Interaction of Androgen Receptors with Androgen Response Element in Intact Cells

ROLES OF AMINO- AND CARBOXYL-TERM RAL REGIONS AND THE LIGAND

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Promoter interference assay was employed to examine in intact cells the roles of the functional domains of androgen receptor (AR) and the ligand for specific DNA interactions using a cytomegalovirus-(androgen response element)-chloramphenicol acetyltransferase reporter (pCMV-ARE$_{2}$-CAT). Native rat and human ARs interfered with pCMV-ARE$_{2}$-CAT expression in a hormone-dependent fashion. Low steroid-independent interference seemed to occur because of the ligand binding domain (LBD), which was transcriptionally inhibitory also in a heterologous context. AR devoid of LBD (rAR$_{4641-902}$) decreased pCMV-ARE$_{2}$-CAT activity by 50%. The rAR$_{464-408}$ mutant devoid of the NH$_{2}$-terminal transcription activation region exhibited ligand-dependent promoter interference of a similar magnitude. Ligand and DNA binding-deficient mutants (hARM807R and rARC562G, respectively) did not influence pCMV-ARE$_{2}$-CAT expression, although hARM807R binds to ARE in vitro. Non-steroidal anti-androgens casodex and hydroxyflutamide antagonized agonist-dependent promoter interference, whereas cyproterone acetate, RU 56187, RU 57073, and RU 59063 were partial agonists/antagonists. Collectively, interaction of ARs with ARE in intact cells does not require the presence of the COOH-terminal or NH$_{2}$-terminal domain and/or their interaction. In the context of native AR, however, the androgen-induced conformational change in LBD is mandatory for generation of a transcriptionally competent receptor that binds to DNA in intact cells.

Androgen receptor (AR)$^{3}$ belongs to the nuclear receptor superfamily of ligand-regulated transcription factors (1–4). Although the interaction of AR with specific hormone-responsive DNA elements is usually required for androgen-dependent transcriptional activation, binding of AR to specific DNA motifs is not always necessary for the receptor’s ability to downregulate gene expression (5–7). With regard to the ligand requirement for the recognition of specific DNA elements by AR, in vitro electrophoretic mobility shift assays have yielded conflicting results. AR protein expressed in reticulocyte lysate or produced insect cells is capable of binding to specific androgen response elements (AREs) in vitro even in the absence of androgen (8, 9); however, the ligand requirement for the binding of hAR to AREs in vitro has also been reported (10). Very limited information is available on ARE occupancy by the receptor protein in intact cells. In vivo footprinting of an androgen-dependent enhancer of the mouse slp gene failed to reveal clear protection of hormone response elements by AR (11). While this work was in progress, Kuil and Mulder (12) reported that AR interaction with an “idealized” consensus ARE derived from polymerase chain reaction selection experiments is ligand-dependent in cultured cells.

The promoter interference assay developed by Hu and Davidson (13) is based on competition of DNA-binding proteins, such as nuclear receptors, for binding with essential transcription factors driving a constitutively active heterologous promoter. The interference is achieved by inserting an attachment sequence between the TATA box and the start site of transcription in the promoter. A promoter interference assay has been employed in a few reports to examine the interaction of estrogen receptor (ER) with its cognate response elements in mammalian and frog cells (14, 15) and that of AR with AREs in Chinese hamster ovary cells (12). Because of the use of transiently transfected cells, the physiological chromatin environment is not achieved under these conditions. However, using a pCMV-PRE$_{2}$-CAT construct stably integrated into T47D breast cancer cells, Gass et al. (16) recently reported that progesterone receptor (PR) is able to interfere with expression of this reporter also in the context of stable chromatin conformation, albeit less well than in transiently transfected cells.

In our previous studies, we constructed several NH$_{2}$-terminal deletion mutants of AR to define domains crucial for the activation functions of this protein (5, 8, 9). One of the mutants, rAR$_{464-408}$, devoid of a region mandatory for transcriptional activation (8), bound to ARE in vitro with somewhat lower affinity than the wild-type AR (9) but behaved as a dominant negative regulator of the native protein, possibly through forming transcriptionally inactive heterodimers with the latter (8, 9). The ability of rAR$_{464-408}$, or comparable mutants of the glucocorticoid receptor subfamily of nuclear receptors, to interact with specific DNA sequences in intact cells has, however, not been addressed previously. The COOH-terminal ligand binding domain (LBD) provides the means to regulate the function of native AR in a steroid-dependent fashion. Removal of this region generates a constitutively active receptor form.

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$^{*}$ The abbreviations used are: AR, androgen receptor; hAR, human AR; rAR, rat AR; ARE, androgen response element; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; DBD, DNA binding domain; ER, estrogen receptor; ERE, estrogen response element; LBD, ligand-binding domain; MMTV, mouse mammary tumor virus; Nterm, NH$_{2}$-terminal region of rAR; PR, progesterone receptor; PRE, progesterone response element; SV, simian virus.

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(17, 18), whose activity is dependent on the promoter context. However, in vivo DNA binding characteristics of LBD-deficient AR forms or those of other members of the glucocorticoid receptor subfamily have not been examined.

In the present work we have used promoter interference assays to investigate whether binding of AR to specific DNA sequences is ligand-dependent in intact CV-1 cells. We have also examined which functional domains of the wild-type receptor, in addition to the DNA binding domain (DBD), are mandatory for specific DNA binding in vivo. To assess the role of the ligand, several non-steroidal and steroidal anti-androgens alone or together with an androgen agonist were used. Finally, immunocytochemical analysis of cultured cells was employed to rule out the possibility that some of the results were compromised by altered subcellular distribution of receptor proteins.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from Sigma, Bio-Rad, and Qiagen GmbH (Hilden, Germany). Testosterone was purchased from Makrogen (Rehovot, Israel). [3H]Acetyl-coenzyme A was obtained from DuPont NEN. DNA-modifying enzymes were purchased from Pharmacia Biotech Inc. and Promega Corp. (Madison, WI). Oligonucleotides were synthesized using Gene Assembler Plus (Pharmacia). Non-steroidal anti-androgens casodex [(2R)-4’-cyano-3,4(4-fluorophenyl)-1,2-hydroxy-2-methyl-3’-[trifluoromethyl]propiononilide] and hydroxyflutamide (4-hydroxy-a,α,α-trifluoro-2-methyl-4-nitro-a-propiolotidizide) were obtained from Zenea Pharmaceuticals (Macchesne, U. K.) and Schering Corp. (Bloomfield, NJ), respectively. Cyproterone acetate (6-chloro-1,2-methylene-17α-hydroxy-4,6-pregnadiene-3,20-dione acetate) was from Schering AG (Berlin, Germany), and RU 56187 (4-(5-oxo-2-thioxo-3,4,4-tri-methyl-1-imidazolidinyl)-2-trifluoro-2-methyl-4-nitroflutamide (4-hydroxy-α,α,α-trifluoro-2-methyl-4-nitro-α-propiolotidizide) was obtained from Zenea Pharmaceuticals (Macchesne, U. K.) and Schering Corp. (Bloomfield, NJ), respectively. Cyproterone acetate (6-chloro-1,2-methylene-17α-hydroxy-4,6-pregnadiene-3,20-dione acetate) was from Schering AG (Berlin, Germany), and RU 56187 (4-(5-oxo-2-thioxo-3,4,4-tri-methyl-1-imidazolidinyl)-2-trifluoro-2-methyl-4-nitroflutamide (4-hydroxy-α,α,α-trifluoro-2-methyl-4-nitro-α-propiolotidizide) was obtained from Zenea Pharmaceuticals (Macchesne, U. K.) and Schering Corp. (Bloomfield, NJ), respectively. Cyproterone acetate (6-chloro-1,2-methylene-17α-hydroxy-4,6-pregnadiene-3,20-dione acetate) was from Schering AG (Berlin, Germany), and RU 56187 (4-(5-oxo-2-thioxo-3,4,4-tri-methyl-1-imidazolidinyl)-2-trifluoro-2-methyl-4-nitroflutamide (4-hydroxy-α,α,α-trifluoro-2-methyl-4-nitro-α-propiolotidizide) was obtained from Zenea Pharmaceuticals (Macchesne, U. K.) and Schering Corp. (Bloomfield, NJ), respectively. Cyproterone acetate (6-chloro-1,2-methylene-17α-hydroxy-4,6-pregnadiene-3,20-dione acetate) was from Schering AG (Berlin, Germany), and RU 56187 (4-(5-oxo-2-thioxo-3,4,4-tri-methyl-1-imidazolidinyl)-2-trifluoro-2-methyl-4-nitroflutamide (4-hydroxy-α,α,α-trifluoro-2-methyl-4-nitro-α-propiolotidizide) was obtained from Zenea Pharmaceuticals (Macchesne, U. K.) and Schering Corp. (Bloomfield, NJ), respectively.

Constructs and Reporter Genes—pCMV-ARE-CAT reporter was constructed from pCMV-0-CAT (a gift from Dr. B. Katzenellenbogen, University of Illinois, Urbana) by inserting a double-stranded 45-base pair oligonucleotide, 5'-CATATGACGCTAGTTCTCCGACATCGTGAATGATTTCCGACT-3', containing duplicated high affinity AREs (half sites underlined) of the C3(1) gene of prostatic binding protein (9, 19) into the Sall site between the TATA box and the transcription start site of pCMV-0-CAT. pCMV-ERE-CAT was constructed in the same way except that the inserted 45-base pair oligomer contained two estrogen response elements in lieu of AREs (14). For transcriptional activation experiments, pARE-Cat reporter vectors pARE-CAT and pMMTV-CAT were used (20). Expression vectors pSGrAR, pSGrHAR, pSGrARC562G, pSGrHAR325, pSGrAR46–408, pSGrAR46–408/C562G, pSGrAR63–461 and pSGrAR641–902 were used (5, 8, 21, 22). Expression vectors pSGrAR, pSGrHAR, pSGrARC562G, pSGrHAR325, pSGrAR46–408, pSGrAR46–408/C562G, pSGrAR63–461 and pSGrAR641–902 were used (5, 8, 21, 22).

RESULTS

Constructs for Promoter Interference Assays—An AR-dependent promoter interference reporter plasmid, pCMV-ARE-CAT, was constructed in a fashion similar to that described for ER (14) by inserting a duplicated high affinity ARE sequence between the TATA box and transcription start site of pCMV-0-CAT (Fig. 1A). Two AREs were inserted, as the presence of more than one palindromic response element facilitates AR-ARE interactions in vitro (9). pCMV-0-CAT devoid of ARE sequences was initially used as the control reporter, but its basal activity in CV-1 cells was several times higher than that of pCMV-ARE-CAT. This result was in agreement with a previous report (13) that insertion of sequences into a similar location (between the TATA box and the start site of transcription) decreases CAT expression. For this reason, we used pCMV-ERE-CAT reporter (Fig. 1A) as control to assess the specificity of AR-ARE interaction in intact cells. CV-1 cells were cotransfected with various AR expression plasmids (Fig. 1B) and pCMV-ARE-CAT (or pCMV-ERE-CAT) reporter driven by the constitutively active CMV promoter.

Specific Promoter Interference by the Native AR Protein Requires Intact Ligand and DNA Binding Domains—Native rAR and hAR interfered with pCMV-ARE-CAT expression in such a way that both ligand-dependent and ligand-independent inhibition of the reporter activity was observed (Fig. 2, A and B). rAR showed somewhat higher ligand-independent promotor-interfering activity than hAR. Since expression levels of rAR and hAR proteins were not compared in these experiments, the reason for this species difference remains to be elucidated. Both ARs also elicited some inhibition of pCMV-ERE-CAT expression which was, however, not increased by androgen (Fig. 2, C and D). Even though the weak ligand-independent activity of

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AR was specific for the hormone response element inserted in the promoter interference construct, it was specific for the promoter interference construct itself, as a similar androgen-independent activity was not observed in transactivation experiments. It is also worth pointing out that maximal promoter interference was not observed in transactivation experiments (see Fig. 2, A and B). AR forms incapable of DNA or hormone binding (rARC562G and hARM807R, respectively) were used to assess the importance of these regions for specific AR-ARE interaction in vivo. rARC562G with a Cys→Gly substitution at codon 562 does not bind to DNA in vitro and is transcriptionally inactive (22). hARM807R is incapable of hormone binding because of a Met→Arg substitution at codon 807 (21). In electrophoretic mobility shift assays under cell-free conditions, hARM807R bound to ARE with approximately the same affinity as the wild-type receptor in the absence of androgen (21), but exposure to androgen did not elicit a conformational change in the LBD of hARM807R in a fashion similar to that in the wild-type LBD (21, 27).

rARC562G and hARM807R mutants did not interfere with pCMV-ARECAT activity in a steroid-dependent fashion, but they exhibited weak hormone-independent interference, essentially indistinguishable from that of the wild-type apo-AR (Fig. 3, A and B). The fact that rARC562G behaved like apo-AR both in the presence and absence of testosterone implies that specific binding of the wild-type AR to DNA in intact cells is totally dependent on the integrity of the first zinc finger and that the conformational change elicited by an active androgen in the LBD does not abolish the ability of this region to interfere weakly with pCMV-ARECAT expression.

Influence of COOH-terminal and NH2-terminal Regions of AR on Receptor-ARE Interaction in Intact Cells—Previous studies have shown that AR forms devoid of the entire LBD behave as constitutively active transactivators and are capable of binding to specific DNA elements in vitro (17, 18); the rARΔ641–902 mutant lacks the LBD reduced expression of pCMV-ARECAT by 50% but did not influence that of pCMV-ERE-CAT (Fig. 4A). It is of note that maximal promoter interference was achieved with the lowest amount of pSGrARΔ641–902 expression vector used (0.5 μg of DNA/10-cm plate), and no additional increase in the interference occurred when the amount of plasmid was augmented 5-fold (Fig. 4A) or 10-fold (data not shown). Interaction of rARΔ641–902 with DNA was, as expected, independent of the presence of ligand, whether an agonist (Fig. 4A) or an antagonist (54 ± 5% of control in cells transfected with 0.5 μg of pSGrARΔ641–902 in the presence of 1 μM casodex).

Fig. 2. The ability of rat and human AR to bind to ARE sequences in intact cells as determined by promoter interference assay. CV-1 cells were transfected with increasing amounts (0.5–2.5 μg) of pSGrAR (panels A and C) or pSGrAR (panels B and D) and pCMV-ARECAT (panels A and B) or pCMV-ERE-CAT (panels C and D) promoter interference reporter as described under “Experimental Procedures.” Eighteen h after transfection, the cells received fresh medium with (solid symbols) or without (open symbols) 10 nm testosterone. Enzyme activities in transfected cells were measured 30 h later. Control reporter activity in the absence of AR expression plasmids was set as 100% in each case, and the values (mean ± S.E., n = 4) are expressed as a percentage of this activity.
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To examine the role of the NH2-terminal region, rARΔ46–408 and rARΔ46–408/C562G mutants were used. rARΔ46–408 expressed in insect cells binds to specific DNA sequences in vitro, albeit with a somewhat lower affinity than the wild-type AR (8, 9). When expressed in CV-1 cells, rARΔ46–408 elicited promoter interference in an androgen-dependent fashion (Fig. 4B). This interference required the presence of ARE sequences, as a similar steroid-dependent inhibition of pCMV-ERE<sub>2</sub>-CAT expression failed to occur under the same conditions (Fig. 4B). Likewise, an intact DBD of the receptor was required, as judged by the inability of rARΔ46–408/C562G to interfere with pCMV-ARE<sub>2</sub>-CAT activity. Some hormone-independent inhibition of pCMV-ARE<sub>2</sub>-CAT activity was observed with rARΔ46–408 when higher amounts of expression plasmid were used in transfections (Fig. 4B).

Taken together, interaction of AR with specific DNA sequences in intact cells can take place in the absence of LBD or most of the NH2-terminal region. DNA binding of the receptor, even in the presence of LBD and steroid, is not sufficient to elicit proper physiological responses, as the rARΔ46–408 mutant is transcriptionally inactive (8). Comparison of the dose-response curves between rARΔ641–902 and rARΔ46–408 suggests that LBD is responsible for the weak hormone-independent interference of AR with pCMV-ARE<sub>2</sub>-CAT or pCMV-ERE<sub>2</sub>-CAT expression (Fig. 4, A and B). The results with full-length AR forms (Fig. 2) were in general agreement with those obtained with rARΔ641–902 and rARΔ46–408 mutants.

Replication of Transcription at a Distance by LBD in a Heterologous System—The ability of LBD to interfere with transcription was examined by additional experiments, in which pG5SV40CAT reporter containing five GAL4 binding sites upstream of the SV40 promoter was used. CV-1 cells were co-transfected using this reporter or an appropriate control reporter (pSV40CAT) and expression vectors encoding GAL4-DDB, GAL4-LBD, or GAL4-Nterm fusion proteins. The NH2-terminal region of AR (residues 5–538) fused to GAL4-DBD did not influence the activity of the latter; however, when the fusion partner was LBD (residues 641–902), repression of transcriptional activity took place (Fig. 5A). It is of note that the ability of ligand-free LBD to repress transcription at a distance exceeded that of the ligand-occupied LBD in the GAL4-DNA-binding construct (Fig. 5A). The repressive action of LBD-containing fusion protein was dependent on its tethering to DNA, as no inhibition was detected in the absence of GAL4-binding elements (Fig. 5B).

Antagonistic and Agonistic Activities of Anti-androgens—Several anti-androgens (casodex, hydroxyflutamide, RU 56187, RU 57073, RU 59063, and cyproterone acetate) were examined by promoter interference assays using rARΔ46–408, as differentiation between ligand-dependent and -independent activities was more clear-cut with this mutant than with native ARs. All other anti-androgens examined but hydroxyflutamide were able to confer some promoter-interfering activity upon rARΔ46–408, with cyproterone acetate and the RU compounds being as potent as an agonist (10 nM testosterone) at the concentration used (1 μM) (Table 1). Additional studies with casodex revealed that its low agonist-like activity in the promoter interference assay was also detectable with wild-type AR and at varying receptor levels (Fig. 6A). When present concomitantly with androgen, casodex inhibited the agonist-induced promoter interference significantly but not completely (Fig. 6B).
COS-1 cells were transfected with 1 \mu g of pSGhAR and were harvested 30 h later for measurement of CAT and \beta-galactosidase activities. The values (mean \pm S.E., \( n = 3 \)) are expressed relative to that in CV-1 cells transfected without pSGhAR.

The results of this work show that sequence-specific DNA binding of the native AR in cultured CV-1 cells requires the presence of ligand. In this respect, our data agree with a recent report by Kuil and Mulder (12), who also used the promoter interference assay to examine the interaction of AR with a synthetic consensus ARE in Chinese hamster ovary cells. Our data show further that neither LBD nor a major part of the NH2-terminal transactivation region of the receptor is needed for its interaction with specific DNA sequences in vivo. LBD is not dispensable but also appears to be responsible for the ability of apo-AR to interfere weakly with pCMV-ARE-CAT expression. In addition, unoccupied LBD fused to GAL4-DBD was capable of repressing transcriptional activity of a heterologous SV40 promoter at a distance. Should AR bind to AREs as a homodimer in intact cells, as it does in vitro (8, 9), the results with rARΔ46–902 illustrate that this dimerization utilizes sequences other than those residing in LBD. Moreover, the interaction between COOH- and NH2-terminal regions of AR, which has been shown to occur in mammalian cells (31),2 is not mandatory for the ability of the receptor to recognize specific

### Table I

| Compound | pCMV-ARECAT activity (%) |
|----------|--------------------------|
| Vehicle  | 85.5 \( \pm 2.9 \) |
| Testosterone (10 nM) | 46.0 \( \pm 3.5 \) |
| Casodex (1 \mu M) | 77.0 \( \pm 3.1 \) |
| Hydroxyflutamide (1 \mu M) | 99.5 \( \pm 6.1 \) |
| RU 56187 (1 \mu M) | 43.5 \( \pm 4.0 \) |
| RU 57073 (1 \mu M) | 48.0 \( \pm 1.5 \) |
| RU 59063 (1 \mu M) | 42.5 \( \pm 1.9 \) |
| Cyproterone acetate (1 \mu M) | 40.0 \( \pm 1.6 \) |

DISCUSSION

The results of this work show that sequence-specific DNA binding of the native AR in cultured CV-1 cells requires the presence of ligand. In this respect, our data agree with a recent report by Kuil and Mulder (12), who also used the promoter interference assay to examine the interaction of AR with a synthetic consensus ARE in Chinese hamster ovary cells. Our data show further that neither LBD nor a major part of the NH2-terminal transactivation region of the receptor is needed for its interaction with specific DNA sequences in vivo. LBD is not dispensable but also appears to be responsible for the ability of apo-AR to interfere weakly with pCMV-ARE-CAT expression. In addition, unoccupied LBD fused to GAL4-DBD was capable of repressing transcriptional activity of a heterologous SV40 promoter at a distance. Should AR bind to AREs as a homodimer in intact cells, as it does in vitro (8, 9), the results with rARΔ46–902 illustrate that this dimerization utilizes sequences other than those residing in LBD. Moreover, the interaction between COOH- and NH2-terminal regions of AR, which has been shown to occur in mammalian cells (31),2 is not mandatory for the ability of the receptor to recognize specific

Previous studies have shown that casodex and hydroxyflutamide are completely devoid of inherent transactivation ability (22, 28), whereas cyproterone acetate is a partial agonist/antagonist (29).

Three N-substituted arylthiohydantoin anti-androgens (RU 56187, RU 7073, and RU 59063) were as effective as testosterone in conferring promoter-interfering activity on rARΔ46–408 (Table I). This is in agreement with binding affinities of these compounds for rat and human ARs in vitro, which are 92–300% of that of testosterone (30). When CV-1 cells cotransfected with pSGhAR and pMMTV-CAT vectors were exposed to 1 \mu M of each RU anti-androgen alone, CAT activity was 50% with RU 56187, 28% with RU 57073, and 35% with RU 59063 of that with 10 nM testosterone. Concomitant exposure of the cells to 10 nM testosterone and 1 \mu M RU anti-androgens resulted in reporter gene activities that were 39–66% of those achieved with androgen alone, indicating that the three RU compounds were partial agonists/antagonists under these conditions. As the RU compounds promoted AR-ARE interaction in CV-1 cells as well as testosterone (Table I), conformational requirements on LBD for transcriptional activation and recognition of specific DNA sequences (promoter interference) are not identical in intact cells.

**Localization of Wild-type and Mutant ARs by Immunocytochemistry in Transfected Cells**—The polyclonal antiserum (K183) used in these experiments was specific for AR proteins, as no extra bands were observed in mock-transfected cells, and the polyclonal antiserum recognized rAR with RU 59063 of that with 10 nM testosterone. Concomitant interference assay to examine the interaction of AR with a synthetic consensus ARE in Chinese hamster ovary cells. Our data show further that neither LBD nor a major part of the NH2-terminal transactivation region of the receptor is needed for its interaction with specific DNA sequences in vivo. LBD is not dispensable but also appears to be responsible for the ability of apo-AR to interfere weakly with pCMV-ARE-CAT expression. In addition, unoccupied LBD fused to GAL4-DBD was capable of repressing transcriptional activity of a heterologous SV40 promoter at a distance. Should AR bind to AREs as a homodimer in intact cells, as it does in vitro (8, 9), the results with rARΔ46–902 illustrate that this dimerization utilizes sequences other than those residing in LBD. Moreover, the interaction between COOH- and NH2-terminal regions of AR, which has been shown to occur in mammalian cells (31),2 is not mandatory for the ability of the receptor to recognize specific

**Fig. 6.** Influence of the anti-androgen casodex alone or together with testosterone on the binding of AR to ARE sequences in intact cells. CV-1 cells were transfected with increasing amounts (0.5–2.5 \mu g) of pSGAR (panel A) or pSGARΔ46–408 (panel B) expression vectors and pCMV-ARE-CAT promoter interference reporter. Eighteen h after transfection, the cells received fresh medium with (open circles, panel A) or without (open squares, panels A and B) 1 \mu M casodex or with 10 nM testosterone (closed squares, panels A and B) or together with 1 \mu M casodex (closed circles, panel B). The cells were harvested 30 h later for measurement of CAT and \beta-galactosidase activities.

**Fig. 7.** Immunoblot analysis of AR proteins expressed in COS-1 cells. The cells were transfected with 15 \mu g of pSG5 vector alone (lane 1), pSGAR (lane 2), pSGARΔ641–902 (lane 3), or pSGARΔ46–408 (lane 4) and harvested 48 h later. Each lane contained 15 \mu g of cellular protein. Conditions for protein extraction and immunoblotting were as described previously (8) except that antiserum K183 at a dilution of 1:1,500 was used as the primary antibody.

was strictly nuclear after exposure to testosterone (data not shown). Likewise, the LBD-deficient mutant (rARΔ641–902) was nuclear in the absence of the hormone (Fig. 8I). CV-1 cells transfected with pShAR expression vector and exposed to casodex or hydroxyflutamide exhibited only nuclear localization of the receptor protein (Fig. 8, I and J). Taken together, immunocytochemical studies of transfected CV-1 cells expressing wild-type and mutant AR proteins revealed that the receptor forms were mainly nuclear even in the absence of ligand, indicating that differences in subcellular localization do not explain their dissimilar behavior in the promoter interference assay.
in the LBD. The findings that hARM807R binds well to AREs in vitro (21) and is localized in nuclei of transfected cells (this study) are interpreted to mean that the steroid-induced change in LBD conformation is primarily required for the release of AR from associated inhibitory protein complexes, rather than for generation of a receptor form capable of ARE recognition.

The mutant rARΔ46–408, which has a region mandatory for transcriptional activation deleted (8), recognized specific DNA sequences in CV-1 cells in a fashion indistinguishable from that of the wild-type receptor, implying that the NH2-terminus is not essential for this interaction. This result is in agreement with our previous studies on AR-ARE interaction in vitro, in that NH2-terminal internal deletions compromised the affinity of rAR for AREs only to a moderate extent; the $K_D$ of interaction increased from 0.5–1.0 nM to 3.0 nM (8, 9). AR protein produced in insect cells has been suggested to require intracellular hormone exposure to overcome the inhibition imposed by the NH2-terminal domain on dimerization and DNA binding of the aporeceptor (10). The present experiments showed, however, that unliganded rARΔ46–408 was incapable of sequence-specific DNA binding in CV-1 cells (Fig. 4B), implying that a major part of the NH2-terminal region is not critical for maintaining the receptor in a non-DNA-binding form.

Anti-androgens that were examined in this work can be divided into two groups on basis of their ability to modulate the binding of AR to AREs. The first group is not capable of converting the receptor to a form that binds to specific DNA sequences in CV-1 cells, or it does this poorly. When present concomitantly with an agonist, these compounds inhibit the agonist-dependent promoter interference. Non-steroidal anti-androgens casodex and hydroxyflutamide belong to this group. The second group includes the steroidal anti-androgen cyproterone acetate and three N-substituted arythiohydantoin anti-androgens (RU 56187, RU 57073 and RU 59063). These compounds, at least at the concentrations used, converted AR to a form capable of ARE binding in CV-1 cells. Moreover, the compounds of the second group behaved as partial agonists/antagonists in transactivation experiments. Kuil and Mulder (12) have also reported that in Chinese hamster ovary cells, casodex and hydroxyflutamide inhibited DNA binding of AR, but cyproterone acetate failed to do so.

Ligand-free human and rat ARs exhibited weak promoter-interfering activity. In this respect, our results on AR differed from those reported by Reese and Katzenellenbogen (14) and Xing and Shapiro (15) on human and frog ERs, respectively. In these latter cases, unoccupied wild-type ER showed moderate to high interfering activity, and some ligand-free ER mutants were even more potent than the estrogen-bound wild-type ER (14, 15). Likewise, apo-PR elicited strong interference with pCMV-PRE-CAT expression, which was almost 50% of that of the progestin-bound PR (16). The observed differences in the behavior of nuclear receptors in promoter interference assays could result, at least in part, from dissimilar affinities of their LBDs for auxiliary proteins communicating with transcription machinery. A COOH-terminal region of PR has recently been reported to contain a transcriptional repressor domain that functions through a putative corepressor (33); however, a similar function has not so far been described for the COOH-terminus of AR. The use of a heterologous GAL4 system supported the notion that LBD is responsible for the weak ligand-independent promoter-interfering activity of AR. The activity of this LBD in the context of GAL4 fusion protein was not dependent on the ligand-induced conformational change; rather, ligand-free GAL4-LBD bound to elements upstream of SV40 promoter exhibited stronger repressive action than the ligand-occupied form, suggesting that apo-LBD is the favored

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**Fig. 8.** Immunocytochemical localization of wild-type and mutated AR proteins in transfected CV-1 cells. The cells were plated on glass coverslips on 10-cm cell culture dishes, transfected, and treated as described under “Experimental Procedures.” Detection by immunofluorescence was performed using Ki88 antiserum (dilution 1:200) as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. The panels correspond to the following receptor forms and treatments: rAR in the absence (A) and presence of 10 nM testosterone (B); hAR without (C) and with 10 nM testosterone (D); rARC562G (E), hARM807R (F), rARΔ46–408 (G), and rARΔ46–902 (H) without androgen; and hAR in the presence of 1 μM casodex (I) or 1 μM hydroxyflutamide (J). Original magnification: A–H, × 400; I and J, × 630.

DNA elements in vivo, as shown by appropriate deletion mutants in promoter interference assay. Studies with rARΔ46–408 that lacks most of the NH2-terminal region and with anti-androgens also provided compelling evidence that mere DNA binding in vivo is not sufficient for the AR to become a competent transcriptional activator.

The hARM807R mutant is incapable of hormone binding, totally inactive in transactivation assays, and the reason for complete androgen insensitivity of a patient (21). It could be speculated that inappropriate folding of this mutant’s LBD may render it free of intracellular chaperones such as heat shock proteins (32), thereby permitting its binding to AREs even in vivo. However, our present data and those of Kuil and Mulder (12) demonstrate that specific DNA binding in living cells is not achieved without a ligand-induced allosteric change
conformation in recruitment of putative repressor proteins.

In conclusion, the results of this work indicate that interaction of AR with specific DNA sequences in CV-1 cells does not involve COOH-terminal or NH2-terminal regions of the receptor, even though the NH2-terminal domain contains sequences required for transcriptional activation. In the context of wild-type AR, the androgen-induced conformational change in the COOH terminus is, however, mandatory for generation of a transcriptionally competent receptor protein that binds to ARE sequences in intact cells, even though mere DNA binding in vivo is not sufficient for the AR to acquire competence in transcriptional activation. And finally, LBD appears to be responsible for the weak promoter-interfering activity of apo-AR.

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