0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 1
Printed in U.S.A.

Substitution of Ala^{564} in the First Zinc Cluster of the Deoxyribonucleic Acid (DNA)-Binding Domain of the Androgen Receptor by Asp, Asn, or Leu Exerts Differential Effects on DNA Binding*

HENNIE T. BRÜGGENWIRTH, ANNEMIE L. M. BOEHMER, JEAN MARC LOBACCARO, LAURENT CHICHE, CHARLES SULTAN, JAN TRAPMAN, AND ALBERT O. BRINKMANN

Departments of Endocrinology & Reproduction (H.T.B., A.L.M.B., A.O.B.) and Pathology (J.T.), Erasmus University, Rotterdam, The Netherlands; and Pathologie Molèculaire des Recepteurs Nucléaires, INSERM U-439 (J.M.L., C.S.), and Centre de Biochimie Structurale, UMR 9955, Faculté de Pharmacie, CNRS-INSERM-Université Montpellier I (L.C.), Montpellier, France

ABSTRACT

In the androgen receptor of a patient with androgen insensitivity, the alanine residue at position 564 in the first zinc cluster of the DNA-binding domain was substituted by aspartic acid. In other members of the steroid receptor family, either valine or alanine is present at the corresponding position, suggesting the importance of a neutral amino acid residue at this site. The mutant receptor was transcriptionally inactive, which corresponded to the absence of specific DNA binding in gel retardation assays, and its inactivity in a promoter interference assay. Two other receptor mutants with a mutation at this same position were created to study the role of position 564 in the human androgen receptor on DNA binding in more detail. Introduction of asparagine at position 564 resulted in transcription activation of a mouse mammary tumor virus promoter, although at a lower level compared with the wild-type receptor. Transcription activation of an $(ARE)_2$-TATA promoter was low, and binding to different hormone response elements could not be visualized. The receptor with a leucine residue at position 564 was as active as the wild-type receptor on a mouse mammary tumor virus promoter and an $(ARE)_2$-TATA promoter, but interacted differentially with several hormone response elements in a gel retardation assay. The results of the transcription activation and DNA binding studies could partially be predicted from three-dimensional modeling data. The phenotype of the patient was explained by the negative charge, introduced at position 564. (*Endocrinology* **139:** 103–110, 1998)

THE ANDROGEN receptor (AR) gene is composed of eight exons and encodes a protein of 910 amino acids with an apparent molecular mass of 110 kDa (1). The AR belongs to a superfamily of nuclear receptors for steroid hormones, thyroid hormones, vitamin D, and retinoids. These receptors are characterized by distinct functional domains: an N-terminal part, involved in transcription activation, a DNA-binding domain (DBD), a hinge region, and a C-terminal part involved in ligand binding, dimerization, and transcription activation (2, 3). The DBD of steroid receptors is encoded by two exons and consists of two functionally different DNA-binding zinc clusters (4). Steroid receptors bind to hormone response elements (HREs) as homodimers, in contrast to several other nuclear receptors that can heterodimerize with the retinoid X receptor (4). Although the structure of the DBD is well conserved between nuclear receptors, several groups of receptors bind to specific DNA sequences (5). The glucocorticoid receptor (GR) and estrogen receptor (ER) DBDs interact with distinct, although related, HREs (4, 6). Three amino acid residues located in the so-called P box (proximal box) are essential for specific interaction with base pairs from the HRE, located in the major groove of DNA (7). The GR, the AR, the mineralocorticoid receptor, and the progesterone receptor recognize the same HRE (AGAACAnnnTGTTCT) (5). Specificity with respect to transcription activation is probably introduced by auxillary factors, which can change the affinity and specificity of binding sites (4). However, recently Claessens *et al.* (8) reported an androgen response element (ARE) in the probasin promoter that is AR specific. The consensus HRE for steroid receptors is an imperfect palindromic sequence, consisting of two half-sites, spaced by three nucleotides (9). Binding of the first receptor molecule enhances binding of the second molecule. Important determinants for this so-called cooperativity of binding are the spacing between the two half-sites of the HRE and protein-protein contacts (9).

Male sex differentiation and development proceed under direct control of the male sex hormones testosterone and 5α-dihydrotestosterone, and the actions of both androgens are mediated by the AR. Mutations in the AR gene of 46,XY individuals are associated with the androgen insensitivity syndrome (AIS), a disorder of sex differentiation. Many abnormalities have been described, causing a wide spectrum of phenotypes, ranging from subjects with an external female

Received June 10, 1997.
Address all correspondence and requests for reprints to: Dr. H. T. Brüggenwirth, Department of Endocrinology & Reproduction, Faculty of Medicine and Health Sciences, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: bruggenwirth@endov.fgg.eur.nl.
* This work was supported by the Netherlands Organization for Scientific Research, through Gebied-Medische Wetenschappen.

phenotype and absence of Mullerian and Wolffian duct derivatives, which is the complete form of AIS, to a phenotype with ambiguous genitalia, called partial AIS (10). The most frequently reported defects are point mutations in the ligand- and DNA-binding domains of the AR (10, 11).

In the present study a mutation in exon 2 of a subject with complete AIS is reported. The alanine residue at position 564 in the DBD was substituted into aspartic acid (mutant A564D). The effect of the A564D mutation on AR function was investigated, as was the effect of an asparagine substitution (mutant A564N) and a leucine substitution (mutant A564L) at this same position. These studies were completed with molecular modeling.

Materials and Methods

Materials

Primers were obtained from Pharmacia Biotech Benelux (Roosendaal, The Netherlands). [γ-^{32}P]ATP (SA, 3000 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). 17β-Hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]R1881; SA, 85 Ci/mmol), and unlabeled R1881 were purchased from New England Nuclear-DuPont de Nemours (s'Hertogenbosch, The Netherlands). The double stranded probe, containing an ARE, derived from the tyrosine aminotransferase (TAT) promoter 5′-TCGACTGTACAGGATGTTCTAGCTACT-3′ (half-sites are *underlined*) was obtained from Promega (Woerden, The Netherlands). The 27-bp oligonucleotides, used to produce two other double stranded probes, 5′-TCGACGTTACAAACTGTTCTAGCTACT-3′ and 5′-TCGACGGTACAGTTTGTTCTAGCTACT-3′ (half-sites are *underlined*), containing, respectively, the strongest ARE from the mouse mammary tumor virus (MMTV) promoter (12) and a consensus ARE (13), were obtained from Pharmacia Biotech Benelux.

Clinical data

The patient exhibiting female external genitalia, atrophic epididymides, and vasa deferentia was diagnosed as having AIS at the age of 1 yr in the absence of a positive family history. A blind ending vagina was present, the uterus was absent, and testes with a normal histology for a boy of this age were present in the inguinal canal. Testosterone synthesis disorders were excluded as a cause of the 46,*XY* sex reversal. Genital skin fibroblasts (GSF) were obtained from the index patient for Scatchard analysis and structural analysis of the AR gene. AR sequence analysis of relatives was performed on white blood cell genomic DNA.

Mutation detection

PCR-single strand conformation polymorphism analysis and direct sequencing were performed as described previously (14).

Ligand binding study

For determination of ligand binding characteristics of the AR of the AIS subject, GSF were incubated with serial dilutions [^{3}H]R1881 (0.02, 0.05, 0.3, 1.0, and 3.0 nM, respectively) in serum-free medium. The binding assay was performed as described previously (14).

Western blot analysis

AR protein, derived from GSF or transiently transfected Chinese hamster ovary (CHO) cells was immunoprecipitated and analyzed by Western immunoblotting according to the method of Ris-Stalpers *et al.* (15).

Construction of AR expression vectors

pAR(0), a human wild-type AR complementary DNA expression plasmid, was described previously (1). Expression plasmids encoding the various mutants, pAR(A564D), pAR(A564N), and pAR(A564L), respectively, were constructed by site-directed mutagenesis. The *Kpn*I-*Asp*I-digested fragment of pAR(0) was exchanged with mutated *Kpn*I-*Asp*I fragments, generated in two separate PCR amplifications (16). Sense primer 470A (14), located upstream of the *Kpn*I site in exon 1, was combined with an antisense primer, containing the mutation (antisense primers: construct A564D, 5′-CATGTGAGAtCTCCATAGTGACAC-3′; construct A564N, 5′-CATGTGAGAttTCCATAGTGACACCC-3′; construct A564L, 5′-CATGTGAGAagTCCATAGTGACACCC3′), and a sense primer, introducing the mutation (sense primers: construct A564D, 5′-GTGTCACTATGGAGaTCTCACATG-3′; construct A564N, 5′-GGTGTCACTATGGAaaTCTCACATGTGG-3′; construct A564L, 5′-GGTGTCACTATGGActTCTCACATGTGG-3′), was used in combination with an antisense primer, 14NB (14), located downstream of a unique *Asp*I site in exon 4. One microliter of both PCR products was used as template in a second PCR reaction using primers 470A and 14NB. The resulting PCR fragment was digested with *Kpn*I and *Asp*I, and exchanged for the corresponding wild-type fragment in pAR0.

pSG5AR(0), a human wild-type AR complementary DNA expression vector (provided by Dr. A. C. B. Cato, Karlsruhe, Germany) was used for transient transfection of COS-1 cells. pSG5AR(A564D), pSG5AR(A564N), and pSG5AR(A564L) were constructed by exchanging the 472-bp *Kpn*I-*Asp*I insert from pSG5AR(0) and the *Kpn*I-*Asp*I fragment from the pSVAR plasmids, encoding the variant ARs.

MMTV-LUC reporter plasmid, cytomegalovirus (CMV)-LUC, the CMV-$(ARE)_3$-LUC reporter plasmids, and pJH4-$(ARE)_2$-TATA-LUC, containing the TATA box and an Sp1 site derived from the Oct-6 gene promoter have been described previously (17–19).

Cell culture conditions and transfections

GSF and COS-1 cells were cultured as previously described (20). CHO cells were cultured according to the COS-1 cell culture protocol. The CHO cells used for transcription activation studies and promoter interference assays were plated in 7- or 11-cm^2 (promoter interference assay) wells and grown for 24 h. Cells were cotransfected, using the calcium-phosphate method, with AR expression plasmid (10 ng/ml precipitate) and reporter plasmid (2 μg/ml precipitate) (21). Carrier DNA (pTZ19) was added to a total of 20 μg DNA/ml precipitate, and 90 μl precipitate were added per well. In the promoter interference assay, 300 ng AR expression plasmid and 30 ng reporter plasmid [CMV-LUC or CMV-$(ARE)_3$-LUC], respectively, were added per ml precipitate. pTZ19 was added to a total of 20 μg DNA/ml precipitate, and 250 μl precipitate were added to 11-cm^2 wells. The transfection and luciferase assay were performed as described previously (22). Both transcription activation studies and the promoter interference assay were performed at least three times in triplicate, using three independent isolates of expression plasmid. In the case of transcription activation studies, luciferase activity was expressed relative to basal activity measured after culture in the absence of hormone. For promoter interference studies, luciferase activity in cells, transfected with CMV-$(ARE)_3$-LUC and AR expression plasmid and cultured in the absence of hormone was set at 100%. Inhibition of promoter activity in the presence of hormone was expressed relative to this 100% activity. CHO cells used for expression studies by Western blotting were also transiently transfected by the calcium-phosphate method. To this end, cells were plated in 175-cm^2 culture flasks and transiently transfected with 20 μg expression plasmid. COS-1 cells were plated in 80-cm^2 culture flasks and transiently transfected with 9.4 μg expression plasmid, using the diethylaminoethyl-dextran method (23). Twenty-four hours before harvesting, CHO and COS-1 cells were washed and incubated with medium containing 1 nM R1881.

Gel retardation assays

Transfected COS-1 cells were collected in 5 ml PBS, and the pellet was resuspended in extraction buffer [10 mM NaH_2PO_4 (pH 7.4), 0.4 M KCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM bacitracin, 0.5 mM leupeptin, 0.6 mM phenylmethylsulfonylfluoride, and 10 mM dithiothreitol] and subjected to four freeze-thaw cycles, followed by 10-min centrifugation at 400,000 $\times$ *g* at 4 C in a TLA120.2 rotor (Beckman, Fullerton, CA) in a Beckman Optima TLX ultracentrifuge. The TAT ARE-containing probe (5′-TCGACTGTACAGGATGTTCTAGCTACT-3′) (halfsites are *underlined*) was obtained from Promega. Two other probes were produced by annealing a 27-bp oligonucleotide with an oligonucleotide of

complementary sequence. One of them (5′-TCGACGTTACAAACTGTTCTAGCTACT-3′) (half-sites are *underlined*) contains the strongest ARE from the MMTV promoter (12), and the other probe (5′-TCGACGGTACAGTTTGTTCTAGCTACT-3′) (half-sites are *underlined*) contains a consensus ARE (13). The ARE-containing probes were end labeled using T4 polynucleotide kinase, and double stranded probe was purified from a 4% acrylamide gel in 0.5 × TBE (1 × TBE = 50 mM Tris base, 50 mM boric acid, and 1 mM EDTA, pH 8.6). Cellular extracts were incubated in binding buffer [10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 4% Ficoll] and 1 μg polydeoxyinosinic-deoxycitidylic acid in the absence or presence of the polyclonal AR antibody Sp197 (10-fold diluted) (24). After an incubation period of 10 min on ice, 2 μl purified DNA probe (50,000 cpm/μl) were added, and incubation was continued for 20 min at room temperature. The 20-μl sample was separated on a 4% polyacrylamide gel in 0.5 × TBE. Gels were fixed for 10 min in 10% acetic acid-10% methanol, and subsequently dried and exposed.

Molecular modeling

The crystal structure of the rat GR DBD bound to a glucocorticoid response element was used as a basis upon which the three-dimensional (3-D) AR models were built. The 3-D model of AR bound to a glucocorticoid response element has previously been described (25). The A564D, A564N, and A564L mutants were built according to the same strategy as that used to build the wild-type model. Briefly, the side-chains of the AR mutants that were substituted in the GR model were placed in energetically favorable conformations using the SMD program (26). The whole system was then energy minimized with the AMBER program (Pearlman *et al.*, 1991, University of California, San Francisco, CA). During the optimization process, the oligonucleotide was kept frozen to prevent unrealistic deviation from the initial crystal structure. Moreover, positional restraints on the backbone and on side-chains of conserved residues were applied and gradually released during the optimization. Figure 5A was generated with the Insight II viewer (Biosym Technologies, San Diego, CA), and Fig. 5B was generated using the program MOLSCRIPT (27).

Results

Mutation detection

Genomic DNA of the index patient was used to amplify the coding part and intronic sequences flanking the exons of the AR gene, followed by single strand conformation polymorphism analysis performed under two different conditions. An aberrant banding pattern was found for exon 2, which encodes the first zinc cluster of the DBD. Direct sequencing showed a single nucleotide substitution at codon 564 (C to A) that resulted in substitution of alanine to aspartic acid (Fig. 1). The numbering of amino acid residues throughout the text is based on a total number of 910 amino acid residues in the human AR (1). The mutation created a *Bgl*II site, which was used to investigate the segregation of this mutation in the family of the index patient. The mother and grandmother of the index patient were heterozygous carriers of this AR mutation (results not shown).

Functional properties of the mutant receptor

The AR protein was isolated from GSF obtained from the index subject. Molecular mass was checked by immunoblotting after immunoprecipitation. The AR protein migrated as a normal 110/112-kDa doublet on SDS-PAGE (data not shown). For Scatchard analysis, cultured GSF were incubated for 1 h with increasing concentrations of [^{3}H]R1881 in either the presence or absence of a 200-fold molar excess of nonradioactive R1881. Both the K_d (0.07 nM) and the maximal

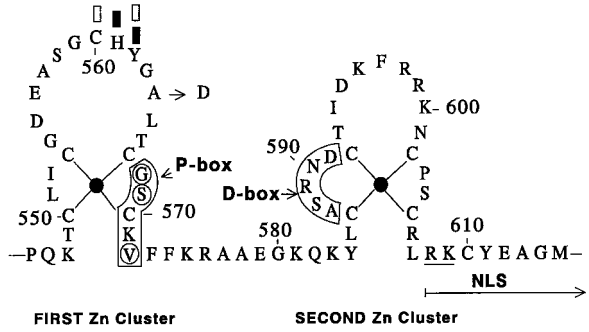

FIG. 1. Sequence of the AR DBD with the A→D mutation, located in the first zinc cluster. The mutation found in the index patient's AR is present at position 564 of the AR DBD, located near the P box, of which the *circled* amino acid residues are involved in ARE recognition. The *boxes* indicate amino acid residues that interact with the phosphate backbone of DNA either at specific (black boxes) or at nonspecific sites (*open boxes*) (25, 28). The second zinc cluster contains the D box, which is involved in dimerization with the other AR receptor. The first part of the nuclear localization signal (NLS) is also shown (*underlined*). The numbering of the various codons is based on a total of 910 amino acid residues in the human AR (1).

binding capacity (58 fmol/mg protein) values were within the normal range (K_d, 0.03–0.13 nM; binding capacity, 39–169 fmol/mg protein), indicating that ligand binding was not affected by the mutation.

Transcriptional activity of AR A564D

AR A564D was inactive in CHO cells cotransfected with $(ARE)_2$-TATA-LUC, in contrast to the wild-type AR (Fig. 2A). The promoter of this reporter construct contains a TATA box and an Sp1 site derived from the Oct-6 gene promoter, and two AREs (19). In general, comparable levels of wild-type and mutant receptor protein were expressed in transiently transfected CHO cells, as verified by SDS-PAGE and immunoblotting (for example, see Fig. 3C). AR A564D was also unable to activate transcription from the complex MMTV promoter in CHO cells (Fig. 2B).

In vivo DNA binding of AR A564D

In vivo DNA binding was studied using a promoter interference assay. CHO cells were cotransfected with CMV-$(ARE)_3$-LUC. Three consensus AREs were inserted between the TATA box of the constitutively active CMV promoter and the transcription start site of the luciferase gene (13). Binding of the AR hinders the assembly of a transcription initiation complex and, therefore, also interferes with the expression of the luciferase gene (18). The level of inhibition is taken as a measure of specific DNA binding. In the presence of 1 nM R1881, the wild-type AR showed a 48% reduction of luciferase activity, whereas no reduction was seen in cells cotransfected with AR A564D (Fig. 2C). The AR could sequester factors that are essential for the transcriptional activity of the CMV promoter (squelching). However, no reduction of luciferase expression was seen in cells cotransfected with CMV-LUC (Fig. 2C).

Transcriptional activities of AR A564N and AR A564L

To investigate whether the inactivity of AR A564D was caused by steric hindrance or by a conformational change

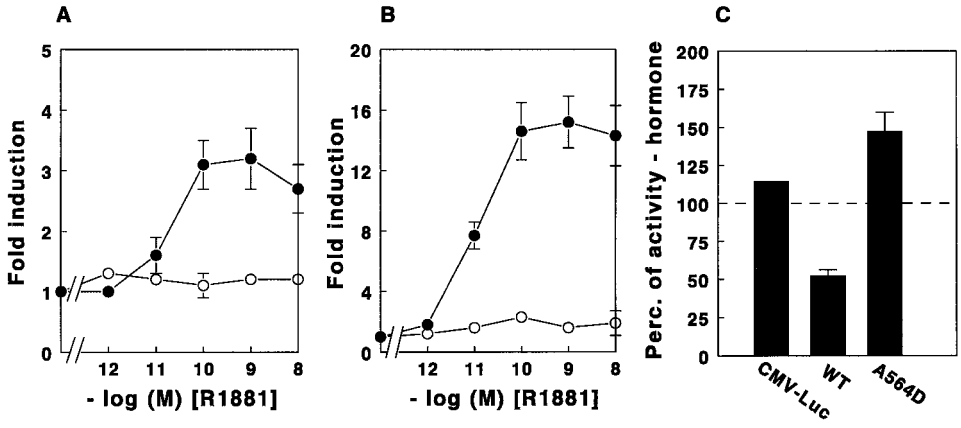

FIG. 2. A, Transcriptional activity of AR A564D at the $(ARE)_2$-TATA promoter. CHO cells were cotransfected with $(ARE)_2$-TATA-LUC reporter plasmid and the wild-type or mutant expression plasmid. Twenty-four hours after transfection, cells were cultured with medium containing increasing concentrations of R1881 for another 24 h. Each *data point* was tested in triplicate. Induction of luciferase activity was calculated from five different experiments (n = 5); symbols represent the mean ± SEM (●, wild type; ○, A564D). Transcription activation by AR A564D was significantly different from that by the wild-type AR (by Student's *t* test, $P = 0.05$). B, Transcriptional activity of AR A564D at the MMTV promoter. Transcription activation properties were determined by cotransfection of CHO cells with MMTV-LUC reporter plasmid and either the wild-type or mutant ARA564D expression plasmid. After 24 h, the cells were incubated with different concentrations of R1881 and incubated for another 24 h before a luciferase assay was performed. Each *data point* was tested in triplicate. Induction of luciferase activity was calculated from three different experiments; symbols represent the mean ± SEM (●, wild type; ○, A564D). Transcription activation by AR A564D was significantly different from that by wild-type AR (by Student's *t* test, $P = 0.05$). C, Promoter interference of wild-type AR and AR A564D. CHO cells were transiently transfected with expression plasmid and CMV-$(ARE)_2$-LUC reporter plasmid. Twenty-four hours after transfection, cells were cultured in the absence or presence of 1 nM R1881 and incubated for another 24 h before performing a luciferase assay. Each *data point* was tested in triplicate. CMV-LUC reporter construct cotransfected with pAR0 was taken as a control (n = 2). The luciferase signal measured in the absence of R1881 was arbitrarily set at 100%, and activities measured in the presence of 1 nM R1881 were related to these values. The mean promoter activity ± SEM in the presence of hormone is represented.

due to the introduction of a negative charged amino acid residue, the alanine residue was replaced by either a leucine residue (A564L) or an asparagine residue (A564N). Leucine has, like the aspartic acid residue, a larger side-chain, but is a neutral amino acid, as is the alanine residue present in the wild-type receptor. Asparagine has also a larger side-chain, but is a polar amino acid residue. CHO cells were transiently cotransfected with AR expression plasmids and $(ARE)_2$-TATA-LUC. Wild-type AR and AR A564L showed comparable activation of the minimal promoter at increasing amounts of R1881, whereas AR A564N showed strongly reduced transcription activation compared with the wild-type AR (Fig. 3A). On the more complex MMTV promoter, AR A564L showed activity comparable to that of the wild-type receptor, and AR A564N displayed a low level of hormone-induced transcription activation (Fig. 3B). All proteins were expressed, and in general, expression levels were comparable (Fig. 3C).

In vivo DNA binding of AR A564N and AR A564L

DNA binding was studied in CHO cells cotransfected with CMV-$(ARE)_3$-LUC (Fig. 3D). In contrast to the wild-type AR, luciferase expression was not lowered after cotransfection of cells with AR A564N and culture in the presence of hormone. AR A564L showed 38% inhibition, which was significantly different ($P < 0.05$) from the 48% inhibition observed for the wild-type receptor. Protein expression levels were identical for all mutant receptors (see also Fig. 3C).

In vitro DNA binding comparing different AREs

The DNA-binding capacities of the wild-type and the different AR mutants were tested *in vitro* in gel retardation assays, using probes containing various AREs. AR was produced in transfected COS-1 cells. The amount of receptor protein was checked by Western blotting, followed by immunostaining. Comparable amounts of AR in COS-1 cellular extracts were incubated with a ^{32}P-labeled probe in either the absence or presence of polyclonal antibody Sp197, which stabilizes AR dimers bound to the DNA (24). Wild-type AR and AR A564L did bind to the ARE derived from the TAT promoter (Fig. A, lanes 3 and 9), whereas binding of AR A564D and AR A564N could not be detected (Fig. 4A, lanes 5 and 7). Gel shift assays were also performed with two other probes. One of the probes contained the strongest ARE from the MMTV promoter (12), which was also present in $(ARE)_2$-TATA-LUC. The other probe contained a consensus ARE (13), which was also cloned behind the constitutively active CMV promoter, that was used for promoter interference studies. Wild-type AR was able to shift the probes in the presence of antibody, indicative of specific DNA binding (Fig. 4B, lane 3, and Fig. 4C, lane 3). Neither probe could be shifted with AR A564D (Fig. 4B, lane 5, and Fig. 4C, lane 5) or AR A564N (Fig. 4B, lane 7, and Fig. 4C, lane 7), although A564N showed transcription activation on a complex MMTV promoter and even on a minimal ARE promoter. AR A564L interacted with both probes, although less efficiently than the wild-type AR, which is in agreement with the results of the promoter interference assay (Fig. 4B, lane 9, and 4C, lane 9).

Molecular modeling

The alanine residue at position 564 is buried, as it is involved in a hydrophobic cluster that is mainly formed by leucine 551, isoleucine 552, cysteine 610, alanine 613, and methionine 615 (Figs. 1 and 5, A and B). The Cα-Cβ bond of

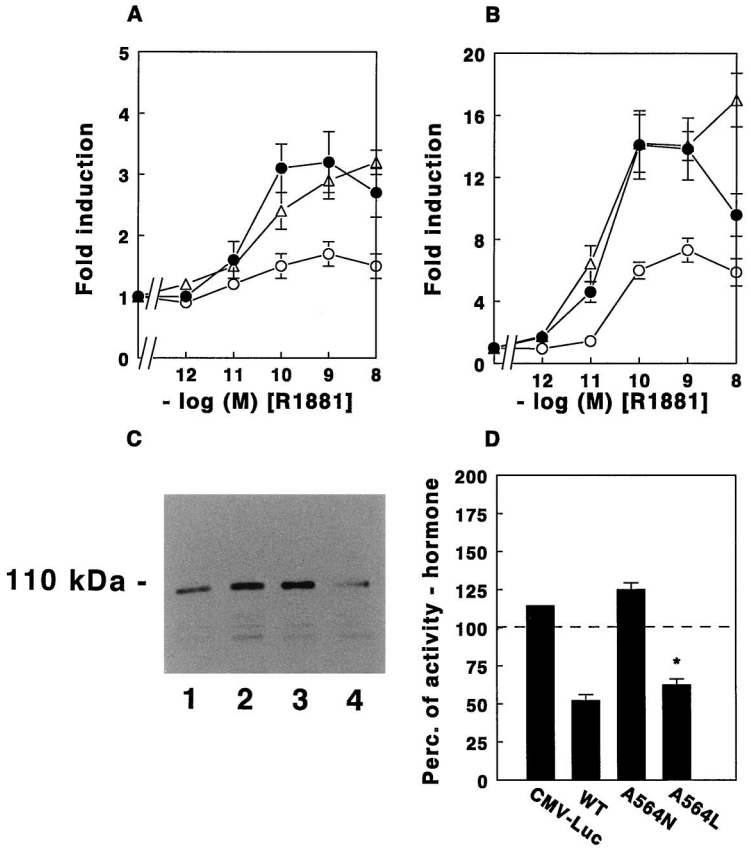

FIG. 3. A, Transcription activation by the wild-type receptor and A564N and A564L using an $(ARE)_2$-TATA-LUC reporter gene. CHO cells were cotransfected with $(ARE)_2$-TATA-LUC reporter plasmid and either the wild-type or one of the mutant expression plasmids. Twenty-four hours after transfection, cells were cultured in medium containing increasing concentrations of R1881 for another 24 h. Each *data point* was tested in triplicate. Induction of luciferase activity was calculated from five different experiments (n = 5); symbols represent the mean ± SEM (●, wild type; ○, A564N; △, A564L). Transcription activation by AR A564N was significantly different from that displayed by the wild-type AR (by Student's *t* test, $P = 0.05$). B, Transcription activation by the wild-type receptor, AR A564N, and AR A564L, using an MMTV-regulated reporter gene. CHO cells were cotransfected with MMTV-LUC reporter plasmid and either the wild-type or one of the mutant expression vectors. After 24 h, cells were cultured with increasing concentrations of R1881 for 24 h before a luciferase assay was performed. Each *data point* was tested in triplicate. Induction of luciferase activity, presented relative to basal activity in the absence of R1881, was calculated from five different experiments; symbols represent the mean ± SEM (●, wild type; ○, A564N; △, A564L). Transcription activation by AR A564N was significantly different from that displayed by the wild-type receptor (by Student's *t* test, $P = 0.05$). C, Western blot analysis of wild-type and mutated ARs after transient transfection in CHO cells. Culture flasks with CHO cells were transfected with expression plasmid, and after 48 h, cell lysates were prepared. From these lysates, the receptor was immunoprecipitated with monoclonal antibody F39.4.1 and separated on a SDS-PAGE gel. After blotting, receptor protein was detected with polyclonal antibody Sp061 and an alkaline phosphatase-coupled goat antirabbit antibody. Lane 1, Wild-type AR; lane 2, AR A564D; lane 3, AR A564N; lane 4, AR A564L. D, Promoter interference assay of AR A564N and AR A564L. The DNA binding properties of the mutated receptors were also tested in intact cells. CHO cells were transiently transfected with expression plasmid and CMV-$(ARE)_2$-LUC reporter plasmid. Twenty-four hours after transfection, cells were cultured in the absence or presence of 1 nM R1881 and incubated for another 24 h before performing a luciferase assay. Each *data point* was tested in triplicate. The CMV-LUC reporter construct cotransfected with pAR0 was taken as a control (n = 2). The luciferase signal measured in the absence of R1881 was arbitrarily set at 100%, and activities measured in the presence of 1 nM R1881 were calculated relative to these values. The mean promoter activity ± SEM in the presence of hormone is represented (n = 4). *, Significantly different from inhibition shown by the wild-type receptor (by Student's *t* test, $P < 0.05$).

the alanine residue at position 564 is directed toward the cysteine residue at position 610 in the protein core. The backbone of residue 564 is hydrogen bonded with the backbone of histidine residue 561, as both residues belong to a β-hairpin (Fig. 5B). They are located at the same side of the hairpin at facing positions. The histidine residue at position 561 is involved in direct contacts with DNA and participates in ARE recognition (Fig. 5B). Molecular modeling showed that in the A564D mutant, the aspartic acid residue is still buried. However, burying of charged residues is unfavorable unless a compensatory charge forms a salt bridge and is also buried at the same site. The only way AR A564D can take a more favorable conformation is by breaking of the β-hairpin and the hydrogen bonding with the histidine residue at position 561. This most likely affects the conformation of the histidine residue 561, resulting in disturbed ARE recognition. The asparagine residue in A564N should lead to smaller perturbations because it will remain buried, although asparagine is a polar residue. The modeling showed that hydrogen bonding of asparagine with threonine 566 may roughly com-

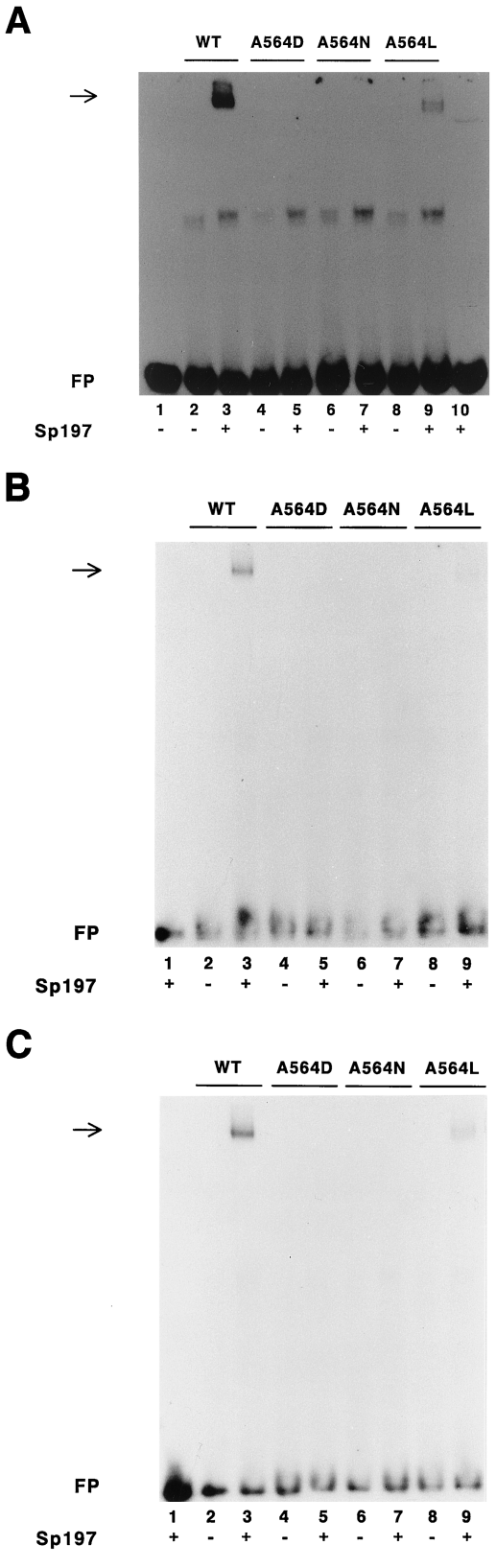

FIG. 4. Gel retardation assay with three different ARE probes. A labeled ARE probe (50,000 cpm) was incubated with nuclear extracts prepared from transiently transfected COS-1 cells. Incubations were performed in the absence (−) or presence (+) of the polyclonal antibody Sp197. The complexes were analyzed by PAGE as described in *Materials and Methods*. The position of the shifted complex is indicated by an *arrow*; FP indicates the position of the free ^{32}P-labeled probe. A, The probe contained an ARE, derived from the TAT promoter. Lane 1, No receptor protein; lanes 2 and 3, wild-type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L; lane 10, free probe. After incubation of the probe with the antibody, no specific shifted band could be seen. B, The probe contained the strongest ARE from the MMTV promoter (12). Lane 1, No receptor protein; lanes 2 and 3, wild-type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L. C, The probe contained a consensus ARE (13). Lane 1, No receptor protein; lanes 2 and 3, wild-type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L.

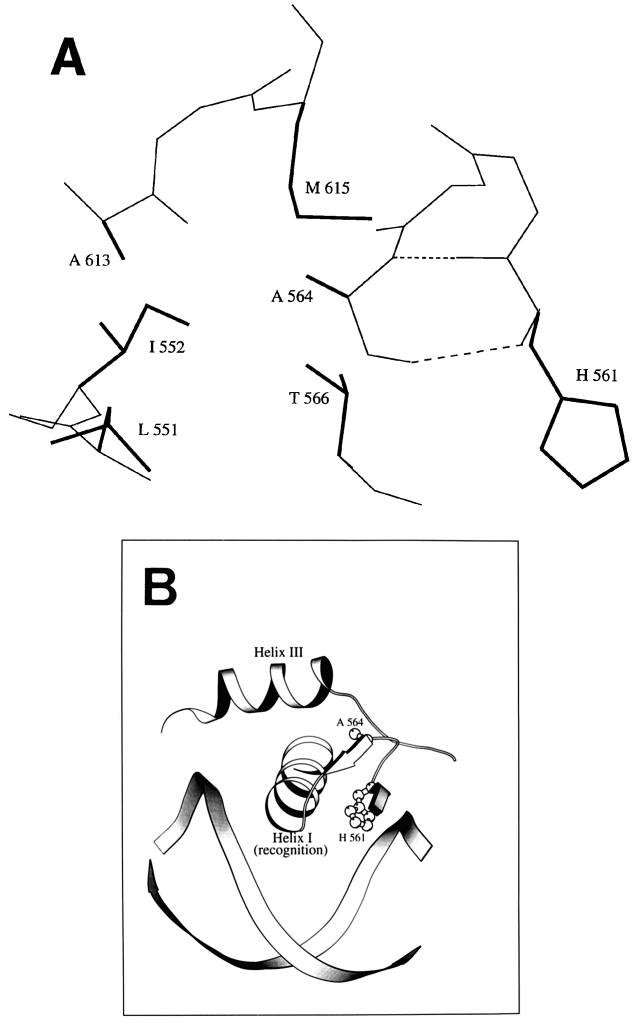

FIG. 5. A, View of the hydrogen bonding between the alanine residue at position 564 and the histidine residue at position 561. The hydrogen bonding is shown by the *dashed lines*. Also shown are residues constituting the hydrophobic pocket in which the alanine residue at position 564 is buried (*i.e.* leucine 551, isoleucine 552, threonine 566, alanine 613, and methionine 615). The backbone is shown by *thin lines*, and the side-chains are shown as *thick lines*. B, Schematic view of part of the ARE and the wild-type AR. Helix I, helix III, and the side-chains of the histidine residue at position 561 and the alanine residue at position 564 (ball and stick) are shown. The histidine residue and the alanine residue belong to a small β-hairpin (strands shown as *thick arrows*), and the histidine residue makes direct contacts with DNA. Part of the ARE is displayed as *long thin antiparallel arrows*.

pensate for the unfavorable burying of polar atoms. Modeling of the A564L mutant showed that the larger leucine side-chain can be accommodated without difficulties in the hydrophobic pocket. Burying of the larger hydrophobic surface may even provide additional stability to the AR. Therefore, the leucine mutant was not expected to significantly perturb DNA recognition.

Discussion

The DBD is the most conserved region within the nuclear receptor family. Characteristic are the eight cysteines in this domain, forming the two zinc clusters. Zinc ion-coordinated binding is essential for proper folding and DNA binding. The structure of the DBDs of the GR and ER, respectively, in complex with their response element on the DNA was solved by x-ray crystallography. Almost similar conformations were displayed (28, 29). The AR DBD is more closely related to the GR DBD sequence than to the ER DBD sequence. Fifteen amino acid residues in the AR DBD, which is defined as a 65-amino acid domain starting at lysine 548, are different from the GR DBD (30, 31). Only 5 of them are located in the first zinc cluster, and 1 of these should be considered a conservative change according to the chemical properties of its side-chain (31). Therefore, it seems reasonable to deduce the structural consequences of mutations in the AR DBD from the 3-D structure of the GR DBD. In the present paper, the characterization of a mutation at position 564 in the first zinc cluster of the DBD of the human AR is reported. Mutations in the first zinc cluster of the AR have been described previously (11). Some of them disrupt the zinc cluster structure because 1 of the cysteines is substituted, resulting in a complete AIS phenotype (11). Alanine 564 is partially conserved in other members of the receptor family. At the corresponding position in the vitamin D receptor, an alanine residue is present as well (32). In the human ER, human GR, human progesterone receptor, and human mineralocorticoid receptor, a valine residue is located at the corresponding position, implying that the presence of a neutral amino acid residue at this position is critical for proper interaction of the receptor with DNA (6, 30, 33, 34). In the AR of the patient, described in this report, alanine was substituted by the negatively charged aspartic acid residue. The aspartic acid residue is located upstream of an α-helical region that is exposed to the major groove of DNA and downstream of cysteine 560, histidine 561, and tyrosine 562, which are involved in specific as well as nonspecific contacts with the phosphate backbone of the DNA (28). Substitution of the latter residues by nonconservative amino acids in the GR resulted in loss of function (*in vivo*), and *in vitro* reduced DNA binding affinity was seen (35). Warriar *et al.* (36) substituted the cysteine residue at position 560 in the human AR by a serine residue. Although this is a relatively conservative change, decreased DNA binding and transcription activation were observed, which were attributed to the instability of the AR mutant-DNA complex.

We showed that AR A564D displayed defective transcription activation. Specific binding to DNA, which was studied *in vitro* by gel retardation assays with oligonucleotide probes containing different AREs and *in vivo* with a promoter interference assay, could not be detected. From 3-D modeling studies, it became clear that the alanine residue at position 564 is buried in a hydrophobic cluster (Figs. 1 and 5A). A hydrogen bond is formed between the backbone of alanine 564 and the backbone of histidine 561. This latter residue is involved in direct interaction with the phosphate backbone of the DNA and is conserved in other nuclear receptors. Yagi *et al.* (37) reported a patient with hereditary 1,25-dihydroxyvitamin D-resistant rickets caused by substitution of the conserved histidine at position 35 of the vitamin D receptor, comparable to histidine 561 in the AR. The phenotype of the patient was caused by perturbation of the conserved site that contacts the phosphate backbone of DNA. For AR A564D, the computer model displayed almost the exact conformation as that seen for the wild type, showing that there is no steric hindrance due to the mutation. However, buried charged residues are only observed in proteins when they can form salt bridges with residues of opposite charge. Therefore, the modeled conformation appears unlikely. Probably, aspartic acid 564 adopts a more favorable conformation, and as a result, the main chain hydrogen bond between aspartic acid 564 and histidine 561 will be broken, which has consequences with respect to DNA binding.

To investigate the role of the alanine residue at position 564 in more detail, the residue was also replaced by an asparagine or a leucine residue. 3-D modeling predicted that introduction of an asparagine residue should have intermediate effects with respect to transcription activation. Hydrogen bonding between asparagine 564 and threonine 566 might compensate for unfavorable burying of the polar amino acid residue. Substitution by an asparagine residue resulted in a less stable AR-DNA complex, which had clear consequences for transcription activation on a minimal $(ARE)_2$-TATA promoter and to a lesser extent on a complex MMTV promoter. DNA binding was not observed *in vitro* by gel retardation analysis. DNA-binding capacity remained undetectable, even when studied in whole cells. However, the functionality of AREs is determined by additional transcription factor-binding sites in the vicinity of AREs. Interaction with other proteins might stabilize the AR-DNA complex. This might explain as well the stronger activation of AR A564N on the MMTV promoter compared with its activity on the minimal $(ARE)_2$-TATA promoter (8, 38, 39).

Modeling showed that no particular constraint resulted from the larger size of the leucine residue. It was predicted that the leucine residue, because of its larger hydrophobic surface, provides even more favorable stability to the AR. However, reduced DNA binding affinity, *in vitro* as well as *in vivo*, was observed for AR A564L. Transcription activation was comparable with activation displayed by the wild-type AR. Apparently, molecular modeling has some limitations with respect to the prediction of complex interactions, which might be explained by the fact that the model was based upon the crystal structure, which was solved for the GR DBD. In addition, functional studies were performed with the intact receptor and not only the DBD.

In conclusion, the negative charge introduced by the aspartic acid residue destabilizes the normal conformation of the AR DBD, resulting in disturbed ARE recognition, in agreement with the phenotype of the patient expressing this

mutant receptor. Results from the functional assays were partially supported by predictions made by 3-D modeling. Although not predicted by molecular modeling, steric hindrance might have an impact on the DNA-binding capacities of AR A564N and AR A564L.

Acknowledgments

We thank Dr. C. W. Kuil for providing the CMV-LUC and the CMV-ARE(3)-LUC constructs, Dr. A. C. B. Cato for pSG5AR0, Dr. L. J. Blok for pJH4-$(ARE)_2$-TATA-LUC, D. P. E. Satijn for excellent technical assistance, and Dr. J. A. Grootegoed for helpful discussions.

References

1. **Brinkmann AO, Faber PW, van Rooij HCJ, Kuiper GGJM, Ris C, Klaasen P, van der Korput JAGM, Voorhorst MM, van Laar JH, Mulder E, Trapman J** 1989 The human androgen receptor: domain structure, genomic organization and regulation of expression. J. Steroid Biochem 34:307–310
2. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM** 1995 Overview: the nuclear receptor superfamily: the second decade. Cell 83:835–839
3. **Beato M, Herrlich P, Schütz, G** 1995 Steroid hormone receptors: many actors in search of a plot. Cell 83:851–857
4. **Freedman LP, Luisi BF, Korszun ZR, Basavappa R, Sigler PB, Yamamoto KR** 1988 The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature 334:543–546
5. **Forman BM, Samuels HH** 1990 Interaction among a subfamily of nuclear hormone receptors: the regulatory zipper model. Mol Endocrinol 4:1293–1301
6. **Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P** 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-*erb*-A. Nature 320:134–139
7. **Danielsen M, Hinck L, Ringold GM** 1989 Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. Cell 57:1131–1138
8. **Claessens F, Alen P, Devos A, Peeters B, Verhoeven G, Rombouts W** 1996 The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors. J Biol Chem 271:19013–19016
9. **Dahlman-Wright K, Wright A, Gustafsson J-A, Carlstedt-Duke J** 1991 Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. J Biol Chem 266:3107–3112
10. **Quigley CA, DeBellis A, Marschke KB, El-Awady MK, Wilson EM, French FS** 1995 Androgen receptor defects: historical, clinical and molecular perspectives. Endocr Rev 16:271–321
11. **Gottlieb B, Trifiro M, Lumbroso R, Pinsky L** 1997 The androgen receptor gene mutations database. Nucleic Acids Res 25:158–162
12. **Ham J, Thomson A, Needham M, Webb P, Parker M** 1988 Characterization of response elements for androgens, glucocorticoids and progestins in mouse mammary tumour virus. Nucleic Acids Res 16:5263–5276
13. **Roche PJ, Hoare SA, Parker MG** 1992 A consensus DNA-binding site for the androgen receptor. Mol Endocrinol 6:2229–2235
14. **Brüggenwirth HT, Boehmer ALM, Verleun-Mooijman MCT, Hoogenboezem T, Kleijer WJ, Otten BJ, Trapman J, Brinkmann AO** 1996 Molecular basis of androgen insensitivity. J Steroid Biochem Mol Biol 58:569–575
15. **Ris-Stalpers C, Trifiro MA, Kuiper GGJM, Jenster G, Romalo G, Sai T, van Rooij HCJ, Kaufman M, Rosenfield RL, Liao S, Schweikert H-U, Trapman J, Pinsky L, Brinkmann AO** 1991 Substitution of aspartic acid-686 by histidine or asparagine in the human androgen receptor leads to a functionally inactive protein with altered hormone-binding characteristics Mol Endocrinol 5:1562–1569
16. **Higuchi R, Krummel B, Saiki RK** 1988 A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 16:7351–7367
17. **de Ruiter PE, Teuwen R, Trapman J, Dijkema R, Brinkmann AO** 1995 Synergism between androgens and protein kinase-C on androgen-regulated gene expression. Mol Cell Endocrinol 110:R1–R6
18. **Kuil CW, Mulder E** 1996 Deoxyribonucleic acid-binding ability of androgen receptors in whole cells: implications for the action of androgens and antiandrogens. Endocrinology 137:1870–1877
19. **Blok LJ, Themmen APN, Peters AHFM, Trapman J, Baarends WM, Hoogerbrugge JW, Grootegoed JA** 1992 Transcriptional regulation of androgen receptor gene expression in sertoli cells and other cell types. Mol Cell Endocrinol 88:153–164
20. **Ris-Stalpers C, Kuiper GGJM, Faber PW, Schweikert HU, van Rooij HCJ, Zegers ND, Hodgins MB, Degenhart HJ, Trapman J, Brinkmann AO** 1990 Aberrant splicing of androgen receptor mRNA results in synthesis of a nonfunctional receptor protein in a patient with androgen insensitivity. Proc Natl Acad Sci USA 87:7866–7870
21. **Chen C, Okayama H** 1987 High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745–2752
22. **Kuil CW, Berrevoets CA, Mulder E** 1995 Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization: studies on the mechanism of antiandrogen action. J Biol Chem 270:27569–27576
23. **Gerster T, Matthias P, Thali M, Jiricny J, Schaffner W** 1987 Cell type-specificity elements of the immunoglobulin heavy chain gene enhancer. EMBO J 6:1323–1330
24. **Kuiper GGJM, de Ruiter PE, Trapman J, Jenster G, Brinkmann, AO** 1993 *In vitro* translation of androgen receptor cRNA results in an activated androgen receptor protein. Biochem J 296:161–167
25. **Lobaccaro JM, Poujol N, Chiche L, Lumbroso S, Brown TR, Sultan C** 1996 Molecular modeling and *in vitro* investigations of the human androgen receptor DNA-binding domain: application for the study of two mutations. Mol Cell Endocrinol 116:137–147
26. **Tuffery P, Etchebest C, Hazout S, Lavery R** 1991 A new approach to the rapid determination of protein side chain conformations. J Biomol Struct Dyn 8:1267–1289
27. **Kraulis P** 1991 MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Crystallogr 24:946–950
28. **Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB** 1991 Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497–505
29. **Schwabe JWR, Chapman L, Finch JT, Rhodes D** 1993 The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell 75:567–578
30. **Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM** 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318:635–641
31. **Trapman J, Klaassen P, Kuiper GGJM, van der Korput JAGM, Faber PW, van Rooij HCJ, Geurts van Kessel A, Voorhorst MM, Mulder E, Brinkmann AO** 1988 Cloning, structure and expression of a cDNA encoding the human androgen receptor. Biochem Biophys Res Commun 153:241–248
32. **Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW** 1988 Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA 85:3294–3298
33. **Misrahi M, Atger M, d'Auriol L, Loosfelt H, Meriel C, Fridlansky F, Guiochon-Mantel A, Galibert F, Milgrom E** 1987 Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. Biochem Biophys Res Commun 143:740–748
34. **Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM** 1987 Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237:268–275
35. **Schena M, Freedman LP, Yamamoto KR** 1989 Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Genes Dev 3:1590–1601
36. **Warriar N, Yu C, Pagé N, Govindan MV** 1994 Substitution of Cys-560 by Phe, Trp, Tyr, and Ser in the first zinc finger of human androgen receptor affects hormonal sensitivity and transcriptional activation. J Biol Chem 269:29016–29023
37. **Yagi H, Ozono K, Miyake H, Nagashima K, Kuroume T, Pike JW** 1993 New point mutation in the deoxyribonucleic acid binding domain of the vitamin D receptor in a kindred with hereditary 1,25-dihydroxyvitamin D-resistant rickets. J Clin Endocrinol Metab 76:509–515
38. **Schüle R, Muller M, Kaltschmidt C, Renkawitz R** 1988 Many transcription factors interact synergistically with steroid receptors. Science 242:1418–1420
39. **Rundlett SE, Miesfeld RL** 1995 Quantitative differences in the androgen and glucocorticoid receptor DNA binding properties contribute to receptor-selective transcriptional regulation. Mol Cell Endocrinol 109:1–10