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Deoxyribonucleic Acid-Binding Ability of Androgen Receptors in Whole Cells: Implications for the Action of Androgens and Antiandrogens

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ABSTRACT

In whole cells, the effects of several androgens and antiandrogens on the induction of DNA binding for the human wild-type androgen receptor (AR) and a mutant receptor ARL (LNCaP mutation; codon 868, Thr to Ala) were examined and related to the transcription activation ability of these receptors. To study DNA binding, an AR expression vector was cotransfected in Chinese hamster ovary cells with a promoter interference plasmid cytomegalovirus-(androgen-responsive element)$_3$-luciferase, containing one or more androgen-responsive elements between the TATA box of the cytomegalovirus promoter and the start site of luciferase gene transcription. Expression levels of the AR are up-regulated by some agonists, but receptor expression levels are comparable for all antiandrogens studied. In the presence of androgens, the wild-type AR is able to reduce promoter activity of the cytomegalovirus-(androgen-responsive element)$_3$-luciferase plasmid, indicating androgen-dependent DNA binding of the AR. The full antagonists hydroxyflutamide, ICI 176.334, and RU 23908 block AR binding to DNA. The antagonists cyproterone acetate and RU 38486 induce approximately 50% of the DNA binding found for androgens. In a transcription activation assay, the RU 38486-bound receptor was almost inactive, and the receptor complexed with cyproterone acetate showed partial agonistic activity. Interaction of the antagonists cyproterone acetate, hydroxyflutamide, and RU 23908 with the mutant receptor ARL resulted in both a DNA-bound and a transcriptionally active receptor.

In conclusion, transformation of the AR to a DNA-binding state in whole cells is blocked by several antiandrogens. Furthermore, studies with the antiandrogens cyproterone acetate and RU 38486 show that DNA binding alone is not sufficient to accomplish full transcriptional activity. Full activity requires additional changes, presumably in the protein structure of the receptor. (*Endocrinology* **137:** 1870–1877, 1996)

THE ANDROGEN receptor (AR) belongs to a superfamily of ligand-inducible transregulators that includes receptors for steroid and thyroid hormones, and vitamins (1). As for the other members of the superfamily, molecular genetic analysis has identified separable domains responsible for DNA binding, hormone binding, and transactivation (2, 3). Upon androgen binding, the AR undergoes several sequential processes to interact with specialized regions on the DNA. These so-called androgen-responsive elements (AREs) are commonly located in the regulating regions of target genes. Binding of the androgen-bound receptor to its response element results in the formation of a stable preinitiation complex that allows efficient transcription initiation by RNA polymerase II (4, 5).

Steroid receptor antagonists inhibit the biological effects of agonists, although the precise molecular mechanism(s) of these antagonists is unknown. In a model explaining the mechanism of antagonist action described recently by O'Malley and collaborators (6–8), the importance of a conformational change in the ligand-binding domain is emphasized. The model argues that antagonists recognize regions of the ligand-binding domain that result in dimerization and DNA binding, but leave the C-terminus of the ligand-binding domain in a form still available for protease (6) and antibody recognition (9, 10). As a result, a repressor function is not removed, and the receptor is not able to induce transcription. In accordance with this model, binding of RU 38486 to an *in vitro* translated AR, resulted in both protease digestion of the C-terminus of the ligand-binding domain (11, 12) and dissociation of the heat shock protein-receptor complex (12). Other results suggested that more steps could be involved in the mechanism of antiandrogen action; upon binding of the AR antagonists cyproterone acetate, hydroxyflutamide, ICI 176.334, or RU 23908, no indications were found for involvement of the C-terminus of the AR in the mechanism of antiandrogen action (12, 13). As an intact heat shock protein-receptor complex is still present upon binding of these antiandrogens *in vitro* (12), we hypothesized that dissociation of the receptor from this complex and subsequent binding of the receptor to DNA, would be blocked partly or completely by some antiandrogens within intact cells. Dissociation of the oligomeric receptor complex and subsequent binding of the receptor to DNA have previously been proposed to be impaired by antagonists (14–16). However, recent observations of DNA-bound receptors do not favor these steps as critical for the actions of antiprogestagens and antiestrogens (17–21).

To find experimental support for the inhibition of DNA binding by several antiandrogens, we applied a promoter interference assay, described by Reese and Katzenellenbogen for the estrogen receptor (17). This assay makes it possible to examine the effects of ligands, both hormones and antihormones, on AR binding to DNA within mammalian cells. The promoter interference assay uses the principle of steric hin-

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drance between the AR and basal transcription factors on a constitutive [cytomegalovirus (CMV)] promoter. Furthermore, the binding of the AR to DNA was related to the transactivation capacity of the various ligand-receptor complexes. The mutant receptor ARL (mutation in codon 868; Thr to Ala) was also investigated, as binding of several antiandrogens resulted in an active receptor complex for this mutant.

Materials and Methods

Materials

R1881 (methyltrienolone) was purchased from New England Nuclear Corp. (Boston, MA). RU 38486 (mifepristone) and RU 23908 (nilutamide) were gifts from Roussel-UCLAF (Paris, France), cyproterone acetate was obtained from Schering (Berlin, Germany), hydroxyflutamide was obtained from Schering (Bloomfield, NJ), and ICI 176.334 (Casodex) was obtained from ICI Pharmaceuticals (Macclesfield, UK). All other steroids were purchased from Steraloids (Wilton, NH). The BM chemiluminescence Western blotting kit was obtained from Boehringer Mannheim (Mannheim, Germany). The plasmids pcDNAI and pGL2 were obtained from Invitrogen (San Diego, CA) and Promega (Madison, WI), respectively. The mouse mammary tumor virus-luciferase (MMTV-LUC) reporter plasmid was kindly provided by Organon (Oss, The Netherlands).

Preparation of AR and reporter plasmids

Preparation of the MMTV-LUC reporter plasmid (22) and the expression plasmids for the wild-type human AR (encoding an AR of 910 amino acids) (23), mutant ARL (LNCaP mutation; codon 868, Thr to Ala) (24), and mutant AR64 (codon 567, Cys to Ser, and codon 570, Cys to Phe) (25) have been described previously. The CMV-LUC plasmid was constructed by cloning a *Sac*I-*Bam*HI fragment that spanned the luciferase transcription unit from pGL2 into pcDNAI (a plasmid containing the CMV promoter), which had been digested with *Sac*I and *Bam*HI. CMV-$(ARE)_{1-3}$-LUC promoter interference plasmids (containing one to three androgen-responsive elements) were prepared by ligation of double stranded oligonucleotides containing a consensus ARE (GGTACAgtt-TGTTCT) (26) into the *Sac*I site of the CMV-LUC plasmid.

Cell culture and transfections

Chinese hamster ovary (CHO) cells were maintained in DMEM-Ham's F-12 tissue culture medium supplemented with 5% charcoal dextran-treated FCS. For promoter interference assay, CHO cells were plated at 1.5×10^5 cells/well (10 cm^2), grown for 24 h, and transfected overnight by calcium phosphate precipitation, as described previously (27). Cells were transfected with 75 ng expression plasmid, encoding AR, ARL, or AR64, and 7.5 ng CMV-LUC or CMV-$(ARE)_{1-3}$-LUC plasmid. Carrier DNA (pTZ19) was added in each case to a total of 5 μg/well. For transcription regulation studies, CHO cells were plated at 0.6×10^5 cells/well (4 cm^2) and transfected with 10 ng AR or ARL expression plasmid, 200 ng MMTV-LUC reporter plasmid, and 1.8 μg pTZ19/well. After transfection, the cells were washed, and experimental media were added. Upon an incubation period of 24 h, cells were harvested for the LUC assay, as described previously (12).

Western immunoblot analysis

Whole cell lysate was prepared by resuspending the cell pellet from a well (10 cm^2) in 200 μl 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.08% (wt/vol) SDS, 0.6 mM phenylmethylsulfonylfluoride, and 0.5 mM bacitracin at 4 C. The lysate was centrifuged (10 min; $1700 \times g$), and 20 μl of the supernatant were used for Western immunoblot analysis, essentially as described previously (27). The polyclonal rabbit antiserum SP197, recognizing the AR (epitope: amino acids 1–20) (28) was used as the primary antibody in a chemiluminescence protein detection method, performed as described by the manufacturer (Boehringer Mannheim).

Results

Transcription activation studies of wild-type AR and mutant ARL in CHO cells

To compare DNA binding of the AR with effects on transcription activation, both an *in vivo* DNA binding assay (described in the next section) and a transcription activation assay were used. Either wild-type AR or mutant ARL expression plasmids were transiently transfected into CHO cells together with the AR-sensitive reporter plasmid MMTV-LUC. The mutant receptor ARL contains a mutation in the ligand-binding domain (amino acid 868, Thr replaced by Ala) that leads to a decrease in steroid binding specificity (24). The ligands, both androgens and antiandrogens, differ in their binding affinities for the AR (24, 27, 29, 30); therefore, added concentrations of ligand were standardized accordingly. The nonmetabolizable synthetic androgen R1881 (1 nM) and the natural androgens dihydrotestosterone (1 nM) and testosterone (10 nM) induced LUC activity to the same level (Fig. 1A) and in a dose-dependent manner (results shown for R1881 in Fig. 3). LUC activities induced by 100 nM of the steroidal antihormones cyproterone acetate and RU 38486 were approximately 55% and 15% of that observed with 1 nM R1881. [RU 38486 has antiandrogenic properties, besides its effect as an antiprogestagen/antiglucocorticoid (31, 32).] Partial agonistic activity was not observed for the nonsteroidal antiandrogens hydroxyflutamide, ICI 176.334, and RU 23908 (used in concentrations up to 1 μM). All antiandrogens inhibited the LUC activity induced by 0.1 nM R1881 (Fig. 1B). In contrast, in cells with the mutant receptor ARL, cyproterone acetate and hydroxyflutamide induced LUC activity to the same level as that observed with R1881, dihydrotestosterone, and testosterone (Fig. 1C). Some partial agonistic activity of RU 23908 on ARL was found, whereas RU 38486 and ICI 176.334 did not activate ARL. The lack of agonistic activity of RU 38486 and ICI 176.334 was not due to the absence of binding to the mutant ARL, as LUC activity induced by 0.1 nM R1881 could be inhibited with both antihormones (Fig. 1D). CHO cells contain a limited amount of glucocorticoid receptors, and RU 38486 is able to bind to these receptors. However, the effect of RU 38486 on transcription activation was not due to this receptor system; in the absence of AR or ARL expression vectors, RU 38486 failed to induce LUC activity from the transfected MMTV-LUC reporter plasmid (not shown).

Promoter activity of constructs containing multiple AREs

DNA binding of the estrogen receptor in whole cells was studied previously by Reese and Katzenellenbogen (17) with a promoter interference assay, based on the principle of steric hindrance between a transcription factor and basal transcription factors on a constitutively active promoter. In the present study, an AR-dependent promoter interference reporter plasmid was constructed by inserting consensus AREs (26) into the *Sac*I site that lies between the TATA box of the constitutively active CMV promoter and the start site of

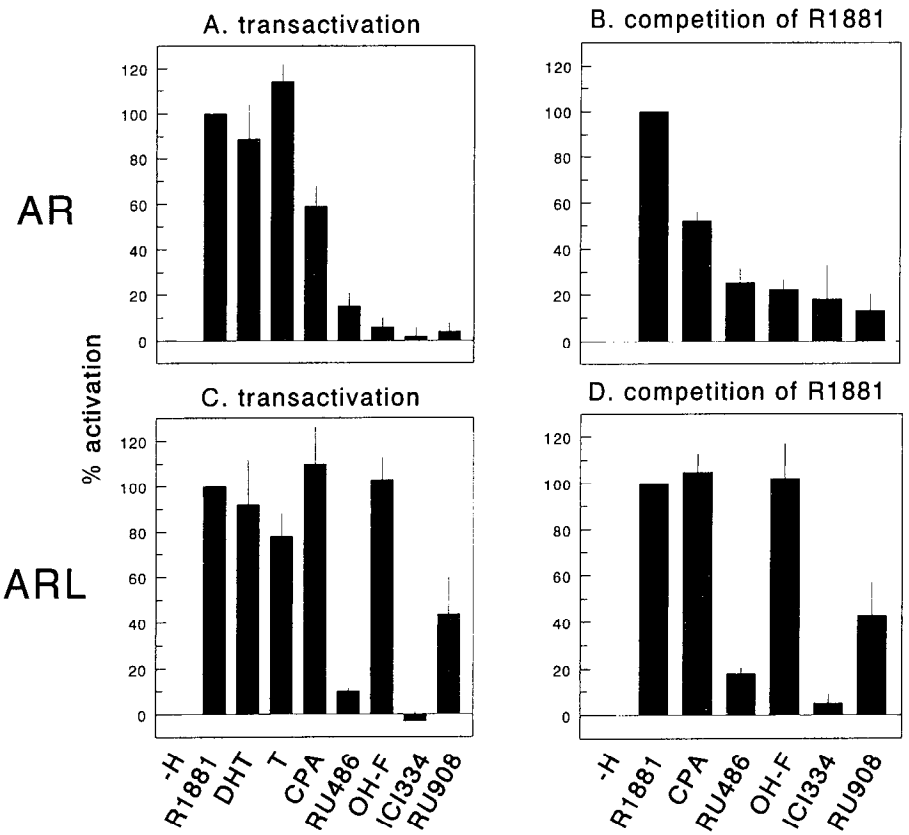

FIG. 1. Transcriptional activity of the wild-type AR and LNCaP AR (ARL) in the presence of androgens and antiandrogens. LUC expression was determined in CHO cells transiently cotransfected with AR (A and B) or ARL (C and D) expression plasmids and the reporter plasmid MMTV-LUC. A and C, After transfection, cells were incubated without hormone (−H) or with R1881 (1 nM), dihydrotestosterone (DHT; 1 nM), testosterone (T; 10 nM), cyproterone acetate (CPA; 100 nM), RU 38486 (RU486; 100 nM), hydroxyflutamide (OH-F; 1 μM), ICI 176.334 (ICI334; 1 μM), or RU 23908 (RU908; 1 μM) for 24 h. LUC activity in the presence of 1 nM R1881 was set at 100%. Values are the mean (±SEM) of three or four determinations; each determination was performed in triplicate. B and D, For competition studies, the various antiandrogens were added simultaneously with 0.1 nM R1881. The *second bar* shows the activity of 0.1 nM R1881 alone (set as 100%). Values are the mean (±SEM) of three determinations.

transcription of the messenger RNA, encoding the luciferase protein. Binding of AR at that position should hinder the assembly of the transcription initiation complex and hence reduce the expression of the LUC gene. The functionality of the CMV-$(ARE)_{0-3}$-LUC plasmids was verified by cotransfection of the construct with the expression vector for the wild-type AR into CHO cells. The promoter activity was reduced in all ARE-containing promoter interference plasmids in the presence of R1881, whereas no influence on LUC expression was seen in cells cotransfected with the original CMV-LUC plasmid (Fig. 2A). The down-regulation of promoter activity was dependent on the number of inserts; it was the largest with the CMV-$(ARE)_3$-LUC plasmid (remaining activity compared to control, 47%). In the absence of hormone, no differences in LUC activity between the different reporter plasmids were observed.

Overexpression of a transcription factor could sequester other factors necessary for the transcriptional activity of a promoter (*i.e.* squelching) (33). As squelching does not require specific DNA binding or an intact DNA-binding domain, the receptor mutant AR64 was studied to further verify that the reduction in promoter activity was actually due to DNA binding of the AR to the CMV-$(ARE)_3$-LUC plasmid. In this receptor mutant, the structure of the first zinc cluster is disrupted by the replacement of two of the four cysteine residues. Mutagenesis experiments have shown that these cysteine residues are essential for DNA-binding capacity of the receptor (34). No effect on LUC expression was seen, when CMV-$(ARE)_3$-LUC plasmid was cotransfected with the AR64 expression plasmid in CHO cells in the presence of ligand (Fig. 2B). As expression levels of wild-type AR and mutant AR64 were comparable, as analyzed by Western blotting (Fig. 2C), a squelching phenomenon could not account for the repression of the promoter activity in the CMV-$(ARE)_3$-LUC plasmid. (The effect of ligand on AR expression level is discussed further in the section *AR expression levels and migration pattern* below.)

To exclude an effect on promoter activity due to a limited amount of glucocorticoid receptor in CHO cells, we also examined the effect of R1881 and dexamethasone on cells transfected only with either the CMV-LUC or the CMV-$(ARE)_3$-LUC plasmid. In the absence of AR expression plasmid, no reduction of LUC expression from both plasmids could be demonstrated either in the presence of 1 nM R1881 or 100 nM dexamethasone (result shown for R1881; Fig. 2B).

In summary, the results presented in Fig. 2, show that interference with promoter activity is dependent on the presence of AREs in the reporter plasmid, a functional AR, and the androgen R1881. This permits the conclusion that the LUC activity measured with this assay system reflects DNA binding of the AR in whole cells.

Comparison of transcription activation and DNA-binding ability of AR

In the previous sections, we reported that the complex of androgen and receptor both activated transcription from a MMTV-LUC plasmid and interfered with the constitutively active CMV promoter. To investigate the dose dependency of the ligand in both processes, we determined the dose-response curves of R1881 in both assays. Maximal effects of the AR on both transcription activation and promoter inter-

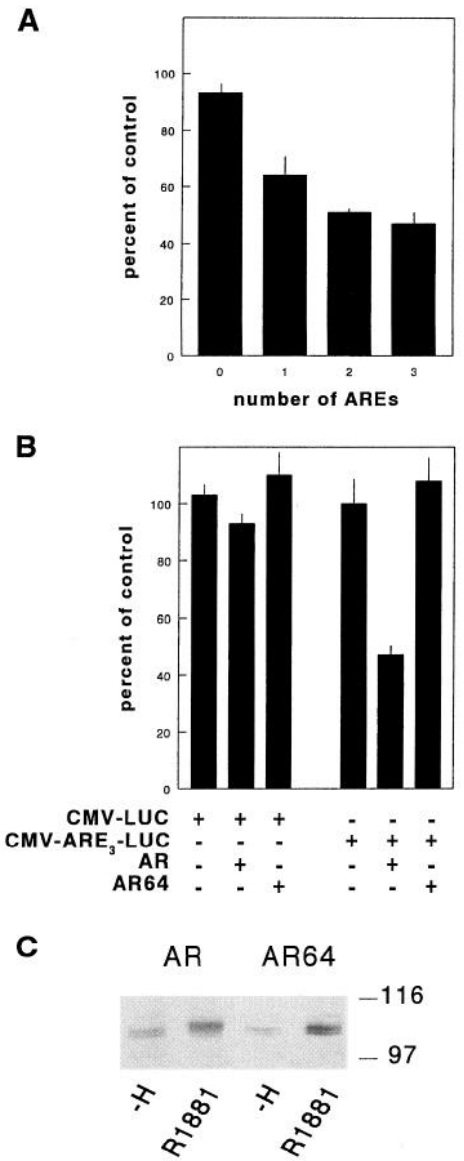

FIG. 2. Effects of multiple AREs and different receptors on CMV promoter activity. A, CHO cells were cotransfected with AR expression plasmid and the CMV-LUC construct containing zero to three AREs. After transfection, cells were treated with control vehicle or 1 nM R1881. Control values were determined as LUC activity in cells transfected with each CMV-$(ARE)_{0-3}$-LUC construct and AR expression vector in the absence of ligand and were set at 100%. Data represent the mean (±SEM) of four to six determinations; each determination was performed in triplicate. B, Promoter activity of CMV-LUC and CMV-$(ARE)_3$-LUC plasmid, cotransfected in CHO cells without or with the expression plasmids for wild-type AR or mutant AR64 (mutations in DNA-binding domain; codons 567 and 570, Cys to Ser) as indicated on the *x*-axis. Cells were treated with control vehicle or 1 nM R1881, and LUC activity was measured. Control values were determined as described in A, whereas the results are the mean (±SEM) of five to six determinations. C, Western blot analysis of lysates from cells, transfected with wild-type AR or the mutant AR64 and incubated with or without 1 nM R1881. Whole cell lysates were fractionated with gel electrophoresis, and separated proteins were transferred to nitrocellulose and visualized with a chemiluminescence protein detection method, using the AR-specific polyclonal antiserum SP197 as primary antibody. Molecular mass markers (kilodaltons) were run on a parallel lane, and their positions are indicated on the *right* of the blot.

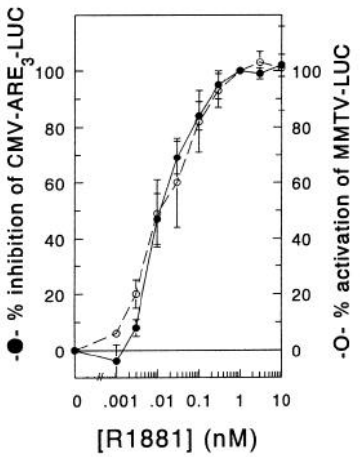

FIG. 3. Effect of R1881 concentration on the promoter activity of the CMV-$(ARE)_3$-LUC plasmid. The CMV-$(ARE)_3$-LUC promoter interference plasmid was cotransfected with the AR expression plasmid in CHO cells, and increasing concentrations of R1881 were added (●). Inhibition of promoter activity in the presence of 1 nM R1881 was set at 100%. The effect of the R1881 concentration on transcription activation of the MMTV-LUC reporter plasmid in transfected CHO cells is also shown (○). Transcription activation in the presence of 1 nM R1881 was set at 100%. The data are the mean of two or three determinations (±SEM).

ference were reached at 1 nM R1881 and set at 100% activation or inhibition, respectively. The dose-response curve of R1881 on transcription activation matched the effect of R1881 on promoter interference (Fig. 3). Because promoter interference in this assay system reflects DNA binding of the AR, these results also indicate a direct correlation between the percent DNA binding and transcription activation.

Influence of different hormones and antihormones on DNA-binding ability of AR

The promoter interference assay permits the study of DNA binding of the receptor in whole cells. Therefore, we examined the effects of several hormones and antihormones on their ability to reduce promoter activity of the CMV-$(ARE)_3$-LUC plasmid in the presence of either the wild-type AR or the mutant ARL. Neither receptor interacted with the promoter interference construct in the absence of R1881, and treatment with R1881 (1 nM) resulted in a similar decrease (to 50%) of LUC activity. This implies a similar dependence on the ligand R1881 for DNA binding of both the AR and ARL to the AREs in the promoter interference construct. CHO cells exposed to the natural androgens dihydrotestosterone (1 nM) and testosterone (10 nM) displayed a comparable reduction in the promoter activity of the CMV-$(ARE)_3$-LUC plasmid, as found with 1 nM R1881 (defined as 100% inhibition; Fig. 4, A and B). Cells expressing the wild-type AR and incubated with the antagonists cyproterone acetate (100 nM) and RU 38486 (100 nM) also showed promoter interference, although less than that in the presence of androgens (Fig. 4A). The complete antagonists hydroxyflutamide (1 μM), ICI 176.334 (1 μM), and RU 23908 (1 μM) only slightly affected the promoter activity of the reporter plasmid (Fig. 4A). For the mutant receptor ARL, binding of cyproterone acetate, hydroxyflutamide, and RU 23908 resulted in a comparable re-

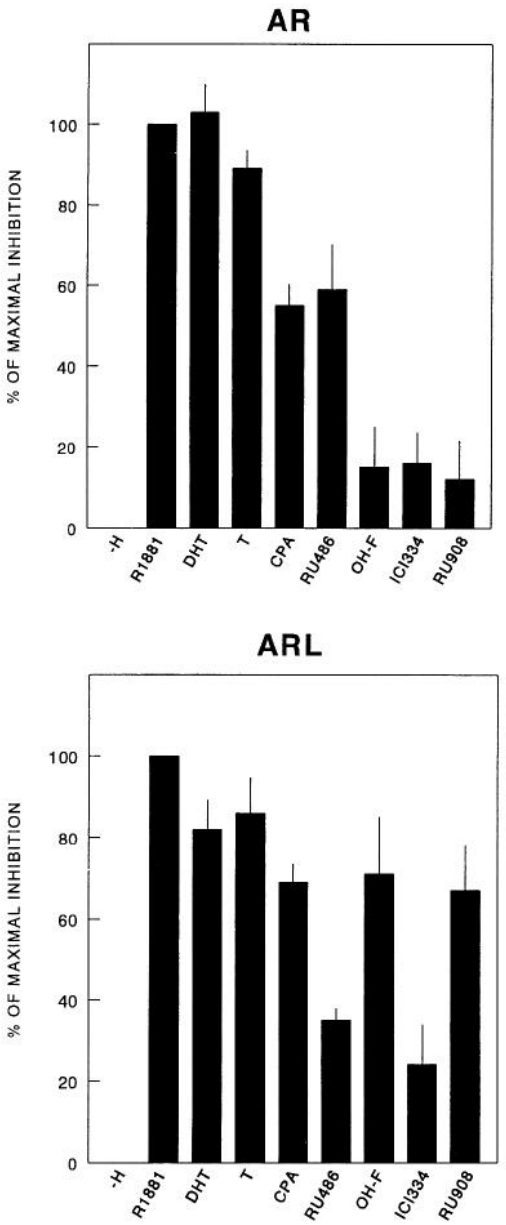

FIG. 4. Promoter interference of the wild-type AR and mutant receptor ARL in the presence of several androgens and antiandrogens. CHO cells were cotransfected with CMV-$(ARE)_3$-LUC promoter interference plasmid and wild-type AR or mutant ARL expression plasmid, and incubated without hormone (−H) or with 1 nM R1881, 1 nM dihydrotestosterone (DHT), 10 nM testosterone (T), 100 nM cyproterone acetate (CA), 100 nM RU 38486 (RU486), 1 μM hydroxyflutamide (OH-F), 1 μM ICI 176.334 (ICI334), or 1 μM RU 23908 (RU908). Inhibition of promoter activity in the presence of 1 nM R1881 was set at 100%. Values are the mean (±SEM) of four or five determinations.

duction of LUC activity of the CMV-$(ARE)_3$-LUC plasmid as observed for the androgens (Fig. 4B). The full antagonist ICI 176.334 showed only a minor effect with the mutant ARL, resembling its effect on the wild-type receptor. For the RU 38486-bound ARL, a slightly smaller reduction in promoter activity was observed than that for the wild-type receptor.

The results on transcription activation and promoter interference of both wild-type AR and mutant ARL, complexed with different ligands, are summarized in Table 1. A correlation between DNA binding and transcription activation was observed for most compounds, with the exception of RU 38486.

AR expression levels and migration pattern

As hormone addition was shown to have effects on the receptor stability (29) and phosphorylation status of the receptor (37), we wanted to exclude the possibility that the differences in the promoter interference assay were due to differences in receptor expression levels. Therefore, extracts were prepared from transfected cells after treatment with the various ligands, and receptors were analyzed by Western blotting. In the absence of hormone, the wild-type receptor and the mutant receptor AR64 migrated as two protein bands (110–112 kDa; Fig. 2C). The difference in electrophoretic mobility represents differences in the degree of phosphorylation of the receptor (35–37). An additional decrease in the electrophoretic mobility of the receptor protein was ob-

TABLE 1. Summary of the effects of ligands on the wild-type and mutated receptors

	AR		ARL	
	DNA binding	*Trans*-activation	DNA binding	*Trans*-activation
Androgen	++	++	++	++
CPA	+	+	++	++
RU486	+	−	+	−
OH-F	−	−	++	++
ICI334	−	−	−	−
RU908	−	−	++	+

−, No effect; +, limited effect; ++, full effect; CPA, cyproterone acetate; RU486, RU 38486; OH-F, hydroxyflutamide; ICI334, ICI 176.334; RU908, RU 23908.

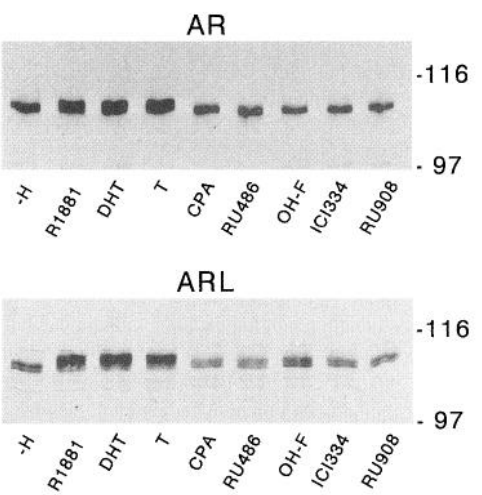

FIG. 5. Effect of androgens and antiandrogens on expression levels of wild-type AR and mutant ARL. Western blot analysis of lysates from CHO cells, transfected with either AR or ARL expression plasmid and incubated with the ligands, as described in Fig. 4. Whole cell lysates were fractionated with gel electrophoresis, and separated proteins transferred to nitrocellulose and visualized with a chemiluminescence detection method, using the AR-specific polyclonal antiserum SP197 as primary antibody. Molecular mass markers (kilodaltons) were run on a parallel lane, and their positions are indicated on the *right* of each blot.

served, when CHO cells, expressing the wild-type AR, were incubated in the presence of R1881 (Fig. 2C). These results are in agreement with observations described by others (37) and suggest the appearance of an additional protein form in the presence of R1881. The additional decrease in electrophoretic mobility was not observed for the mutant receptor AR64 in the presence of R1881 (Fig. 2C) (37). The expression levels of wild-type receptor (Fig. 5A) and mutant ARL (Fig. 5B) also increased in the presence of the natural androgens dihydrotestosterone and testosterone. In contrast, binding of antagonists to both receptors, even those antagonists that showed agonist activity with ARL, did not affect receptor expression levels (Fig. 5, A and B). This indicates that for all antiandrogens studied, the receptor expression levels were comparable, and variations in promoter interference between the various antiandrogen-bound receptors could not be explained by differences in receptor expression levels.

Discussion

An essential step in androgen action is transformation of the AR from a complex with heat shock proteins (27) to a DNA-binding state. DNA binding of full-length AR has been studied previously by *in vitro* electrophoretic mobility shift assays with oligonucleotides containing a consensus ARE. In these studies, performed with wild-type AR produced in a variety of systems (*in vitro* transcription/translation, transiently transfected COS-7 cells, or recombinant baculovirus-infected Sf9 cells), DNA binding of the receptor was either hormone independent (13, 38, 39) or required intracellular hormone exposure (40, 41). Demonstration of hormone-dependent DNA binding in the electrophoretic mobility shift assay is complicated by the isolation procedure of the AR protein, which may cause artificial receptor activation. In addition, the results of *in vitro* studies generally do not account for receptor-ARE interactions under equilibrium conditions. In the present study, we used a promoter interference assay that permits study of AR interaction with DNA in whole cells.

The promoter interference assay was originally described by Hu and Davidson (42), who used it to show that *lac* repressor, bound to its operator near a transcription initiation site, strongly repressed the activity of a reporter gene. This same principle, also used to study interaction of estrogen receptor with DNA (17), was applied by us to examine DNA binding of the AR in whole cells. In these studies, with a promoter interference CMV-$(ARE)_3$-LUC construct transfected into CHO cells, it was shown that reduced LUC activity resulted from inhibition of transcription due to androgen-dependent binding of the AR to its response elements. First, interference of CMV promoter activity was dependent on the presence of AREs in the reporter construct; second, on the expression of functional receptors; and third, on the addition of androgens. Furthermore, we observed reduced LUC activity in transiently transfected CHO cells at AR concentrations that were functional within the cells; the amounts of AR expression plasmid needed to suppress LUC activity from the promoter interference plasmid were comparable to those necessary to activate transcription from a MMTV-LUC reporter plasmid. In addition, we could exclude that the decrease in LUC activity was due to squelching, a phenomenon that has been shown to occur at high expression levels of steroid receptors (43).

The observation that the AR is unable, in the absence of ligand, to bind to AREs in the CMV-$(ARE)_3$-LUC reporter plasmid is in agreement with results from studies that showed that the unliganded forms of the progesterone (16, 44), glucocorticoid (45), and androgen (40, 41) receptors are unable to bind to their response elements on DNA. The androgen-dependent binding of the AR to DNA, as observed in the present study, supports a model in which binding of the ligand to the receptor causes dissociation of the heat shock protein-receptor complex and exposes the major dimerization region of the receptor. This enables the liganded receptor to dimerize and bind with high affinity to its response element. These structural changes and related covalent modifications enable the bound receptor to function as a ligand-dependent transcription activator [reviewed by Tsai and O'Malley (8)]. Results from other studies performed on progesterone (20) and estrogen (17, 21) receptors do not support this model. These observations indicate that progesterone and estrogen receptors are capable of binding to DNA within whole cells in the absence of ligand and that the ligand is needed to enhance or stabilize the interaction of the receptor with response elements. We feel that the differences with respect to hormone dependency of DNA binding between androgen and glucocorticoid receptors, on the one hand, and the estrogen receptor, on the other, may warrant some caution of generalization of steroid hormone receptor transformation from an inactive toward an active state.

The inability of AR antagonists to induce DNA binding of the receptor has been postulated as one of the molecular mechanisms of antiandrogen action (27, 40). The full antagonists, hydroxyflutamide, ICI 176.334, and RU 23908, which lack agonist activity [previously shown (12, 24, and 29)], failed to induce DNA binding of the wild-type AR in whole cells. The antagonistic activity of these compounds stems, therefore, from their inability to induce DNA binding of the receptor. Presumably, these compounds are unable to induce the necessary changes in the conformation of the AR to release the associated proteins (12). The antihormone-receptor complexes formed with cyproterone acetate and RU 38486 were capable of binding to DNA within the cell. RU 38486, which was transcriptionally almost inactive, reduced promoter activity in the promoter interference assay to the same extent as the partial agonist cyproterone acetate, indicating that DNA binding is a prerequisite for *trans*-activation, but that DNA binding alone is not sufficient to ensure a transcriptionally active receptor. ARs, when associated with RU 38486, bind to their responsive elements in whole cells and *in vitro* (40). However, RU 38486 apparently induces an altered conformation of the C-terminus of the receptor compared to the agonist-induced conformation, reflected by differences in proteolytic digestion pattern (11, 12), migration on sucrose gradients (12), and electrophoretic mobility shift assays (40). Similar observations were made in studies with the RU 38486-bound progesterone receptor (6, 9, 10, 18, 20, 46, 47). Therefore, the failure of the antagonist RU 38486 to

trans-activate is due to structural alterations in the ligand-binding domain.

The progesterone and estrogen receptor antagonists have been tentatively divided into two classes depending on their level of action (48, 49). The so-called type I antagonists interfere with binding of the receptor to DNA. The other class of antagonists (type II; including, for example, RU 38486) (48) induces stable, high affinity DNA binding of the receptor, but blocks the interaction of the receptor with the transcription initiation complex. Recently, McDonnell *et al.* (50) distinguished the estrogen receptor agonists from partial agonists on the bases of molecular criteria. Additionally, a classification of the known estrogen receptor antagonists into three distinct classes was proposed. These researchers hypothesized that the estrogen receptor might exist in the cell in multiple conformations, representing the inactive state, the active state, and several intermediate states. Antagonists exert their action by stabilizing a specific structure. These distinct conformations could result as a consequence of the ability of these compounds to keep the receptor in a specific conformation by blocking a progressive change from inactive to active. The effects of AR agonists and antagonists in our analysis suggest that the antiandrogens also promote stabilization of several different conformations. As a consequence, the known AR antagonists can be classified into three distinct categories. When we adopt the convention established by Klein-Hitpass *et al.* (48), the compounds hydroxyflutamide, ICI 176.334, and RU 23908 are type I antagonists, blocking the process that leads to DNA binding. We propose to add a further classification of the type II antiandrogens in two subtypes: the C-terminal end of the receptor is either involved (type IIa) or not involved (type IIb) in the mechanism of antagonist action. According to this classification, RU 38486 is a type IIa antagonist that induces an incorrect conformational change at the C-terminus of the ligand-binding domain of the AR (11, 12). Cyproterone acetate represents a type IIb antagonist that stabilizes the AR in a conformation that allows it to exhibit some transcriptional activity. Protease sensitivity studies did not reveal an abnormal conformation for the C-terminal part of the ligand-binding domain after binding of this ligand (12).

For the mutant receptor ARL, some antiandrogens should be classified differently. RU 23908 induced DNA binding of ARL without the exhibition of full agonistic activity. As the C-terminus of the ligand-binding domain is not involved in its antagonistic behavior (12), this compound represents a type IIb antagonist for ARL. Hydroxyflutamide and cyproterone acetate are full agonists for ARL. It could be speculated that the single amino acid change in the mutant ARL contributed to the ligand-induced changes in ARL conformation that potentiated transactivation (with cyproterone acetate), DNA binding (with RU 23908), or both (with hydroxyflutamide).

In conclusion, transformation of the wild-type AR to a DNA-binding state in whole cells is blocked by several antiandrogens (hydroxyflutamide, ICI 176.334, and RU 23908). Although DNA binding is a necessary step to accomplish transcriptional activity, studies with another antiandrogen (RU 38486) show that DNA binding alone is not sufficient; full transcriptional activity requires additional changes, presumably in the protein structure of the receptor. The classification of AR antagonists into several types according to the site of action, as discussed above, might be useful for the development of new compounds for clinical use.

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