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A Mutant Androgen Receptor from Patients with Reifenstein Syndrome: Identification of the Function of a Conserved Alanine Residue in the D Box of Steroid Receptors

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Reifenstein syndrome is an eponymic term that describes partial androgen-insensitive disorders. Androgen receptor isolated from five patients with this syndrome contains a specific mutation in the DNA binding domain of the receptor. This mutation converts an alanine to a threonine at position 596 next to the zinc catenation site at the second finger. The threonine 596 mutant receptor mediated normal androgen response at promoters with closely positioned multiple regulatory elements for the androgen receptor and other transcription factors. Promoters with single isolated androgen response elements were not transactivated by the mutant receptor. In in vitro receptor-DNA binding studies, interaction with DNA by the mutant receptor was achieved only in the presence of an anti-androgen receptor antibody. Exchanging alanine 596 in the wild-type androgen receptor with serine or valine produced mutants with properties indistinguishable from those of the naturally occurring threonine 596 mutant receptor. These results indicate that an alanine residue at position 596 contributes important structural and functional activities to the androgen receptor. In the androgen receptor from the patients with Reifenstein syndrome, in which this alanine is converted to a threonine, wild-type receptor properties can be restored by exchanging an additional threonine at position 602 to an alanine. An alanine residue at position 596 or 602 in the DNA binding domain of the androgen receptor is therefore important for the full function of this receptor. In all steroid receptors that bind the core sequence AGAACANNNTGTTCT, an alanine residue is also present at a position equivalent to alanine 596 in the androgen receptor.

The androgen receptor (AR) belongs to a large family of ligand-binding transcription factors which include receptors for steroid/thyroid hormones (1, 13), retinoic acids (13), peroxisomal activators (22), vitamin D (13), certain regulators of early development in *Drosophila melanogaster* (28, 29), and other proteins of unknown function (13, 34). These receptors consist of carboxy-terminal ligand binding domains, N-terminal modulator domains, and conserved central portions that bind DNA (1). The DNA binding domains contain two zinc atoms tetrahedrally coordinated by conserved cysteine residues required for proper folding and sequence-specific DNA binding (17).

Like other steroid receptors, the AR is thought to induce gene expression by binding to DNA (4, 7, 31). High-affinity DNA binding is enhanced by the ability of the receptors to dimerize, a function encoded by specific sequences in both the DNA and hormone binding domains. In the DNA binding domain, this function is mediated by sequences in the stem of the second zinc finger in a region designated D box (9, 13, 26). In the hormone binding domain, the relevant sequences are included in a heptamer repeat of hydrophobic residues conserved in all members of the steroid hormone receptor superfamily (14, 16). How these two dimerization regions function is not known, and whether one of the two suffices for DNA binding is yet to be determined.

We have studied the transactivation and DNA binding properties of an AR isolated from patients with an androgen resistance disorder known as Reifenstein syndrome. This syndrome describes a variety of incomplete androgen resistance disorders with various degrees of incomplete virilization. Characteristic clinical features are perineoscrotal hypospadias, small testes, micropenis, azoospermia, infertility, and gynecomastia (for reviews, see references 19 and 20). Five patients with this syndrome from two unrelated families possess a mutation that converts an alanine at position 596 in the D box of the AR to a threonine (25). This naturally occurring mutation provides a tool for the study of the contribution to androgen action of specific amino acids in the D box of the AR.

Transactivation studies using reporter plasmids with multiple androgen response elements (AREs) showed almost wild-type properties for the mutant receptor. However, the mutant receptor did not mediate androgen response at promoters containing single AREs. In receptor-DNA binding studies, DNA binding activity of the mutant receptor was detected only in the presence of anti-AR antibodies. Alternatively, an additional amino acid change of a threonine at position 602 to an alanine restores DNA binding and full transactivation function to the mutant receptor. These experiments demonstrate that for efficient function of the AR, an alanine residue must be present at positions 596 and/or 602 in the DNA binding domain of the receptor.

MATERIALS AND METHODS

Cell culture and transfection. Simian CV-1 and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. The transient transfection assay was carried out by the calcium phosphate coprecipitation procedure. Unless otherwise stated, transfections were carried out with 9 μg of indicator plasmid and 1 μg of receptor expression vector. In some experiments, 2 μg of plasmid pCH110, consisting of the

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simian virus 40 promoter driving the expression of the β-galactosidase gene, was cotransfected as an internal control. Immediately after transfection and a 2-min shock with 15% glycerol in phosphate-buffered saline, the transfected cells were treated with hormones and cultured for 2 days. Thereafter, the cells were harvested and disrupted and a chloramphenicol acetyltransferase (CAT) assay was performed as described previously (3, 5). Transfection of the AR for gel retardation experiments was carried out in Cos-7 cells by electroporation as previously described by Klocker et al. (25).

Plasmid constructs. The indicator plasmid pHCwt and the plasmids in the ERE^+ARE^+ series (originally ERE^+PRE^+) have already been described (3, 5). The construct ARE_2 TATA CAT was derived from $(PRE)_2$pLovTATA (24) by replacing the G-free cassette with a CAT cassette. The wild-type and mutant AR expression vectors have already been described (25). The mutant AR constructs AR[serine]^{596} and AR[valine]^{596} (superscript numbers indicate amino acid positions) were made by polymerase chain reaction (PCR) mutagenesis starting with the wild-type AR expression vector. Two PCR fragments were synthesized by using the primer pairs $AR^{2113/19}$ (5'AGCTACTCCGGACCT TACG3') plus $AR^{2321/21asSer}$ (5'CTGCTGCTGCACAGGT ACTTC3') and $AR^{2302/20Val}$ (5'AAGTACCTGTGCGTCAG CAG3') plus $AR^{2959/25/as}$ (5'GCAGTGCTTTCATGGACAG GAATTC3'), respectively. The two fragments were isolated, ligated in a second run of PCR, and amplified by using the primers $AR^{2113/19}$ and $AR^{2959/25/as}$. A *Hin*dIII-*Asp*I restriction fragment containing the mutations was generated and replaced in the AR expression vector. Individual clones were isolated and analyzed by DNA sequencing.

The amino acid exchanges at position 602, in mutants AR[alanine^{596}-isoleucine^{602}] and AR[alanine^{596}-alanine^{602}], were constructed by the same procedure, but the two primers containing the mutations were $AR^{2352/25asIle}$ (5'CC TTCGGAATTTATCAATAATGCAA3') and $AR^{2315/25Ala}$ (5'CCAGCAGAAATGATTGCGCTATT3'). Mutants AR [threonine^{596}-isoleucine^{602}] and AR[threonine^{596}-alanine^{602}] were constructed in the same way as AR[alanine^{596}-alanine^{602}] and AR[alanine^{596}-isoleucine^{602}], the only difference being that the starting template was AR[threonine]^{596}. Numbering of the PCR primers is according to the AR cDNA sequence published by Chang et al. (6). The first number indicates the 5' nucleotide position on the cDNA, and the second number represents the length of the primer. "as" indicates primers on the antisense strand. The mutated triplets are underlined.

Gel retardation and methylation interference studies. Whole cell extracts were prepared as described by Eul et al. (12) except that the buffers used contained the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 0.6 μg of aprotinin per ml, 1 μg of pepstatin per ml, 1 μg of leupeptin per ml, and 1 μg of antipain per ml. Gel retardation studies were carried out as previously described (27). The oligonucleotide used for the gel retardation studies was obtained by annealing the single-stranded oligonucleotides 5'AGCTTAGAACACAGTGTTC TCTAGAG3' and 5'GATCCTCTAGAGAACACTGTGTT CTA3'. The resulting double-stranded oligonucleotide was phosphorylated by using T4 polynucleotide kinase (Pharmacia) in the presence of [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham). Gel retardation experiments with duplicated AREs were carried out by using a *Hin*dIII-*Bam*HI DNA fragment isolated from plasmid ARE_2pBLCAT8$^+$. The construct was digested with *Bam*HI and labeled at the 3' end with DNA polymerase I, and the labeled duplicated ARE was isolated by digestion with *Hin*dIII. ARE_2pBLCAT8$^+$ was constructed by cloning a duplicated ARE with *Hin*dIII and *Bam*HI sites into the *Hin*dIII and *Bam*HI sites of the promoter of the plasmid pBLCAT 8$^+$ (23). The fragment containing the duplicated AREs was obtained by annealing the following two single-stranded oligonucleotides: 5'GCT TAGAACACAGTGTTCTCTAGATCTAGAACACAGT GTTCTCTAGAG3' and 5'GATCCTCTAGAGAACACTG TGTTCTAGATCTAGAGAACACTGTGTTCTA3'.

Methylation interference studies were carried out as described by Mink et al. (27).

RESULTS

Transactivation by the threonine 596 mutant AR at promoters containing multiple or single AREs. To determine the effect of the threonine 596 mutation on the action of the AR, we analyzed by transfection experiments in receptor-negative simian CV-1 cells the ability of the mutant receptor to mediate androgen responses of various indicator genes. The indicator genes consisted of the long terminal repeat (LTR) region of mouse mammary tumor virus (MMTV) cloned in front of a bacterial CAT gene (MMTV LTR CAT), a duplicated ARE cloned directly in front of a TATA box driving the expression of the CAT gene (ARE_2 TATA CAT), and an ARE cloned in front of the herpesvirus thymidine kinase (*tk*) promoter driving the expression of the CAT gene (ARE tk CAT). In transfection experiments with the MMTV LTR CAT indicator plasmid, CAT activity was enhanced by both the wild-type and mutant AR expression vectors in the presence of the androgen mibolerone, albeit with slight differences in the level of activation (Fig. 1A, lanes 4 and 6). No CAT activity was measured in cells containing an empty expression vector even in the presence of hormone (Fig. 1A, lane 2), indicating that the androgen response was mediated by the cotransfected receptors. Similar results of enhancement of CAT activity by the wild-type and mutant expression vectors were detected at the promoter of the ARE_2 TATA CAT construct in the presence of hormone (Fig. 1B, lanes 4 and 6). Other androgens such as dihydrotestosterone or R1881 (17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one) behaved similarly in stimulating the activity of both the wild-type and mutant receptors at the MMTV LTR and ARE_2 TATA promoters (results not shown). In these studies, the inducing hormones R1881 and mibolerone have been used interchangeably.

In contrast to the transactivation of indicator constructs with multiple AREs, the mutant AR is incapable of enhancing the activity of genes with single AREs. The expression of the ARE tk CAT construct is not enhanced by the mutant AR or by an empty vector in the presence of mibolerone (Fig. 1C, lanes 8 and 12). The wild-type receptor, on the other hand, increased the expression of the ARE tk CAT construct in the presence of hormone (Fig. 1C, lane 10). A quantitation of these results in four independent experiments shows clearly that the mutant AR is indeed incapable of transactivating at the promoter of the ARE tk CAT construct (Fig. 1C). In control experiments, neither the two receptor expression vectors nor the empty expression vector mediated androgen response at the promoter without the ARE (Fig. 1C, lanes 1 to 6). The ARE is therefore an important component in mediating androgen response. The inability of the mutant AR to transactivate the ARE tk CAT construct could be due to a defect in the affinity of the mutant receptor for a single ARE. This could also explain the inability of the mutant receptor to

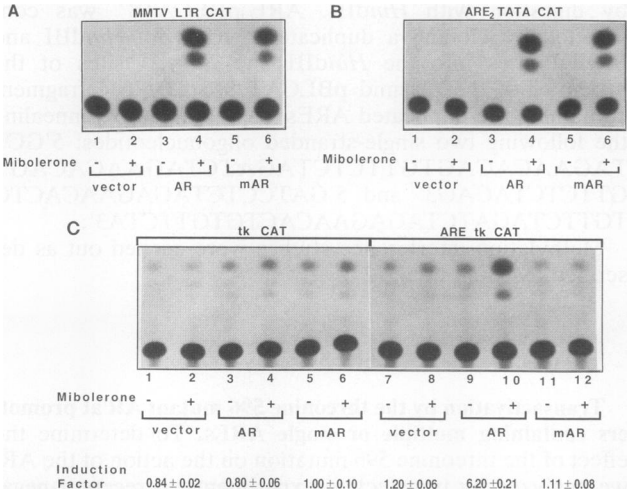

FIG. 1. Transactivation by the wild-type and threonine 596 mutant ARs of reporter genes with single or multiple AREs. Simian CV-1 cells were transiently transfected with 9 μg of the indicated reporter plasmids and 1 μg each of expression vector alone or containing the wild-type AR (AR) or mutant AR[threonine]^{596} (mAR) cDNA sequence. Transfection was carried out by the calcium phosphate coprecipitation method, and the cells were cultured thereafter in the absence or presence of 10^{-7} M mibolerone. Two days later, the cells were harvested and CAT was activity determined. In some experiments, 2 μg of the construct pCH110 was cotransfected as an internal control. In these experiments, extracts containing equal amounts of β-galactosidase activity were used for the CAT assays. The results of experiments with the internal control do not differ significantly from those without this control. The results show representative CAT assays from four independent experiments that produced identical results. Quantitation of these results was carried out by aggregation of the results of assays performed with or without internal controls.

transactivate a construct containing a single ARE linked directly in front of a TATA box (results not shown).

The threonine 596 mutant AR has a defective DNA binding activity. To investigate whether the mutant AR can bind DNA, we carried out gel retardation studies with a radioactively labeled ARE and extracts from transfected simian Cos-7 cells. The Cos-7 cells were transfected with the threonine 596 mutant and wild-type AR expression vectors and treated with or without androgens. Simian Cos-7 cells but not CV-1 cells were used for these transfections, as our receptor constructs are highly expressed in Cos-7 cells. In the absence of hormone, a complex (AR1) was formed by extracts from cells transfected with the wild-type AR (Fig. 2A, lane 1). This complex was quantitatively and qualitatively altered in the presence of the androgen mibolerone or R1881 (Fig. 2A; compare position labeled AR1 in lanes 1 to 3). In contrast, extracts from cells transfected with the mutant AR failed to bind DNA in the absence or presence of hormones (Fig. 2A, lanes 4 to 6). Extracts from nontransfected cells produced results indistinguishable from those obtained from cells transfected with the mutant receptor expression vector. The bands seen in lanes 4 to 6 at the position of the receptor-DNA complex and at the position marked "unspecific" are generated by nonreceptor proteins extracted from the Cos-7 cells. These proteins vary in amount between cell extract preparations, as will become apparent in later experiments.

We wondered whether a weak DNA binding activity of the mutant receptor to a single ARE had escaped our detection

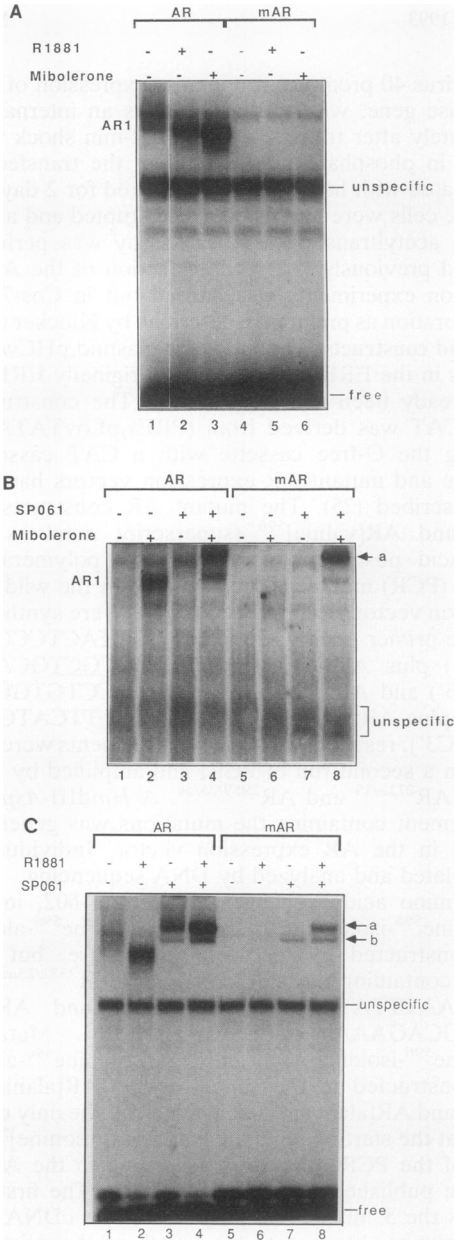

FIG. 2. DNA binding activities of the wild-type and the threonine 596 mutant ARs. Fifteen micrograms of expression vector containing the wild-type or mutant AR[threonine]^{596} cDNA or an empty expression vector was transfected by electroporation into Cos-7 cells and treated with or without androgens. Whole cell extracts were prepared and used for gel retardation experiments. (A) Gel retardation experiment with a labeled ARE oligonucleotide and extracts from cells transfected with wild-type (AR) and mutant AR (mAR) expression vectors, cultured with or without the androgens R1881 and mibolerone. (B) Gel retardation experiments with labeled duplicated ARE and extracts from cells treated with or without the hormone mibolerone in the presence or absence of the anti-AR antibody SP061. This antibody was incubated for 10 min on ice with poly(dI-dC), after which extract and labeled DNA were added. The whole reaction mixture was then incubated for 20 min at 20°C and analyzed by gel electrophoresis and autoradiography. (C) Gel retardation experiments with extracts from cells transfected with the wild-type and mutant AR expression vectors and cultured without or with R1881. After incubation of the labeled DNA and cell extracts for 10 min on ice, the anti-AR antibody SP061 was added, and the mixture was incubated for 30 min at 20°C. The DNA-receptor complex is indicated as AR1. a, the position of migration of the antibody-receptor-DNA complex; b, a complex formed by antibody and DNA alone. Unspecific binding and free oligonucleotides are indicated.

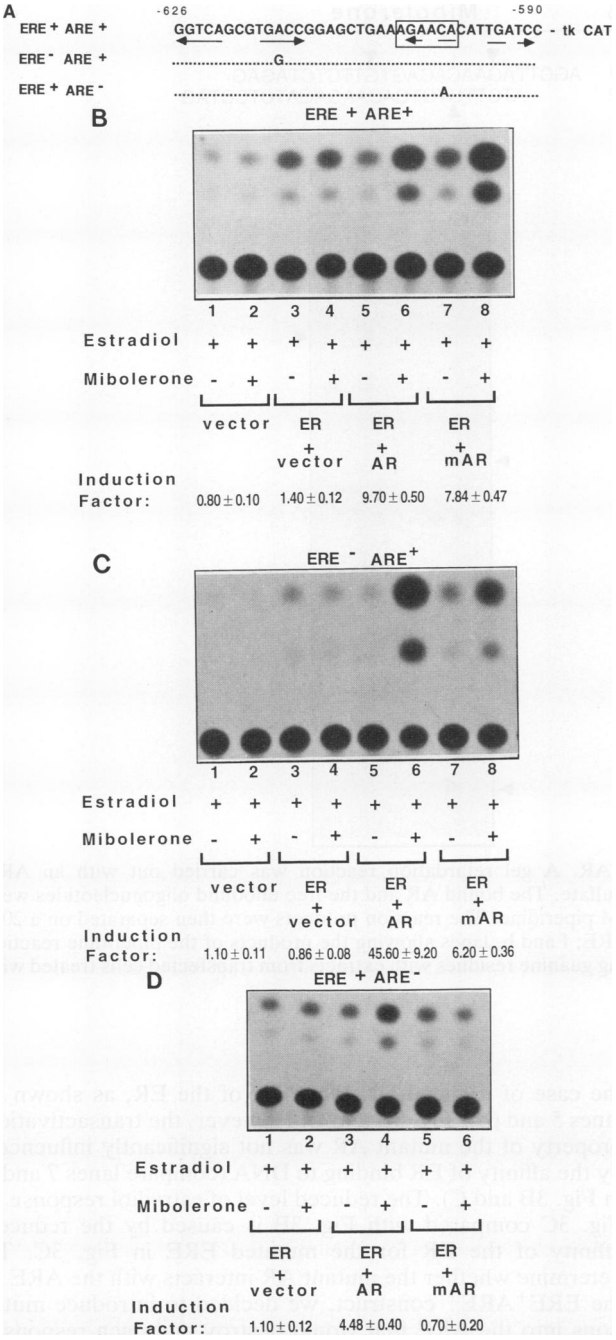

FIG. 3. Transactivation by the wild-type and threonine 596 mutant ARs of a chimeric construct containing EREs and AREs. (A) An oligonucleotide containing ERE and ARE from positions −626 to −590 of the chicken vitellogenin II gene was cloned in front of the *tk* promoter controlling the transcription of a CAT gene as described by Cato et al. (3). Single nucleotide changes in the ERE and ARE are indicated. The dotted lines represent nucleotide sequences identical to the wild-type ERE^+ARE^+ sequence. The hexanucleotide AGAACA corresponding in the upper strand to the conserved motif TGTTCT is boxed, and the palindromic sequences are indicated by horizontal arrows. (B to D). Nine micrograms of the indicated reporter plasmids was cotransfected with 0.2 μg each of expression vectors containing the wild-type (AR) and mutant (mAR) AR cDNAs, ER cDNA, and/or empty receptor expression vectors. In some cases, the internal control vector pCH110 was cotransfected as described in the legend to Fig. 1. The transfected cells were treated with 10^{-8} M estradiol and 10^{-7} M mibolerone. Two days after transfection, the cells were harvested and a CAT assay was performed with extracts from the transfected cells. The results show representative CAT assays from four independent experiments which produced similar results. The induction factors obtained in the four experiments are been presented with their degree of reproducibility.

in the DNA-receptor binding studies. As steroid receptors are known to bind with greater affinity to duplicated response elements (30), we carried out gel retardation experiments with a labeled oligonucleotide containing duplicated AREs. In these experiments, no DNA binding by the mutant AR was detected in the absence or presence of hormone (Fig. 2B, lanes 5 and 6). With the wild-type AR, a hormone-induced DNA binding was observed on the duplicated ARE (Fig. 2B, lane 2, AR1), but no DNA binding was detected in the absence of hormone (Fig. 2B, lane 1). This differs from our finding that the wild-type AR forms complex AR1 with a single ARE even in the absence of hormone (Fig. 2A, lane 1; Fig. 2C, lane 1). The reason for this difference is not known, but it is certainly not due to the quality of the receptor preparation, as the same extract that lacked the ability to bind to a duplicated ARE in the absence of hormone bound a single ARE in a parallel experiment (results not shown). The lack of DNA binding activity of the mutant receptor both with single and duplicated AREs could simply be due to a defective expression of this receptor in the recipient cells used in the transfection experiments. However, this was proven not to be the case, as equal amounts of radioactively labeled R1881 were bound by extracts from Cos-7 cells transfected with either the wild-type or the mutant receptor expression vector (results not shown). Additionally, gel retardation experiments performed with an anti-AR antibody (SP061) that recognizes N-terminal sequences of the AR also showed that the mutant AR is expressed in the Cos-7 cells. In the presence of this antibody, the mutant receptor bound DNA as shown by supershift a, which was observed in gel retardation experiments in which either the single ARE (Fig. 2C, lane 8) or the duplicated ARE (Fig. 2B, lane 8) was used. The antibody also caused supershifts of the receptor-DNA complexes generated by the wild-type receptor with the single (Fig. 2C, lanes 3 and 4) or duplicated ARE (Fig. 2B, lane 4). The conferral of DNA binding activity to the mutant AR by the anti-AR antibody SP061 is specific for this antibody and does not occur with an unrelated anti-glucocorticoid receptor (anti-GR) antibody (results not shown). Note that complex b in Fig. 2C, lanes 3, 4, 7, and 8, is formed by a direct interaction of antibody with labeled DNA. This is observed whenever the antibody is added together with the labeled oligonucleotide. Preincubation of the antibody with poly(dI-dC) prior to its addition to the gel retardation mixture did not produce complex b (Fig. 2B).

Taken together, the results indicate that the mutant AR can bind DNA in vitro in the presence of an anti-AR antibody that possibly stabilizes DNA binding activity of the mutant receptor.

Binding sites for other transcription factors adjacent to an ARE permit interaction of the threonine 596 mutant AR with DNA. The ability of the mutant AR to enhance transcription at promoters containing multiple AREs may not arise from binding of the receptor on its own to DNA but may be due to the ability of the receptor to interact with DNA through the help of neighboring bound factors. This was demonstrated in transfection experiments with an indicator gene containing a

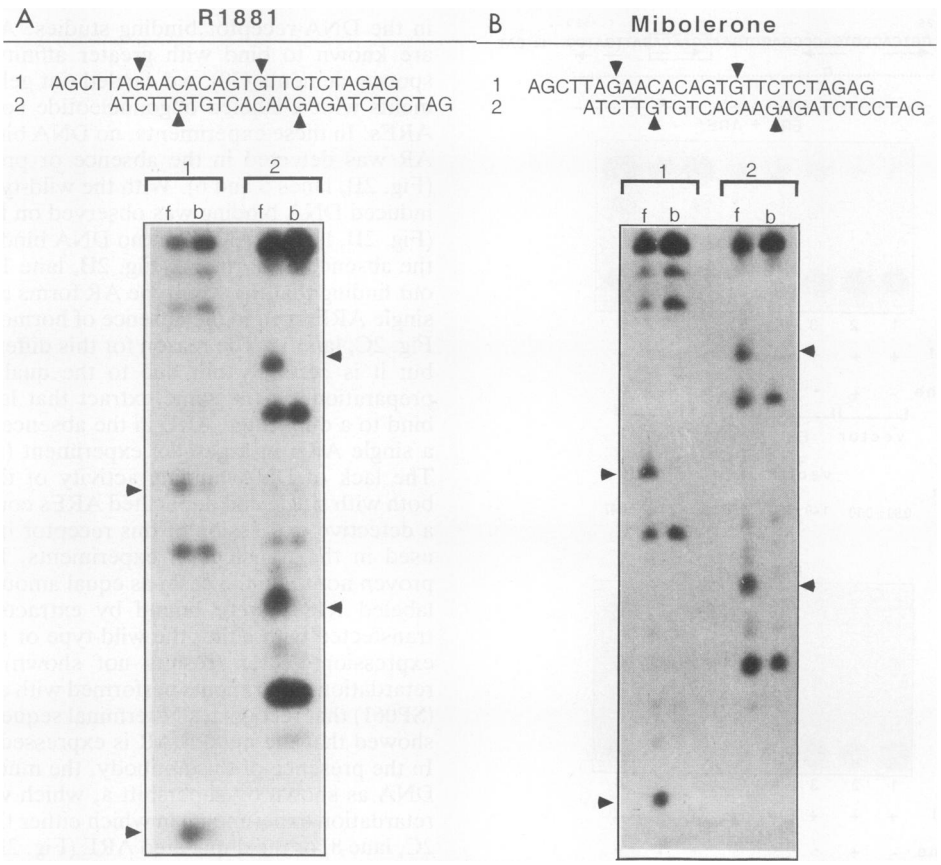

FIG. 4. Guanine moieties on an ARE contacted by the wild-type AR. A gel retardation reaction was carried out with an ARE oligonucleotide labeled at one end and partially methylated by dimethyl sulfate. The bound AR and the free unbound oligonucleotides were isolated and subjected to a strand cleavage reaction in the presence of 1 M piperidine. The reaction products were then separated on a 20% denaturing polyacrylamide gel. 1 and 2, upper and lower strands of the ARE; f and b, lanes showing the products of the piperidine reaction of the free and bound oligonucleotides. The arrowheads show the contacting guanine residues with extracts from transfected cells treated with R1881 (A) and mibolerone (B).

piece of DNA from the chicken vitellogenin II gene on which an ARE occurs close to an estrogen response element (ERE) but in an opposite orientation to the ERE (Fig. 3A). The vitellogenin II fragment was cloned in front of the *tk* promoter of the tk CAT construct to generate the indicator plasmid ERE^+ARE^+ (Fig. 3A). With use of this indicator plasmid in the presence of the estrogen receptor (ER) and estradiol, mibolerone enhanced CAT activity through both the wild-type and mutant ARs (Fig. 3B, lanes 6 and 8). That transactivation in the presence of mibolerone was indeed mediated by the AR is shown by the lack of androgen action in cells that do not contain the AR expression vector (Fig. 3B, lanes 1 to 4). A low level of CAT activity was observed in cells transfected only with the ER and treated with estradiol alone (Fig. 3B, lanes 3, 4, 5, and 7), indicating transactivation by the ER through the ERE. When the ERE^+ARE^+ sequence was replaced by an ERE^-ARE^+ sequence which contains a point mutation in the ERE that reduces the affinity of the ER for its cognate binding site (23), the transactivation function of the mutant AR compared with the wild-type receptor was repressed (Fig. 3C; compare lane 8 with lane 6 and the induction factors of the wild-type and mutant receptors). The affinity of the ER for the ERE appeared to influence androgen responsiveness at the *tk* promoter. Transactivation by the wild-type AR was higher in the case of reduced DNA binding of the ER, as shown in lanes 5 and 6 of Fig. 3B and C. However, the transactivation property of the mutant AR was not significantly influenced by the affinity of ER binding to DNA (compare lanes 7 and 8 in Fig. 3B and C). The reduced level of estradiol response in Fig. 3C compared with Fig. 3B is caused by the reduced affinity of the ER for the mutated ERE in Fig. 3C. To determine whether the mutant AR interacts with the ARE at the ERE^+ARE^+ construct, we decided to introduce mutations into the ARE that would destroy androgen response. To decide which sequences to mutate, we first performed methylation interference experiments to determine which nucleotide sequences are important for AR binding. Binding of the AR to methylated DNA in the presence of either the androgen R1881 or mibolerone showed that the guanine residue in the conserved hexanucleotide sequence TGTTCT of the ARE is a contact point for the receptor (Fig. 4A and B). To destroy the ability of the AR to contact this site, we introduced a mutation into the cytosine residue of the sequence AGAACA on the ARE motif of the ERE^+ARE^+ construct. This mutation produced the sequence AGAAAA, which on the opposite strand would be TTTTCT instead of the conserved TGTTCT. In transfection experiments with the resulting ERE^+ARE^- tk CAT construct, the transactivation function of the wild-type AR was repressed (Fig. 3D;

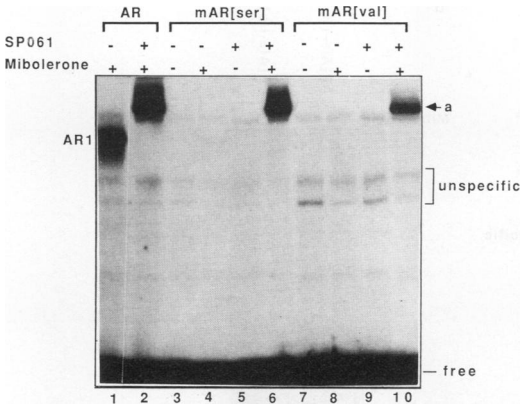

FIG. 5. Exchange of an alanine at amino acid 596 of the AR for a serine or valine residue destroys the DNA binding activity of the receptor. Gel retardation studies were carried out with an end-labeled ARE and extracts from cells transfected with the wild-type AR or the mutants AR[serine][596] and AR[valine][596] and cultured in the absence and presence of the hormone mibolerone. The assay was performed with or without the anti-AR antibody SP061. The position of migration of the androgen receptor-DNA complex AR1 is indicated; supershift complex a, formed by the addition of the anti-AR antibody, is also shown.

compare lanes 3 and 4). More importantly, the transactivation potential of the mutant receptor was completely abolished (Fig. 3D, lanes 5 and 6). Quantitation of the results of this assay and three other similar experiments showed that indeed the mutant receptor does not enhance expression at the promoter of the ERE^+ARE^- construct (Fig. 3D). As the guanine residue which was changed to thymine is an important contact site for the AR on the ARE, the results obtained with the ERE^+ARE^- construct imply that the mutant AR interacts in vivo with the ARE. Whether the mutant AR under the stabilizing influence of the bound ER interacts with the ARE the same way as the wild-type receptor does is not yet known.

An alanine residue following the zinc catenation site in the second finger of the AR is crucial for DNA binding. In all steroid receptors that recognize the idealized sequence AGAACANNNTGTTCT, the D box on the second finger contains an alanine residue next to the zinc catenation site. To investigate whether it was the exchange of the alanine residue or the presence of a threonine that altered the action of the mutant AR, the alanine residue was replaced by serine and valine in in vitro mutagenesis experiments. These new constructs were expressed in Cos-7 cells, and the extracts from the transfected cells were examined in gel retardation experiments to determine whether they bind to a single ARE. As with the defective DNA binding activity of the threonine 596 mutant, the new mutants AR[serine][596] and AR[valine][596] did not bind DNA in these studies (Fig. 5, lanes 3, 4, 7, and 8). Again, as with mutant AR[threonine][596], DNA binding activity of the new mutants was restored in the presence of hormone and the antibody SP061, as shown by supershift a (Fig. 5, lanes 6 and 10). The two new mutant ARs also did not mediate androgen response at the ARE tk CAT promoter but conferred androgen responsiveness to constructs such as MMTV LTR CAT and ARE_2 TATA CAT (results not shown). In fact, the serine and valine substitutions at the position of alanine 596 of the AR produced mutant receptors that were indistinguishable in function from the naturally occurring mutant AR[threonine][596]. Thus, an alanine and not just any amino acid next to the zinc catenation site in the second finger of the AR is required for DNA binding and transactivation at promoters containing single isolated AREs.

Distinct pairs of amino acid residues in the DNA binding domain of the AR are required for DNA binding and transactivation. In the crystal structure of GR bound to DNA, the alanine residue following the zinc catenation site in the second finger of one receptor monomer forms an intersubunit hydrogen bond with an isoleucine six amino acids downstream from this alanine residue on another receptor monomer (26). In the wild-type AR, this isoleucine is replaced by threonine 602. If the DNA binding domain of the AR dimerizes similarly to the GR, then alanine 596 would have to interact with threonine 602 to generate alanine 596-threonine 602 hydrogen bonds in the receptor dimer. To investigate the functional significance of threonine 602 in the AR action, we converted this site to an isoleucine to yield the intersubunit hydrogen bond as it occurs in the GR. The resulting mutant, AR[alanine[596]-isoleucine[602]], bound DNA with wild-type properties (Fig. 6B; compare lanes 1 and 2 with lanes 3 and 4), implying that other amino acids can replace the residue at position 602 without affecting the activity of the receptor. Conversion of threonine 602 to isoleucine in the context of the mutant receptor, AR[threonine[596]-isoleucine[602]], did not have any effect on the inability of the mutant receptor to bind DNA (Fig. 6A; compare lanes 3 and 4 with lanes 5 and 6).

In contrast, the replacement of threonine 602 with an alanine restored DNA binding activity to the mutant receptor (Fig. 6A; compare lanes 3 and 4 with lanes 7 and 8). This result indicates that intersubunit hydrogen bonds between amino acids at positions 596 and 602 can be formed by alanine and threonine independently of which residue occupies which position. An alanine at position 602 in the context of the wild-type AR did not change the ability of this receptor to bind DNA in the presence of hormone (Fig. 6A; compare lane 2 with lane 10) but reduced the DNA binding activity in the absence of hormone (Fig. 6A, lane 9). These results together with the finding that DNA binding activity of the wild-type AR in the absence of hormone differs with single and duplicated AREs demonstrate that a particular conformation of the receptor favors DNA binding activity in the absence of hormone. Our results also demonstrate that an alanine at position 596 confers DNA binding activity to the AR in the presence of hormone with either an alanine, isoleucine, or threonine at position 602. However, a threonine substitution at position 596 inhibits DNA binding activity. This defect can be corrected for only by the presence of an alanine at position 602. The interaction of particular amino acid residues at positions 596 and 602 is crucial for the DNA binding activity of the AR.

To determine the effects of the combinations of amino acids at positions 596 and 602 on the ability of the AR to transactivate, we used transfection experiments with the ARE tk CAT construct as an indicator plasmid. In agreement with the effects of the various mutations on the DNA binding property of the AR, the conversion of the threonine at position 602 to an isoleucine or an alanine in the context of the wild-type AR did not inhibit the transactivation potential of the receptor (Fig. 7). In fact, both substitutions at position 602 even enhanced the transactivation potential of the wild-type receptor nearly twofold (Fig. 7). In contrast, it is only an alanine, not an isoleucine, at position 602 that can restore the transactivation property of the mutant receptor at the ARE tk CAT promoter. The mutant AR with an isoleucine at position 602 behaved in the transactivation experiments

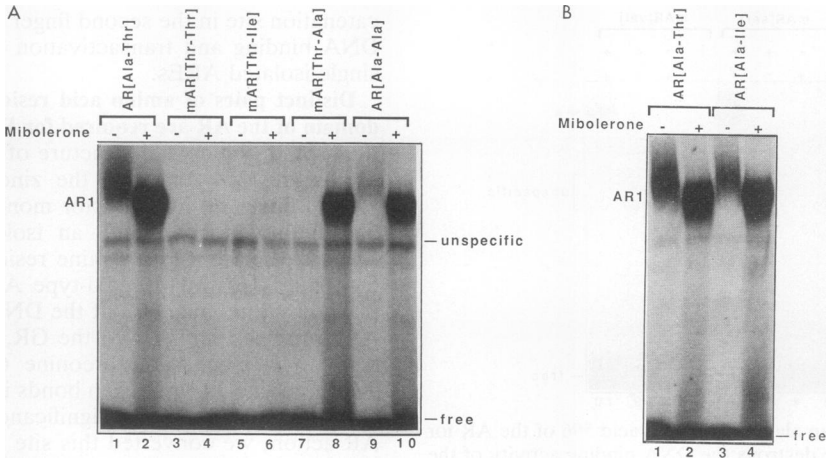

FIG. 6. A second mutation at amino acid 602 in the DNA binding domain of mutant AR[threonine][596] restores DNA binding activity. Gel retardation studies were carried out with a labeled ARE and extracts of cells transfected with the indicated mutant receptors. AR1 is the receptor-DNA complex formed by the extracts from transfected cells cultured in the absence and presence of hormone. Unspecific binding and the unbound labeled oligonucleotide are indicated.

exactly as the empty expression vector did (Fig. 7). These results show the importance of an alanine at position 596 in the AR and demonstrate that only particular combinations of amino acids at positions 596 and 602 permit DNA binding and transactivation at promoters with single isolated AREs.

DISCUSSION

We have analyzed the molecular mechanism of action of a naturally occurring mutant AR isolated from five patients with Reifenstein syndrome. Naturally occurring mutations in steroid hormone receptors are interesting sources for the investigation of structure-function relationships of steroid hormone receptors. In addition, clinical symptoms emerging from the disturbance of steroid action offer the possibility of linking specific impairment of physiological function to mutations in steroid hormone receptors. For example, hypocalcemic vitamin D-resistant rickets in two different families has been linked with point mutations that result in the exchanges of conserved amino acid residues in the first and second fingers in the DNA binding domain of the vitamin D receptor (21).

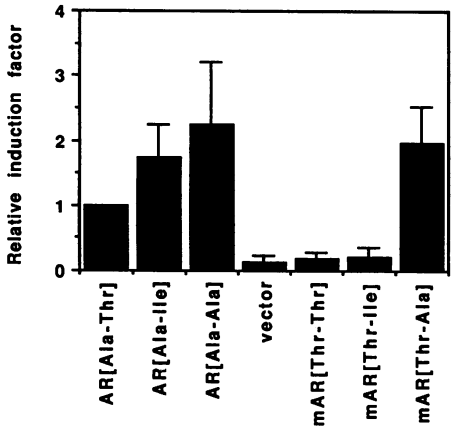

FIG. 7. Transactivation properties of mutant ARs at the promoter of the chimeric construct ARE tk CAT. Nine micrograms of the chimeric construct ARE tk CAT was transfected with 1 μg each of the indicated AR expression vectors and treated with or without the androgen mibolerone. The level of induction of CAT activity by the wild-type AR in the presence of hormone was assigned a value of 1. All induced levels of expression by the other receptor mutants were calculated with reference to this value. The bar diagrams represent for the mean values of four determination; the error bars show the standard deviation. AR[Ala-Thr], AR[Ala-Ile], and AR[Ala-Ala] are based on the wild-type AR with the exchanges alanine 596-threonine 602, alanine 596-isoleucine 602, and alanine 596-alanine 602. The receptor expression vector mAR[Thr-Thr], mAR[Thr-Ile], and mAR[Thr-Ala] represent the mutant AR series with the exchanges threonine 596-threonine 602, threonine 596-isoleucine 602, and threonine 596-alanine 602.

The glucocorticoid-resistant phenotype in most mutant S49 cell lines has been linked with molecular lesions in the GR (18, 36). Several classes of mutations associated with the glucocorticoid insensitivity in these cells have been identified. One of them, termed nt[i], arises from aberrant RNA splicing resulting in a loss of 404 N-terminal residues of the GR (10). In the case of the AR, most of the described naturally occurring mutations are mainly at the carboxy terminus and some are in the DNA binding domain (for a review, see reference 2). They consist predominantly of amino acid exchanges at conserved sequences, a few splicing defects, and stop codons that produce truncated receptors.

In this work, we have analyzed a mutation that exchanges an alanine for a threonine at a position following the zinc catenation site in the D box of the second finger. This mutant receptor does not transactivate from a chimeric construct consisting of an ARE linked in front of the *tk* promoter. However, chimeric genes whose promoters possess AREs fairly close to other transcription factors are transactivated. This pattern of inactivation of some but not all genes may be the reason for the partial androgen insensitivity in patients that carry this mutant receptor.

Mechanistically, the inability of the mutant AR to transactivate genes with single AREs in their promoter regions is derived from the inability of the receptor to bind on its own to DNA. DNA binding activity can be established in vitro by a stabilizing factor such as an anti-AR antibody or in vivo by a bound factor close to the single ARE. In the latter case, we have demonstrated that the mutant receptor does not transactivate when the guanine residue in the hexanucleotide motif TGTTCT is mutated to a thymine. We take this result to indicate that mutation of the hexanucleotide motif de-

stroys the ability of the mutant AR to bind to the ARE. This inference would therefore suggest that in the presence of a bound factor closely positioned to the ARE, the mutant AR interacts with its response element.

Studies on the binding of glucocorticoid and progesterone receptors to single glucocorticoid-responsive elements (GREs) or progesterone-responsive element showed that binding by one receptor monomer occurs preferentially at one of the two half-sites (the conserved TGTTCT sequence) that constitute the receptor binding site. This is followed by interaction of the second receptor molecule to the other half-site in a cooperative manner (8, 32). In transactivation studies with the mutant AR at the *tk* promoter containing an ERE close to an ARE, it is not clear whether androgen response occurs from stabilization of binding of the mutant AR by the ER only to the hexanucleotide motif or whether it allows the formation of stable dimers of AR on the ARE. It is also not clear which domain of the ER is involved in this proposed stabilizing function. Future experiments to clearly demonstrate in vivo stabilization of DNA binding by the mutant receptor would be invaluable for understanding the action of the mutant AR. This is particularly important in view of recent data showing that the GR binds DNA in a fashion different from the classical dimer interaction to negatively regulate the expression of certain genes (11).

Experiments on interaction of GR with single or duplicated GREs showed that the stability of the GR-DNA complex measured in terms of half-life ($t_{1/2}$) of the complex is 180 to 200 min for a duplicated GRE. This is over 10-fold higher than the $t_{1/2}$ of the complex formed with a single GRE, which has a $t_{1/2}$ of only 12 to 15 min (30). These results indicate an increased affinity of the GR and possibly other steroid receptors for a duplicated response elements. The reason for a higher affinity of steroid receptors for duplicated response elements is a combination of protein-protein interaction and alteration of the DNA structure between the two response elements (30, 35). The duplicated AREs that we used in this study are separated from each other by 22 bp on a center-to-center distance basis. At such a distance, the two response elements would face the same side of the DNA helix, providing an ideal opportunity for protein-protein interaction. We would therefore expect binding by the mutant AR to be stabilized by protein-protein interaction if this receptor possesses the slightest affinity for its response elements. The fact that no binding to duplicated AREs was detected in in vitro studies demonstrates that protein-protein interaction of the mutant AR alone cannot explain transactivation by the mutant receptor at promoters with multiple AREs. More likely, other proteins that bind close to the ARE may be required to stabilize the DNA binding activity of the mutant AR. The role of these other proteins in mediating transactivation by the mutant AR may be to alter the DNA conformation and thereby generate the desired DNA structure for the mutant receptor to bind DNA. Experiments are in progress to determine whether the mutant AR can bind DNA sequences that differ from the classical palindromic hormone response elements.

DNA binding by the mutant AR was achieved in vitro through the use of specific antibodies. A similar experiment in which a bivalent antibody promotes DNA binding of a dimerization-defective ER has already been reported (15). Our results with the mutant AR differ from that of the dimerization-defective ER in that the AR antibody induces DNA binding only in the presence of hormone. This finding suggests that a function activated by the steroid hormone is required for the antibody to restore DNA binding activity destroyed by the mutation. Whether this function has anything to do with receptor transformation or the activation of the dimerization signal in the hormone binding domain of the AR is not yet known. It could be that the hormone increases the level of the mutant receptor. Although no Western blotting (immunoblotting) has been carried out to show that equal amounts of receptor are present in the absence and presence of hormone, we consider it unlikely that the hormone alters the level of expression of our transfected receptor constructs. Instead, it is likely that dimerization of the steroid hormone receptors require the elements both in the D box and in the hormone binding domain. The mutation in the D box in the AR can be made functional only when the dimerization elements in the hormone binding domain have been activated by the steroid.

An alanine residue next to the zinc catenation site in the D box of the second finger is conserved in all steroid receptors that bind to the consensus sequence AGAACANNNTGT TCT. In the GR, replacement of the five-amino-acid sequences in the D box of the GR by corresponding sequences from the human β-thyroid receptor abolished the cooperative binding of two receptor monomers to a GRE (9). This finding provides evidence for the role of the D-box sequences in stabilization of receptor dimer structure. Interestingly, one of these five amino acids in the D box is alanine 596, whose mutation to threonine we have analyzed. We have therefore demonstrated that the exchange of only one of these residues suffices to abolish the protein-protein contact necessary for the establishment of active dimers for DNA binding. The function of the other amino acids in the D box is not known, but with only one exception, serine 597 in the AR, all amino acids in the D box are well conserved among the GR, the AR, and the progesterone and mineralocorticoid receptors. We therefore suggest that strong evolutionary pressures to conserve the residues in the D box have yielded conserved surfaces that have been exploited for intermolecular interactions such as contact between the receptor monomers. Whatever the function of the other amino acid sequences in the D box, our results have shown clearly that exchanging alanine 596 for serine or valine destroyed the DNA binding activity of the AR. Thus, an alanine next to the zinc catenation site in the D box of the AR is important for efficient DNA binding.

In the rat GR, this alanine is at position 477 and forms an intersubunit backbone hydrogen bond with an isoleucine at position 483. An isoleucine six amino acids downstream from the alanine in the D box is conserved in all receptors that bind the core sequence AGAACANNNTGTTCT with the exception of the AR that possesses a threonine instead of the isoleucine (1). Exchanging this threonine in the mutant AR with an alanine established a double mutation in the receptor. The sequence threonine 596-alanine 602 is obtained instead of the wild-type AR sequence alanine 596-threonine 602. This exchange reestablished the DNA binding activity and full transactivation function of the mutant AR. Through the use of different combinations of amino acids at positions 596 and 602, we have shown that intersubunit hydrogen bonds in the AR need to be formed by particular amino acids to confer DNA binding activity to the AR. These bonds could be formed by alanine 596-alanine 602, alanine 596-isoleucine 602, alanine 596-threonine 602, and threonine 596-alanine 602 but not by threonine 596-threonine 602, threonine 596-isoleucine 602, serine 596-threonine 602, or valine 596-threonine 602.

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