

A Naturally Occurring Steroidogenic Factor-1 Mutation Exhibits Differential Binding and Activation of Target Genes*

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Steroidogenic factor-1 (SF-1) is an orphan nuclear receptor that binds DNA as a monomer and regulates the transcription of multiple target genes. A mutation in the proximal (P)-box of the first zinc finger of SF-1 (G35E) has been reported to cause complete XY sex reversal and adrenal insufficiency. Because this P-box region dictates DNA binding specificity, we investigated the effect of this mutation on DNA binding and regulation of target genes. Binding of the P-box mutant was markedly impaired for most native SF-1 response elements. However, mutant SF-1 bound to a subset of response elements containing a CCA AGGTCA motif. Mutagenesis studies of response elements revealed that the first nucleotide position in the 5'-flanking sequence triplet and the central part of the half-site dictate DNA binding specificity by the mutant SF-1. Further, introduction of a mutation into the SF-1 A-box, which has been proposed to bind to the 5'-flanking sequence triplet, eliminated binding by mutant SF-1 to all response elements tested. These data support the idea that the A-box stabilizes monomeric binding by nuclear receptors. This action may be particularly important when P-box binding affinity is compromised either by mutations in SF-1 or by sequence alterations in its binding site.

Steroidogenic factor-1 (SF-1)¹ (*FTZ/F1*) (1, 2) is an orphan nuclear receptor that plays an essential role in the development of the adrenal gland, testis, ovary, pituitary gonadotropes, and hypothalamus (3). SF-1 regulates the transcription of a variety of target genes involved in steroidogenesis and reproduction including *DAX-1* (*AHC*) (4), steroidogenic acute regulatory protein (*StAR*) (5), cholesterol side chain cleavage enzyme (SCC, *Cyp11a*) (6), aromatase (*Cyp19*) (7, 8), Müllerian inhibiting substance (*MIS*, *AMH*) (9), and luteinizing hormone (LH) β subunit (10).

SF-1 belongs to the nuclear receptor superfamily (NR5A1) and shares several well conserved domains with other family

members. These regions include an amino-terminal, two zinc finger DNA-binding domain, a putative ligand-binding/dimerization domain, and a carboxyl-terminal AF2 transactivation domain (see Fig. 1A) (11, 12). SF-1 regulates gene transcription through its interactions with nuclear receptor coactivators such as steroid receptor coactivator-1 and CREB-binding protein (12, 13) as well as by direct interactions with other transcription factors such as *DAX-1*, *Egr-1*, *SOX9*, *WT-1*, and *CREB* (10, 14–17).

In general, members of the nuclear receptor superfamily bind to DNA as dimers. Each monomer within these dimer pairs interacts with a half-site sequence, AGNNCA (18–20). Based on half-site specificity, nuclear receptors can be divided into two subgroups. The estrogen receptor (ER) subgroup, which also includes the thyroid hormone receptor (TR) and retinoic acid receptor among others, recognizes the core sequence, AGGTCA. The glucocorticoid receptor (GR) subgroup, which includes the androgen receptor and progesterone receptor, has distinct half-site specificity for AGAACA. The two central nucleotides in the half-site sequence therefore largely determine DNA sequence recognition by the two groups of nuclear receptors. Structure-function analyses reveal that nuclear receptors distinguish these two nucleotides via three amino acid residues in the proximal (P)-box of the first zinc finger (21–23). These P-box residues are located within a DNA recognition α -helix that interacts with the major groove of the DNA response element (18).

In contrast to these dimeric receptors, a small group of orphan nuclear receptors binds to DNA as monomers. These factors recognize the estrogen response element (ERE)-type half-site, AGGTCA, and include SF-1, FTZ-F1, NGFI-B, RevErbA, and ROR α (24–29). Several of these receptors share a 30-amino acid basic region carboxyl-terminal to the second zinc finger designated the FTZ-F1 box or A-box (25–27). This A-box is proposed to recognize additional nucleotide sequences 5' to the AGGTCA motif in the minor groove of the response element DNA. For example, SF-1 binds preferentially to the 5'-flanking sequence PyCA AGGTCA (where Py is pyrimidine), whereas NGFI-B recognizes AA AGGTCA.

Recently, we reported a SF-1 mutation in a patient with XY sex reversal, primary adrenal insufficiency, dysgenetic testes, and persistent Müllerian structures (30). The heterozygous G35E mutation involves the last amino acid in the P-box, an area shown previously to be critical for DNA binding of other nuclear receptors (21, 22). As expected, this mutant SF-1 protein failed to bind to SF-1 response elements such as those found in the proximal promoter of SCC (*Cyp11a*), and no transactivation of this gene was seen (30). However, because SF-1 response elements are variable, we considered the possibility that this P-box mutant might recognize other SF-1 response elements.

In this report, we examined mutant SF-1 binding to response

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¹ The abbreviations used are: SF-1, steroidogenic factor-1; SCC, side chain cleavage enzyme; LH, luteinizing hormone; CREB, cAMP-response element-binding protein; ER, estrogen receptor; TR, thyroid hormone receptor; GR, glucocorticoid receptor; P, proximal; ERE, estrogen response element; EMSA, electrophoretic mobility shift assays; MIS, Müllerian inhibiting substance.

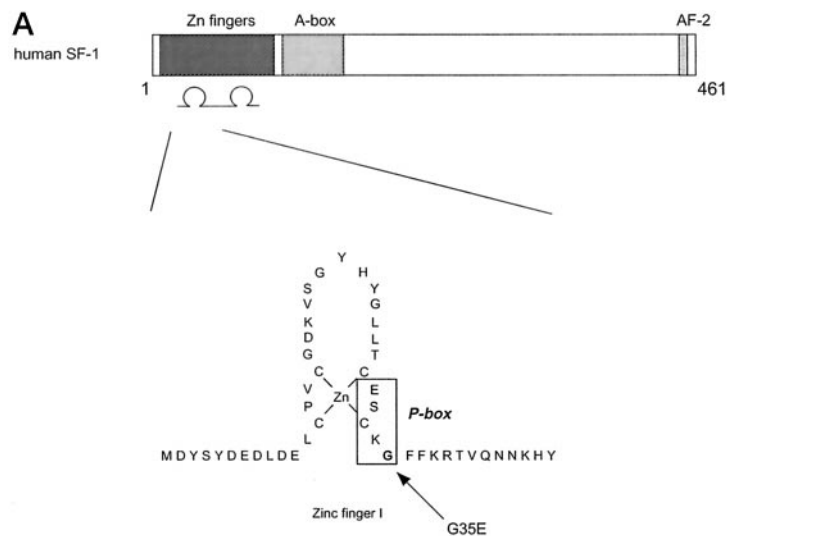


FIG. 1. DNA binding of the G35E P-box mutant to various SF-1 response elements. *A*, schematic representation of the human P-box mutation (30) is shown. Glycine, at the last position of the P-box, was changed to glutamic acid (G35E). *B*, EMSAs were performed using *in vitro* translated wild-type (WT) and mutant (MT) SF-1 (3 μ l) and 20 fmol of radiolabeled probes corresponding to SF-1 response elements from murine SCC, murine MIS, rat aromatase, rat LH β , and murine DAX-1 gene promoters. DNA-protein complexes were resolved on a 0.5 \times TBE polyacrylamide gel.

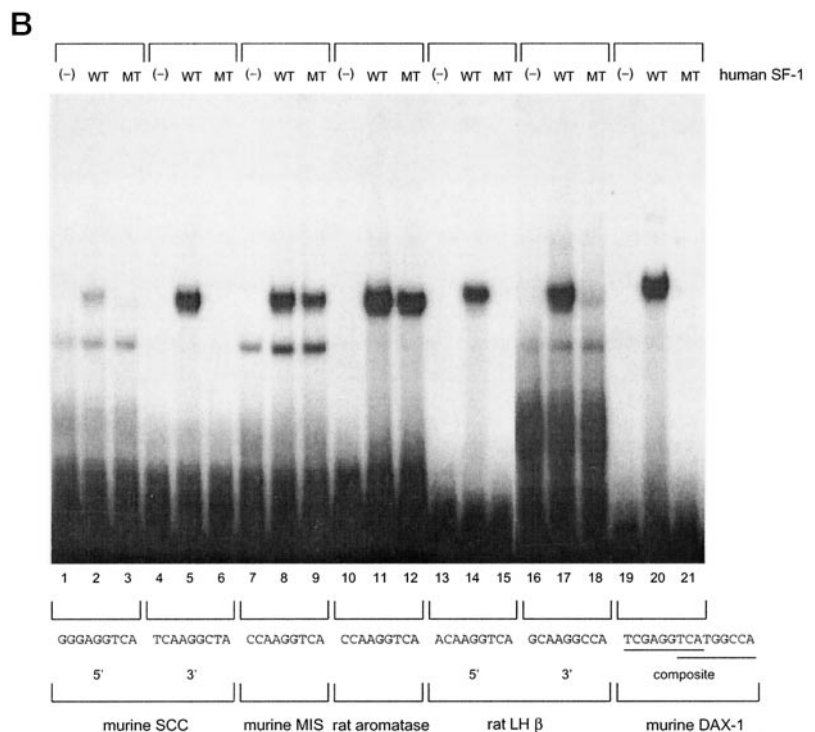


TABLE I
Functional analyses of the G35E SF-1 P-box mutant

Gene	Location	SF-1 response element	DNA binding	Transactivation
Murine SCC	5'	GGG AGGTCA	(-)	(-)
Murine SCC	3'	TCA AGGCTA	(-)	(-)
Murine MIS		CCA AGGTCA	(+)	(+) ^a
Rat aromatase		CCA AGGTCA	(+)	(+)
Rat LH β	5'	ACA AGGTCA	(-)	(-)
Rat LH β	3'	GCA AGGCCA	(-)	(-)
Murine DAX-1 composite		TCG AGGTCA TCA TGGCCA	(-)	(-)

^a Denotes artificial reporter system. The murine DAX-1 composite enhancer element contains two SF-1 binding sites that overlap each other.

elements from a variety of target genes. These studies revealed surprising heterogeneity in the SF-1 binding interactions with various target sequences, thereby allowing detailed analysis of the structural determinants in SF-1 and its recognition sites

that mediate receptor binding. We find that interactions between the A-box and the 5'-flanking sequence of the response element stabilize monomeric binding by SF-1, especially in situations where binding affinity is compromised by P-box mutations.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Mutant SF-1 cDNAs were synthesized by overlapping polymerase chain reaction from wild-type human SF-1 cDNA and cloned into the pCMX mammalian expression vector (31). The DNA sequence of the cloned cDNA was confirmed using a dRhodamine terminator cycle sequencing kit and an ABI377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The pA3 luciferase reporter construct for the SCC gene (-81 to +42) was described previously (30). The rat aromatase (-294 to +20) and LH β (-154 to +5) promoter regions were amplified by polymerase chain reaction and cloned into the pA3 vector. The pGL3 basic luciferase reporter construct (Promega, Madison, WI) for the murine DAX-1 gene (-134 to +26) was described previously (4).

Electrophoretic Mobility Shift Assays—*In vitro* translation of wild-type and mutant SF-1 was performed using the TnT reticulocyte lysate

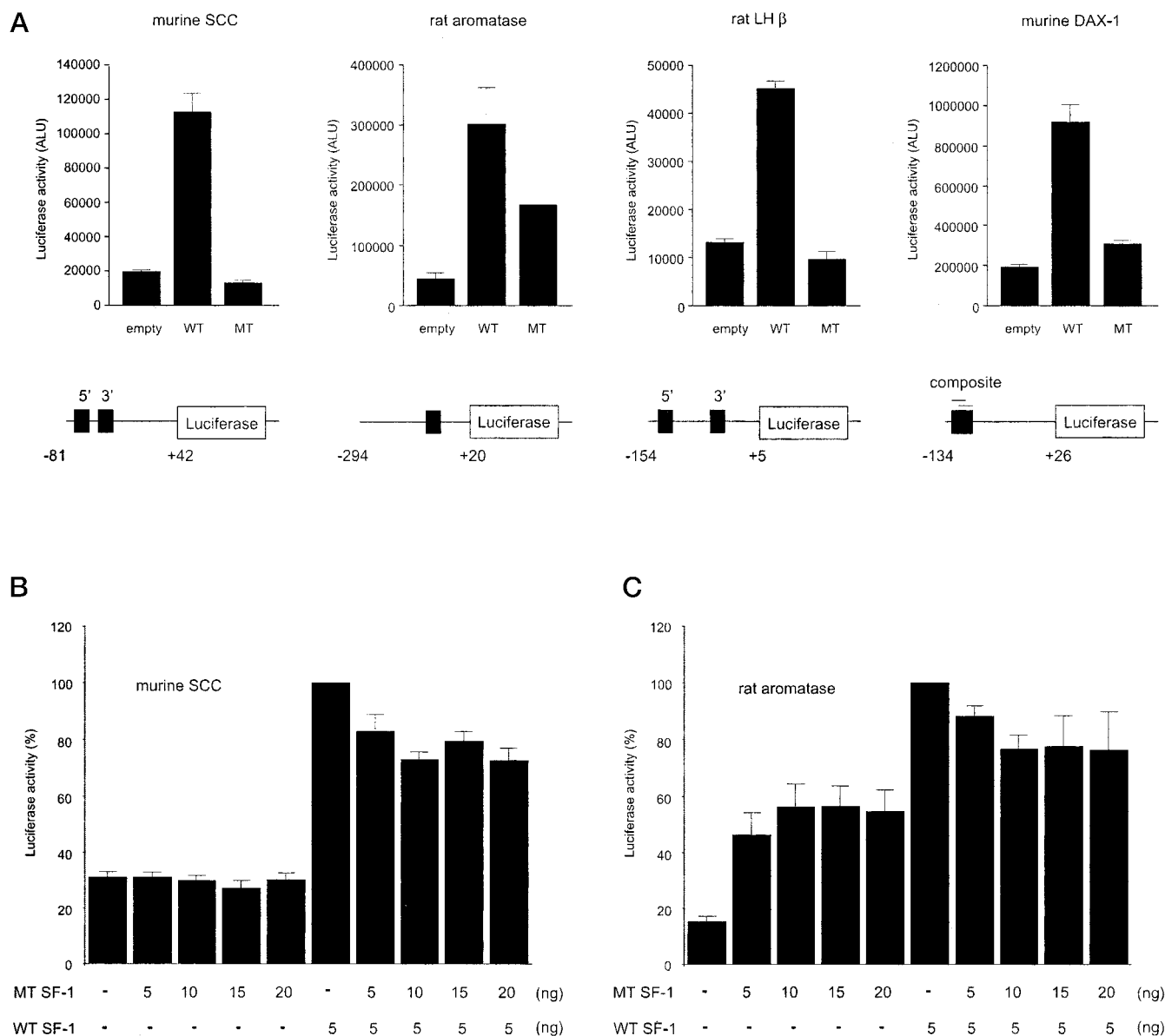


FIG. 2. Effect of the G35E mutant on SF-1 responsive promoter activity. *A*, reporter constructs containing the promoter regions of murine SCC (–81 to +42), rat aromatase (–294 to +20), rat LH β (–154 to +5), and murine DAX-1 (–134 to +26) genes (0.5 μ g) were transfected into tsa cells along with wild-type or G35E mutant expression vectors (5 ng). Results are the mean \pm S.E. of triplicate transfections. The location of the SF-1 response elements is shown by closed boxes. *B* and *C*, the SCC or aromatase reporter gene (0.5 μ g) was transfected into cells with increasing amounts of mutant SF-1 expression vector (0, 5, 10, 15, 20 ng) in the absence or presence of a constant amount of wild-type SF-1 (5 ng). The total amounts of transfected plasmids were adjusted with an empty vector. Results are expressed as a percentage of wild-type SF-1 activity. WT, wild-type; MT, mutant type; ALU, arbitrary light units.

system (Promega). Synthetic oligonucleotides corresponding to the SF-1 response elements of the murine SCC, murine MIS, rat aromatase, rat LH β , and murine DAX-1 promoters were created as follows (SF-1 response element is underlined): SCC 5', 5'-GGAGGAAGGGGGGAGGT-CACCGCTCATC-3'; SCC 3', 5'-GCTTCTCTTAGCCTTGAGCTGGTT-3'; MIS, 5'-GCCAGGCACTGTCCCAAGGTCACCTT-3'; aromatase, 5'-CCTGAGTCTCCAAGGTCATCCTTGTTT-3'; LH β 5', 5'-TCCTTTCTGACCTTGCTGTCT-3'; LH β 3', 5'-TGCTTAGTGGCCTTGC-CACCCCA-3'; DAX-1, 5'-AGCTTTCGAGGTCATGGCCACACAC-3'. These probes were labeled with [32 P]dCTP by Klenow polymerase, and electrophoretic mobility shift assays (EMSA) were performed as described previously (12). Briefly, 3 μ l of lysates were incubated with 20 fmol of 32 P-labeled oligonucleotides, and the DNA-protein complexes were resolved on 4% native polyacrylamide gels in 0.5 \times Tris-borate-EDTA (TBE) buffer.

Cell Culture, Transfection, and Luciferase Assays—SF-1-deficient human embryonic kidney tsa 201 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected by the calcium phosphate method. The composition and amounts of plasmid DNA used for transfections are described in the

figure legends. Forty-eight hours after transfection cell extracts were prepared, and luciferase assays were performed (32).

RESULTS

DNA Binding Specificity of the G35E SF-1 Mutant—DNA binding specificity of the naturally occurring G35E SF-1 mutant (Fig. 1A) was studied using probes corresponding to the SF-1 response elements of a variety of target genes. These probes were: the murine SCC gene, which has two SF-1 binding sites (GGG AGGTCA, TCA AGGCTA) within the proximal promoter region (6); the murine MIS and rat aromatase genes, which have a single SF-1 binding site of the same sequence (CCA AGGTCA) (7–9); the rat LH β promoter, which contains two SF-1 binding sites (ACA AGGTCA, GCA AGGCCA) (10); and the murine DAX-1 gene, which has an enhancer element containing two composite SF-1 binding sites (TCG AGGTCA, TCA TGGCCA) (4) (Table I, Fig. 1B).

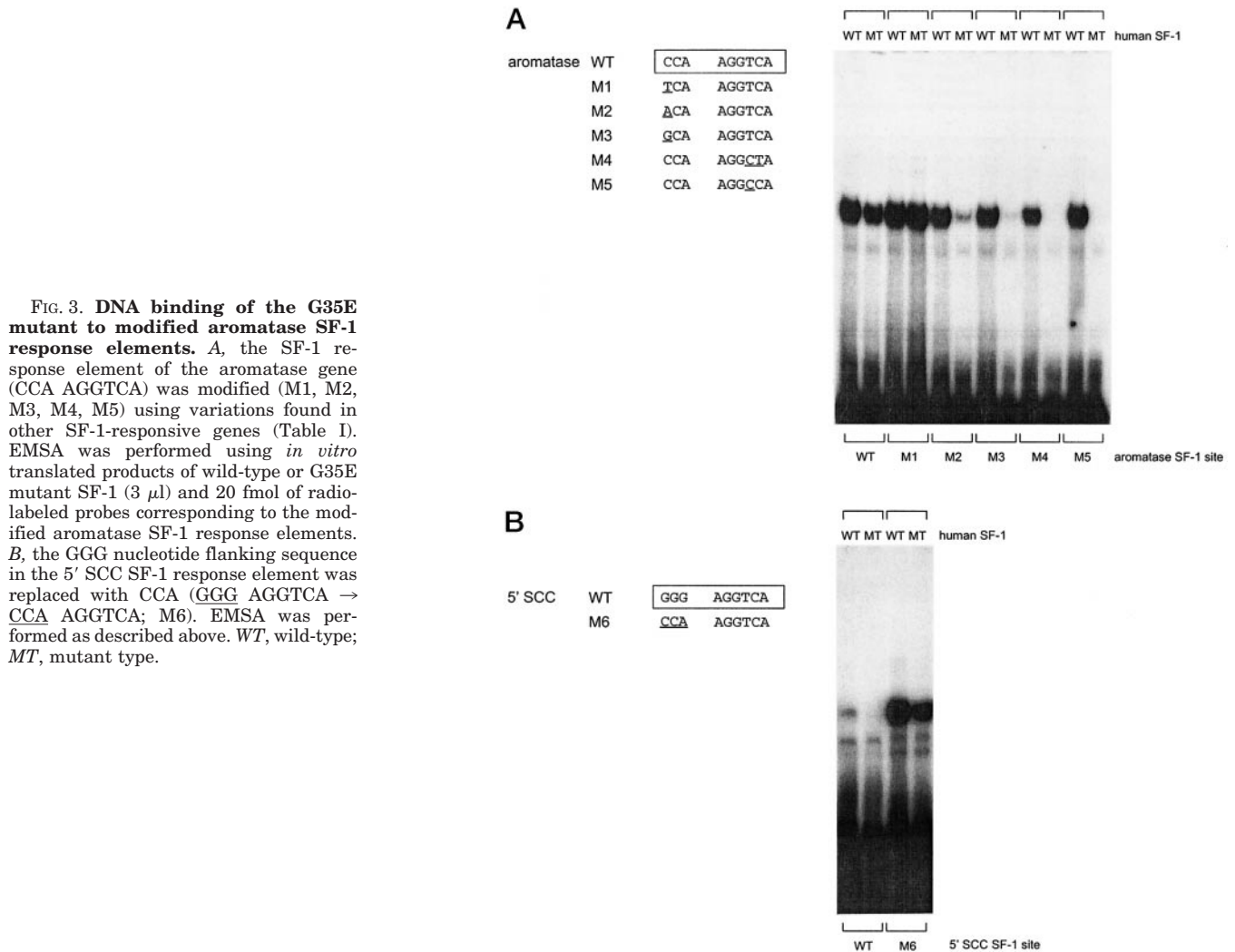


FIG. 3. DNA binding of the G35E mutant to modified aromatase SF-1 response elements. *A*, the SF-1 response element of the aromatase gene (CCA AGGTCA) was modified (M1, M2, M3, M4, M5) using variations found in other SF-1-responsive genes (Table I). EMSA was performed using *in vitro* translated products of wild-type or G35E mutant SF-1 (3 μ l) and 20 fmol of radiolabeled probes corresponding to the modified aromatase SF-1 response elements. *B*, the GGG nucleotide flanking sequence in the 5' SCC SF-1 response element was replaced with CCA (GGG AGGTCA \rightarrow CCA AGGTCA; M6). EMSA was performed as described above. *WT*, wild-type; *MT*, mutant type.

As expected, wild-type SF-1 bound to each of these response elements (Fig. 1*B*, lanes 2, 5, 8, 11, 14, 17, and 20). The G35E mutant SF-1 did not bind to either of the response elements in the SCC promoter (lanes 3 and 6) as reported previously (30). Consistent with these findings, little or no mutant SF-1 binding was seen to probes corresponding to the 5' and 3' SF-1 response element from the LH β promoter (lanes 15 and 18) or the DAX-1 composite enhancer element (lane 21). Surprisingly, however, mutant SF-1 protein retained binding to the response elements from the MIS or aromatase promoters (CCA AGGTCA) (lanes 9 and 12).

To test the functional effects of these observations, reporter genes containing the SF-1 response elements were transfected into SF-1-deficient tsa 201 cells along with wild-type or mutant SF-1 cDNAs (Fig. 2). SF-1 responses of the native SCC, aromatase, LH β , and DAX-1 promoters were studied. In contrast to wild-type SF-1, mutant G35E SF-1 did not activate the SCC, LH β , and DAX-1 gene promoters (Fig. 2*A*) even when increasing doses (0, 5, 10, 15, 20 ng) of mutant SF-1 expression vectors were used (Fig. 2*B*). This observation is consistent with deficient binding of this mutant SF-1 to these response elements.

In contrast, mutant SF-1 protein did activate the native aromatase promoter with about 50% of wild-type function (Fig. 2*A*). Similar results were obtained using an artificial reporter system containing two copies of the SF-1 response element from the MIS promoter (data not shown). Increased expression of mutant SF-1 did not increase transactivation further (Fig. 2*C*). These data indicate that the SF-1 mutant is not fully

functional when bound to this response element, presumably because of conformational changes.

To investigate a possible dominant negative interaction between the mutant G35E and wild-type SF-1, increasing amounts of the mutant vector (0, 5, 10, 15, 20 ng) were co-transfected with a constant amount of wild-type vector (5 ng). Though a small reduction in aromatase promoter transcriptional activity was seen when mutant SF-1 was co-transfected with wild-type SF-1 (Fig. 2*C*), the degree of inhibition was similar to that seen with the murine SCC promoter (Fig. 2*B*), which does not bind the mutant protein. Similarly, in competitive gel shift binding assays for the aromatase and MIS probes mutant SF-1 did not significantly displace the binding of wild-type protein even when used at a 4-fold excess (data not shown). Taken together, these data suggest that mutant SF-1 is not a potent dominant negative inhibitor of wild-type SF-1 on promoters where partial mutant binding and transactivation occur.

Identification of Positions in the Response Elements Critical for DNA Binding of the G35E P-box Mutant—The pattern of transactivation of native promoters by mutant SF-1 was consistent with its DNA binding specificity (Table I). Comparison of the SF-1 response elements in the MIS and aromatase promoters (CCA AGGTCA) with those in other promoters suggested that the first position of the three preceding nucleotides and the nucleotides at the fourth and fifth positions of the nuclear receptor half-site might be important for determining P-box mutant binding. To address this hypothesis, various

mutations were introduced into the aromatase SF-1 response element (Fig. 3A). The cytosine at the first position of the 5'-flanking sequence of the response element (CCA) was changed to thymine (TCA; SCC 3' SF-1 site, M1), adenine (ACA; LH β 5' SF-1 site, M2), or guanine (GCA; LH β 3' SF-1 site, M3). Also, nucleotides at the fourth and fifth positions of the aromatase SF-1 response element (AGGCTCA) were changed to those found in the SCC 3' SF-1 site (AGGCTA; M4) and LH β 3' SF-1 site (AGGCCA; M5).

Wild-type SF-1 protein bound to each of the response elements although binding to the M1 sequence (TCA AGGCTCA) was somewhat greater and binding to the M4 probe (CCA AGGCTA) was weaker than to the wild-type response element (Fig. 3A). Binding of mutant SF-1 to the wild-type and M1 probes was comparable with that of wild-type SF-1. In contrast, binding of mutant SF-1 to the M2, M3, M4, and M5 probes was markedly reduced compared with binding of wild-type protein (8, 2, 0, 0%, respectively). These results indicate that the first position of the three nucleotides in the 5'-flanking sequence, as well as the central part of the nuclear receptor half-site, mediate binding specificity of the G35E SF-1 mutant.

To investigate this hypothesis further, the GGG trinucleotide sequence in the 5' SCC SF-1 response element was replaced with CCA (GGG AGGTCA \rightarrow CCA AGGTCA; M6) (Fig. 3B). This conversion increased wild-type SF-1 binding and facilitated binding by the G35E SF-1 mutant. These data confirm the importance of the 5'-flanking sequence for monomeric binding by SF-1.

Identification of Amino Acids within the SF-1 P-box Critical for DNA Binding—Three amino acid residues within the P-box have been shown previously to be critical for DNA binding of other nuclear receptors (21, 22). These amino acids correspond to codons 31, 32, and 35 in SF-1 (Fig. 4A). To characterize the function of the P-box of SF-1 further, various amino acid substitutions were introduced into these positions. Glutamic acid at position 31 was changed to glycine, an amino acid found at the corresponding position of the GR (E31G). Valine was also introduced into this codon position (E31V), which was shown previously to eliminate DNA binding of TR (33, 34). At codon 32, serine was replaced with either glycine, as found in the ER and TR (S32G), or valine, which disrupts TR binding (33, 34) (S32V). Finally, glycine at position 35 was changed to alanine or valine, as found in the ER (G35A) and GR (G35V), respectively.

The two SF-1 mutants with amino acid residues found in other ER-type receptors (S32G, G35A) bound to the wild-type aromatase probe as effectively as wild-type SF-1, but DNA binding of the E31G GR-type mutant was reduced (Fig. 4B). In contrast, the G35V GR-type mutant did bind to the wild-type aromatase probe, although binding of this mutant to probes with modified half-sites (M4, M5) was significantly reduced (data not shown). As expected, only minimal binding was detected with the E31V and S32V artificial mutants, and introduction of E31V or S32V mutations into the G35E SF-1 protein impaired binding to the wild-type aromatase promoter (data not shown). These findings suggest that selected P-box sequence variations, analogous to those found in other nuclear receptors, are relatively well tolerated in SF-1.

Effect of A-box Mutations on DNA Binding Specificity of the G35E Mutant—The A-box is thought to influence the recognition of the 5'-flanking sequence of the SF-1 response element. Because variations in the 5'-flanking sequence appear to affect the binding affinity of the G35E mutant, we hypothesized that A-box interactions with SF-1 might stabilize binding of the G35E mutant. Two mutations (R92Q, K94Q), which have been shown previously to have little effect on DNA binding, were

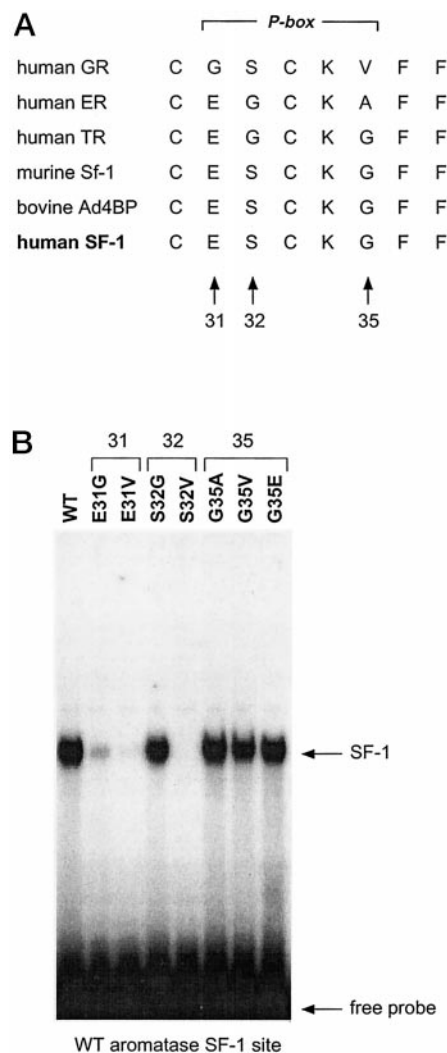


FIG. 4. DNA binding of various P-box mutants to the wild-type aromatase SF-1 response element. A, the P-box amino acid sequences of various nuclear hormone receptors were aligned with the P-box sequence of SF-1. Amino acid positions 31, 32, and 35 of human SF-1 correspond to those critical for DNA binding of GR, TR, and ER. B, mutations were introduced into positions 31 (E31G, E31V), 32 (S32G, S32V), or 35 (G35A, G35V) of the SF-1 P-box. EMSAs were performed using *in vitro* translated products of wild-type SF-1 and various P-box mutants (3 μ l) and 20 fmol of radiolabeled probe corresponding to the wild-type aromatase SF-1 response element. WT, wild-type.

introduced into the A-box sequence (Fig. 5A) (25). Double mutants having the G35E mutation as well as the A-box mutations (G35E/R92Q, G35E/K94Q) were created to investigate possible interdependence of these domains with respect to DNA binding. As expected, binding of the R92Q and K94Q mutants to the wild-type response element was similar to that of wild-type SF-1 (Fig. 5B). In addition, the G35E/K94Q double mutant bound similarly to the G35E mutant alone. However, introduction of the R92Q mutation into the G35E construct (G35E/R92Q) nearly eliminated DNA binding, suggesting that these sequences may act cooperatively to stabilize SF-1 binding.

DNA Binding Specificity of Double Mutants on Modified Aromatase SF-1 Response Elements—When all the mutant response elements are considered, the DNA binding specificity of the R92Q and R94Q mutants was similar to wild-type SF-1 except for slightly reduced binding of the R92Q mutant to the M4 probe (Fig. 6). In contrast, introduction of the R92Q mutation into the G35E mutant dramatically reduced or eliminated DNA binding in all cases, whereas the K94Q mutation had no effect on DNA binding of the G35E mutant.

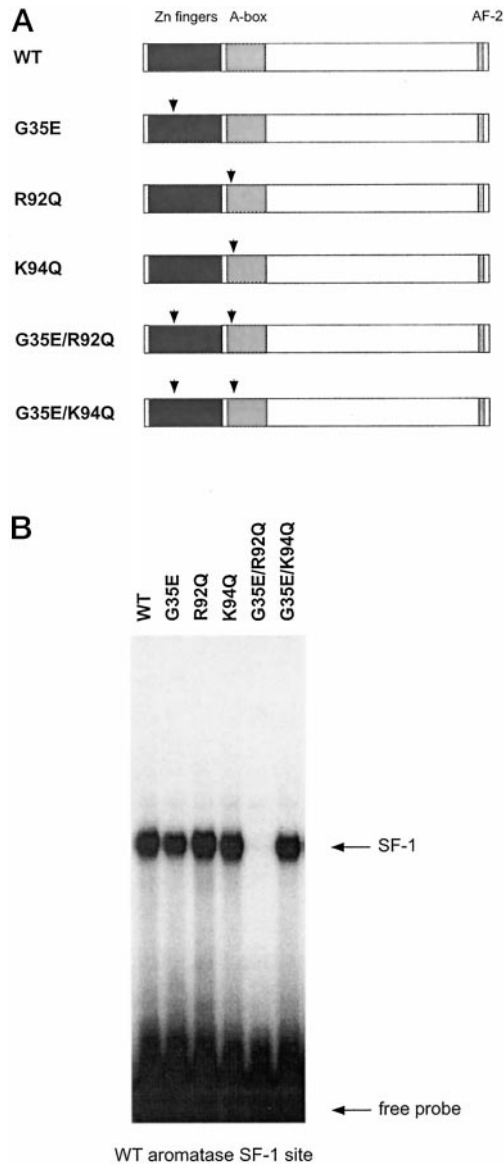


FIG. 5. Effect of A-box mutations on G35E P-box mutant binding to the wild-type aromatase SF-1 response element. A, mutations were introduced into the A-box of wild-type SF-1 (R92Q, K94Q) or the G35E mutant (G35E/R92Q, G35E/K94Q). B, EMSAs were performed using *in vitro* translated products of wild-type SF-1 and the mutants described above in A (3 μ l) and radiolabeled probe (20 fmol) corresponding to the wild-type aromatase response element. WT, wild-type.

DISCUSSION

Nuclear receptors regulate gene transcription in diverse biological systems. DNA binding specificity is crucial, therefore, so that different receptors can recognize their specific target genes appropriately. Several studies have shown that the DNA binding specificity of classic ligand-dependent nuclear receptors is determined by the P-box amino acid sequence (for example, GSV for the GR, EGA for the ER, and EGG for the TR (Fig. 4A) (18–22)). The GS sequence of the GR recognizes the GRE-type half-site, AGAACA, whereas the EG residues of the ER and TR recognize the ERE-type half-site, AGGTCA. Comparatively little is known about the DNA binding specificity of SF-1, an orphan nuclear receptor that has no known ligand and one of the few nuclear receptors that binds DNA as a monomer. SF-1 has a hybrid P-box sequence (ESG) and recognizes a consensus response element consisting of an ERE-type half-site and three preceding nucleotides (PyCA AGGTCA) (35). The

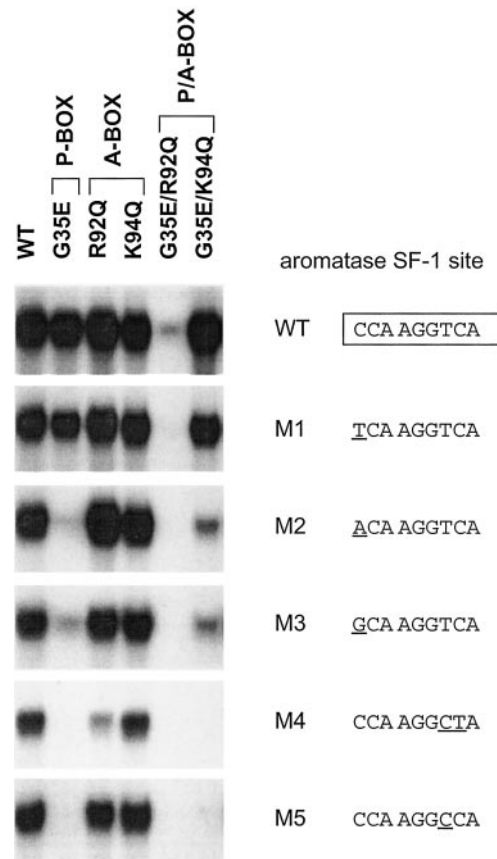


FIG. 6. Effect of A-box mutations on DNA binding of the G35E P-box mutant to modified aromatase SF-1 response elements. EMSAs were performed using *in vitro* translated products of wild-type SF-1 and the mutants described in Fig. 5A (3 μ l) and radiolabeled probes (20 fmol) corresponding to the modified aromatase response elements (Fig. 3A). DNA-protein complexes were resolved on a 0.5 \times TBE polyacrylamide gel. WT, wild-type.

recent discovery of a naturally occurring human SF-1 P-box mutation (G35E) (30) led us to investigate the structural determinants of DNA binding specificity of SF-1 further.

Initial studies showed that the G35E SF-1 mutant did not bind to either of two SF-1 response elements present in the proximal promoter region of the SCC gene (30). This lack of binding might be predicted, as a similar glycine to glutamic acid mutation at the third P-box position resulted in loss of TR binding (33, 34). Also, the patient's phenotype of primary adrenal failure is consistent with impaired transcription of steroidogenic enzymes. However, SF-1 regulates the transcription of a wide array of target genes (35). Many of these have response elements that deviate from the consensus SF-1 sequence such as the murine DAX-1 composite enhancer (Table I; TCG AGGTCA, TCA TGGCCA) (4) or the inhibin α gene, where SF-1 regulates gene transcription through its interaction with CREB bound to the cAMP-response element (17). We hypothesized therefore that the G35E mutant might exhibit different DNA binding specificity with respect to other SF-1 response elements.

Among the SF-1 response elements studied, the G35E mutant was found to bind to the MIS and aromatase promoters with an affinity approaching that of wild-type SF-1. These promoters share a common SF-1 response element, CCA AGGTCA. Comparison of this sequence with other native SF-1 response elements (Table I) led us to speculate that the first position of the three nucleotides in the 5'-flanking sequence and the fourth and fifth nucleotides of the nuclear receptor half-site itself may influence the DNA binding specificity of the G35E mutant. These nucleotides were replaced with those found in other native pro-

motors to investigate this further. Substitution of the flanking cytosine (CCA) with thymine (TCA) in the aromatase SF-1 response element actually strengthened binding by the mutant SF-1, highlighting the importance of a pyrimidine in this flanking sequence. However, binding was impaired significantly following substitution of this nucleotide with a purine (ACA, GCA) or alteration of the central part of the half-site, supporting the hypothesis that the first nucleotide of the flanking sequence, as well as the composition of the half-site, influence DNA binding by the P-box motif.

The role of each of the three P-box amino acid residues in half-site recognition has been studied previously for a variety of nuclear receptors, which allowed us to predict the outcome of mutating these codons in SF-1 (36). The first P-box residue (position 31 for SF-1) is a glutamic acid in SF-1, ER, and TR and a glycine in GR. This glutamic acid facilitates binding to response elements containing guanine at the third position of the nuclear receptor half-site (ERE, AGGTCA) and inhibits binding to those containing adenine (GRE, AGAACA) (37). Reduced binding of the SF-1 E31G mutant is consistent with these observations. At the second P-box position (32 for SF-1) SF-1 and GR have a serine, and ER and TR have a glycine. Replacement of this serine with glycine has been shown to increase binding of GR to an ERE-type half-site (37). Consistent with these findings, the S32G SF-1 mutant showed similar DNA binding specificity to wild-type SF-1. Finally, the third P-box residue (position 35 for SF-1) is a glycine in SF-1 and TR, an alanine in ER, and a valine in GR. Like the glutamic acid in the first P-box position, this valine has a dual function of facilitating binding to response elements containing adenine at the fourth position of the nuclear receptor half-site (GRE, AGAACA) and inhibiting binding to those containing thymine (ERE, AGGTCA) (37). Contrary to our prediction, the G35V mutant bound reasonably well to the wild-type response element (half-site; AGGTCA). However, binding of this mutant to the modified half-sites (M4, AGGCTA; M5, AGGCCA) was reduced dramatically presumably due to the mutation introduced into the fourth nucleotide position of the half-site. Taken together, these data demonstrate that the three amino acid residues in the P-box are critical for recognition of the half-site by SF-1, a nuclear receptor that binds to DNA as a monomer. It is also notable that the P-box sequence of SF-1 (ESG) showed the same DNA binding specificity as that of the TR (EGG) because monomeric TR binding has been demonstrated in certain circumstances (38).

Mutations of the first nucleotide in the 5'-flanking sequence affected binding of several P-box mutants in addition to the G35E mutant protein. This observation provides further evidence that SF-1 binding can be influenced by the 5'-flanking sequence as well as the central part of the nuclear receptor half-site itself. However, it is unclear whether this represents a direct interaction between the P-box motif and the 5'-flanking sequence or whether the flanking sequence has an indirect effect by stabilizing the bound protein-DNA complex. Because it has been suggested that the FTZ-F1/SF-1 A-box can interact directly with this 5'-flanking sequence without interacting with the half-site (25), we introduced two mutations into the SF-1 A-box based on previous studies of FTZ-F1. The A-box mutations, R92Q (R589Q in FTZ-F1) and K94Q (K591Q in FTZ-F1), did not affect DNA binding on their own. However, introduction of the R92Q mutation in the background of the G35E mutant reduced binding quite dramatically. Taken together, these data support the idea that the A-box of SF-1 can stabilize monomeric binding to half-sites through an interaction with the 5'-flanking sequence in the minor groove of DNA. The additional binding energy provided by this contact may

facilitate monomeric binding by receptors, thereby compensating for the stability otherwise provided by receptor dimerization. This interaction may be particularly important when P-box binding is compromised either by mutations in SF-1 or by sequence alterations in its binding site.

In this particular patient, activation of the aromatase promoter by the G35E SF-1 mutant probably had little clinical significance because of multiple other deficiencies in steroidogenesis. In addition, activation of the MIS promoter may have had little effect if the dysgenetic testes had reduced capacity to produce MIS. Nevertheless, this study does indicate that a single mutation in SF-1 can exert differential effects on various target genes. This concept may also apply to other transcription factors that regulate multiple downstream target genes with variant response elements.

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