Functional Characterizations of the Androgen Receptor Confirm that the Molecular Basis of Androgen Action Is Transcriptional Regulation

Stephen E. Rundlett, Xi-Ping Wu, and Roger L. Miesfeld*

Departments of Molecular and Cellular Biology (S.E.R., R.L.M.), Biochemistry (X.-P.W., R.L.M.)
Arizona Cancer Center (R.L.M.)
University of Arizona
Tucson, Arizona 85721

In an effort to understand the molecular basis of androgen action in the prostate, we isolated androgen receptor (AR) cDNA from rat ventral prostate cells and analyzed the transcriptional regulatory activity of the encoded protein in a cotransfection assay. We found that AR is capable of inducing chloramphenicol acetyltransferase activity more than 20-fold using the mouse mammary tumor virus LTR as a source of androgen response elements. This induction was observed in both monkey CV1 cells and human HeLa cells, neither of which contains endogenous functional AR, and was entirely dependent on added androgens. Deletion mapping studies showed that carboxy-terminal deletions of ~250 amino acids convert AR into a constitutive activator of transcription. In addition, a chimeric receptor protein containing the amino-terminus and DNA-binding domains of AR fused to the previously defined ligand domain of the glucocorticoid receptor was found to be fully functional based on dexamethasone-induced chloramphenicol acetyltransferase activity. Our results support the prediction that androgens modulate rates of transcriptional initiation, suggesting that posttranscriptional effects of androgens are secondary responses. Moreover, these data reveal that, like other steroid receptors, AR contains a number of distinct regulatory regions important for normal activity. The isolation and characterization of fully functional AR sequences will facilitate the use of molecular genetics to study complex androgen responses in target tissues such as the prostate. (Molecular Endocrinology 4: 708-714, 1990)

INTRODUCTION

The androgen receptor (AR) belongs to a family of steroid receptor proteins that mediate cellular responses to extracellular signals through transcriptional control (1-3). Although both the human and rat ARs have been studied by biochemical methods for many years, cDNA sequences that encode them have only recently been isolated (4-9). Sequence analysis of these AR cDNAs reveals significant homology to the DNA-binding domain of the glucocorticoid (GR) and progesterone (PR) receptors (6, 8-10). This finding suggests that functional domains similar to those previously described for GR and PR may also be present in AR. Other segments of the AR sequence are less conserved, but based on functional studies with GR, are thought to encode an amino-terminal modulatory domain and a carboxy-terminal ligand-binding domain (11, 12).

The majority of evidence to date suggests that AR acts as a ligand-regulated transcription factor. The most convincing data have come from transient transfections of mouse mammary tumor virus (MMTV) reporter genes into the AR-containing cell line T47D (13-17). These studies have demonstrated that androgen-dependent induction of MMTV-directed transcription requires androgen response elements (AREs) which apparently overlap with glucocorticoid/progesterone response elements (GRE/PRE) in the MMTV LTR (16, 17). However, T47D cells are not optimal for studies of AR function because they contain various amounts of AR, GR, and PR (14). This could complicate transcriptional studies of MMTV reporter genes, in that potential interactions between these closely related receptors may effect the results. To study AR in detail, structure-function analyses need to be performed in appropriate heterologous cell types which contain no endogenous receptors, such as monkey CV1 cells, which have been used to map GR and PR functional domains (18, 19).

Unlike the rapid progress realized in the molecular characterization of GR (20-24), progress in the field of androgen regulation has been lagging (3). In addressing the problem of androgen regulation several findings must be considered. First, there has been some debate over the molecular basis of androgen action in the...
prostate and kidney, specifically as it relates to the role of transcriptional initiation in the observed response (3). It is presumed that the androgen receptor acts as a transcriptional initiation factor (12-17), but several in vivo studies of known androgen target genes have suggested that androgens primarily control mRNA stability (25, 26). Secondly, results from studies using transient transfections of tissue culture cells (14) or transgenic mice (27) have suggested that control of some androgen target genes may require cell-specific factors in addition to AR, implying that androgen responses are more complicated than what has been observed with other steroids. Thirdly, it has been found that tissue-specific forms of the thyroid (28) and retinoic acid (29) receptors exist. It is, therefore, conceivable that more than one AR species (as defined by ligand binding) is expressed in various target tissues.

We are interested in studying the transcriptional control of androgen-regulated prostate growth and regression. In an effort to confirm that the molecular basis of androgen action is at the level of transcriptional initiation and to map critical functional domains present in the receptor, we have cloned and characterized rat ventral prostate (RVP) AR cDNA. We describe in this report our findings that the RVP AR functions as a ligand-regulated transcription factor, and furthermore, that like other steroid receptors, AR contains discrete domains which operate independently. These results are discussed with respect to their impact on the analyses of several AR defects, such as Tfm mutations in mice (30) and androgen insensitivity in humans (31), and the study of complex androgen responses using molecular genetic approaches.

RESULTS AND DISCUSSION

Isolation and Expression of AR cDNA

To isolate cDNA sequences corresponding to rat prostate AR, we synthesized four oligonucleotide primers based on the AR cDNA sequence reported by Chang et al. (8) for use in the polymerase chain reaction (PCR) and specific cDNA priming. A combination of random hexanucleotide primers and specific primers was used to synthesize cDNA from size-fractionated RVP mRNA to enrich for large transcripts (>4 kilobases (kb)). A cloned amplified AR cDNA fragment was used as a probe to screen the prostate-specific cDNA library. The location of this probe (p6-3) relative to the putative AR DNA-binding domain (hatched box) is shown in Fig. 1A. Approximately 3 x 10^5 primary recombinants were screened; Fig. 1B shows two of the overlapping cDNA inserts analyzed by DNA sequencing. The composite map of a full-length AR cDNA (p6R-AR) cloned into a eucaryotic expression vector is illustrated in Fig. 1C.

We next examined the transcriptional regulatory activity of this cloned AR. Figure 2 shows the results of a transient cotransfection assay using p6R-AR and a reporter plasmid containing 1.4 kb of the MMTV LTR linked to the chloramphenicol acetyltransferase (CAT) gene. We observed that 10^{-7} M testosterone or dihydrotestosterone (DHT) or testosterone (TST) for 48 h before extracts were prepared.
(GMLO) likewise resulted in androgen-dependent induction of β-galactosidase activity (Rundlett, S., unpublished data). Based on the results from these transfection experiments, we conclude that p6R-AR encodes an androgen-dependent transcription factor similar to other steroid receptors. In addition, these data indicate that all functions required for androgen-dependent MMTV transcriptional regulation are encoded within the cloned AR cDNA sequence, thus making it possible to map functional domains using this cotransfection assay.

**Constitutive Activity of AR Carboxy Deletion Mutants**

Deletion of the carboxy-terminal portion of GR constitutively activates DNA binding and transcriptional regulatory functions (20–22). This suggests that native GR (ligand free) is inactive or repressed, and that ligand binding [which has been mapped to this region independently (23)] alters the protein conformation in a way that derepresses specific DNA binding and transcriptional activation (24). To test the possibility that AR carboxy deletion mutants function in a way similar to GR deletions, we truncated the AR cDNA at the carboxy-terminus and subcloned the deleted AR sequences into an RSV expression vector containing downstream stop codons in all three reading frames (Fig. 3A). The results of transient cotransfection assays using the MMTV CAT reporter plasmid and the AR carboxy-terminal deletion mutants are shown in Fig. 3B. Removal of 201 amino acids from the AR carboxy-terminus (N701) leads to partial activation of the native receptor. If an additional 33 residues are removed (N668), the deleted AR is constitutively active to the same level observed with ligand-bound full-length AR (N902). Note that the N629 deletion retains near wild-type levels of activity, indicating that DNA binding has not been compromised in this mutant.

This deletion end point for constitutive activation maps to the same general region in AR, as has been reported for GR (20), relative to the lysine-rich sequence located in the functionally defined DNA-binding domain (23). One possible explanation for the observed constitutive activity in the N668 and N629 mutants may be that AR sequences have been deleted which interact with heat shock protein-90, a protein thought to repress steroid receptor activation (24). If this is true, then the putative heat shock protein-90-binding site would overlap with amino acids 668–701 in AR, since it is between these two deletion end points that constitutive activity increases dramatically (Fig. 3). By analogy, this would correspond to amino acids 556–595 in GR based on a similar rise in activity of the N556 deletion relative to N595 (20).

The finding that N629 retains high level constitutive activity by this assay maps not only the DNA-binding domain, but also positive enhancement sequences which may be localized in this portion of AR. At least two classes of activation sequences appear to be present in the amino-terminal modulatory domain. First, there is an uninterrupted stretch of glutamines between residues 172–194. Such sequences have been implicated as transcriptional regulatory elements important to SP1 function (32). Second, there are abnormally high numbers of aspartate and glutamate residues in the AR amino-terminus, similar to those found to be required for maximal GR activity (22, 33). Another potential transcriptional activation sequence may be interdigitated within the DNA-binding domain itself as described for GR (18, 34).

**Activity of an AR-GR Chimeric Receptor Protein**

To further characterize functional domains of the cloned AR we constructed an AR-GR fusion protein using the modified PCR technique of overlap extension (35). This fusion receptor was used to compare relative transcriptional regulatory activities of AR and GR under conditions in which the same ligand [dexamethasone (dex)] and expression vector could be used in transiently transfected CV1 cells. Green and Chambon (36) replaced the estrogen receptor (ER) DNA-binding domain

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**Fig. 3. Transcriptional Activity of AR Carboxy-Terminal Deletions**

A, Map of AR deletion mutants showing the terminal amino acid position, as determined by DNA sequencing. These deleted cDNAs were cloned into an expression vector containing translational stop codons in all three reading frames located at the 3′ cloning site. The amino acid sequence of a lysine-rich region located near the putative DNA-binding domain in AR is shown (KLKKL). B, CAT assay results from CV1 cotransfections containing 50 μg each of the deleted AR expression plasmids and MMTV CAT grown in the presence or absence of 10⁻⁹ m dihydrotestosterone (DHT). The amino acids contained within each expression plasmid are shown for the full-length AR 1–902 (N902), and the three deletions: 1–701 (N701), 1–668 (N668), and 1–629 (N629).
with the analogous region from GR to create a functional hybrid ER-GR receptor. However, in our AR-GR fusion protein we maintained the integrity of the AR amino-terminus and DNA-binding domain and, therefore, measured the combined activities of these two domains. Figure 4 illustrates that the chimeric receptor AGR contains amino acids 1–623 of AR fused to the functionally autonomous steroid-binding domain of GR (residues 520–795).

The results of our cotransfection experiments using the MMTV CAT reporter plasmid and either the GR or AGR expression plasmids are shown in Fig. 4. We found that AGR functions as a heterologous receptor by inducing CAT activity only in the presence of dex, thus demonstrating that GR carboxy sequences convert an AR constitutive activator into a glucocorticoid-regulated chimeric receptor (compare N629, Fig. 3, to AGR, Fig. 4). Figure 5 summarizes quantitative comparisons of AR, GR, and AGR activities using the results of these and other cotransfection assays. It can be seen that GR induction of MMTV CAT is substantial, resulting in 73% conversion of the substrate. In contrast, activation of MMTV CAT by AGR is significantly lower (11% conversion). Assuming similar levels of AR and GR expression in CV1 cells, these data suggest that GR is approximately 15 times more active than AR in this assay. The 2-fold difference between AR and AGR is very reproducible and may reflect the contribution of enhancer activation sequences from this region of GR (37).

The reduced AR induction of MMTV CAT relative to GR suggests that interactions of these receptors with the MMTV LTR may not be identical. To further examine this, we cotransfected the receptor expression plasmids with another reporter gene, referred to here as TyTCO, containing the tyrosine aminotransferase (TAT) GRE linked to the thymidine kinase promoter and CAT (38). The results of these transfections indicate that while GR is capable of activating TyTCO up to 20-fold, both AR and AGR induce CAT activity only 2-fold (see Figs. 4 and 5). As a control, additional experiments were performed in which the MMTV β-galactosidase plasmid GMLO was included in the transfection. We found that in the same extracts, AR and AGR induced β-galactosidase activity as expected, but, again, only minimal induction of TyTCO was observed (data not shown). These results are consistent with a recent report by Dennison et al., (39), in which a 2-fold induction of CAT activity was observed using a reporter plasmid very similar to TyTCO transfected into the AR-containing human prostate cell line LNCaP.

Comparative Analyses of AR and GR

It has been proposed that AREs and GREs are functionally identical based on transfection studies using MMTV-CAT reporter genes introduced into AR-containing cell lines (12–17). Therefore, we found it surprising
that GR activates MMTV CAT and TyTCo approximately 15 times better than AR or AGR in our cotransfection assay. Although there may be dissimilarities in protein stability between AR and GR in CV1 cells that could explain our results, it is also possible that in the earlier transfection studies, the CAT activity should be normalized to reflect the observed difference in steady state levels of AR and GR in T47D cells (AR > GR). If this is done, then GR is found to be 10–20 times more active than AR in inducing MMTV CAT, which is consistent with our results in the cotransfection assay.

Inspection of the amino acid sequence in the DNA-binding domains of AR, GR, and PR reveals that there are 9 of 66 amino acids unique to the conserved DNA-binding domain of AR compared to GR or PR (40). These changes are clustered in a proposed α-helix structure situated between the two fingers (41). These may be significant, since GR and PR bind to a nearly identical DNA sequence (42), and AR and GR do not activate MMTV CAT and TyTCo to the same extent (Refs. 39 and 43 and results in this paper). Interestingly, one of these nine amino acids lies within the recently defined D box (44), changing Gly-478 in GR to Ser-580 in AR. There are a number of other changes in the amino-terminus of AR that may contribute to the observed differences in transcriptional activation of MMTV by AR and GR. These could involve protein-protein interactions important for regulation of certain cell-specific steroid target genes (43) or in cooperative interactions between receptors (45, 46).

Molecular Genetic Approaches to the Study of Androgen Action

Our observation that the RVP AR is functionally and mechanistically similar to the other steroid receptors provides clear direction to future studies aimed at deciphering androgen effects in both the kidney and prostate systems. One very useful finding from these studies is that AR can function in cell lines containing no endogenous AR. This raises the possibility that while cell-specific factors may be involved in the regulation of gene expression, they are not always necessary for AR function. Studies designed to investigate multihormonal regulation of overlapping steroid-regulated gene networks can now be performed using cloned AR and GR sequences. For example, Strahle et al. (47) recently reported that stable transfection of PR cDNA into hepatocytes can lead to progesterone induction of glucocorticoid target genes, implying that, at least in this case, cell-specific glucocorticoid and progesterone gene networks are controlled at the level of receptor expression. Similarly, expression of AR in cell lines normally containing only GR may result in androgen regulation of glucocorticoid target genes, potentially identifying any overlap in the androgen and glucocorticoid gene networks (48).

Finally, AR defects in cases of human androgen insensitivity have recently been examined using AR cDNA probes and PCR (49). Initially, these studies have concentrated on mutations in the ligand-binding domains, but with the development of a cotransfection assay using AR and MMTV reporter plasmids, it should now be possible to study a wider variety of AR mutants. In addition, for the analyses of molecular determinants of androgen resistance in prostate tumor cells, experimental manipulation of functional AR sequences can be used to investigate genetic alterations in the androgen response of malignant cells. One of the most challenging problems in prostate cancer research is the identification of factors that contribute to the inevitable failure of endocrine therapy. Knowledge of AR structure and function, and identification of key androgen target genes in the prostate of normal and neoplastic cells will be important in understanding the molecular basis of androgen resistance in this prevalent human disease.

**MATERIALS AND METHODS**

**Complementary DNA Cloning**

Poly(A)* RNA isolated from 2-day castrated rats was size fractionated on sucrose gradients as previously described (50). cDNA was synthesized using gradient-purified RNA of more than 4 kb and a primer mixture containing random hexanucleotides and the specific primers AR2 and AR4. Size-selected double strand cDNA (>0.6 kb) was ligated to EcoRI adaptors and cloned into XZAPII (Stratagene, San Diego, CA) following the manufacturer’s recommendations. The cDNA library was screened with the insert from p6–3 (see below), which was labeled using a random primer kit (Amersham, Arlington Heights, IL) and [α-32P]dCTP. Two of the isolated clones, Zap R2–7 and Zap R2–25, were digested with H/ndlll and AR and 541–598 in GR using the plasmids pAR-SK and p6R-AR, respectively. The carboxy-terminal deletions were made by treating Stü-di-gested pAR-SK with Exoll and mung bean nuclease and subcloning deleted fragments as previously described (50).

**PCR Reactions**

All primers were made on a Cyclone DNA synthesizer (Milligen, Bedford, MA) and correspond to the following nucleotides (5’ to 3’) in the rat AR cDNA reported by Chang et al. (8); AR6, 1050–1071; AR2, 1730–1704; AR3, 2590–2565; and AR4, 2803–2780. First strand cDNA synthesis was performed in a 20-μl volume using 1 μg poly(A)* RNA from the RVP of 2-day castrated rats (Sprague-Dawley; 250–300 g), 10 pmol of the 3’ primer (AR3), 1 mw of each dNTP, and AMV reverse polymerase (Cetus) to a final reaction volume of 100 μL. Most PCR amplifications required 25 cycles, with annealing temperatures ranging from 46–58 °C. The AR 6–3 PCR product was digested with AccI and EcoRI and cloned into the SK+ (Stratagene) or p6R (28) vectors to create pAR-SK or p6R-AR, respectively. The carboxy-terminal deletions were made by treating Stü-di-gested pAR-SK with Exoll and mung bean nuclease and subcloning deleted fragments as previously described (50).

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appropriate fragments from pAR-SK and pRD93 into p6R. A majority of this final construct was analyzed by DNA sequencing to confirm its structure.

Cotransfection Assays

Transfections were performed using the monkey kidney cell line CV1 or the human carcinoma cell line HeLa. Cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal calf serum (testosterone concentration is $10^{-11}$ M in this media). Electrophoreses were performed using a BRL Cell Porator at 275 mV and 330 microfarads as described by the manufacturer (Life Technologies, Inc., Gaithersburg, MD). Logarithmically growing cells were resuspended in HBSS (21 mM HEPES, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM Na2PO4; and 6 mM glucose) at a concentration of $6 \times 10^6$ cells/ml, including expression plasmid (10--50 μg), reporter plasmid (50 μg), and, in some experiments, 50 μg of a β-galactosidase internal control plasmid (GMLO). After electroporation and a 10-min recovery period, cells from a single cuvette were split into two equal portions and plated in Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum with and without $10^{-7}$ M testosterone, dihydrotestosterone, or dex. After 48 h in culture the cells were harvested using trypsin. CAT assays were performed using 60 μg protein in a volume of 200 μl as previously described (51). The reaction mixture was incubated at 37°C for 16 h, and acetylated forms of chloramphenicol were separated by TLC on Silica Plates (EM Science) in chloroform-methanol (95:5). The reporter plasmids pMMCAT and pGMLO contain 1.4 kb of the MMTV LTR linked to the CAT or β-galactosidase genes, respectively. pTYTCC, pOTC, and pOMCO have been previously described (38, 52).

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Address requests for reprints to: Dr. Roger Miesfeld, Arizona Cancer Center, University of Arizona, Tucson, Arizona 85724.

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