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Transcriptional Regulation of the Human *FTZ-F1* Gene Encoding Ad4BP/SF-1[1]

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Ad4BP, also known as SF-1, is a steroidogenic tissue–specific transcription factor that is also essential for adrenal and gonadal development. Two mechanisms for the transcriptional regulation of the mammalian *FTZ-F1* gene encoding Ad4BP in adrenocortical cells have been proposed in the previous studies: the crucial role of a *cis*-element, an E box for the steroidogenic cell–specific expression of mouse and rat *FTZ-F1* genes, and a possible autoregulatory mechanism of the *rFTZ-F1* gene by Ad4BP itself through binding to the Ad4 (or SF-1) site in the first intron. In the present study, the transcriptional regulation of the human *FTZ-F1* gene in adrenocortical cells was investigated from several angles, including the above two mechanisms. Using a series of deletion analyses of the 5′-flanking region of the *hFTZ-F1* gene and site-directed mutagenesis for transient transfection studies, an E box element, CACGTG at –87/–82 from the transcriptional start site, was also found to be essential for the transcription of the *hFTZ-F1* gene in mouse or human adrenocortical cell lines as well as in non-steroidogenic CV-1 cells. Despite the presence of a corresponding Ad4 site, CCAAGGCC at +163/+156 in the first intron of the *hFTZ-F1* gene, an autoregulatory mechanism through the Ad4 site was found to be unlikely in the *hFTZ-F1* gene mainly due to site-directed mutagenesis. In addition, the forced expression of Ad4BP had little effect on *hFTZ-F1* gene transcription in non-steroidogenic CV-1 cells. Such Ad4BP-independent regulation of the *hFTZ-F1* gene was in striking contrast to the regulation of steroidogenic CYP genes, such as the human *CYP11A* gene, in which the proximal promoter activity is Ad4BP-dependent and the transactivation by Ad4BP is silenced by DAX-1. Even though the Ad4BP-dependent transcriptional regulation of the *DAX-1* gene has been reported, DAX-1 did not affect the transcriptional activity of the *hFTZ-F1* gene in our study. Taken together, these observations suggest that the E box is indeed required for the expression of the *FTZ-F1* gene, at least in mammalian species, but may not determine the tissue-specific expression of the *hFTZ-F1* gene, and that, unlike the steroidogenic *CYP* gene, the regulation of the *hFTZ-F1* gene appears to be independent of both Ad4BP and DAX-1.

Key words: Ad4BP/SF-1, *FTZ-F1*, gene regulation.

The biosynthesis of steroid hormones begins with the mitochondrial transport of cholesterol from the outer to inner membrane by the steroidogenic acute regulatory protein (StAR) (*1*). Subsequently, five distinct steroidogenic cytochrome P450 hydroxylases, as well as 3β-hydroxysteroid dehydrogenase (3β-HSD), are involved in the adrenocortical steroidogenic pathways, leading to the production of glucocorticoids, mineralocorticoids and adrenal androgens (*2*, *3*).

A steroidogenic tissue-specific transcription factor, adrenal 4-binding protein (Ad4BP), also known as steroidogenic factor 1 (SF-1), is a mammalian homologue of Drosophila fushi-tarazu factor 1 (FTZ-F1) (*4*), a member of the nuclear receptor superfamily (*5–9*). Ad4BP controls the tissue-specific or cAMP-responsive expression of several genes encoding for steroidogenic enzymes and StAR (*9*). In addition, this factor is also involved in the gonadal tissue–specific expression of aromatase P450 (P450arom) (*10*) and Müllerian inhibiting substance (MIS) (*11*). Ad4BP is also a critical developmental regulator in the pituitary-adrenal and gonadal axis, because mice targeted for disruption of the *ftz-f1* gene lack adrenal glands and gonads (*9*, *12*, *13*), as

[1] Based on a unified nomenclature system for the nuclear receptor superfamily, Ad4BP and DAX-1 correspond to NR5A1 and NR0B1, respectively (*54*).

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Abbreviations: Ad4BP, adrenal 4-binding protein; SF-1, steroidogenic factor 1; FTZ-F1, fushi-tarazu factor 1; m, mouse; r, rat; h, human; AHC, adrenal hypoplasia congenita; DSS, dosage-sensitive sex reversal; DAX-1, DSS-AHC critical region on the X chromosome, gene 1; StAR, steroidogenic acute regulatory protein; 3β-HSD, 3β-hydroxysteroid dehydrogenase; ELP, embryonal long terminal repeat-binding protein; HHG, hypogonadotropic hypogonadism; cAMP, cyclic adenosine 3′,5′-monophosphate; RT, reverse transcriptase; PCR, polymerase chain reaction; bHLH, basic helix-loop-helix; SCC, side chain cleavage enzyme; CAT, chloramphenicol acetyltransferase.

well as show a selective loss of gonadotropin-specific markers in the pituitary (*9*, *14*, *15*). Recently, Ad4BP was demonstrated to interact functionally with a novel type of nuclear receptor, DAX-1 (*16–18*), whose gene is mutated in the human X-linked disorders adrenal hypoplasia congenita (AHC) and hypogonadotropic hypogonadism (HHG) (*19*, *20*). Namely, DAX-1 represses the Ad4BP-dependent transcription of the *StAR*, *DAX-1*, and *MIS* genes (*16–18*). Ad4BP is thus considered to play a pivotal role in steroidogenesis, sex determination and sexual differentiation.

The structure of the human *FTZ-F1* gene encoding Ad4BP has been clarified by us (*21*) and others (*22*), and is at least 28 kb long and contains 7 exons including a non-coding exon 1 (*21*). The deduced amino acid sequence of human Ad4BP consists of 461 amino acid residues and is highly homologous to the sequences of rat, mouse, and bovine Ad4BP. In addition, the analysis of the *hFTZ-F1* gene in our study revealed that at least the classical and most common subtype of ELP (embryonal long terminal repeat–binding protein), originally identified as a splicing variant of the *mftz-f1* gene (*23*, *24*), is not likely to be present in humans (*21*). The sequence of the proximal 5′-flanking region of the *hFTZ-F1* gene is remarkably homologous to those of other species and most of the consensus sequences for the transcription factor recognition sites are preserved (*21*, *24–26*).

Regarding the regulation of mammalian *FTZ-F1* gene expression, two important mechanisms have been proposed. One is that a *cis*-element, the E box located at around –80 in the 5′-flanking region, and its binding protein are essential for the steroidogenic tissue–specific expression of the rat (*25*) or mouse *FTZ-F1* gene (*26*). Second, for the steroidogenic tissue–specific expression, an autoregulatory mechanism of the *rFTZ-F1* gene, namely the positive regulation of the *rFTZ-F1* gene by Ad4BP itself through its binding to the Ad4 site located in the first intron, has also been proposed (*27*), although this mechanism has been not observed in the mouse gene (*26*). However, there is as yet no report demonstrating the transcriptional regulation of the *hFTZ-F1* gene.

In the present study, deletion and/or site-directed mutagenesis analyses were first performed to define the functional significance of the 5′-flanking region of the *hFTZ-F1* gene, in order to determine whether or not the above two mechanisms also exist in humans. Secondly, to clarify the functional association between DAX-1 and Ad4BP, we investigated whether or not DAX-1 can modulate the expression of the *hFTZ-F1* gene at the transcriptional level.

MATERIALS AND METHODS

5′-Flanking Sequence of the Human and Bovine FTZ-F1 Gene Encoding Ad4BP—A *hFTZ-F1* gene clone No. 120, which contains exons 1–3 and 8.5 kb of the 5′-flanking sequence, was isolated in a previous study (*21*), and the nucleotide sequence of the 1.5 kb 5′-upstream region from the non-coding exon 1 was characterized in the present study. A 1.1 kb segment of the bovine *FTZ-F1* gene (GenBank accession No. AF140357) containing non-coding exon 1 was obtained by inverse PCR (*28*) using primers based on the sequence of the 5′-UTR of the bovine Ad4BP cDNA (*8*). PCR (*29*) was performed using *Klen Taq* DNA polymerase (Clontech, Palo Alto, CA) and a DNA Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT). DNA sequences were determined by the dideoxynucleotide method (*30*) using an ALFexpress DNA Autosequencer (Pharmacia Biotech, Uppsala, Sweden).

Plasmid Construction for Transient Transfection—An 8.5 kb long fragment containing the 5′-upstream region from the *Fsp*I site in the first exon (clone 120) was subcloned into a reporter plasmid, pSV00CAT (*31*), to yield hAd4-CAT8.5K. Various lengths of recombinant plasmids were constructed from hAd4CAT8.5K as indicated in Fig. 3. For the construction of the recombinant plasmids, hAd4CAT-7.0K, hAd4CAT4.0K, hAd4CAT2.2K, hAd4CAT781, hAd4-CAT591, hAd4CAT97, and hAd4CAT84, the following restriction endonuclease sites in the upstream region were used: *Ban*III site at 7.0 kb, the *Sse*8387I site at 4.0 kb, the *Kpn*I site at 2.2 kb, the *Xba*I site at 781 bp, the *Sph*I site at 591 bp, the *Stu*I site at 97 bp, and the *Pma*CI site at 84 bp, respectively.

A construct, hAd4ECAT8.5K, containing the 5′-upstream 8.5 kb sequence plus the sequence of the first intron, was also made. hAd4ECAT8.5K comprised a total of 12.5 kb DNA fragments including the 5′-upstream 8.5 kb sequence plus the 4 kb intronic sequence encompassing the region from the transcriptional start site to the initiation methionine in exon 2. As shown in Fig. 3, hAd4ECAT7.0K, hAd4-ECAT4.0K, hAd4ECAT2.2K, hAd4ECAT781, hAd4ECAT-591, hAd4ECAT97, and hAd4ECAT84 were also constructed using the same restriction endonuclease sites in the upstream region, respectively.

The 4.0 kb fragment was also subcloned into the *Hin*dIII site of a reporter plasmid, pGL3-Basic Vector (Promega, Madison, WI), to yield hAd4LUC4.0K. Recombinant plasmids of various lengths were constructed from hAd4LUC-4.0K as indicated in Fig. 4. For the construction of the recombinant plasmids hAd4LUC4.0K, hAd4LUC2.2K, hAd4-LUC1.0K, hAd4LUC781, hAd4LUC591, hAd4LUC480, hAd4LUC230, hAd4LUC97, hAd4LUC91, and hAd4LUC-82, the above restriction endonuclease sites, plus the *Bam*-HI site at 1.0 kb, the *Nco*I site at 480 bp, the *Bal*I site at 230 bp, the *Pst*I site at 91 bp, and the *Pma*CI site at 82 bp, respectively, were used. The plasmid, hAdLUC77 was constructed from hAd4LUC82 by deleting 5 nucleotides using T4 DNA polymerase.

Plasmids lacking some sequences in the first intron were also made as shown in Fig. 3. hAd4ECATΔ2.4 K, hAd-4ECATΔ1.7 K, hAd4ECATΔ158, and hAd4ECATΔ30, were constructed using the *Sac*I site at 2.4 kb, the *Xho*I site at 1.7 kb, the *Sma*I site at 158 bp, and the *Nae*I site at 30 bp upstream from the acceptor splice site, respectively. A construct, hAd4ECAT591ΔNN, was constructed from hAd4-ECAT591 by the internal deletion of a *Nae*I–*Nae*I fragment spanning the region from 71 bp downstream of the donor splice site to 30 bp upstream of the acceptor splice site and the branchpoint site in the first intron.

A construct, hAd4ECAT591ΔES, was obtained by internal deletion of the 3.9 kb sequence from the *Eco*47III site in the first exon to the *Sac*II site in the second exon. Next, for the construction of hAd4ECAT591Ci, which contains a foreign chimeric intron, the *Eco*47III–*Sac*II fragment of hAd-4ECAT591 was replaced by a 181 bp *Pst*I–*Afl*II fragment from plasmid pRL-SV40 (Promega, Madison, WI), which contains the 5′-donor splice site from the first intron of the human β-globin gene and the branchpoint and 3′-acceptor

splice site from an intron preceding an immunoglobulin gene heavy chain variable region (*32*). Two additional chimeric constructs, hAd4ECAT591int2F and hAd4ECAT591int2R, in which the first intron of the *hFTZ-F1* gene was replaced by 130 bp of the sense and antisense *Pma*CI-*Pma*CI fragments containing the second intron of the *hFTZ-F1* gene, respectively, were made.

The expression vector for bovine Ad4BP cDNA (RSV/Ad4BP), its null expression control vector for the antisense cDNA (RSV/RevAd4BP), an expression vector for the catalytic subunit of protein kinase A (PKA), a CAT construct, pS2.3HCAT, containing a 2.3 kb upstream region from the transcriptional initiation site of the human *CYP11A* gene encoding P450scc, and the luciferase expression vector (RSV/luc) driven by the Rous sarcoma virus enhancer/promoter, were generous gifts from Dr. K Morohashi (National Institute for Basic Biology, Okazaki) and all have been described previously (*33*). The expression vector for the human DAX-1 cDNA (RSV/hDAX-1) and its null expression control vector for the antisense cDNA (RSV/RevhDAX-1) were obtained basically by RT (reverse transcription)-PCR. Namely, total RNA was isolated using Isogen (Waco Pure Chemical, Osaka) from a human adrenal gland obtained from a patient who underwent adrenalectomy for renal cancer. The first-stranded cDNA was synthesized using total RNA by a RT-PCR kit (Strategene, La Jolla, CA). The cDNA was then amplified by PCR using 10 pmol of sense/antisense DAX-1 primers (5′-AGGAAGCTTCCACTGGGCAGAACTGGGCTA-3′/5′-ATCTAGAACTGCACTACTGCACTTGTGTGG-3′; *Hin*dIII and *Xba*I sites are underlined) and *Klen Taq* DNA polymerase. The hDAX-1 cDNA fragment was then subcloned into an RSV-driven expression vector, pRc/RSV (Invitrogen, Carlsbad, CA), and sequenced on an ALFexpress DNA Autosequencer to verify its sequence.

Site-Directed Mutagenesis—Four constructs, hAd4CAT591M, hAd4CAT591m, hAd4ECAT591M, and hAd4ECAT591m, carrying a two nucleotide substitution within the E box (wild type, CACGTG at –87/–82; M, mutant CCCGGG; m, mutant CATATG) were generated by the recombinant PCR method (*34*) using 526 bp of the *Sph*I–*Mun*I fragment (Fig. 1). hAd4ECAT591A, containing a three nucleotide substitution at the Ad4 site within the first intron of the *hFTZ-F1* gene (wild type, CCAAGGCC at +163/+156; A, mutant CCAATATCC), was also constructed by the recombinant PCR method (*34*) using 301 bp of *Pma*CI–*Nae*I fragment (Fig. 1). Two constructs, hAd4ECAT591MA and hAd4ECAT591mA, carrying a two-nucleotide substitution within the E box, as well as a three-nucleotide substitution at the Ad4 site within the first intron of the human *FTZ-F1* gene, were basically constructed by the recombination of hAd4ECAT591M, hAd4ECAT591m, and hAd4ECAT591A (Fig. 5). The mutated sequences were finally verified by sequencing on an ALFexpress DNA Autosequencer.

Cell Culture and Transfection Assay—A Y1 mouse adrenocortical tumor cell line, SW-13 human adrenocortical tumor cell line, and CV-1 monkey kidney cell line were purchased from the Japanese Cell Research Bank (Tokyo). Y1 cells and CV-1 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Tokyo) supplemented with 10% fetal bovine serum at 37°C. SW-13 cells were maintained in L-15 (Life Technologies) supplemented with 10% fetal bovine serum at 37°C. Transient transfection was performed by the lipofection method using LipofectAMINE reagent (Life Technologies).

For CAT assay, 3 μg of the recombinant CAT plasmid and 0.2 μg of RSV/luc were co-introduced into the cultured cells. When the CAT constructs were transfected, the efficiencies of transfection were normalized by the luciferase activities of RSV/Luc as previously described (*25*). The cells were harvested 48 h after transfection, and CAT assays were performed (*25*) using the 1-deoxy-[dichoroacetyl-1-^{14}C] chloramphenicol (56 mCi/mmol, Amersham). The CAT activity of each construct is expressed relative to pSV2CAT, which contains the SV40 enhancer/promoter.

For luciferase assay, 2 μg of the recombinant pGL3-derived plasmid and 0.2 μg of pRL-CMV (Promega, Madison, WI) were co-introduced into the cultured cells. The cells were harvested 36 h after transfection, and luciferase activity was normalized using a Dual Luciferase Reporter Assay System (Promega). The luciferase activity of each construct is expressed as a relative activity to pGL3-Control containing SV40 enhancer/promoter.

Gel Mobility Shift Assay—Nuclear extracts from cultured cells were prepared using the previously described method (*35*). Gel mobility shift assays were performed essentially as described with 2 μg poly (dI. dC) as a nonspecific competitor and 5 μg nuclear extract (*25*). Double stranded oligonucleotides, dhENC, containing the putative Ad4 site in the first intron (5′-gTAAGTGAGGGCCTTGGCCCAGGCCT-3′/3′-ATTCACTCCCGGAACCGGGTCCGGAg-5′) and dAd4, containing an authentic Ad4 site in the promoter region of the bovine *CYP11B* (5′-ggACATACCCAAGGTCCCCTTT-3′/3′-TGTATGGGTTCCAGGGGAAAgg-5′), were used as the probes. One or two guanine nucleotides (g) were added to the 5′ end of the synthetic nucleotides and used for labeling with the Klenow fragment in the presence of [α-^{32}P]dCTP (110 Tbq/mmol, Amersham). For a competition analysis, a 50-fold molar excess of the nonradiolabeled double stranded oligonucleotides, dhEM1-M6 (see Fig. 6B), and dENC, containing the rat intronic Ad4 sequence (5′-gTAAGTGAAGGCCGGGGCCCAGGCCT-3′/3′-ATTCACTTCCGGCCCCGGGTCCGGAg-5′) (*27*), respectively, were added prior to the addition of the probe. Of the above oligonucleotides, dhEM1–M6 has a three sequential nucleotide substitution in dhENC (underlined). Antiserum to Ad4BP was added after the addition of the probe according to the previously described procedure (*25*).

RESULTS

The Sequence of Promoter Region of hFTZ-F1 Gene—The nucleotide sequence of the 1.3 kb *hFTZ-F1* gene containing the 5′-flanking region from –2.2 kb to –0.9 kb (Fig. 1) and that of the 1.1 kb bovine *FTZ-F1* gene containing non-coding exon 1 and 0.6 kb of the 5′-flanking region (Fig. 2) were newly determined. The major transcriptional start site of the *hFTZ-F1* gene in the normal human adrenal gland and in the adrenocortical adenoma from a patient with Cushing's syndrome was mapped around the guanine nucleotide in our previous study (*21*) (Fig. 1). The start site was 5 bases downstream of that of the rat gene (*25*) and a few bases upstream of that of the mouse gene (*26*). The initiator motif (Inr) was almost completely conserved among all species including bovine (Fig. 2). The promoter sequence of the

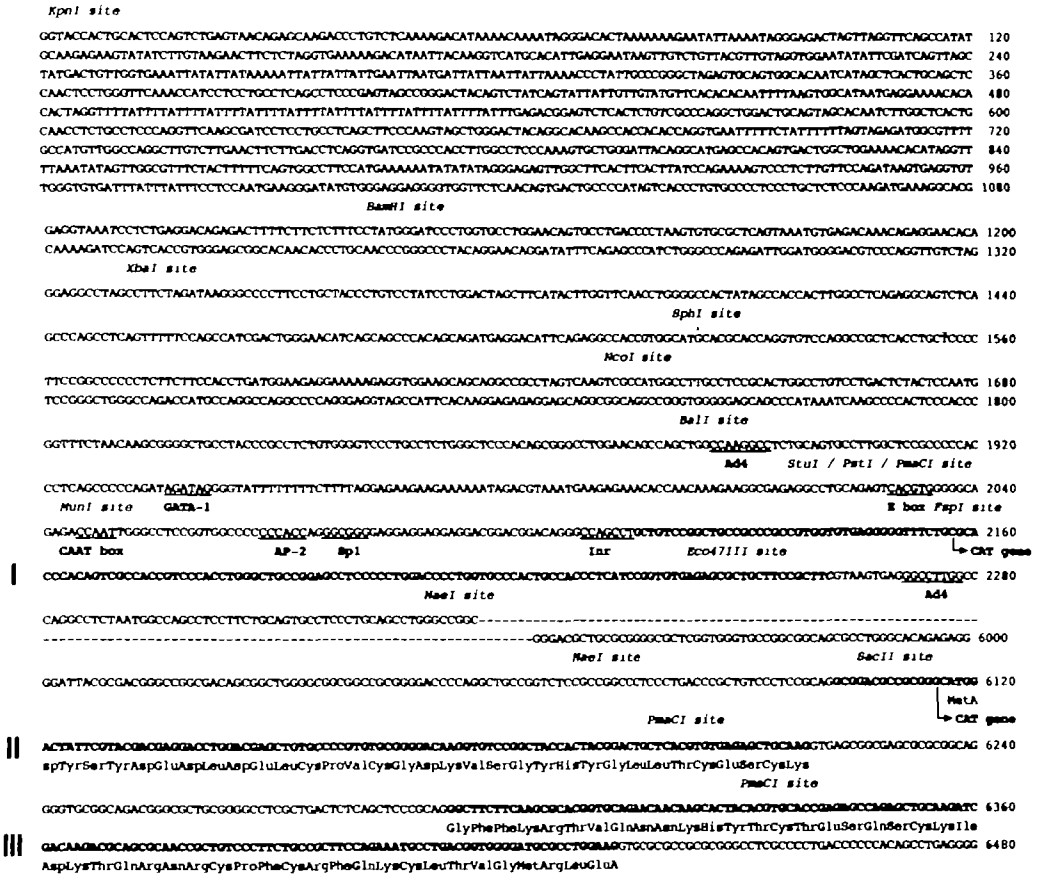

Fig. 1. **Sequences in the *hFTZ-F1* gene including 2.2 kb of the 5′-flanking region, non-coding exon 1, exon 2 and exon 3.** The nucleotide sequence of the *hFTZ-F1* gene and the deduced amino acid sequence are shown. The nucleotide sequences of the exons are written in bold letters. The numbers to the left indicate *hFTZ-F1* gene exons. The connecting site with the CAT gene is indicated in the first and second exons. The potential regulatory elements, Ad4, GATA-1, E box, CAAT box, AP-2, Sp1, and Inr are underlined. The restriction enzyme sites used for deletion analysis and subcloning in this study are indicated by dots.

hFTZ-F1 gene upstream of non-coding exon 1 was also highly homologous to those of the bovine, rat and mouse genes, and most of the consensus sequences for the recognition sites of transcription factors, such as GATA-1, E box, CAAT box, AP-2, and Sp1, were preserved (Fig. 2), suggesting a shared and essential regulatory mechanism in the expression of this gene among species. The proximal Ad4 site, CCAAGGPyC, is located at around –210 bp in the human, bovine and mouse *FTZ-F1* gene upstream regions, whereas it is not completely preserved in the corresponding region of the *rFTZ-F1* gene. In mouse and rat, the Ad4 site has been reported to be present near the donor splice junction of the first intron (*8*, *24*, *25*). An antisense Ad4 site (GGCCTTGG) was surprisingly present in the corresponding region of the first intron of the *hFTZ-F1* gene (Fig. 1), whereas no consensus Ad4 site was found in the corresponding region of the bovine *FTZ-F1* gene.

Promoter Activity of the hFTZ-F1 Gene in the Absence or Presence of the First Intron in Y1 Mouse Adrenocortical Cells—To investigate the promoter activities of the *hFTZ-F1* gene, various lengths of each promoter region were constructed in a CAT vector, and then expressed transienty in Y1 cells. The CAT activity of each construct was expressed as the activity relative to pSV2CAT. As indicated by the open box in Fig. 3, the addition of the 8.5 kb upstream sequences to the CAT vector produced a significant amount of CAT activity. The activity was relatively higher than that after adding 2.0 kb of the *rFTZ-F1* gene promoter in Y1 cells (data not shown). The CAT activity was essentially preserved until the truncation of the promoter region reached –97 bp. However, a dramatic decrease in the CAT activity was observed when the deletion reached –84 bp, at which point half of the E box sequence, CACGTG, was destroyed, suggesting that the E box is also be essential for the transcription of the *hFTZ-F1* gene.

Next, the effect of the first intron on the basal transcriptional activity of the *hFTZ-F1* gene was examined, since a previous study using the *rFTZ-F1* gene showed a dramatic increase in the transcriptional activity by the first intron through the Ad4 site as a possible autoregulatory mechanism by Ad4BP in Y1 cells (*27*). The closed box in Fig. 3 indicates the CAT activity when the constructs contained the 4 kb long first intron in addition to various lengths of the upstream promoter sequence. As indicated, each construct caused a dramatic increase in the CAT activity compared with constructs lacking the first intron.

Promoter Activities of the hFTZ-F1 Gene by Luciferase Assay in Mouse Y1 or Human SW-13 Adrenocortical Cell Line and in a Non-Steroidogenic CV-1 Cell Line—The CAT activity of the *hFTZ-F1* gene transfected into non-ste-

roidogenic CV-1 cells was undetectable in the absence of the first intron, despite almost the same transfection efficiency as observed in Y1 cells. In addition, the CAT activity of the *hFTZ-F1* gene transfected into human adrenocortical SW-13 cell was nearly undetectable in the absence of the first intron because of the low transfection efficiency of this cell line (data not shown). As a result, for a more sensitive transcriptional activity assay, several promoter sequences were constructed in a pGL3-Basic Vector, transfected into these cells, and then assayed to determine luciferase activity. By

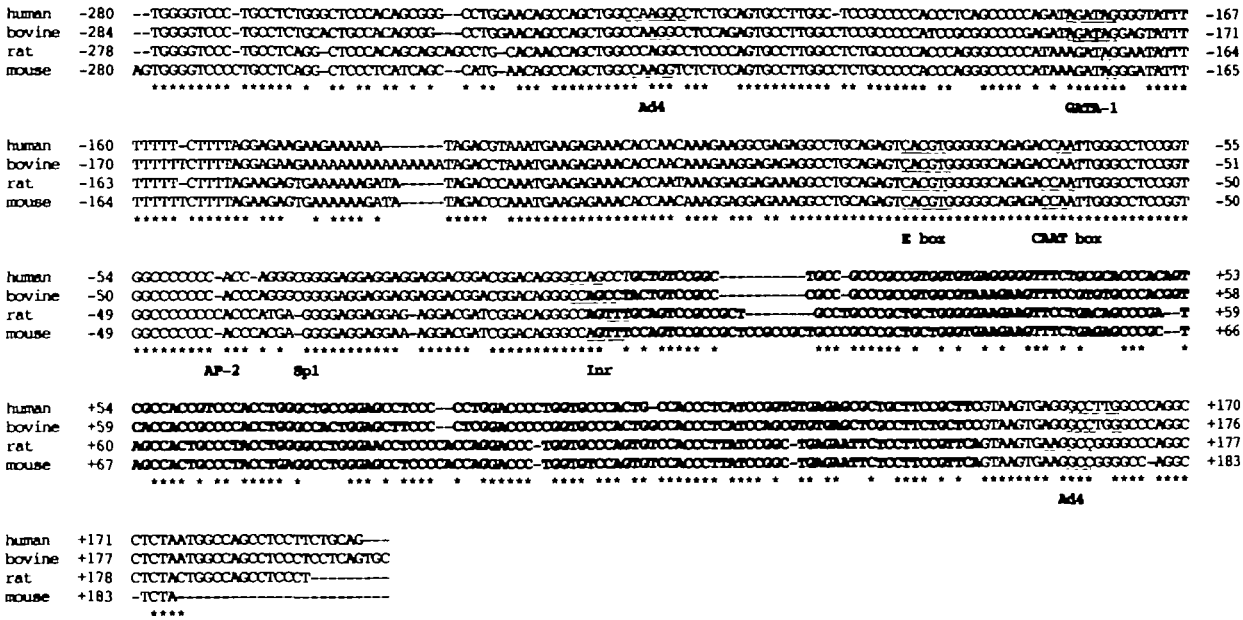

Fig. 2. **Comparison of nucleotide sequences of mammalian *FTZ-F1* genes in the 5′-flanking region and around the boundary of the non-coding exon 1 and the first intron.** Nucleotide sequences of the human and bovine *FTZ-F1* genes were determined as described in "MATERIALS AND METHODS." Nucleotide sequences of the mouse and rat *FTZ-F1* genes are cited from reports by Ikeda *et al.* (*8*) and Ninomiya *et al.* (*24*) and a report by Nomura *et al.* (*25*), respectively. Nucleotide sequences that are identical among the four species are indicated by asterisks (*); nucleotide deletions (alignment gaps) are indicated by dashes. The nucleotides are numbered relative to the transcriptional start site (+1) (*21*). Potential regulatory elements such as Ad4, GATA-1, E box, CAAT box, AP-2, Sp1, and Inr are underlined.

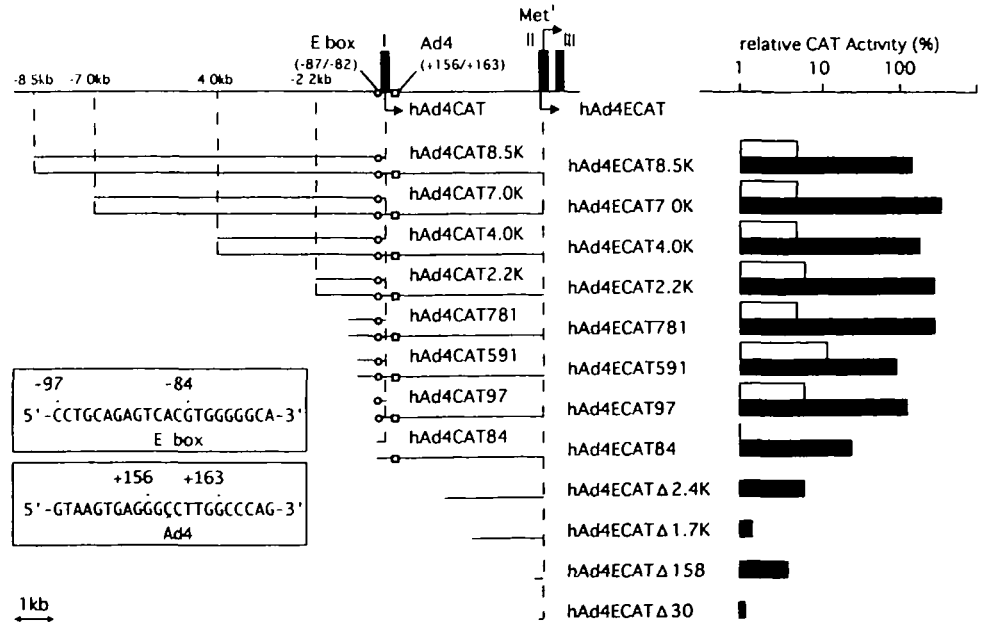

Fig. 3. **Transient transfection assays in Y1 adrenocortical cells with a series of deletion mutant plasmids carrying the 5′-flanking regions of the *hFTZ-F1* gene in the absence or presence of the first intron.** *Constructions of* the recombinant plasmids are indicated below the map of the *hFTZ-F1* gene. The plasmids were transfected into Y1 cells and CAT assays were performed as described in "MATERIALS AND METHODS." The CAT activity of each construct is expressed relative to pSV2CAT. The open box indicates the relative CAT activity of the hAd4-CAT construct lacking the first intron. The closed box indicates the relative CAT activity of the hAd4ECAT construct containing a 4 kb long first intron in addition to the 5′-flanking sequence. In hAd4CAT84 or hAd4-ECAT84, half of the E box sequence, CACGTG, was destroyed. ○, E box (CACGTG) at –87/–82; □, Ad4 site (GGCCTTGG) at +156/+163. The experiment was carried out using triplicate cultures and repeated three times. The values were averaged for the three experiments (Mean ± SD).

this assay, the E box was also found to be essential for the transcriptional activity in SW-13 cells and even in CV-1 cells, although the magnitude of the responsiveness in SW-13 or CV-1 cells was clearly less than that observed in Y1 cells (Fig. 4).

Site-Directed Mutagenesis of E Box and/or Ad4 Site in the First Intron—The significance of the E box at –87/–82 for the transcriptional activity of the *hFTZ-F1* gene in Y1 cells or CV-1 cells was also examined by site-directed mutagenesis (Fig. 5). Two nucleotide substitutions in the E box, CCCGGG instead of CACGTG, dramatically reduced the CAT activity of the construct containing the 0.6 kb upstream promoter sequence, regardless of the presence or the absence of the first intron in Y1 cells (hAd4CAT591M and hAd4ECAT591M in Fig. 5A). In addition, two additional nucleotide substitutions in the E box, CATATG also caused a significant reduction in the CAT activity in Y1 cells (hAd4CAT591m and hAd4ECAT591m in Fig. 5A). Although the bHLH binding motif was demonstrated to be CANNTG (*36*), these results suggest that the *hFTZ-F1* gene promoter strictly requires CACGTG as the core E box sequence for transcriptional activity.

Next, when most of the internal region of the first intron was deleted except for 70 bp nucleotide sequences around both splice junctions (hAd4ECAT591NN), no remarkable changes in the CAT activity were observed. However, when the junctional 70bp sequence was also deleted (hAd4-ECAT591ΔES), the CAT activity was decreased to the same extent as observed for the construct lacking the first intron (hAd4CAT591). These results suggest that the higher CAT activity dependent on the first intron may be associated with some sequences in the junctional 70 bp (Fig. 5A). Since the 70 bp sequence contains an antisense putative Ad4 sequence (GGCCTTGG at +156/+163) near the donor splice junction of the first intron (Figs. 1 and 2), we next examined the possibility that the Ad4 site in the first intron is involved in the autoregulatory mechanism, as suggested for the *rFTZ-F1* gene (*27*). The ability of Ad4BP to bind to the putative Ad4 site in the first intron was investigated by a gel mobility shift assay using ^{32}P-labeled oligonucleotide, dhENC, carrying the putative Ad4 site in the first intron, and the nuclear extract prepared from Y1 cells (Fig. 6). The experiment revealed the formation of a single protein complex that completely disappeared by the addition of nonradiolabeled dhENC. The oligonucleotides dhEM3 and M4, carrying disrupted Ad4 sites, did not function as competitors, whereas the other oligonucleoties, dhEM1, M2, M5, M6, carrying putative Ad4 sites, and dENC, carrying a rat intronic Ad4 site (*27*), were able to function as competitors. The complexes with dhENC showed the same mobility on polyacrylamide gels as those with dAd4 carrying an authentic Ad4 site in bovine *CYP11B*. The signals with two distinct probes were each abolished completely by the addition of either nonradiolabeled oligonucleotide. The single protein complex was inhibited by the addition of antiserum to Ad4BP (Fig. 6). These results clearly indicate that the donor junctional sequence containing an Ad4 site in the first intron can be recognized by Ad4BP *in vitro*.

We next performed site-directed mutagenesis of the Ad4 site in the first intron. The Ad4 sequence, GGCCTTGG, was mutated to GATATTGG produce a complete loss of the binding ability to Ad4BP, as confirmed by gel mobility shift assay (Fig. 6). However, the construct containing the mutated 4 kb intronic sequence in addition to the 0.6 kb upstream promoter sequence (hAd4ECAT591A) showed no dramatic decrease in CAT activity in Y1 cells (Fig. 5A). Constructs containing mutations in both the Ad4 site and the E box (hAd4ECAT591MA and hAd4ECAT591mA) showed CAT activities similar to those of constructs containing a mutation only in the E box (hAd4ECAT591M and hAd4ECAT591m) in Y1 cells (Fig. 5A).

In the case of CV-1 cells, the transcriptional activity of the *hFTZ-F1* gene also increased dramatically due to the presence of the first intron, thus making it possible to eval-

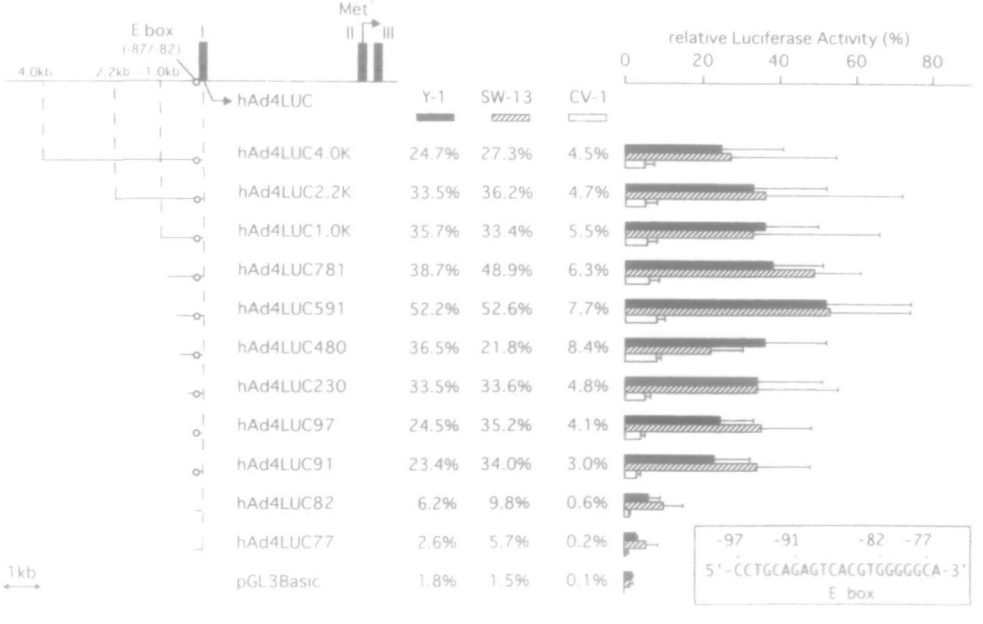

Fig. 4. **Promoter activities of the *hFTZ-F1* gene by luciferase assay using mouse Y1 adrenocortical cells, human adrenocortical carcinoma SW-13 cells, and non-steroidogenic CV-1 cells.** The mutant lacking the upstream promoter sequence was constructed in a promoter-less luciferase vector, pGL3-Basic (Promega, Madison, WI). For luciferase assay, 2 μg of the recombinant plasmid, and 0.2 μg of pRL-CMV (Promega) were co-introduced into Y1 cells, SW-13 cells or CV-1 cells for 36 h. The luciferase activities were assayed using a Dual Luciferase Reporter Assay System (Promega) and the luciferase activity of each construct is expressed relative to pGL3-Control. ○, E box (CACGTG) at –87/–82. The experiment was carrried out using triplicate cultures and repeated three times. The values were averaged for the three experiments (Mean ± SD).

uate the effect of mutagenesis (Fig. 5B). In this condition, the pattern of the changes in the transcriptional activity of the *hFTZ-F1* gene by mutagenesis of the E box and/or the Ad4 site (hAd4ECAT591M, hAd4ECAT591A, and hAd4-ECAT591MA, respectively) in CV-1 cells was quite similar to that observed in Y1 cells (Fig. 5B), suggesting again that the effect of the E box or the effect of the first intron on the transcriptional activity of the *hFTZ-F1* gene is not specific to steroidogenic cells.

Finally, when the first intron was replaced by a 181 bp unrelated foreign chimeric intron (hAd4ECAT591Ci) or by 70 bp of *hFTZ-F1* gene intron 2 (hAd4ECAT591int2F), the CAT activity was also increased in comparison with the construct lacking the intronic sequence in both Y1 and CV-1 cells, while reverse replacement of *hFTZ-F1* gene intron 2 (hAd4ECAT591int2R) caused no increase in CAT activity (Fig. 5, A and B), suggesting that the effect of the first intron in transcriptional regulation is neither sequence- nor tissue-specific in the *hFTZ-F1* gene.

Effect of Ad4BP on the Basal Transcriptional Activity of the hFTZ-F1 Gene—The proximal promoter lesion of the *hFTZ-F1* gene was examined, and the consensus Ad4 site

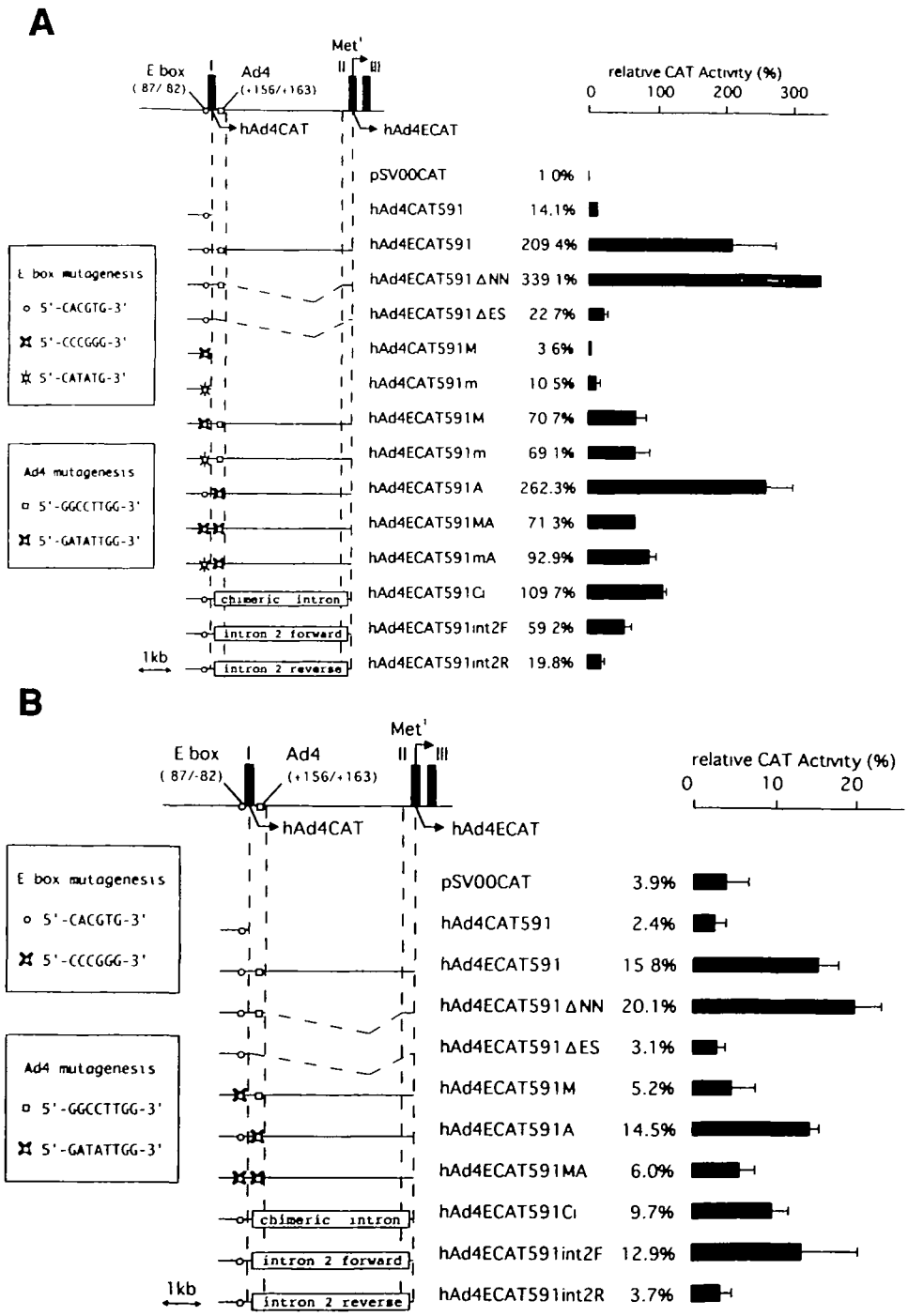

Fig. 5. **Functional analysis of chimeric foreign introns and the E box at –87/–82 and/or the Ad4 site in the first intron of the *hFTZ-F1* gene by site-directed mutagenesis.** hAd4-ECAT591ΔNN was constructed from hAd4ECAT591 by the internal deletion of the *Nae*I–*Nae*I fragment in the first intron. A construct, hAd4ECAT591ΔES was obtained by the internal deletion of the 3.9 kb sequences from the *Eco*47III site in the first exon to the *Sac*II site in the second exon. A chimeric construct, hAd4ECAT591Ci, containing a 181 bp foreign sequence of the donor splice site from the first intron of the human β-globin gene and the branchpoint and acceptor splice site from an intron preceding an immunoglobulin gene heavy chain variable region and two other chimeric constructs, hAd4ECAT591int2F and hAd4-ECAT591int2R, which contain 130 bp of the sense and antisense *hFTZ-F1* gene intron 2 sequences, respectively, were constructed as described in "MATERIALS AND METHODS." The other recombinant plasmids in the figure were constructed from hAd4ECAT591 by nucleotide substitution in either or both the E box and the Ad4 site as described in "MATERIALS AND METHODS." These constructs were transfected into Y1 cells (A) or CV-1 cells (B) for 48 h and assayed for CAT activity. o, E box (CACGTG) at –87/–82; □, Ad4 site (GGCCTTGG) at +156/+163. The crosses indicate the substitutions introduced. The E box was replaced by CCCGGG or CATATG. The Ad4 site was replaced by GATATTGG. The CAT activity of each construct was expressed as activity relative to pSV2CAT. The experiment was carried out using triplicate cultures and repeated three times. The values were averaged for the three experiments (Mean ± SD).

was located around –200 bp in the upstream 5'-flanking region. We next examined whether or not the forced expression of Ad4BP affects the transcriptional activity of the *hFTZ-F1* gene through its Ad4 site in CV-1 cells. As a control, a CAT plasmid, pS2.3HCAT, carrying the 2.3 kb fragment upstream from the transcriptional initiation site of the human *CYP11A* gene, was also examined under the same conditions. The CAT activity in CV-1 cells driven by hAd4ECAT8.5K carrying a 8.5 kb upstream sequence plus the 4 kb sequence of the first intron was essentially unaffected by co-transfection of an Ad4BP expression plasmid (RSV/Ad4BP) (Fig. 7). In contrast, the forced expression of Ad4BP caused a significant induction of the CAT activity driven by the human *CYP11A* gene promoter, as previously reported (*33*). Moreover, while the Ad4BP-dependent promoter activity of the human *CYP11A* gene was further augmented by co-transfection of an expression vector for the catalytic subunit of PKA, the CAT activity of hAd4ECAT-8.5K was affected by neither PKA nor PKA plus Ad4BP (Fig. 7). These results suggest that the transcriptional regulation of the *hFTZ-F1* gene is essentially Ad4BP- or PKA-independent, regardless of the presence of the Ad4 site in the first intron and even in the upstream promoter region.

Effect of DAX-1 on the Expression of the hFTZ-F1 Gene—Since the functional association of DAX-1 and Ad4BP has been implicated, we investigated the effect of DAX-1 on the expression of the *hFTZ-F1* gene in Y1 adrenocortical cells that express Ad4BP. The transcriptional activities of the *hFTZ-F1* gene were measured in Y1 cells co-transfected with a CAT reporter gene plasmid carrying the 8.5 kb upstream promoter sequence plus the 4kb sequence of the first intron (hAd4ECAT8.5K), and a DAX-1 expression plasmid (RSV/hDAX-1) (Fig. 8). A CAT plasmid, pS2.3-HCAT, carrying the 2.3 kb fragment upstream of the human *CYP11A* gene was also co-transfected with RSV/hDAX-1 under the same conditions. As shown in Fig. 8, while the forced expression of DAX-1 significantly suppressed the CAT activity driven by the human *CYP11A* gene promoter, it had little effect on the CAT activity driven by hAd4ECAT8.5K. Under the same conditions, it also had

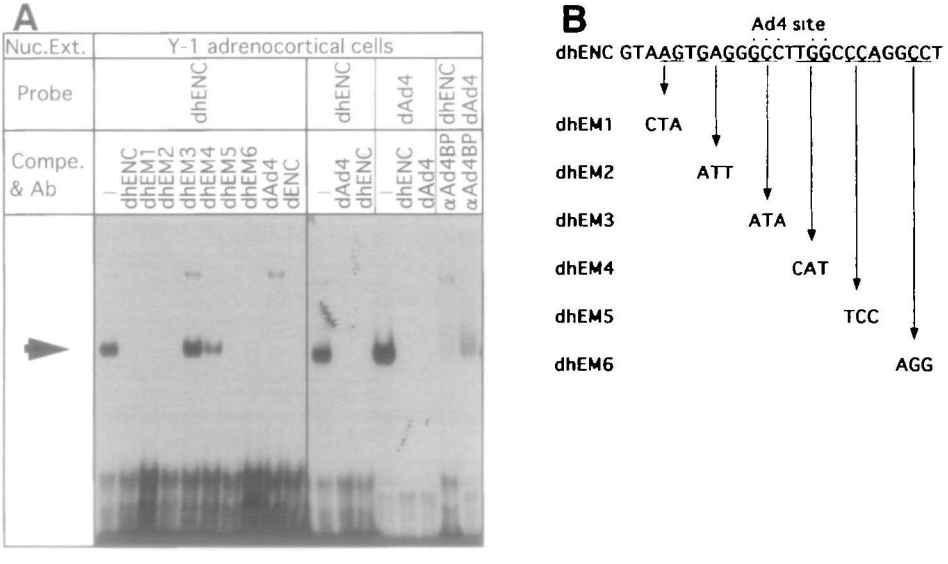

Fig. 6. **Gel mobility shift assays with the nuclear extract prepared from Y1 cells.** Figure 6A shows gel mobility shift assays. ^{32}P-labeled oligonucleotides, dhENC or dAd4, were incubated with 5 μg of nuclear extract. For competition assays, a 50-fold molar excess of each nonradiolabeled oligonucleotide shown in Fig. 6B was added prior to the addition of the probes. Antiserum to Ad4BP(αAd4BP) was added after the probes. The incubation mixtures were then examined on a 4.5% polyacrylamide gel. Complexes with dhENC and dAd4 showing the same mobility are indicated by an arrowhead. In Fig. 6B, each of the three underlined nucleotides is replaced to make dhEM1 to dhEM6.

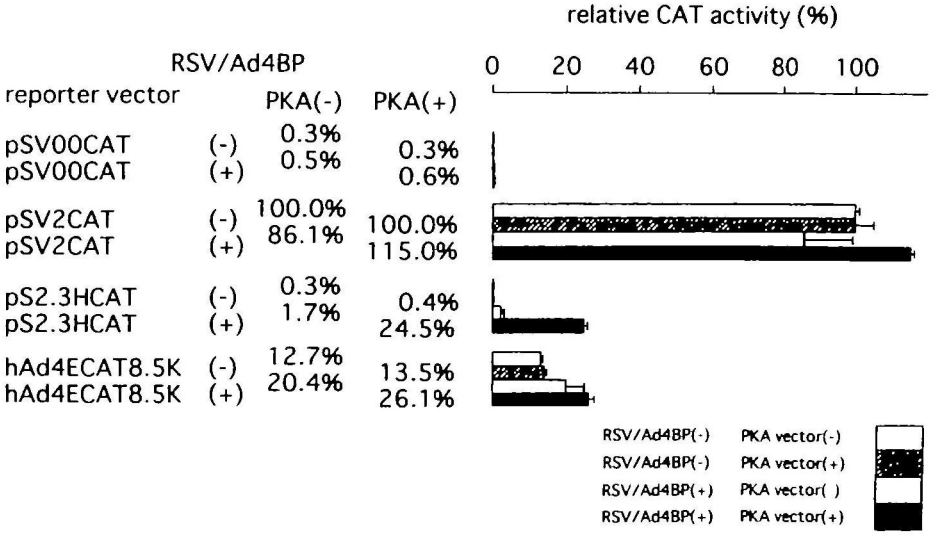

Fig. 7. **Effect of Ad4BP on the human *CYP11A* or *hFTZ-F1* gene promoter.** The CAT construct with the *hFTZ-F1* gene promoter (hAd4ECAT-8.5K) or with the 2.3 kb human *CYP11A* gene promoter (pS2.3HCAT) as a control were co-transfected into CV-1 cells with bovine Ad4BP expression vector, RSV/Ad4BP, or mock (null) expression vector as a negative control. The above experiments were done in the presence or absence of the PKA expression vector into CV-1 cells. The pSV2CAT activity in the absence of RSV/Ad4BP is expressed as 100% and the relative CAT activities are shown. The experiment was done using triplicate cultures and repeated three times. The values were averaged for the three experiments (Mean ± SD).

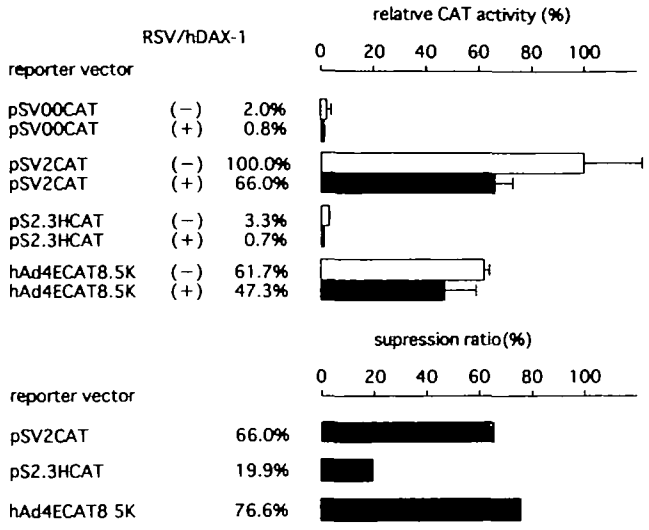

Fig. 8. **Effect of hDAX-1 on the *hFTZ-F1* gene promoter.** CAT constructs with the *hFTZ-F1* gene promoter (hAd4ECAT8.5K) were co-transfected with the hDAX-1 expression vector, RSV/hDAX-1, into Y1 cells. As a negative control, the respective antisense expression vector, RSV/RevhDAX-1, was also used in the above experiments. Experiments with the CAT construct containing the 2.3 kb human *CYP11A* promoter (pS2.3HCAT) were also performed under the same conditions. A CAT assay was performed as described in "MATERIALS AND METHODS." The pSV2CAT activity in the absence of RSV/hDAX-1 is expressed as 100% and the relative CAT activities are shown (upper figure). The lower figure indicates the suppression ratio of CAT activity by the presence of RSV/hDAX-1. The experiment was carried out using triplicate cultures and repeated three times. The values were averaged for the three experiments (Mean ± SD).

little effect on the luciferase activity driven by hAd4-LUC591 (data not shown). In CV-1 cells, the Ad4-dependent transcriptional activation of the human *CYP11A* gene (see Fig. 7) was also dramatically suppressed by the co-expression of DAX-1, whereas the transcriptional activity of the *hFTZ-F1* gene was essentially unchanged regardless of the presence or absence of the forced expression of DAX-1 (data not shown).

DISCUSSION

In previous studies on rat and mouse *FTZ-F1* gene regulation, the class B bHLH family recognition site, E box (5′-CACGTG-3′), located about –80 bp from the transcriptional start site has been reported to be an essential *cis*-element for the regulation of mammalian *FTZ-F1* gene expression in Y1 (*25*, *26*), I-10 (*25*) and DC3 (*26*) steroidogenic cell lines, but not in non-steroidogenic CV-1 or NIH3T3 (*25*, *26*) cell lines that do not express Ad4BP. However, a subsequent study by Harris and Mellon demonstrated that the E box is also required for the expression of the *rFTZ-F1* gene not only in steroidogenic cells but also in a wide variety of cell types including pituitary gonadotrope cell lines and even CV-1 cells (*37*). While E box binding proteins are generally considered to be heterodimers of two types of bHLH comprising both cell-type-restricted and ubiquitously expressed members, steroidogenic cell-specific bHLH has not yet been identified. Based on the ubiquitous expression patterns of nuclear factors that bind to the *rFTZ-F1* gene E box, a class B member of the bHLH family of transcription factors, USF (upstream stimulatory factor), was proposed as a ubiquitous type of bHLH (*37*). In our study, the identical E box, CACGTG, was also found to be preserved in the proximal promoter region of the *hFTZ-F1* gene, and also proven to be functionally important for expression in CV-1 cells as well as in mouse Y1 or human SW-13 adrenocortical cell lines. While the bHLH recognition motif was demonstrated to be CANNTG (*36*), a site-directed mutagenesis study of the E box in our study indicated that for the transcriptional activity of the *FTZ-F1* gene, the class B bHLH recognition motif, E box, CACGTG, is strictly required. These findings by us and by Harris and Mellon (*37*) suggest that although the E box regulates mammalian *FTZ-F1* gene expression as a minimal promoter, it may not fully explain the tissue-specific expression of this gene. Interestingly, it has recently been revealed that the 5′-flanking region of the frog *FTZ-F1* gene encoding Ad4BP does not contain an E box at the corresponding region, although an E box–like sequence (5′-CANNTG) was found further from the region than in mammalian *FTZ-F1* genes (*38*). This suggests that the E box may not be a universal *cis*-element for vertebral *FTZ-F1* gene expression, especially in species other than mammals. Regarding the basal transcriptional activity of the mammalian *FTZ-F1* gene, other than the E box, at least two other elements, a CAAT box, and an Sp1 site in the proximal promoter of the mammalian *FTZ-F1* gene, are also reported to be required for transcriptional activity, but are still not critical for mammalian *FTZ-F1* gene expression in steroidogenic cells (*26*). These results suggest the need for a renewed effort to find the unidentified *cis*-element and factor to determine the steroidogenic tissue–specific expression of the mammalian *FTZ-F1* gene.

Previous studies have reported an increase in the transcriptional activity of the *rFTZ-F1* gene by the first intron through an Ad4 site in Y1 adrenocortical cells, leading to the hypothesis of an autoregulatory mechanism (*27*). Since the first intron of the *hFTZ-F1* gene also contains the antisense Ad4 site in the corresponding region of the first intron, we examined whether or not this mechanism holds true for the *hFTZ-F1* gene. The transcriptional activity of the *hFTZ-F1* gene was strongly enhanced by the presence of the first intron, not only in Y1 adrenocortical cells but also in CV-1 non-steroidogenic cells. An increase in transcriptional activity was also observed when the first intron was replaced by other intronic sequences from either the *hFTZ-F1* gene (intron 2) or a foreign gene. Moreover, the Ad4 site in the first intron of the *hFTZ-F1* gene appeared to be unrelated to the transcriptional activity, since little change in the transcriptional activity was observed by site-directed mutagenesis of the Ad4 site or by the forced expression of Ad4BP in non-steroidogenic CV-1 cells. These results cast doubt on the specificity of the enhancer activity in the first intron and the involvement of the Ad4 site in *hFTZ-F1* gene. Increased transcriptional activity in the presence of the first intron was observed for both the rat *FTZ-F1* gene (*27*, *37*) and the human gene, but not for the mouse gene (*26*), suggesting the phenomenon to be non-specific. The difference may be due to the reporter plasmid, pSV00CAT vector (*27*, *37*, and the present study) *vs.* pLuc vector (*26*). The increase in the trancriptional activity in the presence of the first intron in the previous study (*27*, *37*) and in our study is probably related to the increased

stability of the transcripts according to the splicing event, while cryptic splicing due to the small t-intron within the 3′-region of the luciferase of pLuc vector may disturb such an event (*39*, *40*).

Although a consensus Ad4 site is also located in the upstream promoter region of the *hFTZ-F1* gene, the forced expression of Ad4BP had no effect on the transcriptional regulation of the *hFTZ-F1* gene, indicating an autoregulatory mechanism to be unlikely. Such Ad4BP-independent regulation of the *hFTZ-F1* gene is in striking contrast to the regulation of the human *CYP11A* gene, in which the proximal promoter activity shows Ad4BP-dependent regulation. In the regulation of the *hFTZ-F1* gene, the structural and functional coordination between Ad4BP and the basic transcriptional machinery system may not be adequate to fulfill their function.

Unlike most steroidogenic *CYP* and *StAR* genes (*1–3*), the expression of the mammalian *FTZ-F1* gene is not controlled by cAMP or ACTH (*41*). In our study, *hFTZ-F1* gene regulation was also found to be PKA-independent, in contrast to the regulation of steroidogenic *CYP* genes. These findings further suggest the regulation of the mammalian *FTZ-F1* gene to be uniquely different from those of other steroidogenesis-related genes.

DAX-1 is mutated in patients with X-linked AHC and HHG (*19*, *20*) and is also a candidate gene for DSS (dosage-sensitive sex reversal) (*42*). The role of DAX-1 in DSS was recently supported by the phenotype of *dax-1* transgenic mice, namely genotypic XY mice were found to develop as females (*43*). However, *dax-1*–deficent mice were most recently found to have no abnormalities in female sexual differentiation but instead to demonstrate disturbed spermatogenesis, thus revealing an unexpected male-specific function (*44*). As a result, the function of the *DAX-1* gene in sexual differentiation is currently very puzzling. Ad4BP and DAX-1 are similar with respect to the tissue specificity of expression and the developmental pattern of expression during fetal development, suggesting some functional association exists between them. While the presence of the Ad4 (SF-1) site in the 5′-flanking region of the mouse (*45–47*) or human (*48*) *DAX-1* gene has been noted, the findings on the Ad4BP-dependent regulation of the *DAX-1* gene have been conflicting. While the presence of DAX-1 in *ftz-f1*–disrupted mice, as well as the fact that Ad4BP has no effect on *mdax-1* gene regulation, have been reported (*46*), a positive reguation of Ad4BP on the mouse (*47*, *49*) or human (*48*) *DAX-1* gene has been demonstrated. On the other hand, the overexpression of DAX-1 in steroidogenic cells was found to repress the basal and cyclic AMP–stimulated transcription of the *StAR*, *CYP11A*, and *3β-HSD* genes (*50*), or the Ad4BP-dependent transcription of the *StAR*, *DAX-1*, and *MIS* genes (*16–18*). One mechanism for this suppression by DAX-1 is thought to be through binding to a unique hairpin structure on the *DAX-1* or *StAR* gene (*17*). A second mechanism is through the interaction between Ad4BP and DAX-1, in which the recruitment of the nuclear receptor corepressor (N-CoR) takes place, resulting in a marked reduction in the transcriptional activity of Ad4BP (*51*). A third mechanism for the suppression by DAX-1 was most recently proposed to be through interaction with the corepressor Alien (*52*). In our study, the forced expression of human DAX-1 in Y1 cells caused little change in the transcriptional activity of the *hFTZ-F1* gene, while it caused a marked reduction in the basal or Ad4BP-dependent transcriptional activity of the human *CYP11A* gene. As a result, DAX-1 may modulate steroidogenesis by silencing the expression of some steroidogenic *CYP* and *StAR* genes, but not by affecting Ad4BP at the transcriptional level.

Taken together, it seems that an E box binding protein, Ad4BP itself or DAX-1 do not determine the steroidogenic tissue-specific expression of Ad4BP. Recently, tissue-specific chromatin structure has been suggested to be one mechanism for tissue-specific gene expression. In fact, the chromatin structure of the 5′-flanking region of the mammalian *FTZ-F1* gene is reported to be open in steroidogenic cells such as mouse adrenocortical Y1 cells (*27*, *53*) and rat adrenal tissue (*27*), but closed in non-steroidogenic cells such as NIH3T3 cells (*53*) and rat liver tissue (*27*), supporting the above view. Further investigations are needed to elucidate the true mechanism of the tissue-specific expression of the *hFTZ-F1* gene.

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