinner cultures in Grace medium containing 0.33% yeastolate, 0.33% lactalbumin hydrolysate, 1% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 70 μg/ml gentamicin, and were passaged every 3 days at an initial density of 0.5 million cells/ml. Sf9 cells were plated at 105 cells/10 cm dish, infected with recombinant virus for 40–48 h at m.o.i. 1000, and analyzed on an 8% SDS-polyacrylamide gel as previously described (20).
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RESULTS

Construction of Full-length and Truncated Human AR Recombinant Baculoviruses—The full-length coding sequence of human AR was cloned into the NcoI and BamHI sites of the polyhedrin region of pAcC4 baculovirus transfer vector (Cetus). Cloning was achieved by ligation of the restricted vector with a 517-bp PCR amplified NH2-terminal NcoI/AflII AR fragment and a 2.24-kb AflII/BamHI AR fragment from the mammalian expression vectors pCMVhAR (29). As shown in Fig. 1A, the vector contains sequences required for homologous recombination with the wild type nuclear polyhedrosis virus from A. californica, AcMNPV, and a multiple cloning site that allows placement of the starting AR methionine coincident with that of the polyhedrin protein. Two major deletion mutants of human AR were also constructed (see "Experimental Procedures") such that the coding sequence contained the NH2-terminal and DNA-binding domains (amino acids 1-660, designated AR1-660) or the DNA-binding, hinge, and steroid-binding domains (amino acids 507-919, designated AR507-919) of the 919-amino-acid human AR. Sf9 insect cells were cotransfected with the transfer vectors and wild type AcMNPV DNA. Viral stocks were collected from infected cells and recombinant plaques enriched by serial dilution and dot-blot hybridization. Recombinant plaque purification was achieved by visual screening for absence of polyhedrin-containing occlusion particles. Several plaques for each construction were selected for further characterization.

Properties of Baculovirus-expressed AR from Sf9 Cells—Cultured Sf9 cells maximally express AR within 30–48 h post infection at m.o.i. 2–10 and produce a 118 ± 4-kDa protein that comigrates on an immunoblot with AR expressed in COS cells (see Fig. 6). AR displayed high affinity, saturable binding of [3H]R1881 in a whole cell binding assay, with approximately 6 pmol/mg protein, and the apparent binding affinity constant, Kd 0.2 ± 0.2 nM (Fig. 1, B and C). A Hill coefficient of 1.2 suggested minimal cooperative androgen binding. AR phosphorylation was evident when baculovirus-infected Sf9 cells were incubated with [32P]orthophosphate. Androgen caused an increase in both receptor phosphorylation and stabilization during a 3-h incubation at 27°C similar to that previously reported for receptor expressed in COS cells (20), indicating that AR undergoes post-translation modification in insect cells and is a phosphoprotein (data not shown).

Intracellular Hormone Dependence of in Vitro AR DNA Binding—The intracellular androgen dependence of specific AR DNA binding was determined in an in vitro DNA binding assay using a [32P]-labeled 27-bp oligonucleotide containing a full ARE sequence (see "Experimental Procedures"). A faint

Fig. 1. Recombinant baculovirus transfer vector and binding studies. A, Schematic diagram of the AR baculovirus transfer vector. The pAcC4 transfer vector from Cetus Corporation contained sequences for homologous recombination with the nuclear polyhedrosis wild type AcMNPV baculovirus and a multiple cloning site following the polyhedrin promoter. An NcoI site was coincident with the starting Met of the polyhedrin protein. Full-length human AR 2.8-kb coding sequence was cloned into the NcoI/BamHI site in the polyhedrin region. The 5' end of the human AR was constructed by PCR amplification using a 5' primer containing the NcoI site and the 3' coding sequence of AR and the 3' primer position 3' of the internal AflII site. A triple ligation led to the final construct of 11.9 kb. B and C, saturation binding and Scatchard plot analysis of baculovirus expressed human AR binding of [3H]R1881 in Sf9 cells. Sf9 cells were infected with recombinant human AR baculovirus at m.o.i. 2 as described under "Experimental Procedures." Cells were harvested, washed in PBS, pH 6.4, and aliquoted at approximately 2 × 106 cells/assay. Intact cells were incubated at increasing concentrations of [3H]R1881 in the presence or absence of unlabeled R1881 overnight at 4°C, washed three times in PBS, pH 6.4, the pellet solubilized in 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8, and radioactivity determined by scintillation counting. Specific binding represented the difference between counts/min bound in the presence and absence of unlabeled hormone. Illustrated is total (●), nonspecific (◇), and specific binding (▲) (B) and a Scatchard plot of specific binding (C). Apparent binding constant Kd 0.3 ± 0.2 nM Kd, Hill coefficient 1.2.
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nonspecific band was observed when extracts from uninfected Sf9 cells were combined with ARE DNA, a protein-DNA complex that was unaffected by AR antibody addition (Fig. 3, lanes 1 and 2). No additional DNA complexes were observed using extracts from cells infected with recombinant AR baculovirus in the absence of androgen (Fig. 3, lane 13). However, addition of dihydrotestosterone to cells expressing AR resulted in a dose-dependent formation of an AR-DNA complex that increased in intensity as the concentration increased from 10 to 50 nM dihydrotestosterone (Fig. 3, lanes 3, 5, 7, 9, and 11). The most intense band observed in the absence of antibody occurred following 24-48 h of treatment with 50 nM dihydrotestosterone (Fig. 3, lane 14). Unlabeled ARE DNA in 100-fold excess competed for AR-DNA complex formation (data not shown) indicating that the DNA binding was specific.

Dose-dependent androgen-induced increases in AR-DNA complex formation were also evident in the presence of the AR antipeptide antibody AR52. In the absence of cell exposure to dihydrotestosterone, only weak intensity bands were observed with full-length AR when tested with AR52 IgG at increasing concentrations of Sf9 cell extracts (Fig. 3, lanes 3, 5, 7, 9, and 11). However, addition of 50 nM dihydrotestosterone to the Sf9 cell incubations 48 h prior to harvest and AR52 IgG to the binding reactions caused up to a 120-fold increase in band intensity (Fig. 3, lanes 18-20). A dose-dependent increase in complex formation with increasing androgen concentrations was also observed in the presence of AR antibody (Fig. 3, lanes 4, 6, 8, 10, and 12). Addition of AE52 IgG potentiated androgen-dependent AR-DNA binding by at least 11-fold after cell incubation times of 1-3 h with androgen treatment. Enhancement of AR DNA binding by antibody may reflect antibody stabilization of AR dimerization (see below). Immunoblot analysis revealed similar amounts of AR protein in extracts from cells incubated in the absence and presence of increasing androgen concentrations for 3 h. After 30 h in the presence of androgen, some increase in AR protein was noted (data not shown).

Steroid Specificity of AR DNA Binding—We established whether exposure of Sf9 cells expressing AR to nonandrogenic hormones could affect AR DNA binding. As shown in Fig. 4, hormone-induced ARE binding reflected the ability of that hormone to activate a reporter gene previously reported (20). Strongest DNA binding was noted with the androgens, R1881, dihydrotestosterone, and testosterone (Fig. 4, lanes 1, 3, and 5), and the addition of AR antibody further enhanced DNA binding (Fig. 4, lanes 2, 4, and 6). Estrogen and progesterone promoted weak DNA binding only apparent in the presence of AR antibody (Fig. 4, lanes 7-10). Weakest activities were noted with dexamethasone and flutamide which do not bind AR with significant affinity at the concentrations tested. Somewhat to our surprise, the antiandrogen cyproterone acetate (Fig. 4, lanes 13 and 14) and the antiprogestin RU486 (Fig. 4, lanes 19 and 20) promoted relatively strong AR DNA binding, particularly in the presence of antibody. Both of these antihormones were shown previously to display agonist activity (20).

Of particular interest was hydroxyflutamide, since it binds
pressed AR were incubated with and flutamide

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AR with moderate affinity, causes nuclear transport, but fails to permit DNA binding. Even numbered lanes had 1 µg of AR52 antibody added during the DNA binding reactions.

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Baculovirus: AR1-660 contained the NHn-terminal and DNA-binding domains and AR507-919 contained the DNA-, hinge, and steroid-binding domains (diagrammed in Fig. 7). Expression of the deletion mutants from baculovirus was verified by immunoblot analysis, revealing truncated receptor forms of 85 ± 3 kDa (AR1-660, Fig. 6, lane 5, N) and 45 ± 1.5 kDa (AR507-919, Fig. 6, lane 6, C) relative to 118 ± 4 kDa full-length AR (Fig. 6, lane 4). The smaller sized bands reflect limited proteolytic cleavage prior to analysis. Dimerization was examined using 32P-labeled ARE DNA described above, and initially, receptors extracted from cells exposed to dihydrotestosterone.

Incubation of AR with ARE DNA resulted in a slowly migrating AR-DNA complex (Fig. 7, lane 7). A faster migrating complex was observed with AR507-919 (Fig. 7, lane 3), and AR1-660 formed a weak DNA complex migrating similar to full-length AR-DNA (Fig. 7, lane 5). Addition of antipeptide antibody AR52 caused a further shift in each band, verifying the presence of AR in the protein-DNA complexes (Fig. 7, lanes 4, 6, and 8). No specific bands were observed using extracts of uninfected cells in the presence or absence of antibody (Fig. 7, lanes 1 and 2).

AR dimerization was evident in the mobility shift assay using combinations of truncated and full-length receptors. A major band of intermediate migration formed between deletion mutants AR1-660 and AR507-919 (Fig. 7, lanes 10–12). The intermediate band was 39- and 6-fold greater intensity than either mutant alone, respectively, suggesting a synergistic increase in DNA binding with dimerization. About 15% residual fragment AR507-919-DNA complex migrated in its original position (Fig. 7, lane 12 compared with lane 11). Dimer formation between the dissimilar deletion mutants was therefore stronger than between either mutant alone.

Combining full-length AR and AR507-919 also resulted in strong dimer formation, with an intermediate band (Fig. 7, lanes 14–16) migrating slightly slower than the AR1-660-AR507-919 DNA complex (lane 12). Residual AR-DNA complexes remained detectable at their original positions of migration. The ratio of intensity of the three bands was 2:7:1 (Fig. 7, lane 16), corresponding to full-length AR, dimer, and AR507-919. Thus, dimerization between full-length AR and AR507-919 was preferred to dimerization of either with itself, assuming that AR-DNA complexes observed with each fragment alone represents dimerization (see below).

Dimerization of full-length AR and AR1-660 results in inhibition of DNA binding of full-length AR (Fig. 7, lanes 18–20). The same low level of DNA binding was observed with full-length AR and AR1-660 as with AR1-660 alone, suggesting that, in the absence of one or both hormone-bound ligands...
Arrows with recombinant baculovirus encoding either full-length AR (amino acids 1-660, prepared as described under "Experimental Procedures" and incubated in the full-length AR 919, extraction. Whole cell extracts (20 μg total protein/reaction) were used to determine DNA; reactions with different AR fragments contained 10 μg of protein from each cell extract. Sf9 cell extracts were from uninfected cells (lanes 1, 2, 9, 13, and 17) or from cells infected with the full-length AR (lanes 7, 8, 15, 16, 19, and 20), AR1-660 (lanes 5, 6, 10, 12, 18, and 20), and AR507-919 (lanes 3, 4, 11, 12, 14, and 16). The presence of AR in the DNA complex was established by adding some reactions 1 μg of AR IgG antibody (lanes 2, 4, 6, and 8). Arrows highlight the major DNA-protein complexes in lanes 12 and 16. Below is a schematic diagram illustrating regions of AR expressed by the wild type and mutant vectors.

binding domains, the two NH2-terminal domains in the AR dimer inhibit DNA binding.

Intracellular Androgen Dependence of in Vitro Dimerization—We next determined whether androgen exposure of Sf9 cells expressing AR was required for AR507-919 dimerization with itself or with full-length AR. AR507-919 extracted from cells untreated with androgen produced a strong migrating DNA-protein complex (Fig. 8, lane 3) which migrated slightly faster than when extracted from androgen treated cells (Fig. 8, lane 1). The strong band intensities suggest that AR507-919 is capable of binding DNA independent of androgen exposure in Sf9 cells. The slight difference in migration may result from conformational differences in AR507-919 in the presence or absence of androgen binding. Both AR507-919-DNA complexes shifted with antibody (Fig. 8, lanes 2 and 4).

Further evidence for dimerization of AR507-919 independent of androgen was obtained using an oligonucleotide containing a single right half-site ARE consensus sequence TGTTCCT (see "Experimental Procedures"). A similar oligonucleotide was used previously to demonstrate monomer binding of the glucocorticoid receptor (30). Little or no detectable bands were observed using full-length AR507-919 with the right half-site oligonucleotide (Fig. 9, lanes 6 and 8). In a control experiment, full-length glucocorticoid receptor expressed from baculovirus displayed binding to both the half-site and full ARE (Fig. 9, lanes 3 and 4). Monomer binding of the glucocorticoid receptor was also evident by the change in mobility of DNA complexes formed between the dissimilar oligonucleotides (Fig. 9, lanes 3 and 4). The results suggest that AR, either in its full-length or NH2 terminally truncated form, binds DNA primarily as a dimer and that ARE binding of AR507-919 reflects dimer binding. Furthermore, AR507-919 dimerization and DNA binding occurs independent of androgen exposure.

Full-length AR dimerization with AR507-919 was examined where either one or both were exposed to androgen in Sf9 cells. As shown above, dimerization occurred when both full-length AR and AR507-919 were exposed to androgen (Fig. 8, lanes 9–11). However, if either was untreated with androgen, no dimerization was observed (Fig. 8, lanes 12–20). Thus, unlike AR507-919 dimerization with itself, dimerization of full-length AR with AR507-919 required that each be exposed to androgen in Sf9 cells. The results support the hypothesis that the AR NH2-terminal domain inhibits dimer
formation in the absence of androgen and that androgen treatment relieves this inhibition.

Since AR1–660 and AR507–919 form a strong dimer band (see Fig. 7, lane 12), it was important to establish whether AR507–919 in this complex required androgen exposure as it did in dimerization with full-length AR but not with itself. Fig. 10 shows that androgen exposure of AR507–919 in Sf9 cells is required for dimerization with AR1–660. The results reaffirm the inhibitory influence of the NH2-terminal domain in dimer formation, even in a complex where one fragment contains the steroid-binding domain and the other contains the NH2-terminal domain. The results raise the possibility that regions required for strong dimer formation are located within the domains of these dissimilar AR fragments.

Finally, we tested the hormone specificity of dimerization. In control experiments in the absence of hormone, full-length AR fails to bind the ARE, AR507–919 binding is strong and dimerization between them is undetectable (Fig. 11, lanes 2–4). Strong dimerization occurs with R1881 and DHT (Fig. 11, lanes 5–10). Dimers are also detected with cyproterone acetate (lane 13) and RU486 (lane 16), but DNA binding and dimerization fails to occur with hydroxyflutamide (lanes 17–19). It is noteworthy that the migration of AR507–919 changes markedly depending on hormone treatment. For example, AR507–919 migration with dihydrotestosterone (Fig. 11, lane 9) differs from that with R1881 (lane 6) but is similar to that observed in the absence of hormone (lane 3). Also, it is important to note that hydroxyflutamide inhibits AR binding of both full-length AR (lane 17) and AR507–919 (compare lanes 3 and 18). The change in AR507–919 DNA complex migration likely reflects changes in receptor conformation resulting from hormone binding.

**DISCUSSION**

Overexpression of recombinant steroid receptors provides an important approach with which to determine the molecular properties of this family of transcriptional regulatory proteins. By the criteria of androgen binding, subcellular localization, and phosphorylation, a functional AR is expressed in Sf9 cells from baculovirus. It was determined that the AR requires prior intracellular exposure to androgen in order to acquire the ability to bind an ARE sequence in vitro. The steroid specificity of this hormone-dependent intracellular change in AR correlated with agonist activity demonstrated previously for a variety of hormones (20). Particularly noteworthy was the differential response of the antihormones: those that display agonist activity in transient cotransfection assays (cyproterone acetate and RU486) induce AR DNA binding, while the pure antiandrogen, hydroxyflutamide, which lacks agonist activity, failed to potentiate AR DNA binding, and, in fact, inhibited AR DNA binding when added together with androgen. The antagonistic activity of hydroxyflutamide results, therefore, from its failure to induce changes in AR necessary for DNA binding.

To investigate further the molecular basis for the steroid-mediated changes in AR required for DNA binding, two AR deletion mutants were expressed from baculovirus in Sf9 cells. The results indicate that AR undergoes dimerization in association with ARE binding, and, as a monomer, fails to bind the ARE. Furthermore, by using different combinations of full-length and AR deletion mutants, it was established that dimerization was dependent on intracellular hormone exposure in order to overcome inhibition created by the AR NH2-terminal domain. This conclusion was supported by the observation that deletion mutant AR507–919, which lacked the NH2-terminal region, dimerized and bound DNA efficiently in the absence of hormone, and that the presence of the NH2-terminal domain blocked this hormone independent DNA binding. Finally, it is speculated that the hormone specificity of DNA binding reflects the ability of a given hormone to promote conformational changes in AR that overcome NH2-terminal domain inhibition.

The critical role of hormone in steroidal receptor action is believed to be activation of receptors to a DNA binding state. The demonstration of hormone-dependent DNA binding is
sometimes complicated by isolation procedures that often cause artificial receptor activation so that receptors no longer require hormone for high affinity DNA binding. SF9 cells expressing AR must be exposed to androgen intracellularly in order to render the receptor capable of binding DNA in vitro.

In a recent study using baculovirus-expressed AR, binding to androgen response element DNA occurred independent of androgen exposure (7). An explanation for the differing results is that the AR used in the previous report was extracted using denaturing conditions and was subsequently renatured. Ligand-dependent DNA binding was reported, however, for baculovirus-expressed human progesterone receptor (5, 6).

Antihormones bind AR with moderate affinity, cause nuclear transport (20), but, as shown in this report, differ in their ability to induce DNA binding. These observations suggest that hormone binding may impose different conformations on the receptor, which either allow or disallow DNA binding. That steroid hormones play a major role in receptor conformation is supported by the recent study of Allan et al. (31) where proteolytic cleavage products differed in size after hormone and antihormone exposure of the progesterone receptor. It was proposed that distinct receptor conformations are induced by hormone and antihormone binding. The primary site for conformational modification was thought to be the carboxyl-terminal tail (32). A monoclonal antibody raised against the 14 terminal amino acids of the progesterone receptor only recognized the progesterone receptor when unliganded or bound to the steroid antagonist, RU486, suggesting that agonist binding induces a unique receptor conformation (33). Our studies using hydroxyflutamide support the formation of an improper receptor conformation that interferes with DNA binding. This was evident even with the NH2 terminally truncated mutant AR507-919, that without hormone, displayed strong DNA binding, but with hydroxyflutamide, showed inhibition of DNA binding.

Further evidence for the importance of the carboxyl-terminal tail in conformational effects that differentiate agonist and antagonist activity comes from a switch in the activity of hydroxyflutamide from antagonist to agonist in an AR mutant. Agonist activity of hydroxyflutamide is undetectable with wild type AR but becomes significant with an AR mutant containing a single base mutation at amino acid residue 877, changing threonine to alanine, in the carboxyl-terminal region of the AR steroid-binding domain (34, 35). It could be speculated that the single residue change contributed to a change in AR conformation when bound to hydroxyflutamide which potentiated DNA binding and thus, transactivation. Similarly, a single amino acid change in the progesterone receptor disallowed antagonist RU486, but not agonist binding (36).

Alternative mechanisms for the molecular basis of steroid antagonism include reduced nuclear translocation with concomitant inhibition of 10 S to 4 S conversion, as shown for the glucocorticoid receptor (37). All of the antihormones tested in the present report, including hydroxyflutamide, caused nuclear transport of AR (20) and preliminary studies with baculovirus-extracted receptor indicate that hormone- and nonhormone-treated receptors migrate as 5-6 S complexes on sucrose gradients. Studies on RU486 interaction with the human progesterone receptor indicate that binding to a specific response element was indistinguishable from that with the synthetic agonist, R5020 (38). Although the progesterone receptor binds its response element when associated with RU486, it apparently binds with a somewhat altered conformation reflected by differences in migration on sucrose gradients and in DNA mobility shift assays; failure to transactivate results therefore from structural alterations (39).

Using deletion mutants of the rabbit progesterone receptor, it was demonstrated that RU486-bound receptors interact with the same response element (40). In agreement with these studies, the AR-RU486 dimer appeared to migrate with slightly altered mobility. These studies support the important role of receptor conformation mediated by steroid binding in transcriptional activation. They point out that the molecular basis of hydroxyflutamide antagonism differs from that of most antihormones reported thus far, which bind with high affinity to their respective hormone response elements, as recently summarized (41, 42). Hydroxyflutamide therefore belongs to a new class of "pure" antagonists that lack agonist activity due to their inability to promote specific DNA binding.

AR dimerization and the inhibitory role played by the NH2-terminal domain was revealed using full-length and deletion baculovirus expressed mutants in the DNA mobility shift assay. AR1–660, that contains the NH2-terminal and DNA-binding domains, and AR507–919, the DNA- and steroid-binding domains, showed strong hormone-dependent dimerization. Thus, a major dimerization domain may reside within their overlapping regions in the DNA binding and hinge regions. Alternatively, dimerization may involve distinct regions in each of the dimer components. The DNA-binding domain of the glucocorticoid receptor contains a dimerization region (10, 11) within a so-called D box of the second zinc finger (43, 44). Crystal structure analysis of the glucocorticoid receptor DNA-binding domain was linked directly to DNA binding-induced dimerization (45). A major dimerization domain of the estrogen receptor occurs within the steroid-binding domain (46), and the ligand-binding domain was proposed as a site for a major dimer interface that interacts with the same region in the dimer component (44). Such a mechanism does not comply with our results with AR. A dimerization region within the steroid-binding domain must necessarily interact with a region outside the steroid-binding domain in the other dimer component.

The intensity of DNA-receptor complex formation between AR1–660 and AR507–919 deletion mutants exceeded that observed with AR1–660 and the full-length receptor, both of which contain the NH2-terminal region. The results suggest that the AR NH2-terminal domain within the dimer weakens dimer strength. The results raise the possibility that efficient dimerization and DNA binding might occur when AR heterodimerizes with another protein. In this regard, it was recently reported that a truncated form of AR expressed in Escherichia coli complexes with an AR accessory factor that potentiates DNA binding (47).

After short incubation with androgen (1–3 h), AR displayed relatively weak DNA binding that was potentiated by antibody. AR antibody did not enhance DNA binding using extracts of cells untreated with androgen, even though the extraction buffer contained high salt. Furthermore, more prolonged androgen exposure of SF9 cells (48 h) caused AR to bind DNA strongly independent of antibody. It was noted that addition of androgen to the in vitro DNA binding reaction without prior exposure of SF9 cells was ineffective in promoting DNA binding, even though specific high affinity androgen binding was measured. These results, taken together, indicate that intracellular changes occur in AR in response to androgen that enable the receptor to bind DNA. The molecular basis for this sequential, time-dependent acquisition of DNA binding remains to be determined. Post-translational modification of AR such as phosphorylation occurs in SF9 insect cells and could theoretically have a role in the induction of strong DNA binding. Antibody may stabilize receptor dimerization or a
pure antiandrogens lack agonist activity because they fail to promote dimerization and DNA binding. Failure to promote DNA binding likely results from an altered conformation imposed on the ligand-binding domain. The AR NH$_2$-terminal domain blocks DNA binding in the absence of the appropriate ligand by interfering with receptor dimerization.

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