

Delineation of Two Distinct Type 1 Activation Functions in the Androgen Receptor Amino-terminal Domain*

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Based on the finding that some transcription factors contain multiple transcriptional regulatory activities, we constructed a panel of rat androgen receptor (AR) mutants containing small internal deletions and point mutations within the amino-terminal region of the receptor. Trans-activation assays in CV-1 cells using AR-responsive reporter genes were performed and led to the identification of two noncontiguous trans-activation regions in the AR amino terminus. One of these regions, termed activator function 1a (AF-1a) is a highly-conserved 14-amino acid segment that is predicted to form a β -turn followed by an acidic amphipathic α -helix. Point mutagenesis within AF-1a revealed that two adjacent hydrophobic residues were required for full AR trans-activation function, as arginine substitutions resulted in a 60% reduction in transcriptional activity. A second amino-terminal region was also identified and has been designated AF-1b. Deletion of the 65-amino acid AF-1b segment, which contains numerous glutamate and aspartate residues, caused a 55% decrease in trans-activation function. An AF-1a/AF-1b double mutant retains less than 10% trans-activation function compared with wild-type AR, suggesting that AF-1a and AF-1b may each contribute separately to maximal AR activity. To determine whether AF-1a and AF-1b play a role in AR-mediated trans-repression of AP-1 function, we tested single and double AF-1a/AF-1b mutants in a transient trans-repression assay. Our results showed that neither AF-1a nor AF-1b was required for AP-1 trans-repression, demonstrating that AR-mediated trans-repression and trans-activation are discrete functions.

The steroid hormones testosterone and dihydrotestosterone (DHT)¹ exert their physiological effects through the intracellular androgen receptor (AR). The AR is a member of a large family of transcriptional regulatory factors that modulate expression of their target genes in a hormone-regulated manner. Other members of this steroid and nuclear receptor family

include the glucocorticoid receptor (GR), the progesterone receptor, the mineralocorticoid receptor, the estrogen receptor, and the retinoic acid receptor. These receptors contain several functional regions: a carboxyl-terminal hormone binding domain, a DNA binding domain containing two zinc finger motifs, and a poorly conserved amino-terminal domain that contains one or more transcriptional activation domains (1).

The importance of the amino-terminal domain in mediating the trans-activation function of AR has been demonstrated by a variety of studies. First, complete testicular feminization of genetic males can be caused by a frameshift mutation that results in the deletion of a portion of the amino-terminal domain in the human AR (2). Translation from an internal methionine results in a protein that binds androgen but has severely reduced transcriptional activation function. Second, androgen-specific activation of the mouse sex-limited protein gene is mediated by the amino-terminal domain of the AR, but not by the amino terminus of the related GR (3). Finally, mapping experiments using truncations or large deletions in the AR amino terminus have suggested the presence of one or more extended activation domains (4–7).

We are interested in understanding AR-selective responses in the prostate and have characterized a number of prostate cell lines that display elevated levels of AR trans-activation function relative to a large number of nonprostatic cell lines (8). Preliminary mapping studies identified the AR amino terminus as the region of the receptor most likely to be responsible for this enhanced AR activity in the prostate cell lines, and we have proposed that AR-selective co-regulatory proteins may mediate this effect in prostate cells (8). Previous characterizations of the AR amino terminus have been based on the use of large deletion mutations and AR protein fusions, which lack the hormone binding domain (4, 5, 7). Data from these experiments are somewhat difficult to interpret, since these types of sequence alterations are likely to cause substantial changes in the overall protein structure. Therefore, to obtain a more detailed characterization of the AR amino terminus, we performed fine structure mapping studies to identify amino acids required for the known ligand-dependent transcriptional regulatory functions of AR. In the results reported here, we describe the identification of two distinct activation regions in the AR amino terminus, AF-1a and AF-1b, which we found function together to mediate androgen-regulated transcriptional activation.

EXPERIMENTAL PROCEDURES

Construction of AR Amino-terminal Mutants—All AR derivatives were cloned into the expression vectors p6R (9) and pCMX (10) for chloramphenicol acetyltransferase (CAT) and ligand binding assays, respectively. The mutants Δ 117–326 and Δ 197–266 were created as bidirectional nested deletions (11). Briefly, the rat AR expression vector p6RAR-AB (8) was linearized at the *Avr*II site and treated with 150 units of exonuclease III/pmol ends at 37 °C for 5, 15, and 30 s. The three reactions were each treated with 2 units of mung bean nuclease at 37 °C

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¹ The abbreviations used are: DHT, dihydrotestosterone; AF-1, activation function 1; AR, androgen receptor; CAT, chloramphenicol acetyltransferase; GR, glucocorticoid receptor; TPA, phorbol 12-myristate 13-acetate; TRE, TPA responsive element; R1881, methyltrienolone; β -gal, β -galactosidase; kb, kilobase.

for 30 min, pooled, treated with 3 units of a Klenow fragment of DNA polymerase I at room temperature for 1 h, and intramolecularly ligated at 16 °C for 16 h. The mutant $\Delta 40$ –218 was created by cutting p6RAR-AB with *ApaI* and *AvrII*, gel purifying the 6-kb fragment and ligating it to a synthetic *ApaI*-*AvrII* adaptor (5'-CCGATAGTTAC and 5'-CTAGTAACTATCGGGCC). To make deletion $\Delta 154$ –167, p6RAR-AB was cut with *BfrI*, treated with Klenow, and cut with *XbaI* (in the 3'-multiple cloning site). The 4.1-kb band was gel-purified and ligated to a gel-purified 2.4-kb *Cfr10I* (Klenow)-*XbaI* fragment. The double deletion $\Delta 40$ –148/ $\Delta 168$ –221 was made as follows. p6RAR-AB $\Delta 40$ –148 was first constructed by deleting the *ApaI*-*ApaI* fragment by a partial *ApaI* digest. The mutant p6RAR-AB $\Delta 168$ –221 lacks the *NaeI*-*AvrII* fragment. The $\Delta 40$ –148 and $\Delta 168$ –221 mutations were combined using the central *BfrI* site. The point mutations in S165A and I163N/L164N were introduced into the vector pSKAR-AB by site-directed mutagenesis using the oligonucleotides 5'-ATTAAGACATCCTGCTGAGG-CGGCACCATGCAACTTC-3' and 5'-GCAGACATTAAGACAATAA-TAGCGAGGCCCGCACCATGCAAC-3', respectively. The *BfrI*-*AvrII* fragment of each, which contained the point mutations, was exchanged for the *BfrI*-*AvrII* fragment of p6RAR-AB. The deletion $\Delta 235$ –245 was introduced into pSKAR-AB by site-directed mutagenesis using an oligonucleotide that contained bases flanking the sequence to be deleted (5'-GACAGTCCAAGGAGGGTGTGGAAGCACTGGAACAT-3'). The *AvrII*-*NruI* fragment, containing the deletion, was exchanged with the *AvrII*-*NruI* fragment of p6RAR-AB. The $\Delta 269$ –356 deletion was created by cutting pSKAR-AB with *MluI* and *NruI*, purifying the 5.7-kb fragment, and ligating it to a synthetic *MluI*-*NruI* adaptor (5'-CGCGTCGCTCAATCG-3' and 5'-CGATTGAGCGA-3'). AR-AB $\Delta 269$ –356 was subcloned into p6R. AR $\Delta 411$ –531 was constructed as follows. Two existing *NheI* sites were eliminated by subcloning AR-AB $\Delta 40$ –148 (which lacks one *NheI* site) into pBluescript SK⁺ (Stratagene) via *PstI* and *XbaI* (deleting a portion of the AR 5'-untranslated region containing the second *NheI* site). The resultant plasmid was cut with *NheI* and *AatII*. The 5.2-kb fragment was purified and ligated to a synthetic *NheI*-*AatII* adaptor (5'-CTAGCTTAATTGACGT-3' and 5'-CAATTAAG-3'). The *NruI*-*BssHIII* fragment (containing the $\Delta 411$ –531 deletion) was exchanged with the *NruI*-*BssHIII* fragment in p6RAR-AB. The end points of all mutations were subjected to double strand dideoxy sequencing (Sequenase kit version 2.0, U. S. Biochemical Corp.) to verify the sequence and the integrity of the reading frames.

The reporter plasmid pMM-CAT contains 1.4 kb of the mouse mammary tumor virus long terminal repeat upstream of the CAT gene (12), and the 5XTRE-CAT reporter gene contains five copies of a consensus AP-1 binding site upstream of a minimal promoter linked to CAT (13).

Cell Culture and Transfections—Monkey kidney CV-1 and COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% defined calf bovine serum. For trans-activation assays using the AR deletion mutants, 1×10^6 CV-1 cells were transfected by the calcium phosphate method (11) using equimolar receptor expression plasmid (10 μ g of for p6RAR-AB), 2 μ g of pMM-CAT reporter plasmid, 5 μ g of the β -galactosidase (β -gal) expression plasmid pEQ176 (9), and carrier DNA (sheared calf thymus DNA or pSK⁺ plasmid DNA) at up to 25 μ g/10-cm² plate. After 4 h in the presence of the DNA precipitate, the cells were subjected to a 45-s osmotic shock with 2.5 ml 20% glycerol in PD buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.2). Following two washes with PD buffer, fresh Dulbecco's modified Eagle's medium plus 5% charcoal-stripped calf bovine serum and 1 μ M DHT were added to each plate. The AP-1 trans-repression assays were done in the same manner, except that 60 ng/ml phorbol 12-myristate 13-acetate (TPA; Sigma), a tumor-promoting agent, was added to each culture 16 h prior to harvesting.

For each ligand binding assay and Western blot sample, 15-cm² plates containing 1.8×10^6 COS-7 cells in Dulbecco's modified Eagle's medium plus 5% charcoal-stripped calf bovine serum were transfected as above, except that ~ 30 μ g of each pCMX receptor expression plasmid and 1 μ g of pRSCAT (14) were used per plate, and the DNA precipitates were left on the cells for 16 h. Each plate was rinsed twice with PD buffer, fresh Dulbecco's modified Eagle's medium and 5% charcoal-stripped calf bovine serum were added to the cells.

CAT, β -Galactosidase, and Ligand Binding Assays—Forty hours after transfection, the cells were harvested and assayed for CAT activity (12) following a normalization for transfection efficiency using β -gal activity (15). Substrate and acetylated products were separated by thin-layer chromatography, and the percentage of conversion of [¹⁴C]chloramphenicol to the acetylated forms was quantitated using a Betagen Betascope or a Molecular Dynamics PhosphorImager. CAT activity was expressed as the percentage of conversion per β -gal/unit/h of CAT assay. β -gal units were defined as the A₄₁₀/mg protein/min of

β -gal assay, multiplied by the μ g of protein in the CAT assay. The protein concentration of cell extracts was determined using the BCA assay (Pierce).

For ligand-binding assays, transfected COS-7 cells were harvested 48 h after the removal of the DNA precipitate, and the cell pellets were frozen in liquid nitrogen and stored at -80 °C. Cell pellets were thawed on ice in 300 μ l of TEGN50 (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10%, v/v, glycerol, 1 mM 2-mercaptoethanol, 50 mM NaCl, 10 mM Na₂MoO₄, 1 mM phenylmethylsulfonyl fluoride). Cell extracts were prepared on ice by ultrasonic disruption using a Branson probe sonicator at setting 1, 50% duty cycle for 10 pulses, followed by centrifugation at $4000 \times g$ for 10 min at 4 °C. Ligand-binding assays were conducted in triplicate by incubating 65 μ l of extract with 20 nM [³H]methyltrienolone ([³H]R1881; DuPont NEN), and nonspecific binding was measured by including a 1000-fold molar excess of unlabeled [³H]R1881 in one reaction of each set. After a 90-min incubation on ice, unbound [³H]R1881 was separated from receptor-bound ligand by the addition of 100 μ l of 10 mg/ml activated charcoal (J. T. Baker), followed by centrifugation through 0.45 μ m spin filters (Intermountain Scientific). Filtrate was added to 3 ml of scintillation mixture, and net cpm were used to determine receptor-specific radiolabeling.

Western Blots—Transfected COS-7 cells were harvested, immediately resuspended in 250 μ l of TEGN50, and sonicated and centrifuged as above. A 10- μ l portion of the supernatant was reserved for protein determination. 2-Mercaptoethanol and SDS were added to the remainder of the supernatant, each to a final concentration of 1%, and samples were boiled for 5 min. Samples (100 μ g) were subjected to electrophoresis on a 7.5% (37.5:1) SDS-polyacrylamide gel. Proteins were transferred to Zeta-Probe membrane (Bio-Rad) by electroblotting in 25 mM Tris and 192 mM glycine at 60 V for 5 h at 4 °C. Nonspecific sites were blocked for 1 h in 5% powdered milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05%, v/v, Tween 20). AR derivatives were detected using anti-AR rabbit polyclonal antisera (16) (diluted 1:1000 in 5% powdered milk in TBST), followed by incubations with biotinylated goat anti-rabbit secondary antibody (1:3000; Bio-Rad) and avidin-conjugated horseradish peroxidase (1:1000; Pierce). The membrane was incubated with the peroxidase substrates hydrogen peroxide and ImmunoPure 3-amino-9-ethylcarbazole (Pierce) to visualize the bands.

RESULTS

Deletion Mapping of the AR Amino Terminus Identifies Two Activation Functions—A number of transcriptional activation domains have been characterized that are able to function as discrete units when fused to heterologous DNA binding domains. For example, we (8) and others (7, 17) have shown that Gal4-AR fusion proteins containing segments of the AR amino terminus can stimulate transcription of GAL4-responsive promoters as hormone-independent transcriptional activators. However, based on data that suggest that intramolecular protein folding between the amino terminus and the hormone or DNA binding domains of AR (17, 18) and GR (19) may contribute to receptor function, and that environmental compounds may modulate AR activity by interacting with the hormone binding domain (20), we chose to analyze AR amino-terminal activation functions in the context of an androgen-dependent assay (4, 5).

Simental *et al.* (4) previously showed that deletion of amino acids 1–141 of human AR had no effect on trans-activation function, whereas deletion of residues 1–338 resulted in a complete loss in trans-activation without altering hormone binding or nuclear localization functions. In addition, large internal deletions of amino acids 46–244 in human AR (7) and residues 38–296 in rat AR (6), have been shown to abolish androgen-regulated trans-activation function. To more precisely characterize the amino-terminal region of rat AR that encodes transcriptional trans-activation function, which by convention would be called AF-1 (22), we made a series of internal in-frame AR deletions as detailed under "Experimental Procedures." All of the deletion end points were determined by DNA sequencing, and the encoded AR proteins were biochemically characterized by expression of the mutated AR cDNAs in transfected cells. As shown in Table I, the AR amino-terminal deletants were found

TABLE I

[³H]R1881 binding of wild-type and mutant rat androgen receptors

AR derivatives were subcloned into the high expression vector pCMX and transfected into COS-7 cells by the CaPO₄ method. Forty-eight hours after transfection, cells were harvested for *in vitro* [³H]R1881 binding assays, as described under "Experimental Procedures." Specific binding was calculated by subtracting the cpm measured in a binding reaction containing 20 nM [³H]R1881 and a 1000-fold molar excess of unlabeled R1881 from the average of two binding reactions containing 20 nM [³H]R1881. This value was used to calculate the number of fmol of AR/mg of protein in each binding reaction, and the mean ± S.E. of two to four experiments is shown.

Receptor	Binding activity <i>fmol AR/mg protein</i>
AR	31 ± 7
Δ117–326	31 ± 4
Δ40–218	34 ± 4
Δ154–167	29 ± 2
Δ40–148/Δ168–221	25 ± 5
S165A	23 ± 1
I163N/L164N	28 ± 1
Δ197–266	33 ± 7
Δ235–245	22 ± 4
Δ269–356	40 ± 11
Δ269–295	21 ± 5
Δ295–359	20 ± 3
Δ359–411	38 ± 12
Δ411–531	29 ± 4

to have comparable hormone binding activities based on a [³H]R1881 binding assay. Moreover, Western blot analysis using a polyclonal antibody directed against the AR carboxyl terminus (16), was used to confirm predicted protein molecular weights and to verify equivalent expression levels (Fig. 1 and data not shown).

The abilities of wild-type and mutant ARs to induce transcription from an androgen-responsive promoter were assessed in CV-1 cells by cotransfection of receptor expression vectors with an androgen-responsive reporter plasmid, MM-CAT, that contains the mouse mammary tumor virus long terminal repeat fused to the CAT gene. Cotransfection of the AR expression plasmid and MM-CAT in the presence or absence of DHT resulted in a DHT-dependent 60-fold induction of CAT activity (data not shown). Using this level of DHT-dependent activation by wild-type AR as a reference, we initially tested a rat AR deletant that was missing most of the predicted AF-1 region based on the data of Simental *et al.* (4). As can be seen in Fig. 2A, the AR deletant Δ117–326 retains less than 5% of the trans-activation function of wild-type AR, suggesting that AF-1 maps within this segment. We also tested an AR mutant that lacked amino acids 411–531 and found that it was less active than wild-type AR even though it retained the amino acid 117–326 segment. This region of the receptor could contain a secondary trans-activation function, as has been suggested (7), or it may mediate enhanced AR binding to DNA (23). Since we were interested in delineating functional components of the primary AF-1 region, we focused our efforts on the 117–326 segment.

Fig. 2B shows results from more detailed deletion mapping of AF-1. By comparing the activity of each AR mutant with that of wild-type AR, it was found that although most had reduced activity (Δ40–218, Δ197–266, Δ235–245 and Δ269–356), several of the deletants had reproducibly more activity (Δ40–148 and Δ168–221). The elevated activity observed with the Δ168–221 deletant likely reflects loss of the inhibitory polyglutamine tract (16). The enhanced trans-activation function of the Δ40–148 deletant indicates that the amino-terminal boundary of AF-1 does not extend into this region. The relative activity of each AR variant was found to be the same when it was tested using a simplified hormone response element reporter gene

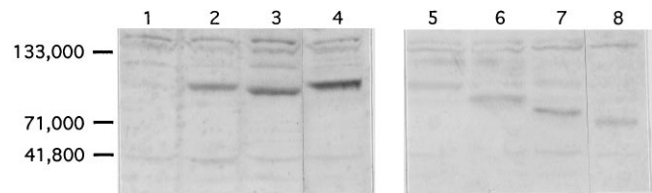


FIG. 1. Western blot analysis of selected AR amino-terminal deletion mutants. Western blot analysis was done using a polyclonal antibody raised against the rat AR carboxyl terminus (16) to analyze extracts from COS-7 cells transiently transfected with wild-type AR and deletion mutants. Lane 1, untransfected COS-7 extract; lane 2, wild-type AR; lane 3, Δ154–167; lane 4, I163N/L164N; lane 5, Δ235–245; lane 6, Δ197–266; lane 7, Δ269–356; and lane 8, Δ411–531. The positions of protein standards (β-galactosidase, 133 kDa; bovine serum albumin, 71 kDa; carbonic anhydrase, 41.8 kDa) run in separate lanes are shown. Note that the results shown in lanes 5–8 were from a separate filter, and the reduced intensity of all bands (specific and nonspecific) likely reflects decreased efficiency of protein transfer from this gel.

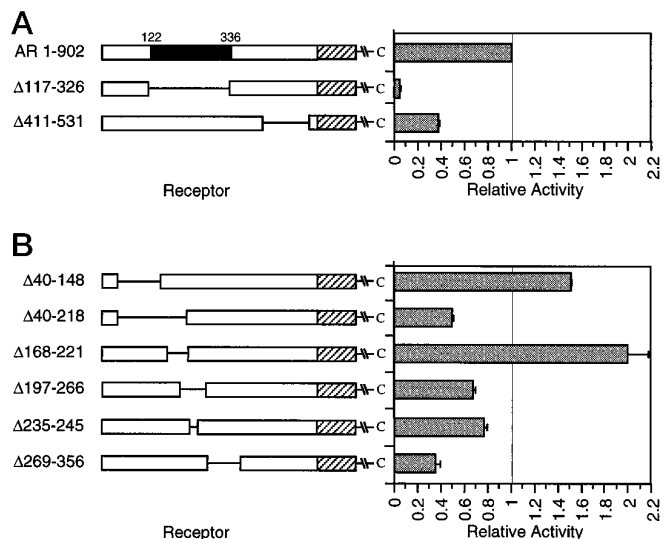


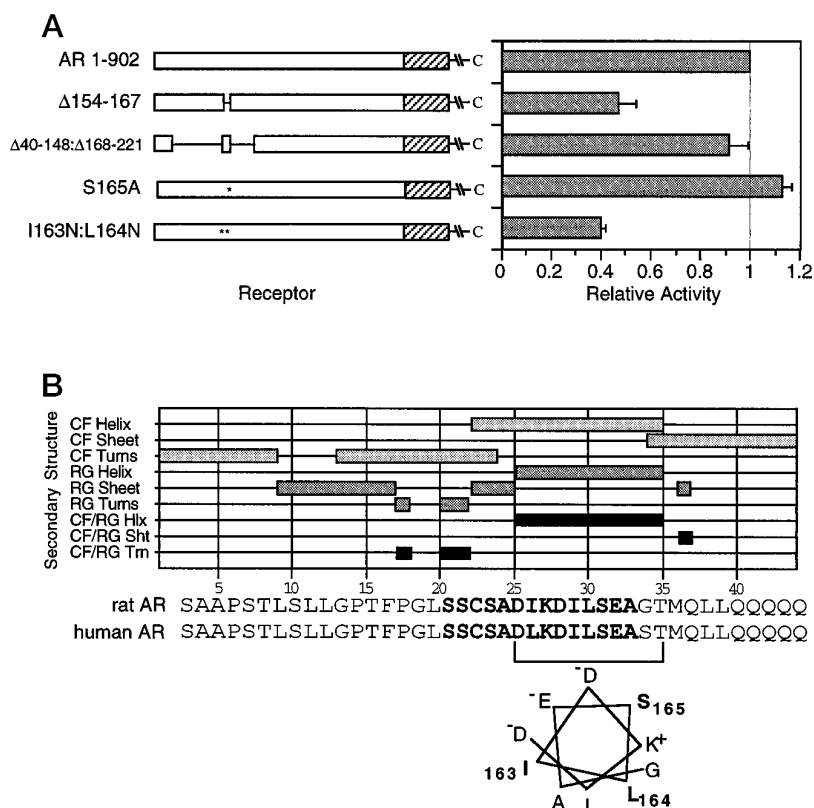
FIG. 2. Delineation of the AF-1 activation domain in the AR amino terminus. A schematic representation of each AR construct is shown alongside the results of CAT assays using extracts from transiently transfected CV-1 cells. Note that all the AR derivatives described in this study contained amino acids 531–902 and were DHT-dependent for activity; however, for clarity, this portion of the receptor is not shown. Each mutant is named using the numbers of the first and last amino acids deleted. The activity of wild-type AR was calculated by subtracting the CAT enzyme activity (percentage of conversion/β-gal unit/h of CAT assay) measured in extracts from cells maintained in the absence of hormone from the CAT activity in extracts of cells treated with DHT. The activities of the mutant ARs relative to the wild-type receptor (activity = 1.0) are expressed as mean ± S.E. (bars). The mean level of DHT-dependent induction by wild-type AR of CAT activity using the MM-CAT reporter plasmid was 60-fold (data not shown). A, results showing that the major AF-1 activation function in the rat AR maps to the region contained within amino acids 117–326. The black box shown in AR 1–902 represents the corresponding region in rat AR that was previously shown to contain the primary activation function in human AR (4). B, results from progressive deletions of the AF-1 region in rat AR.

called TAT3-CAT (8) (data not shown).

The data in Fig. 2B suggests that at least two nonoverlapping regions could be contributing to maximal AF-1 activation function. This can best be seen by comparing the deletant Δ40–218 with that of the more carboxyl-terminal deletions that cover the region containing amino acids 197–356. Since both of these regions map within AF-1 (amino acids 117–326), we refer to them as AF-1a and AF-1b.

AF-1a Activation Function Is Encoded by a Putative Amphipathic α-helix—As shown in Fig. 2, the deletants Δ40–148

FIG. 3. AF-1a activity maps to a 14-amino acid segment. *A*, mutations that define AF-1a function are shown diagrammatically with the corresponding relative CAT activities as in Fig. 2. *B*, computer predictions of secondary structure for amino acids 137–178, encompassing the AF-1a region (shown in *bold*), were conducted using both the Chou-Fasman (*CF*) and Robson-Garnier (*RG*) methods. The 10 amino acids predicted by both methods (*CF/RG Hlx*) to form an amphipathic α -helix are *bracketed* and shown in a *helical wheel*. The three amino acids subjected to site-directed mutagenesis (*I163*, *L164*, and *S165*) are indicated.



and $\Delta 168-221$ have increased activity, suggesting that the region contained within amino acids 148–168 may be critical to AF-1a activity. To test this idea, we deleted amino acids 154–167 and assayed the function of this mutant in the transactivation assay. As shown in Fig. 3A, deletion of this 14-amino acid segment resulted in a 52% reduction in activity, which is similar to what was seen with the much larger $\Delta 40-218$ deletion (Fig. 2B). To confirm that this smaller AF-1a segment has activation function, we constructed the double mutant $\Delta 40-148/\Delta 168-221$ (Fig. 3A). We found that this mutant, which essentially inserts the 148–168 region back into the middle of the $\Delta 40-218$ deletion, had nearly full activity. Taken together, we concluded that the AF-1a activation function, initially identified by the $\Delta 40-218$ deletion, was indeed fully contained within the 14-amino acid segment defined by amino acids 154–167.

As shown in Fig. 3B, both the Chou and Fasman (24) and Garnier *et al.* (25) methods of protein structure predictions indicate that the highly conserved AF-1a region has the potential to form a β -turn followed by an α -helix. When drawn as a helical wheel, residues 159–168 form an amphipathic α -helix, with one side having three acidic amino acids and a serine (Fig. 3B). The other face of the helix has four hydrophobic residues. To identify point mutations in AF-1a that alter AR activity within the context of the full-length receptor, serine 165 was changed to the nonphosphorylatable residue alanine, and two of the hydrophobic amino acids (isoleucine 163 and leucine 164) were changed to asparagines (Fig. 3B). As shown in Fig. 3A, the S165A mutation had no effect on trans-activation function. However, the double point mutation I163N/L164N had 60% less activity than wild-type AR, which is nearly identical to the AF-1a deletion $\Delta 154-167$. These results indicate that hydrophobic residues, perhaps within the context of an amphipathic α -helix, may play a key role in AF-1a function.

The AF-1b Region Resembles an Acidic Activation Domain—To better define AF-1b sequences, we constructed and tested additional deletion mutants within the amino-terminal

region defined by amino acids 269–411. The results in Fig. 4A show that deletants $\Delta 269-295$ and $\Delta 295-359$ had the same level of activity as the larger deletion $\Delta 269-356$, indicating that AF-1b activity maps within this segment. The deletant $\Delta 359-411$ had greater activity than wild-type AR, suggesting that AF-1b activity does not extend beyond amino acid 359.

Fig. 4B shows the amino acid sequence of the AF-1b region as defined by these deletants. Using the same algorithms to predict protein structures in this region as was described for AF-1a (Fig. 3B), we were unable to identify any significant protein structures (data not shown). However, it can be seen that there are numerous glutamate and aspartate residues in the segment of the protein (17% between amino acids 285–358), which is similar to the level found in acidic activation domains (26), including the tau1 acidic activation domain of the GR (27). An amino acid homology comparison between the GR tau1 region and the AR sequences shown in Fig. 4B revealed no significant matches beyond the prevalence of acidic residues (data not shown).

The Phenotype of AF-1a/AF-1b Double Mutants—If AF-1a and AF-1b contribute separately to maximal trans-activation function, then a AF-1a/AF-1b double mutant should have less activity than either one of the single mutants. To test this prediction, we combined the AF-1a point mutations I163N/L164N with the three deletions used to map AF-1b ($\Delta 269-295$, $\Delta 295-359$, and $\Delta 359-411$), as shown in Fig. 5. The trans-activation function of these three AF-1a/AF-1b double mutants was measured and compared with the activity of wild-type AR and the single AF-1 mutants.

The data in Fig. 5 reveal that the mutant I163N/L164N/ $\Delta 295-359$ retained only ~10% of the activity of wild-type AR, which was less activity than either deletion alone. In contrast, the mutant I163N/L164N/ $\Delta 269-295$ had only slightly less activity than either single AF-1 mutation, and I163N/L164N/ $\Delta 359-411$ had more activity than the AF-1a mutation. These data demonstrate that in the background of the AF-1a point mutations, deletion of the amino acid segment 295–359 results

FIG. 4. The sequence of AF-1b resembles an acidic activation domain.

A, AR amino-terminal deletion mutants are depicted next to the results of CAT assays used to map AF-1b. B, amino acid sequence of rat AR in the AF-1b region as defined by the deletant $\Delta 269-356$. Glutamate (E) and aspartate (D) residues are shown in *bold*.

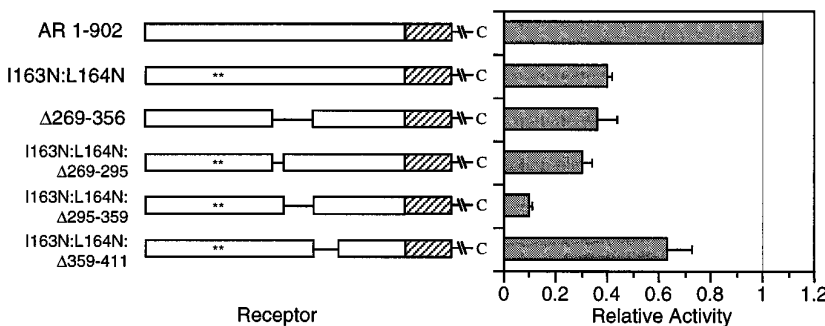
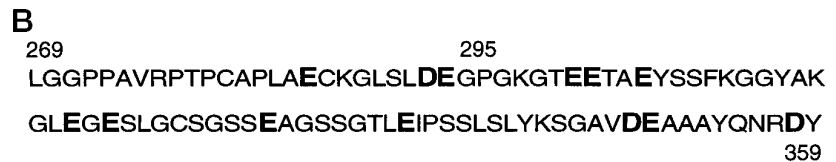
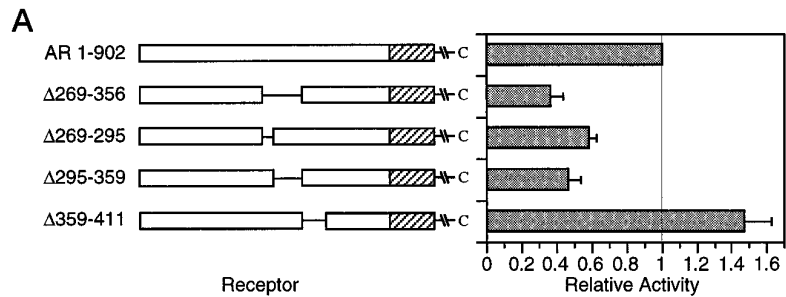


FIG. 5. An AF-1a/AF-1b double mutant is less active than the AF-1a and AF-1b single mutants. The structure of AF-1a, AF-1b, and AF-1a/AF-1b mutants are shown with the corresponding CAT assay results of these mutants compared with wild-type AR. The significantly lower activity of I163N/L164N/ $\Delta 295-356$ compared with I163N/L164N/ $\Delta 269-295$ suggests that AF-1b maps to amino acids 295-356.

in a more severe mutant phenotype than the single mutations, consistent with a model in which AF-1a and AF-1b each contribute to maximal trans-activation function. Moreover, since the AR mutant I163N/L164N/ $\Delta 269-295$ is more active than the I163N/L164N/ $\Delta 295-359$ mutation, we conclude that AF-1b activity is localized primarily to the 65-amino acid segment between residues 295 and 359.

It has recently been shown by Kallio *et al.* (28) that the AR is able to inhibit the transcriptional regulatory activity of AP-1. Using several large AR deletions lacking portions of the amino terminus, they proposed that amino-terminal trans-activation sequences may also be required for AP-1 trans-repression. Since biochemical characterization of the AF-1a and AF-1b mutants had shown no significant effect on hormone binding affinities (Table I), protein expression levels (Fig. 1), or DNA binding properties² indicative of minimal protein structure perturbations, we tested whether the AF-1a and AF-1b mutants were defective in AP-1 trans-repression. CV-1 cells were co-transfected with an AP-1 reporter gene containing five copies of a TPA responsive element (TRE) linked to a minimal promoter upstream of the CAT gene (5XTRE-CAT) and an AR expression plasmid. Transfected cells were cultured for 24 h with or without 10^{-7} M DHT, and then 60 ng/ml of TPA was added to each dish for an additional 16 h. In control experiments using wild-type AR, it was found that DHT treatment caused a 60% reduction in TPA-induced CAT activity (data not shown). As shown in Fig. 6, the relative repressing activity of the AF-1a (I163N/L164N) and AF-1b ($\Delta 269-359$) mutants was equivalent to that of wild-type AR. In addition, the AF-1a/AF-1b double mutant (I163N/L164N/ $\Delta 295-359$) was able to repress AP-1 function to nearly the same level of wild-type AR, even though this variant receptor is 90% deficient in trans-activation function (Fig. 5). When larger amino-terminal dele-

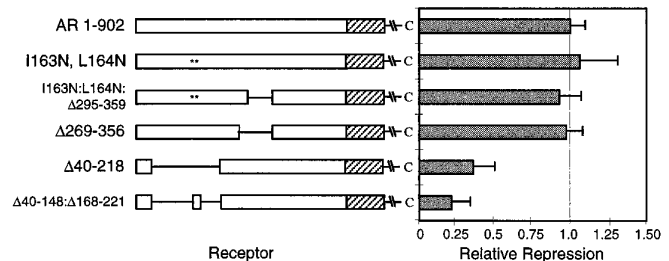


FIG. 6. AF-1a and AF-1b are not required for repression of AP-1 activity. The results of AP-1 trans-repression assays using extracts from CV-1 cells that had been transiently transfected with the 5XTRE-CAT reporter plasmid, the indicated AR expression plasmid, and the β -gal internal control plasmid are shown. Transfected cells were grown in the presence or absence of 100 nM DHT for 24 h and then treated with 60 ng/ml TPA for an additional 16 h before harvesting. CAT activities are shown as percentages conversion after correcting for transfection efficiency. Bars, S.E. of at least three independent experiments. The mean level of DHT-dependent AP-1 trans-repression by wild-type AR in these assays was 60% (data not shown).

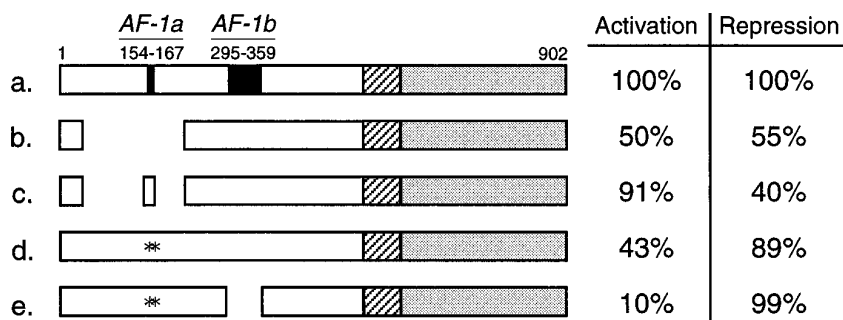
tions ($\Delta 40-218$ and $\Delta 40-218/\Delta 168-221$) were tested in this assay, they were found to be less capable of repressing AP-1 activity.

DISCUSSION

The regulation of gene expression by eukaryotic transcriptional activators is of fundamental importance for cell growth and differentiation (29). Activation domains, the regions of the activators that mediate effects on transcription, have been classified into several general categories based on their amino acid composition (30, 31). We demonstrate in this study that the amino-terminal activation domain of the rat AR is similar to the activation domains of other eukaryotic transcription factors in that it contains multiple subregions that together are required for full trans-activation function. We identified two regions, which we call AF-1a and AF-1b, using functional anal-

² N. L. Chamberlain and R. L. Miesfeld, unpublished data.

FIG. 7. Comparison of trans-activation and trans-repression activities of wild-type AR and selected AF-1a and AF-1b mutants. A schematic drawing of the full-length AR showing the location of AF-1a and AF-1b based on results from this study is shown. Shaded box, hormone binding domain. The relative levels of DHT-dependent MM-CAT activation and 5XTRE-CAT repression by wild-type AR (a), $\Delta 40-218$ (b), $\Delta 40-148/\Delta 168-221$ (c), I163N/L164N (d), and I163N/L164N/ $\Delta 295-356$ (e) are shown on the right. The mean levels of activation and repression functions shown were obtained from the data in Figs. 2–6.



yses of a panel of receptor constructs containing deletions and point mutations. Deletion of a 14-amino acid segment encompassing AF-1a leads to a 60% reduction in transcriptional activation. More importantly, a deletion mutant lacking a total of 161 amino acids on either side of AF-1a ($\Delta 40-148/\Delta 168-221$) is fully active, whereas deletion of the entire region ($\Delta 40-221$) greatly reduces activity. We also found that the AF-1a double point mutant I163N/L164N had the same phenotype as the AF-1a deletion ($\Delta 154-167$), strongly suggesting that AF-1a activity maps to this segment of the AR amino terminus. Protein structure predictions of the AF-1a region indicate that it may form an amphipathic α -helix (Fig. 3B), which would be similar to the predicted structure of several other activation domains (32).

Receptor mutants lacking AF-1a retained approximately 40% activity, suggesting that a second region within AF-1 (amino acids 117–326) must be necessary for maximal trans-activation. To investigate this further, we made a series of progressive deletions downstream of AF-1a. Based on both single and double mutations, we found that the region contained within amino acids 295–359, which we call AF-1b, is responsible for approximately one-half of the total trans-activation function in the AR amino terminus. Although no clear protein structure prediction could be made, there are numerous glutamate and aspartate residues in this region, which is similar to the amino acid composition of acidic activation domains (26). One of the best characterized acidic activation domains is that of the herpesvirus VP16 protein, which contains ~22% glutamate and aspartate residues (33). It was initially proposed that the acidic residues within the VP16 activator domain were specifically required for activity (34), but subsequent mutagenesis studies indicated that hydrophobic residues were also important (21). There are a number of hydrophobic residues scattered throughout the AF-1b region (Fig. 4B), and we are in the process of characterizing their contribution to AF-1b function using site-directed mutagenesis.

Recently, Jenster *et al.* (7) used a panel of amino-terminal human AR deletants to analyze trans-activation function in the context of full-length AR, truncated AR, and Gal4-AR gene fusions using a variety of reporter genes. They concluded that depending on the assay system used, two large regions within the amino terminus could be shown to have activation function. One of the transcription activation units was mapped to amino acids 101–370 by deletion mutagenesis using an androgen-dependent assay, and a second functional region was delineated using a truncated form of AR that exhibited ligand-independent activity. Our results are consistent with the identification of a major activation function contained within amino acids 101–370, as both AF-1a (amino 154–167) and AF-1b (amino acids 295–359) map to this region. Moreover, similar to Jenster *et al.* (7), we found that deletion of amino acids 411–531 decreased AR activation function (Fig. 2A). Interestingly, this more carboxyl portion of the AR amino terminus contains a

large number of prolines, which could mimic a proline-rich activation domain under some conditions, and it is also the interaction site for insulin-degrading enzyme, which Kupfer *et al.* (23) have proposed to be a modulator of AR binding to DNA.

Kallio *et al.* (28) have proposed that overlapping AR amino-terminal sequences were required for both transcriptional trans-activation and AR-mediated trans-repression of AP-1 activity. Since the AF-1a and AF-1b mutations we constructed were more specific than the large amino-terminal deletion used in the study by Kallio *et al.* (28), we tested AF-1a, AF-1b, and AF-1a/AF-1b mutants in an AP-1 trans-repression assay. Fig. 7 summarizes our results of selected trans-activation and trans-repression assays to illustrate the lack of overlap between these two functions. For example, the inclusion of AF-1a sequences within the context of a larger deletion restores activation function (91%) without improving repression activity (40%). Furthermore, point mutations in AF-1a and a double mutation of AF-1a/AF-1b both decrease trans-activation function without affecting trans-repression activity. Based on these data, we conclude that AR-mediated trans-activation and trans-repression functions are distinct activities. One explanation for the differences between our results and that of Kallio *et al.* (28), is that we analyzed smaller mutations, which were less likely to alter overall protein structure. Indeed, when we examined a large amino-terminal deletion ($\Delta 40-148$), we found that both trans-activation and trans-repression functions were defective, whereas with a less disruptive mutation (I163N/L164N/ $\Delta 295-359$), only trans-activation function was affected (Fig. 7).

With the availability of transcriptional activation mutants that contain only minimal alterations in the amino acid sequence, we can now begin to exploit biochemical strategies based on differential protein affinities to isolate co-regulatory transcription factors that may interact specifically with the wild-type AR amino terminus. Moreover, a better understanding of AR-selective transcriptional regulatory properties, both activation and repression, should facilitate additional studies investigating mechanisms of steroid- and cell-specific effects.

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