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DAX-1 Inhibits SF-1-Mediated Transactivation via a Carboxy-Terminal Domain That Is Deleted in Adrenal Hypoplasia Congenita

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X-linked adrenal hypoplasia congenita (AHC) with hypogonadotropic hypogonadism was recently shown to be caused by mutations in a gene referred to as *DAX-1*, which encodes a novel member of the orphan nuclear receptor family. DAX-1 is homologous to other nuclear receptors in its carboxy-terminal region, but it lacks the characteristic zinc finger DNA-binding domain. The tissue distribution of DAX-1 (adrenal cortex, gonads, hypothalamus, and pituitary) is the same as that of another orphan nuclear receptor, steroidogenic factor 1 (SF-1), that is required for development of the adrenal glands and gonads. We examined whether DAX-1 and SF-1 might interact in the regulation of SF-1-responsive target genes. Coexpression of DAX-1 and SF-1 inhibited SF-1-mediated transactivation. DAX-1 was shown to interact directly with SF-1 in in vitro protein binding studies; however, it did not interfere with SF-1 binding to DNA in gel mobility shift assays. Transactivation by GAL4–SF-1 constructs was inhibited by DAX-1, indicating that neither the SF-1 DNA-binding domain nor the SF-1 binding sites are required for inhibition by DAX-1. A series of DAX-1 deletion mutants localized the inhibitory domain to the carboxy-terminal region of the protein. Deletion of this domain also reduced basal transcriptional silencing by GAL4–DAX-1. This inhibitory domain has been deleted in all naturally occurring AHC deletion mutants described to date. In addition, two naturally occurring point mutations in DAX-1 exhibited impaired inhibition of SF-1. We conclude that DAX-1 can inhibit SF-1 transcriptional activity and suggest that the loss of this inhibitory property in DAX-1 may account in part for the phenotype of AHC.

Steroidogenic factor 1 (SF-1) and DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) are orphan nuclear receptors that play an important role in the development of the adrenal gland and the reproductive system (31, 33, 53). Disruption of the *SF-1* gene in mice results in the absence of adrenal gland and gonadal development (31, 43). Genetic males appear sex reversed because of the absence of male external genitalia and the preservation of Müllerian structures. In addition, these mice have hypogonadotropic hypogonadism, reflecting hormonal deficiencies at both the hypothalamic and pituitary gland levels (20, 21). These features are consistent with the expression of SF-1 in the adrenal cortex, testis, ovary, ventromedial nucleus of the hypothalamus, and gonadotrope cells in the pituitary (17, 20, 21, 31).

In addition to its role during development, SF-1 has been shown to function as a transcription factor for a variety of different steroidogenic enzyme genes in the adrenal gland and gonads (10, 18, 29, 32, 36, 37). It binds to, and may also modulate expression of, the Müllerian inhibiting substance promoter (47). SF-1 also regulates several genes that are expressed in pituitary gonadotrope cells, including genes coding for the gonadotropin-releasing hormone receptor (GnRH-R), the glycoprotein hormone α-subunit, and the luteinizing hormone β-subunit (1, 3, 16, 20, 21, 27).

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The functional role of DAX-1 has been elucidated by the identification of deletions and mutations in patients with the syndrome of X-linked adrenal hypoplasia congenita (AHC) (13, 14, 38, 52, 53). These patients lack the permanent zone of the adrenal cortex, and usually develop adrenal insufficiency in early childhood. Hypogonadotropic hypogonadism is also an invariant component of the syndrome, and reflects defects in the production of hypothalamic GnRH and the pituitary gonadotropins (14, 41). These manifestations correlate well with the tissue distribution of DAX-1, which is nearly identical to that of SF-1 (50).

In comparison to SF-1, there is less information concerning how DAX-1 might function to regulate potential target genes. Although the carboxy-terminal region of DAX-1 is homologous to the ligand-binding domain of nuclear receptors, it lacks a characteristic zinc finger DNA binding region (53). Instead, the amino terminus of DAX-1 consists of three and one-half repeats of a 65- to 67-amino-acid motif that has been proposed to serve as a DNA-binding domain.

The overlapping tissue distributions and functional roles of SF-1 and DAX-1 raise the possibility that they might interact in one of several manners. For example, the receptors could interact directly or share cellular partners to facilitate or inhibit the transcription of target genes. Alternatively, they could act in a developmental cascade in which one factor regulates the expression of the other gene. In this report, we examined the effects of DAX-1 on SF-1 binding to DNA and transactivation. We find that DAX-1 is a potent inhibitor of SF-1 action and propose that this effect involves the action of a carboxy-terminal inhibitory domain in DAX-1.

MATERIALS AND METHODS

Cloning and plasmid constructions. Human DAX-1 cDNA was prepared by reverse transcriptase PCR (RT-PCR). Total RNA was isolated from a human adrenal cortical tumor. RNA (1 μg) was reverse transcribed with avian myeloblastosis virus RT (Promega, Madison, Wis.), and full-length DAX-1 cDNA was amplified with *Pfu Taq* polymerase (Stratagene, La Jolla, Calif.) as described previously (23). The amplification primers were 5′-AGGAGAATTCCACTGG GCAGAACTGGGCTA-3′ and 5′-ATGGCTCGAGGCACTACTGCACTTGT GTGG-3′ (the *Eco*RI and *Xho*I sites are underlined). After verification of the DNA sequence by the dideoxy-chain termination method, the 1.5-kb cDNA was cloned into the *Eco*RI-*Xho*I sites of the pBKCMV expression vector (Stratagene).

The mouse SF-1 cDNA was isolated from a lambda ZAP II cDNA library that was constructed with poly(A)$^+$ RNA derived from αT3 cells (51) according to the instructions of the manufacturer (Stratagene). The library was screened with a 690-bp SF-1 cDNA that was generated from the poly(A)$^+$ RNA of αT3 cells by RT-PCR. The primers used were 5′-GGCTTCTTCAAGCGCACAGTCCAGA ACAAC-3′ and 5′-GGCTGAAGGGCGCTGGCTGGTCAGAGCGGCT-3′ (29). After screening, a pBKCMV vector containing mouse full-length SF-1 cDNA was excised in vivo from the phage by the ExAssist/XLOLR system (Stratagene).

Expression vectors for DAX-1 carboxy-terminal deletion mutants (deleted at amino acids 92, 158, 228, 271, 288, 321, 347, 427, and 443) were generated by the removal of carboxy-terminal regions from the pBKCMV DAX-1 expression vector with the appropriate restriction sites (*Bsi*WI, *Pml*I, *Bsp*EI, *Sca*I, *Fsp*I, *Bst*YI, *Nae*I, *Tfi*I, and *Eco*57I, respectively). Expression vectors for naturally occurring DAX-1 mutations (R267P and ΔV269) were made by replacing the 150-bp *Bsp*EI-*Sca*I DNA fragment with a fragment that contained a site-directed mutant prepared by PCR. The pBKCMV expression vectors for the SF-1, DAX-1, and DAX-1 mutants were used for in vitro transcription and translation. However, for expression in eukaryotic cells, the *lac* promoter and *lac*Z ATG were removed from these vectors by restriction digestion to enhance expression. Expression vectors containing TRα and TRβ cDNAs driven by the Rous sarcoma virus promoter have been described previously (7). TRα, TRβ, and RXRα cDNAs were cloned into the pGEM-7Zf(−) vector (Promega) for in vitro transcription and translation.

An expression vector for the GAL4-SF-1 fusion proteins was constructed by introducing a *Bsr*DI site immediately downstream of the GAL4 DNA-binding domain (amino acids 1 to 147) by PCR with the primers 5′-ATGAAGCTACT GTCTTCTAT-3′ and 5′-GCCGGCAATGGGGATACAGTCAACTGTCTTT GACC-3′ (the *Bsr*DI site is underlined). The expression vector for the GAL4 DNA-binding domain, pSG424 (44), was used as a template. The PCR product was digested with *Xho*I and *Bsr*DI after the sequence had been verified (the *Xho*I site is present within the GAL4 DNA-binding domain). The *Bsr*DI site is compatible with the *Bcg*I site in SF-1. The *Xho*I-*Bsr*DI fragment was subcloned into the *Xho*I-*Xba*I sites of pSG424 along with the *Bcg*I-*Xba*I fragment of the SF-1 cDNA (residues 133 to 463). The GAL4–DAX-1 construct was prepared by inserting, in frame, full-length human DAX-1 cDNA (amino acids 1 to 470) downstream of the GAL4 DNA-binding domain. The pSG424 vector was digested with *Eco*RI, treated with T4 DNA polymerase, and digested with *Xba*I. DAX-1 cDNA was digested with *Nco*I, treated with T4 DNA polymerase, and digested with *Xba*I and ligated into the pSG424 vector. The indicated carboxy-terminal DAX-1 mutants were linked to the GAL4 DNA-binding domain in an analogous manner. An expression vector for the DAX-1 amino-terminal deletion mutant (DAX-1 del1–226) was constructed as follows. The pBKCMV SF-1 vector was digested with *Sun*I, blunt ended with T4 DNA polymerase, and subsequently digested with *Xho*I, yielding a vector containing the 5′-untranslated region and coding sequence for the first five residues, MDYSY. DAX-1 cDNA was digested with *Bsp*EI followed by fill-in reaction and digestion with *Xho*I. Ligation of the *Bsp*EI-*Xho*I fragment into the vector cut with *Sun*I and *Xho*I resulted in a DAX-1 expression vector containing a Kozak consensus sequence and translational start site but lacking the amino-terminal region of DAX-1. The GAL4 TRβ construct was created by introducing an artificial *Eco*RI site into the human TRβ cDNA to allow insertion of an *Eco*RI fragment encompassing the ligand-binding domain of TRβ (residues 174 to 461) in frame with the GAL4 DNA-binding domain in pSG424.

A reporter gene for SF-1 was prepared by insertion of one or two copies of the SF-1 binding site from the glycoprotein hormone α-subunit gene (ACA AGGT CA) (3, 20) upstream of the −81 thymidine kinase (TK) promoter linked to the luciferase gene (1xSF-1 TK81luc or 2xSF-1 TK81luc). Reporter constructs containing one or two copies of the palindromic TREs (1xTRE TK109luc or 2xTRE TK109luc) have been described previously (39). The reporter gene, UAS-TK109luc, contains two copies of the GAL4 binding site (UAS) (44) upstream of the −109 TK promoter.

For the expression of $(His)_6$-containing SF-1 fusion proteins in Sf9 insect cells, full-length SF-1 cDNA was subcloned into the *Eco*RI-*Kpn*I site of the pAcHLT baculovirus transfer vector (Pharmingen, San Diego, Calif.).

Cell culture, transfections, and luciferase assays. Human placental JEG-3 cells (American Type Culture Collection, HTB 36), murine gonadotrope αT3 cells (51), and human embryonic kidney tsa201 cells (34) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C. In experiments examining thyroid hormone regulation, cells were cultured in DMEM containing 10% charcoal-stripped fetal bovine serum (7).

Cells were transfected by the calcium phosphate method as described previously (12). Luciferase assays were performed 48 h after transfection (11) and are reported as means ± standard errors in relative light units (RLU). In the experiments in which TRα, TRβ, and GAL4-TR were studied, cells were cultured in the absence or presence of triiodothyronine (T_3) for 24 h before the luciferase assays.

Nuclear extracts, Western blots, and EMSAs. tsa201 cells were transfected with DAX-1 or SF-1 expression vectors, and nuclear extracts were harvested 48 h after transfection (46). Protein concentrations were determined with the Bradford protein assay (Bio-Rad, Hercules, N.Y.).

For Western blots, nuclear proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) and electrotransferred to nitrocellulose membranes. Membranes were blocked in 3% skim milk and analyzed with anti-DAX-1 (Santa Cruz Biotechnology, Santa Cruz, Calif.) or anti-SF-1 (Upstate Biotechnology, Lake Placid, N.Y.) polyclonal antibody at 1 μg/ml. Horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) (Promega) was used in conjunction with an enhanced chemiluminescence detection system (Amersham, Arlington Heights, Ill.) to visualize the bands.

For electrophoretic mobility shift assays (EMSAs), oligonucleotides (5′-ACG ACA AGGTCA-3′ and 5′-GGC TGACCT TGT-3′) corresponding to the SF-1 binding site in the glycoprotein hormone α-gene (ACA AGGTCA) were used. The annealed oligonucleotides were labeled with [^{32}P]dCTP with Klenow polymerase. Nuclear extracts (10 μg) or in vitro-translated proteins (2 μl of lysate) were incubated with 20 fmol of labeled oligonucleotides for 30 min on ice in 20 μl of the binding buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9), 10% glycerol, 50 mM KCl, 1 mM dithiothreitol, and 1 μg of poly(dI-dC). In vitro transcription and translation were performed with the TNT reticulocyte lysate system (Promega) as recommended by the manufacturer. T3 RNA polymerase was used for the transcription of SF-1 and DAX-1. The DNA and protein complexes were resolved with a 4% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer.

In vitro protein interaction assays. Baculovirus was used to express the $(His)_6$-containing SF-1 fusion protein in insect cells. Recombinant baculovirus was generated by cotransfection of the pAcHLT SF-1 vector with Baculogold viral DNA in Sf9 cells (Pharmingen). Sf9 cells were infected with baculovirus, and extracts were prepared 72 h after transfection. SF-1 fusion protein was purified by affinity chromatography with Ni-nitrilotriacetic acid (NTA) agarose (Qiagen, Hilden, Germany) followed by washing in the presence of 40 mM imidazole. In vitro translation was performed with the TNT reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine. T3 RNA polymerase was used for the transcription of DAX-1 and DAX-1 mutants. TRα, TRβ, and RXRα proteins were transcribed with either SP6 or T7 RNA polymerase.

For protein interaction assays, [^{35}S]methionine-labeled, in vitro-translated proteins were incubated with $(His)_6$–SF-1 fusion protein bound to Ni-NTA agarose in binding buffer A (20 mM HEPES [pH 7.9], 10% glycerol, 50 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) containing 40 mM imidazole for 2 h at 4°C. After extensive washing with buffer A containing 100 mM imidazole, bound proteins were eluted from the agarose beads by boiling in SDS sample buffer and were separated by SDS-PAGE (10% polyacrylamide) followed by autoradiography. The results were analyzed with a PhosphorImager to quantitate the amount of radioactivity in individual bands (Fuji Medical Systems, Stamford, Conn.).

RESULTS

DAX-1 inhibits SF-1-induced transcriptional activation. The transcriptional activity of SF-1 was examined with reporter genes containing one or two copies of a consensus SF-1 binding site (ACA AGGTCA) inserted upstream of TK81luc. Transient expression assays were performed with JEG-3 choriocarcinoma cells, which lack endogenous SF-1 and DAX-1 as assessed by RT-PCR and Western blot analyses (data not shown). The reporter gene containing a single copy of the SF-1 site was stimulated fourfold by expression of SF-1. SF-1 induced 12-fold activation of the reporter gene containing two copies of the SF-1 site (Fig. 1A). DAX-1 (threefold excess cDNA) was cotransfected with SF-1 to assess whether it might enhance or inhibit SF-1-mediated transactivation. With the construct containing two copies of the SF-1 site, DAX-1 repressed SF-1-mediated transactivation by 75%. The inhibitory effect of DAX-1 was dose dependent. Coexpression of increasing amounts of DAX-1 (0 to 20 ng) in the presence of a constant amount of SF-1 expression plasmid (100 ng) caused

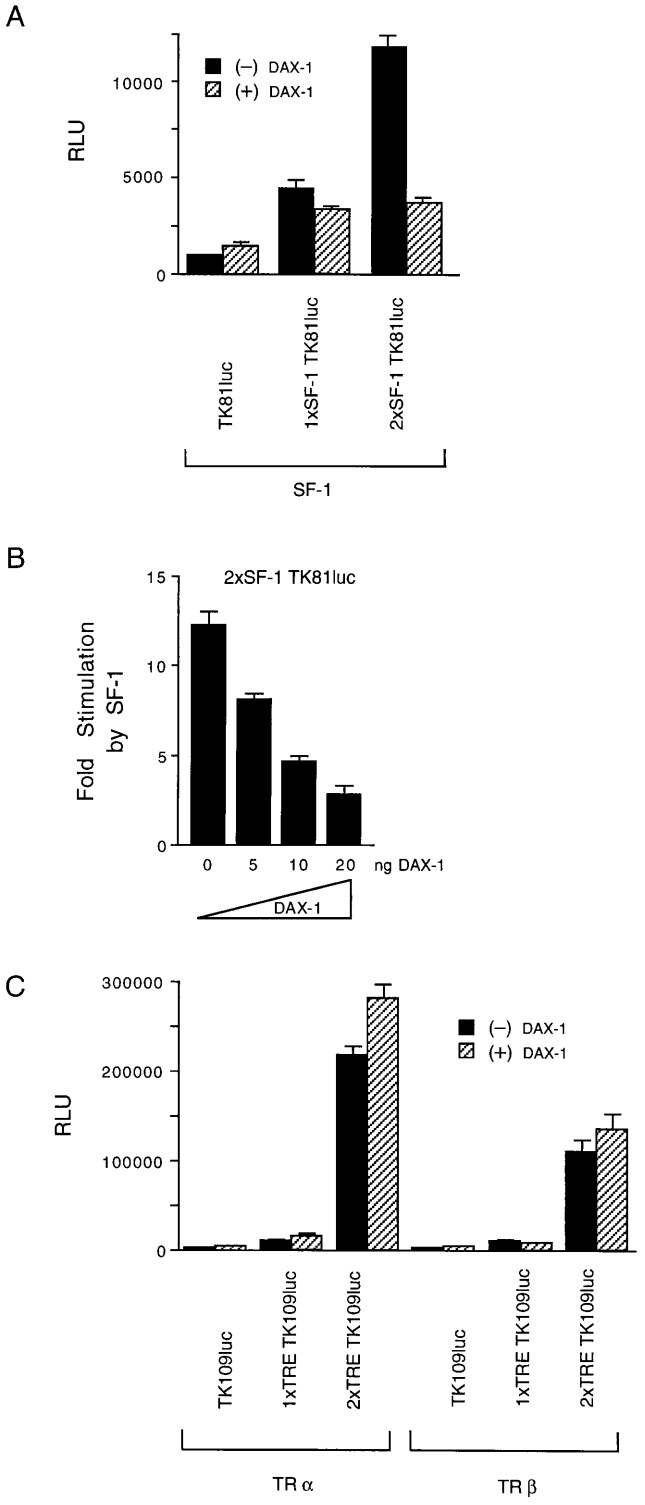

FIG. 1. Effect of DAX-1 on transactivation by SF-1 and TR. (A) SF-1 response element-containing reporter genes (1 μg) (1xSF-1 TK81luc and 2xSF-1 TK81luc) were transfected into JEG-3 cells with an SF-1 expression vector (100 ng) along with 300 ng of the empty vector or the DAX-1 expression vector. (B) Dose response of DAX-1 inhibition of SF-1-mediated transcription. The 2xSF-1 TK81luc reporter gene (1 μg) was transfected with 100 ng of SF-1 expression vector and increasing amounts (0 to 20 ng) of DAX-1 expression vector. The empty DAX-1 expression plasmid was added to each reaction mixture to maintain a constant total amount of expression plasmid. The data are expressed as fold stimulation by SF-1 relative to the basal activity of TK81luc. (C) Effect of DAX-1 on T_3-mediated transcriptional activation by TR. Reporter plasmids (1 μg) (TK109luc, 1xTRE TK109luc, and 2xTRE TK109luc) and TRα or TRβ expression vectors (20 ng) were transfected into JEG-3 cells with a threefold excess (60 ng) of the empty vector or the DAX-1 expression vector. Twenty-four hours after transfection, the cells were treated with 1 nM T_3 and then were harvested 48 h later for luciferase assays. Transcriptional activity is the mean ± standard error of triplicate transfections.

progressive suppression of SF-1-mediated transactivation (Fig. 1B).

As a control for the specificity of DAX-1 effects, the thyroid hormone receptor (TR) was used to assess whether DAX-1 inhibition occurred for other nuclear receptors. As shown in Fig. 1C, the activity of reporter genes containing one or two copies of a thyroid hormone response element (TRE) was increased by expression of TRα or TRβ expression vectors in the presence of T_3. However, coexpression of a threefold excess of DAX-1 had no effect on T_3-induced transactivation. DAX-1 also failed to significantly inhibit transactivation by the estrogen receptor with an estrogen-responsive reporter gene (data not shown). These results suggest that DAX-1 inhibition is relatively specific for SF-1-mediated transactivation.

DAX-1 does not alter SF-1 binding to DNA. EMSAs were used to analyze the effect of DAX-1 on SF-1 binding to its response element. With in vitro-translated receptors, DAX-1 did not bind to the SF-1 site (lane 2), and it did not alter the binding of SF-1 to DNA (lane 3) (Fig. 2A). Because of the possibility that in vitro-translated DAX-1 is not functional or requires other modifications to alter SF-1 binding, the properties of coexpressed DAX-1 and SF-1 were also examined with extracts from transfected tsa201 cells, which express high levels of transfected receptors (Fig. 2B). Nuclear extracts from cells transfected with SF-1 cDNA contained a prominent SF-1–DNA complex (lane 2) that was not detected in extracts transfected with an empty vector (lane 1). The SF-1 complex was eliminated by preincubation with an anti-SF-1 antibody (data not shown). Expression of DAX-1 alone did not result in the formation of new bands (lane 3) in comparison with the control extract (lane 1), confirming that DAX-1 does not bind to the SF-1 site. Mixing of extracts from cells transfected with either SF-1 or DAX-1 did not alter the amount or the mobility of the SF-1 complex (lane 4). In addition, cotransfection of DAX-1 and SF-1 did not result in a heterodimer complex, and there was no evidence that DAX-1 inhibited the binding of SF-1 (lane 5). Expression of DAX-1 and SF-1 in transfected cells was confirmed by Western blot analyses with anti-DAX-1 and anti-SF-1 antibodies (data not shown). These results suggest that DAX-1 does not inhibit SF-1 transactivation by interfering with binding to DNA.

DAX-1 interacts directly with SF-1. Although no SF-1–DAX-1 heterodimer complexes were observed in the EMSAs, direct protein-protein binding assays were used as an alternative approach to detect interactions between these receptors. The protein interaction assay was performed with baculovirus-expressed $(His)_6$–SF-1 fusion proteins linked to Ni-NTA agarose (Fig. 3A). DAX-1 and several other nuclear receptors (TRα, TRβ, and RXRα) were radiolabeled with [^{35}S]methionine during in vitro translation. DAX-1 bound to the $(His)_6$–SF-1 fusion protein linked to Ni-NTA agarose, but no binding was detected with the Ni-NTA agarose beads alone. In comparison with the input radiolabeled protein, approximately 7% of the DAX-1 was bound, which is similar to the interactions of RXRα and TRβ in analogous assays (data not shown). In contrast, there was no significant binding to $(His)_6$–SF-1 with radiolabeled TRα, TRβ, or RXRα, indicating that SF-1 interacts selectively with DAX-1.

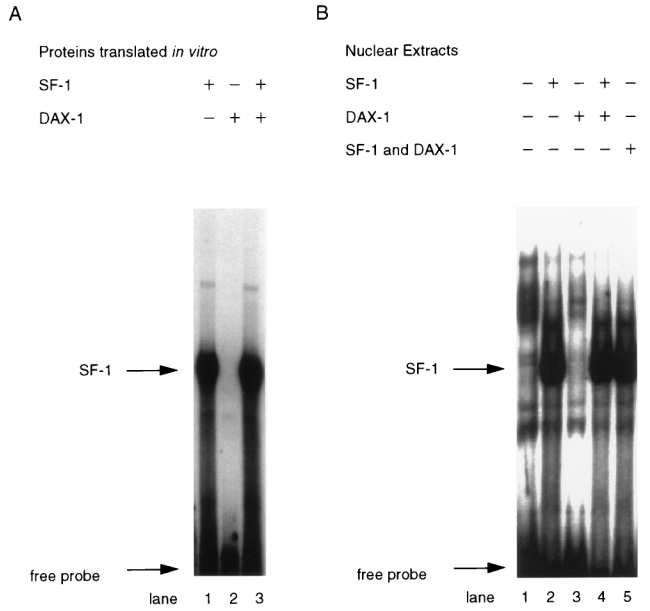

FIG. 2. Effect of DAX-1 on SF-1 binding to its DNA response element. A ^{32}P-labeled SF-1 binding site (20 fmol) was incubated with 2 μl of reticulocyte lysates expressing the indicated in vitro-translated proteins (A) or 10 μg of nuclear extracts derived from tsa201 cells that were transfected with the indicated expression vectors (B). The DNA-protein complexes were resolved on 4% native polyacrylamide gels. The location of the SF-1 complex is indicated by an arrow.

A series of DAX-1 mutants were analyzed in an effort to delineate protein domains involved in interactions with SF-1 (Fig. 3B). Carboxy-terminal deletions between amino acids 470 and 288 failed to significantly reduce DAX-1 binding to SF-1. Similarly, two naturally occurring amino acid substitutions (R267P and ΔV269) did not alter DAX-1 interactions with SF-1. After deletion of all but the first 91 amino acids of DAX-1 or removal of the amino terminus (deletion of amino acids 1 to 226), binding to SF-1 decreased to <2% of input radiolabeled protein. These results suggest that the amino-terminal region of DAX-1 contains an interaction domain for SF-1 and indicate that the naturally occurring carboxy-terminal truncations of DAX-1 do not substantially alter direct interactions with SF-1.

DAX-1 inhibition of SF-1-mediated transactivation occurs independent of DNA binding. The absence of DAX-1 interference with SF-1 binding to DNA prompted functional studies to examine whether DAX-1 inhibition could occur in the absence of the SF-1 DNA-binding domain. A GAL4–SF-1 fusion was created to address this possibility (Fig. 4A). GAL4–SF-1 stimulated the UAS-TK109luc reporter activity 10-fold in comparison to a construct containing the GAL4 DNA-binding domain alone (Fig. 4B). As seen with native SF-1, increasing doses of the DAX-1 expression vector (0 to 20 ng) caused dose-dependent inhibition of GAL4–SF-1 (100 ng) activity. Inhibition was seen with as little as 2 ng of the DAX-1 expression vector, and GAL4–SF-1-induced transcriptional activation was nearly eliminated by cotransfection with 20 ng of DAX-1 cDNA.

Analogous experiments were carried out with GAL4-TRβ to determine whether inhibition was selective for GAL4–SF-1 (Fig. 4C). In the presence of T_3, UAS-TK109luc reporter activity was induced 18-fold by GAL4-TR. However, there was no inhibition by cotransfection with 20 ng of DAX-1 cDNA.

DAX-1 contains a transcriptional silencing domain. Because specific DAX-1 response elements have not been identified, the DAX-1 protein was fused to the GAL4 DNA-binding domain to assess whether it might confer transcriptional silencing or activation upon binding to the UAS-TK109luc reporter gene. Relative to the GAL4 DNA-binding domain alone, full-length GAL4–DAX-1 caused (64%) inhibition of reporter gene activity (Fig. 5). Deletion of the carboxy-terminal 28 amino acids or insertion of either of two naturally occurring amino acid substitutions (R267P or ΔV269) reversed this repression (10 to 21% inhibition). These results suggest that in the context of these chimeric constructs, DAX-1 does not confer transactivation and appears to exert a silencing function.

DAX-1 inhibition localizes to its carboxy terminus and correlates with naturally occurring AHC mutations. A series of DAX-1 deletion mutants were used to identify the domains involved in the inhibition of SF-1-induced transactivation (Fig. 6). As shown above, 20 ng of full-length DAX-1 caused marked inhibition of GAL4–SF-1-mediated transactivation, and there was no effect with the empty DAX-1 expression vector. Sequential deletion of the carboxy-terminal region of DAX-1 revealed a repressor domain near the carboxy-terminal end of the protein. Removal of 28 residues from the end of DAX-1 (DAX-1 del443–470) decreased inhibition by 50%. Further deletions (amino acids 321 to 470) caused complete loss of inhibition.

As shown at the bottom of the figure, all naturally occurring DAX-1 deletions reported to date eliminate this putative carboxy-terminal inhibitory domain. Two more subtle mutations (R267P and ΔV269) (38) reduced DAX-1 inhibition similar to

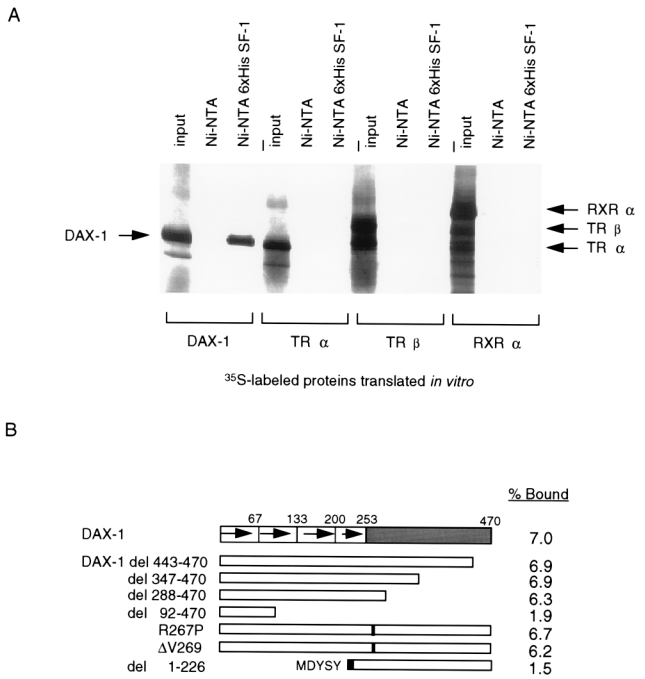

FIG. 3. Protein-protein interaction assays. (A) [^{35}S]methionine-labeled in vitro-translated proteins (4 μl) for DAX-1, TRα, TRβ, and RXRα were incubated with 2 μg of $(His)_6$–SF-1 fusion protein bound to Ni-NTA agarose or with Ni-NTA agarose in the absence of $(His)_6$–SF-1. After extensive washing, bound proteins were analyzed by SDS-PAGE (10% polyacrylamide). For each receptor, 10% of the input radiolabeled protein is shown on the left. The location of DAX-1 is shown on the left, and the locations of TRα, TRβ, and RXRα are shown on the right. (B) Specific binding of DAX-1 mutants to $(His)_6$–SF-1 fusion protein. Binding reactions were performed as described for panel A, and specific binding (bound/input ratio) was determined after quantitation with a PhosphorImager.

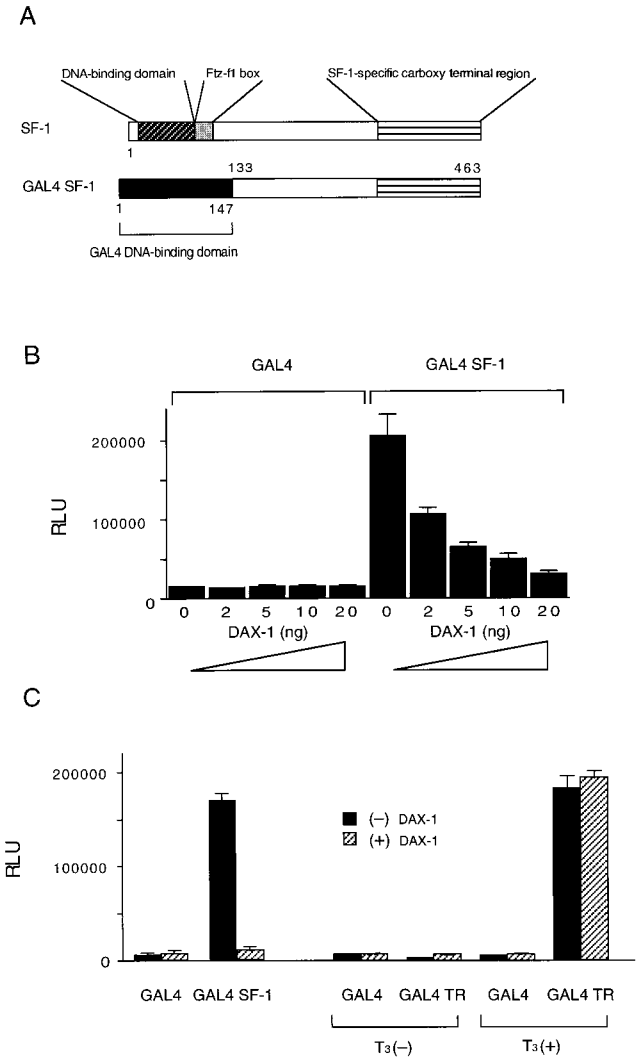

FIG. 4. Effect of DAX-1 on transactivation by a GAL4–SF-1 fusion protein. (A) Schematic representation of the GAL4–SF-1 fusion protein. The DNA-binding domain and Ftz-f1 box of SF-1 were replaced with the GAL4 DNA-binding domain. The resulting GAL4 fusion protein lacks the first 132 residues of SF-1 and contains the carboxy-terminal region that corresponds to the SF-1 splicing variant of the Ftz-F1 gene. (B) Effect of DAX-1 on transactivation by GAL4–SF-1. UAS-TK109luc (1 μg) was transfected into JEG-3 cells with GAL4 or GAL4–SF-1 expression vectors (100 ng) along with increasing amounts of the DAX-1 vector (0, 2, 5, 10, and 20 ng). The total amount of the transfected pBKCMV vector was kept constant in each reaction. JEG-3 cells were harvested 48 h after transfection for luciferase assays. (C) Effect of DAX-1 on GAL4-TR-mediated transcription. UAS-TK109luc (1 μg) and GAL4, GAL4–SF-1, or GAL4-TRβ expression vector (100 ng) were transfected into JEG-3 cells with empty vector or DAX-1 expression vector (20 ng). In experiments in which GAL4 and GAL4-TRβ were cotransfected, cells were cultured in DMEM supplemented with 10% charcoal-stripped serum and treated in the absence or presence of 1 nM T_3 24 h after transfection. Luciferase activity was determined 48 h after transfection. Transcriptional activity is the mean ± standard error of triplicate transfections.

that seen with the carboxy-terminal truncations. Thus, there is a strong correlation between naturally occurring mutations that cause AHC and the ability of DAX-1 to inhibit SF-1.

DISCUSSION

DAX-1 and SF-1 have striking similarities in their tissue and developmental patterns of expression (17, 20, 21, 31, 50). Both proteins are expressed in the developing adrenal gland, gonads, hypothalamus, and pituitary gland. The concordance of expression raised the possibility that these orphan nuclear receptors might converge on common developmental pathways. Because many members of the nuclear receptor superfamily interact as dimers (33), we initially hypothesized that DAX-1 and SF-1 might function as heterodimeric partners. This idea was supported by the observation that the carboxy-terminal region of DAX-1 is homologous to RXR and COUP (53), which are known to heterodimerize with a wide array of other nuclear receptors. In addition to RXR and COUP, this region of DAX-1 is structurally similar to SF-1.

Although the concept of heterodimers is attractive for SF-1 and DAX-1, we were unable to provide evidence for such complexes in traditional gel mobility shift assays (Fig. 2). It should be recognized, however, that these assays are based solely upon interactions with an SF-1 binding site. In experiments not shown, we also found no evidence for DAX-1 heterodimerization when the SF-1 binding sites were duplicated. Ideally, these experiments would utilize DNA containing a combined SF-1 and DAX-1 DNA recognition site. However, the DNA sequences required for high-affinity DAX-1 binding have not been clearly identified. In the initial characterization of DAX-1 (53), there was evidence of binding to a retinoic acid response element, but these experiments used relatively large amounts (5 μg) of recombinant protein, and we have not seen significant DAX-1 binding to this or related elements under the conditions of our binding assays (data not shown). In view of our finding that SF-1 and DAX-1 interact in direct protein binding assays (Fig. 3), it remains possible that the proteins can dimerize when bound to appropriate DNA sequences. The fact that DAX-1 did not appear to "supershift" the SF-1–DNA complex suggests that in the absence of a DAX-1 recognition site, the protein interactions are relatively weak or that the dissociation rate is high enough that multimeric complexes are not observed under the conditions of the gel mobility shift assays.

In parallel with the DNA binding studies, we assessed the functional effects of DAX-1 on SF-1-mediated transactivation. Because deletion of either the *SF-1* or *DAX-1* gene results in similar developmental abnormalities, we anticipated that DAX-1 might enhance SF-1 effects on target genes. However, we observed that DAX-1 caused inhibition, at least for the group of SF-1-responsive reporter genes that were studied (Fig. 1). It should be noted that these are artificial reporter genes that are designed to provide robust responses to SF-1. The effects of DAX-1 on SF-1-responsive promoters, such as the steroidogenic enzyme genes (10, 17, 29, 32, 36, 37) or gonadotropin promoters (3, 21, 27), remain to be investigated. The inhibitory effect of DAX-1 appeared to be relatively specific for SF-1, in that no repression was seen with the empty

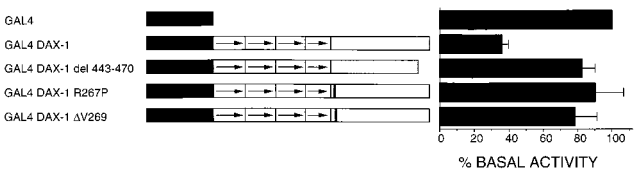

FIG. 5. GAL4–DAX-1 contains a transcriptional silencing domain. UAS-TK109luc (1 μg) was transfected into JEG-3 cells with 100 ng of full-length GAL4–DAX-1 or with expression vectors for the indicated DAX-1 mutants. A carboxy-terminal truncation mutant is designated by "del" and the missing amino acids. Single-amino-acid substitutions are shown by a line. Transcriptional activity is the mean ± standard error of triplicate transfections and is shown relative to the effect of the GAL4 DNA-binding domain alone.

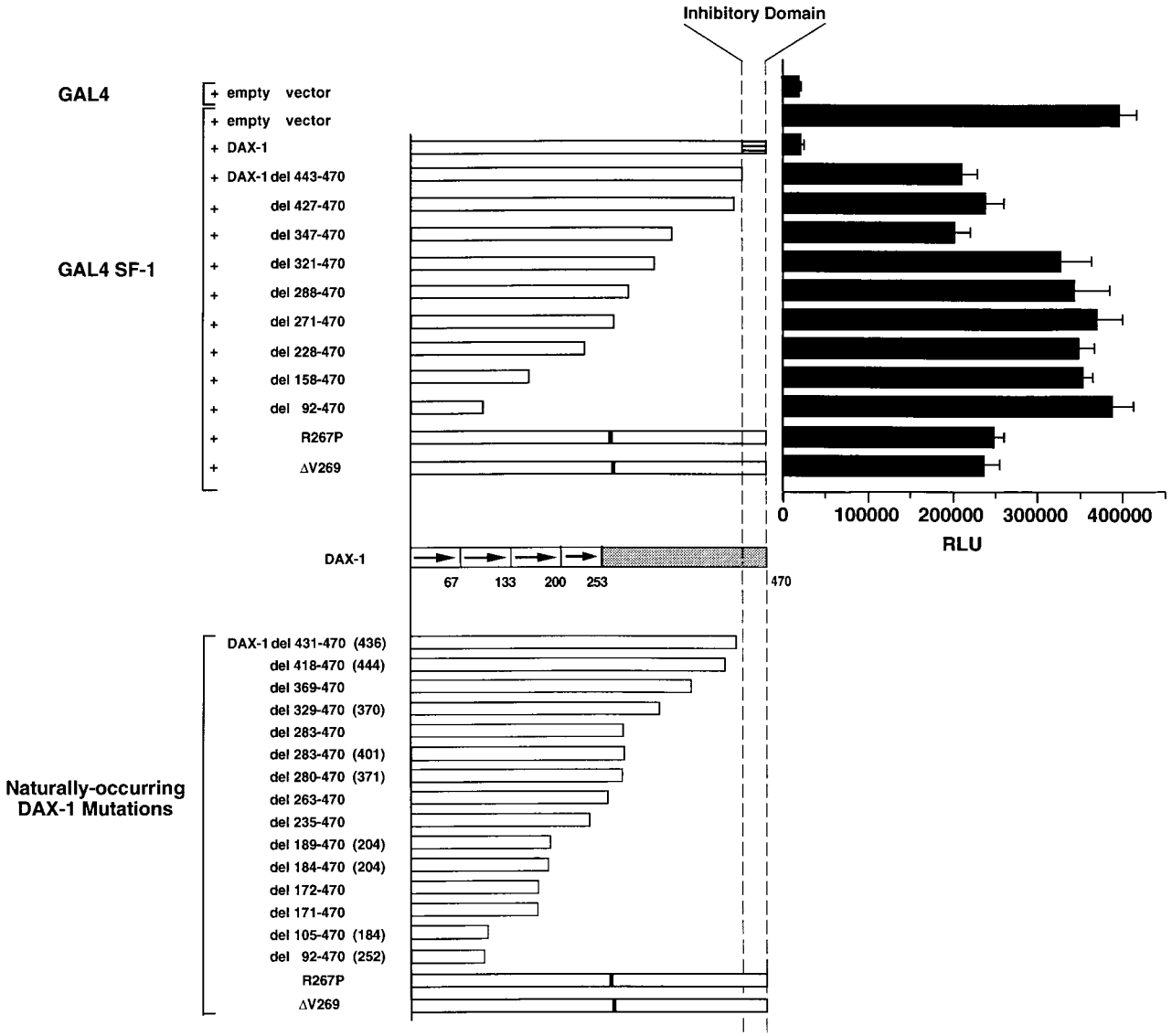

FIG. 6. Effect of DAX-1 mutants on GAL4–SF-1-induced transactivation. UAS-TK109luc (1 μg) was transfected into JEG-3 cells with GAL4–SF-1 (100 ng) and full-length DAX-1 vector (20 ng) or with expression vectors for the indicated mutants (20 ng). Carboxy-terminal truncation mutants are designated by "del" and the missing amino acids. Single-amino-acid substitutions are shown by a line. The activities of the GAL4 DNA-binding domain alone (GAL4) and GAL4–SF-1 are shown at the top of the figure. Naturally occurring DAX-1 mutants are shown in the bottom panel. The amino acid position at which translational termination occurs after a frameshift is shown in parentheses. A putative carboxy-terminal domain involved in DAX-1 inhibition is indicated by the dashed lines. Transcriptional activity is the mean ± standard error of triplicate transfections.

DAX-1 expression vector, and there was no inhibition of TR- or estrogen receptor-mediated transactivation by DAX-1.

At least three different mechanisms might be considered for DAX-1 inhibition: (i) interference with SF-1 binding to its target site; (ii) direct interactions between DAX-1 and SF-1 that interfere with an SF-1 transactivation surface; or (iii) DAX-1 interactions with a critical coactivator for SF-1 to block transactivation or DAX-1 recruitment of a repressor. Two lines of evidence argue against the first mechanism involving interference with SF-1 binding to DNA. First, there was no evidence of DAX-1 inhibition of SF-1 binding in gel shift assays performed with in vitro-translated proteins or with extracts from cells transfected with both proteins (Fig. 2). Second, DAX-1 inhibition was still present when the SF-1 DNA-binding domain was replaced with that of GAL4 and a different target sequence (UAS) was substituted for the SF-1 binding site (Fig. 4). These experiments indicate that the SF-1 binding site and the SF-1 DNA-binding domain are not required for the inhibitory effects of DAX-1.

The second and third mechanisms are not mutually exclusive, although our data currently favor a mechanism in which DAX-1 causes repression indirectly. Although the protein-protein binding assays indicate that DAX-1 and SF-1 can interact directly, the nature of the DAX-1–SF-1 interactions remains to be fully defined. A limited series of DAX-1 mutations reveal that the protein interactions are preserved even after deletion of the entire carboxy-terminal domain. On the other hand, removal of the amino-terminal repeat regions decreased binding to SF-1. These experiments suggest that one or more of the three and one-half repeats contain an important contact region. Further experiments will be required to delineate these domains further and to include mutations in SF-1 as well. Most

significant for the current study is the finding that direct DAX-1-SF-1 interactions do not seem to be crucial for DAX-1 inhibition of SF-1 transactivation. Several of the DAX-1 mutants that greatly reduce inhibition had little effect on binding to SF-1. While these results do not exclude another role for DAX-1–SF-1 interactions, they favor an indirect mechanism for DAX-1 inhibition of SF-1 transactivation.

In principle, DAX-1 could indirectly repress SF-1 action either by recruiting a repressor protein or by competing for a coactivator. We have found that SF-1 contains a strong transactivation domain in its carboxy terminus (22). DAX-1 contains a region which is homologous to the AF-2 domain of SF-1 and other nuclear receptors. The nature of the coactivators that interact with these domains has not been identified for SF-1 or DAX-1. However, the fact that DAX-1 inhibition is relatively specific for SF-1 and is not seen with several other nuclear receptors raises the possibility that they may share an activator that is distinct from factors such as SRC-1 (40), RIP-140 (6), ERAP 160 (15), and CBP (26) that activate multiple receptors. Experiments with GAL4–DAX-1 also raise the possibility that DAX-1 can act more directly to silence transcription, if it is targeted to DNA (Fig. 5). Perhaps like a subset of other nuclear receptors, DAX-1 may be able to interact with corepressors and coactivators, depending upon the cellular environment or conformational changes in the protein.

The inhibitory effects of DAX-1 are reminiscent of those described for a number of other transcription factors. In the nuclear receptor family, a recently described protein referred to as SHP (small heterodimer partner) has been shown to inhibit the activity of certain orphan nuclear receptors (45). Like DAX-1, SHP lacks the characteristic zinc finger DNA-binding domain that is seen in other nuclear receptors. SHP forms heterodimers with several different nuclear hormone receptors, and it inhibits the activity of RAR, TR, and MB67. In other instances, such as PPAR or RAR inhibition of TR function, there is evidence for competition for dimerization partners such as RXR and, perhaps, other coactivators (9, 24, 25, 30). Proteins such as TRUP appear to inhibit nuclear receptor action by interacting with the DNA-binding domain (5), whereas other corepressors interact with the ligand-binding domain and function to actively suppress transcription (8, 19). Apart from the nuclear receptor family, Id inhibition of MyoD and other basic helix-loop-helix proteins may share features of DAX-1 inhibition. In this case, Id lacks a DNA-binding domain, but it retains dimerization and forms an inactive complex with basic helix-loop-helix proteins (4). A variety of other inhibitors of transcription have been described, including IκB inhibition of NFκB (2), CHOP inhibition of C/EBP (42), c-Myc inhibition of YY-1 (48), and MafB inhibition of Ets-1 (49). Like DAX-1 and SF-1, the interactions of these transcription factors are thought to play an important role in developmental or regulatory cascades.

Given that DAX-1 inhibits SF-1 action in our transfection studies, it is somewhat puzzling that patients with AHC, who harbor loss-of-function mutations in DAX-1, share certain clinical features that resemble those caused by a knockout of SF-1. Like the SF-1 knockout mouse, these patients exhibit adrenal insufficiency and hypogonadotropic hypogonadism (14, 28, 53). Despite these similarities, there are also important differences. In AHC, adrenal development is more advanced and appears to involve selective absence of the adult cortex with relatively normal development of the fetal zone (35). The hypogonadotropic hypogonadism in AHC responds poorly to exogenous GnRH (14), whereas administration of GnRH restores gonadotropins in the SF-1 knockout mice (20). In addition, males with AHC are not sex reversed and have relatively normal development of the testis, with preservation of testosterone responses to exogenous luteinizing hormone (14). Some of these differences may reflect comparisons across species (mice versus humans), and this issue should ultimately be clarified by studies of DAX-1 knockout mice or the identification of SF-1 mutations in humans.

Although questions remain concerning how DAX-1 and SF-1 might interact in different tissues and in the context of various target genes, there is a striking correlation between the locations of DAX-1 mutations that cause AHC and the ability of DAX-1 to inhibit SF-1-mediated transactivation (Fig. 6). A relatively large number of AHC truncation mutants have been identified, and all of these delete the carboxy-terminal inhibitory domain that we have identified in DAX-1. In addition, a naturally occurring amino acid substitution (R267P) and a single-amino-acid deletion (ΔV269) were sufficient to impair DAX-1 inhibition of SF-1. It will be useful to examine other naturally occurring DAX-1 mutations in this SF-1 transactivation assay. Once transcriptional cofactors for DAX-1 are identified, it will also be of interest to determine whether these mutations affect DAX-1 interactions with transcriptional cofactors.

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