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Contribution of Ad4BP, a Steroidogenic Cell-Specific Transcription Factor, to Regulation of the Human CYP11A and Bovine CYP11B Genes through Their Distal Promoters

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We analyzed the upstream regions of the human CYP11A and bovine CYP11B genes, and identified a distal promoter in each gene. The distal promoters are located at −1.8 to −1.5 kb in the upstream region of the CYP11A gene and −1.5 to −1.1 kb in the upstream region of the CYP11B gene. Transient transfection of CAT plasmid carrying each of the two distal promoters indicated that the regions had a transcriptional activating function and that the function was stimulated by cAMP. The basal and cAMP-stimulated transcriptional activities were detected only in steroidogenic cells. On structural analyses of the regions, we identified two Ad4 sites and a cAMP responsive element in the distal promoter of CYP11A, and two Ad4 sites and one NF-IL6 binding site in the distal promoter of CYP11B. The presence of an Ad4 site in common suggests its major contribution to the transcriptional activation. We also investigated the functional interactions between the distal promoters and basal promoters of both genes. Interestingly, the two distal promoters showed different requirements as to the basal promoter.

Key words: Ad4 binding protein, CYP11A gene, CYP11B gene, distal promoter, transcriptional regulation.

Steroid hormones synthesized in several organs, adrenal gland, testis, ovary, and placenta, play essential role in sexual differentiation and the maintenance of homeostasis of glucose and ions in the animal body. Six P-450s are involved in the biosynthesis of steroid hormones, and each step of the biosynthetic pathway is regulated by tropic peptide hormones, such as adrenocorticotropin, luteinizing hormone, and follicle-stimulating hormone. These tropic hormones secreted from the anterior lobe of the pituitary gland affect the biosynthesis of steroid hormones by increasing the intracellular cAMP levels of the target organs through receptors on the plasma membrane (*1-3*).

It has been established that there are two modes as to the stimulation of the biosynthesis of steroid hormones as the result of elevation of the cAMP level, *i.e.*, increases in the activities of steroidogenic enzymes (*e.g.*, steroid hydroxylating P-450s), through efficient uptake/transport of cholesterol into mitochondria, and triggering of transcription of the steroidogenic P-450 genes (*1*). Since steroidogenic P-450 genes have been isolated from various animals, transcriptional regulation of the genes has been examined with the use of the promoter regions. Many reports have been published describing the functions of P-450 gene promoters in the cAMP-response and steroidogenic cell specific transcription (*4-17*).

The cAMP responsive element (CRE) is well known as a *cis*-element recognized by members of the CREB/ATF family which confers transcriptional activity responding to stimulation by cAMP (*18*). The presence of functional CRE was confirmed in the promoters of the human CYP11A, bovine and mouse CYP11B, and human CYP21 genes (*6, 19-21*). However, CRE was not found in the promoters of other steroidogenic CYP genes, although they are also activated by cAMP. Other sequences different from CRE have been suggested to be responsible for the cAMP-stimulation of some steroidogenic P-450s (*22-24*). Recently, Wilson *et al.* (*24*) showed that the NGFI-B transcription factor stimulates transcription of the mouse *Cyp* 21 gene rapidly in a cAMP-dependent manner.

On the other hand, we demonstrated that Ad4 site, in addition to Ad1(CRE), was necessary for the full response to cAMP in the case of the bovine CYP11B gene (*20*). This observation was further confirmed by examining the transcriptional activity of a template carrying 4 copies of Ad4 and one copy of Ad1 (CRE). The template clearly showed the essential role of the Ad1(CRE) site as a basal cAMP responsive element and of Ad4 site as an enhancing element (*25*). The significance of Ad4 site was also strongly suggested by the presence of the *cis*-element in the promoter regions of all the steroidogenic CYP genes. Interestingly, many of the Ad4s were mapped to the regulatory regions, which were reported to confer transcriptional activity to the genes (*26-28*). The significant role of the Ad4 site was

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Abbreviations: CAT, chloramphenicol acetyltransferase; CYP11A, gene encoding side-chain cleavage P-450; CYP11B, gene encoding 11β-hydroxylase P-450; CYP21, gene encoding 21-hydroxylase P-450 (*48*).

further supported by the steroidogenic cell specific expression of an Ad4 binding protein, (Ad4BP), a Zn finger transcription factor (*29*). Transactivation experiments involving an Ad4BP expression vector showed the essential role of Ad4 site as well as Ad4BP in steroidogenic specific and cAMP-dependent expression of the CYP11A and CYP11B genes (*25*).

In this study, we identified Ad4-containing distal promoters of the CYP11A and CYP11B genes. Moreover, it was revealed that the regions mainly controlled the transcription of the CYP11A and CYP11B genes, in an Ad4BP-dependent manner.

MATERIALS AND METHODS

Construction of Recombinant Plasmids and the CAT Assay—CAT plasmids carrying the human CYP11A upstream region were generated from pS2.3H-CAT containing the 2.3 kb upstream region of CYP11A as described in the previous papers (*14, 15*). As indicated in Fig. 1, pS1.8C-CAT, pS1.5S-CAT, and pS0.6P-CAT were constructed using the restriction sites of *Cla*I at −1.8 kb, *Sca*I at −1.5 kb, and *Pvu*II at −600 bp, respectively. pS130-CAT, pS93-CAT, pS57-CAT, pS46-CAT, and pS20-CAT were constructed by use of BAL 31 exonuclease (Takara Shuzo, Kyoto). CAT plasmids carrying the bovine CYP11B promoter region were also generated from p7β1.5H-CAT containing the 1.5 kb upstream region of CYP11B as described (*14*). As indicated in Fig. 2, p7β0.4S-CAT, p7β0.1S-CAT, and pΔ1-4M1.5H-CAT were constructed using the restriction sites of *Hin*dIII at −1.5 kb upstream from the transcription initiation site, *Bgl*II at −1.1 kb, *BstE*II at −629 bp, *Sma*I at −407 bp, and *Stu*I at −125 bp, respectively.

S-RR (−1,841 to −1,523 bp of the CYP11A gene upstream region) and B-RR (−1,467 to −1,145 bp of the CYP11B upstream region) were obtained by digestion with *Cla*I–*Sca*I and *Hin*dIII–*Bgl*II, respectively. The fragments were subcloned into *Sma*I site of pUC 19 and then tandemly repeated three times in a head to tail manner to make constructions of 3×[S-RR] and 3×[B-RR]. 3×[S-RR]-B125-CAT and 3×[B-RR]B125-CAT were constructed by insertion of the 3×[S-RR] and 3×[B-RR] fragments, respectively, at *Stu*I site (−125 bp) of p7β0.1S-CAT. 3×[S-RR]S46-CAT and 3×[B-RR]S46-CAT were constructed by insertion of the 3×[S-RR] and 3×[B-RR] fragments, respectively, at −46 bp of pS46-CAT. 3×[S-RR]-S57-CAT and 3×[S-RR]S130-CAT were constructed by insertion of the 3×[S-RR] fragment at −57 bp of pS57-CAT and −130 bp of pS130-CAT, respectively. 3×[S-RR]SV-CAT and 3×[B-RR]SV-CAT were constructed by insertion of the 3×[S-RR] and 3×[B-RR] fragments, respectively, at *Bgl*II site of pCAT (Promega, Madison), which contains SV40 core promoter region (408 to 610 bp) in the upstream region of the CAT gene. Expression vectors of Ad4BP (RSV/Ad4BP) and luciferase (RSV/luc) were constructed as described (*25*). An expression vector of the catalytic subunit of protein kinase A (PKA) was a generous gift from Dr. McKnight (University of Washington, Seattle).

The plasmids for the CAT assay were transfected into Y-1, I-10, and CV-1 cells (supplied by the Japanese Cancer Research Resources Bank, Tokyo) by electroporation using a Gene Pulser (Bio Rad Laboratories, Richmond) (*14*) or the calcium phosphate precipitation method as described previously (*29*). Cells were cultured in the presence or absence of 1 mM Bt_2cAMP for 36 h and then used for the CAT assay as described previously (*14*). The CAT activity expressed in the transfected cells was determined by counting the radioactivity of the acetylated products with a Fuji Image Analyzer (Fuji Photo Film, Odawara).

DNase I Footprinting and the Gel Shift Assay—DNA fragments, −1,523 to −1,841 bp of the CYP11A upstream region and −1,145 to −1,467 bp of the CYP11B upstream region, were end-labeled using [γ-^{32}P]ATP (110 TBq/mmol) (Amersham, U.K.) and used as probes. The binding reaction and *DNase* I (Sigma, St. Louis) digestion were performed as described (*30*).

The nucleotides corresponding to −62 to −30 bp (S-1) and −1,661 to −1,642 bp (HSC-CRE) of the CYP11A gene, −1,304 to −1,278 bp (Ad7) of the CYP11B gene, −129 to −111 bp (αCG-CRE) of the human chorionic gonadotropin-α gene, −61 to −37 bp (NF-IL6) of the C reactive protein gene, 131 to 154 bp (Myc) of the *c-myc* gene, and −150 to −127 bp (E4TF-1) of the adenovirus early region 4 promoter were synthesized with an Applied Biosystems 380B synthesizer (Foster City) as shown in Figs. 4a, 5a, and 9a. Two mutated oligonucleotides, S-2 and S-3, were synthesized by introducing nucleotide substitutions, as shown in Fig. 9a. One guanine nucleotide added to the 5′ end of the synthetic oligonucleotides was used for the labeling reaction with the Klenow fragment (Takara Shuzo, Kyoto) and [α-^{32}P]dCTP (110 TBq/mmol) (Amersham, U.K.). Nuclear extracts were prepared from bovine adrenal cortex and rat liver according to Gorski *et al.* (*31*). Gel shift analysis was performed as described previously (*15*). For competition experiments, a 5 or 50-fold molar excess of an unlabeled probe was added to the reaction mixture.

RESULTS AND DISCUSSION

Identification of Distal Promoters of the CYP11A and CYP11B Genes—In order to determine the promoter function of the human CYP11A gene, several deletion plasmids were generated from pS2.3H-CAT, which contained the 2.3 kb long upstream region of the CYP11A gene (Fig. 1). These plasmids were transfected into two different types of steroidogenic cells, Y-1 and I-10 cells, derived from mouse adrenal and testicular Leydig tumors, respectively. After 36 h incubation in the presence or absence of 1 mM Bt_2cAMP, the cells were used for the CAT assay. The results of the CAT assay with I-10 cells are summarized in Fig. 1. pS2.3H-CAT and pS1.8C-CAT showed 10- and 4-fold induced transcriptional activities, respectively, on treatment with Bt_2cAMP. On the other hand, other plasmids, which contained promoter regions shorter than 1.5 kb, did not show cAMP-responsiveness. Similar results were obtained when the same plasmids were transfected into Y-1 cells (data not shown). It was therefore concluded that the two regions, −2.3 to −1.8 kb and −1.8 to −1.5 kb in the CYP11A upstream region, have a cAMP-responsive function. The presence of cAMP-dependent transcriptional activity in the region from −1.8 to −1.5 kb was observed with Y-1 cells (*7, 11, 19*) and with MA-10 Leydig cells (*21*). However, the presence of the same activity in the other region, from −2.3 to −1.8 kb, is not consistent with

the previous reports (*19, 21*). Similar inconsistency was also seen in the case of the human CYP21 gene (*8, 21*). Such inconsistent observations in several reports are likely to be explained in part by the differences in the reporter gene plasmids and cultured cells used in each experiment. In particular, Y-1 cells maintained in different laboratories showed different levels of transcriptional activation of pS2.3H-CAT and pS1.8C-CAT by Bt_2cAMP (data not shown). Recently, we identified a *cis*-acting element, Ad4, which confers full cAMP responsiveness to the CYP11B promoter in the co-presence of Ad1(CRE) (*15, 20, 25*). Interestingly, the presence of at least an Ad4 site was confirmed in all the steroidogenic CYP gene promoters using the Ad4 binding protein (Ad4BP) purified from bovine adrenal cortex (*27*). Six Ad4 sites are present at −37 to −45, −540 to −532, −678 to −670, −1,637 to −1,629, −1,719 to −1,727, and −2,125 to −2,132 bp in the CYP11A promoter region. The two functional regions determined above, −2.3 to −1.8 kb and −1.8 to −1.5 kb, both have Ad4 sites.

For analysis of the bovine CYP11B promoter region, various deletion plasmids were constructed from p7β1.5H-CAT, which contained the 1.5 kb long upstream region of the bovine CYP11B gene. These plasmids were transfected into Y-1 cells and CAT assays were performed. As shown in Fig. 2, p7β1.5H-CAT showed induced transcriptional activity (10-fold) on treatment with Bt_2cAMP. The inducibility was retained when the CAT plasmids contained 407 bp upstream region (p7β0.4S-CAT), although the induced CAT activity was less than half the level in the case of p7β1.5H-CAT. The 125 bp upstream region did not confer cAMP responsiveness to the CAT plasmid in spite of the presence of Ad1(CRE). These results showed good agreement with those of the deletion experiments on the CYP11B promoter reported previously (*15*). In this study, we investigated the transcriptional activity of the region from −1.5 to −1.1 kb by making four additional CAT constructs carrying internal deletions (Fig. 2). Com-

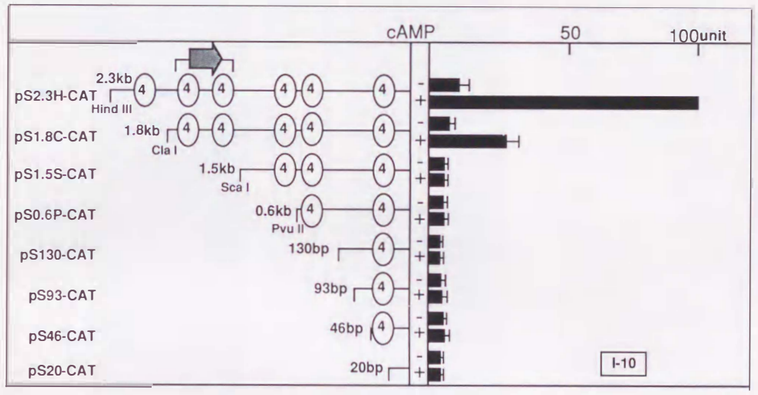


Fig. 1. **Construction of CAT plasmids carrying sequential deletions of the CYP11A gene promoter and their CAT activities in I-10 cells.** The plasmids were constructed from pS2.3H-CAT (*14*) as described under "MATERIALS AND METHODS." The region from −1.8 to −1.5 kb is indicated by a shaded arrow. These plasmids were transfected into I-10 cells by electroporation (*14*), and then the cells were used for the CAT assay after 36 h incubation in the presence (+) or absence ( ) of 1 mM Bt_2cAMP. The CAT activities indicated by the closed bars are the averages±SEM for three transfection experiments. The CAT activity of pS2.3H-CAT treated with Bt_2cAMP was taken as 100 units.

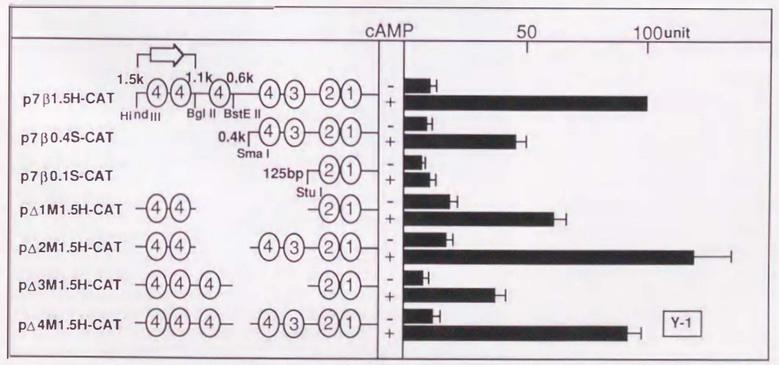


Fig. 2. **Construction of CAT plasmids carrying various regions of the CYP11B gene promoter and their CAT activities in Y-1 cells.** The plasmids for the CAT assay were constructed from p7β1.5H-CAT, which has a 1.5 kb long DNA fragment of the bovine CYP11B gene promoter upstream from the transcription initiation site (*15*). All the plasmids were constructed using the restriction sites of *Hin*dIII at −1.5 kb, *Bgl*II at −1.1 kb, *BstE*II at −0.6 kb, *Sma*I at 407 bp, and *Stu*I at −125 bp. The locations of the *cis*-elements, Ad1, Ad2, Ad3, and Ad4, are indicated by the numbered ovals on each construct. The region from −1.5 to −1.1 kb is indicated by an open arrow. These plasmids were transfected into Y-1 cells and then the cells were used for the CAT assay. The CAT activities indicated by the closed bars are the averages±SEM for three transfection experiments. The CAT activity of p7β1.5H-CAT treated with Bt_2cAMP was taken as one hundred units.

parison between p7β0.1S-CAT and pΔ1M1.5H-CAT indicated that the addition of the upstream region, from −1.5 to −1.1 kb, to p7β0.1S-CAT provided cAMP responsiveness in addition to basal transcriptional activity. Similar results were observed on comparison between p7β0.4S-CAT and pΔ2M1.5H-CAT. Conversely, comparison between pΔ1M1.5H-CAT and pΔ3M1.5H-CAT, or pΔ2M1.5H-CAT and pΔ4M1.5H-CAT indicated that the presence of the region from −1.1 to −0.6 kb reduced the CAT activity in both the presence and absence of cAMP. In a recent report (*20*), we described that the upstream region, from −0.4 to −125 bp, which contained Ad3 and Ad4 sites, was necessary, in addition to Ad1(CRE), for the full activation of the gene by cAMP. The region, from −1.5 to −1.1 kb, which carries two Ad4 sites seemed to have a function equivalent to that of the region from −0.4 to −125 bp.

Several Ad4 sites have been reported to be present in the steroidogenic CYP gene promoters including CYP11A and CYP11B. Many of them were mapped to the promoter regions having transcriptional activity (*27*). As indicated above, some Ad4s of the CYP11A and CYP11B genes are in the region showing the transcriptional activities and the others are not. The −1.1 to −0.6 kb region of the CYP11B promoter carrying an Ad4 site rather seems to have a transcription suppressive function judging from the results of deletion analysis of the region. Similarly, the region from −346 to −573 bp of the CYP11A promoter, which has a transcription suppressive function (*7*), also contains an Ad4 site. In the previous study, we confirmed that the co-existence of Ad3 and Ad4 was required for the cAMP-dependent transcriptional activity of the CYP11B gene promoter (*15*). Our recent experiment with OVEC reporter gene constructs strongly supported the observation. The Ad4 site in an OVEC construct enhanced the transcription depending on the presence of Ad1(CRE), although Ad4 showed little transcriptional activity by itself (*25*). The activating and suppressive functions of the Ad4-containing regions seem to depend on the presence of other *cis*-elements in the regions. As will be described below, other elements, a CRE-like element and Ad7, were present beside Ad4 sites in the distal promoters of CYP11A (−1.8 to −1.5 kb) and CYP11B (−1.5 to −1.1 kb), respectively.

Cis-Elements in the Distal Promoters—We identified the two regions from −1.8 to −1.5 kb of CYP11A, and from −1.5 to −1.1 kb of CYP11B as the distal promoters carrying two Ad4 sites. To determine the structure and function of the Ad4-containing distal promoters, we used DNA fragments corresponding to these two regions for further experiments. First of all, we performed *DNase* I footprint analysis of the fragments using a nuclear extract prepared from bovine adrenal cortex. As shown in Fig. 3a, three portions were protected from *DNase* I digestion in the CYP11A distal promoter. Table I summarizes the nucleotide sequences of the protected regions. Two of them containing Ad4 sites are located at −1,637 to −1,629 bp and −1,727 to −1,719 bp. The former Ad4 site (−1,637/−1,629) was protected more strongly than the latter one (−1,727/−1,719), which was consistent with the binding strength of the purified Ad4BP as to two oligonucleotides carrying −1,642/−1,623 bp and −1,714/−1,733 bp (*27*). The remaining portion, −1,653 to −1,646 bp, adjacent to the former Ad4 site (−1,637/−1,629) contained a CRE-like sequence (tentatively named HSC-CRE), TGATGTCA, carrying one base substi-

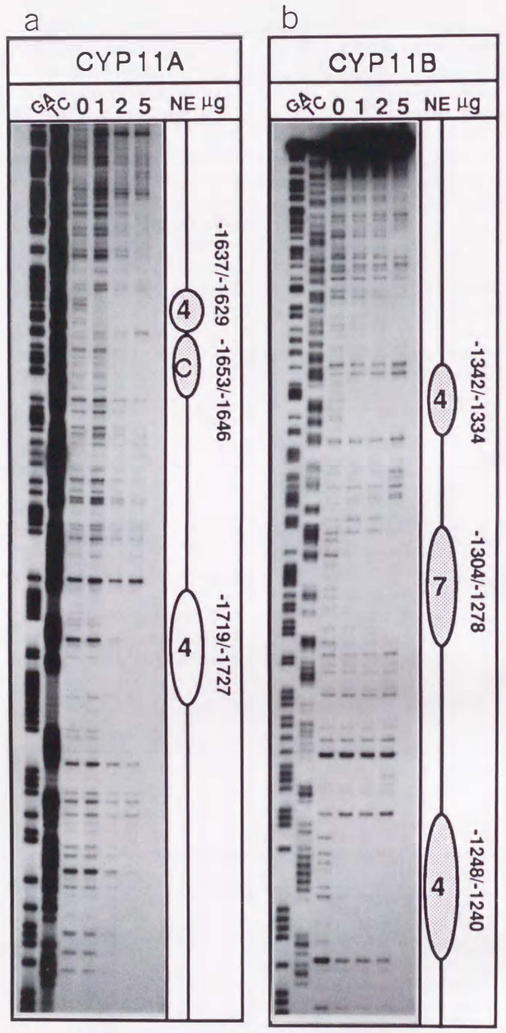


Fig. 3. ***DNase* I footprint analyses of the CYP11A and CYP11B distal promoters with a nuclear extract prepared from bovine adrenal cortex.** (a) The DNA fragment corresponding to the CYP11A distal promoter (−1,841 to −1,523 bp) was end-labeled at −1,841 bp and then used for the footprint analysis. Increasing amounts of the nuclear extract prepared from bovine adrenal cortex (from 0 to 5 μg) were used. G+A and T+C ladders were generated by chemical cleavage (*45*). Shaded ovals indicate the portions protected from *DNase* I digestion and the locations of *cis*-elements (Ad4s and the CRE-like element) are also indicated by nucleotide numbers. (b) The CYP11B distal promoter (−1,467 to −1,145 bp) was analyzed in the same manner. The locations of the protected portions and *cis*-elements (Ad4s and Ad7) are indicated.

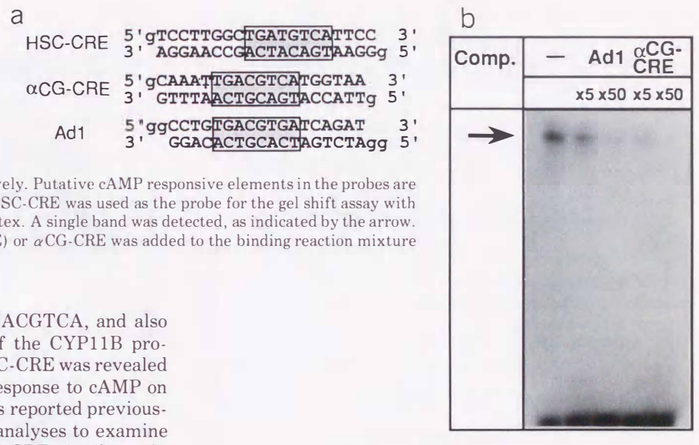


Fig. 4. **Nuclear factor binding to CRE-like element in the CYP11A distal promoter.** (a) Three probes were used for the gel shift assay. The nucleotide sequences of the three probes, HSC-CRE, Ad1(CRE), and αCG-CRE, are correspond to −1,661 to −1,642 bp of the CYP11A gene, −71 to −54 bp of the CYP11B gene, and −129 to −111 bp of the human chorionic gonadotropin-α gene (*32*), respectively. Putative cAMP responsive elements in the probes are indicated by shaded boxes. (b) End-labeled HSC-CRE was used as the probe for the gel shift assay with 10 μg of nuclear extract of bovine adrenal cortex. A single band was detected, as indicated by the arrow. A 5 or 50 molar excess of unlabeled Ad1(CRE) or αCG-CRE was added to the binding reaction mixture as a competitor.

tution from the palindromic CRE, TGACGTCA, and also one from Ad1(CRE), TGACGTGA, of the CYP11B promoter (Fig. 4a) (*13*). Furthermore, HSC-CRE was revealed to exhibit transcriptional activity in response to cAMP on mutational analysis of this sequence, as reported previously (*19*). Then we performed gel shift analyses to examine protein factor(s) interacting with HSC-CRE. As shown in Fig. 4b, when the nuclear extract prepared from bovine adrenal cortex was used, a single retarded signal was detected. The signal with this probe disappeared completely on the addition of a 50-fold molar excess of unlabeled Ad1(CRE) or CRE (αCG) of the human chorionic gonadotropin gene (*32*). Since Ad1(CRE) binds two forms, CREB/ATF proteins (*20*), HSC-CRE of the CYP11A promoter seems to form a complex with the same protein family of CREB/ATF.

Similar experiments were performed with the distal promoter of the CYP11B gene. Three portions were also protected from *DNase* I digestion (Fig. 3b). As in the case of the CYP11A distal promoter, two of them, located at −1,240 to −1,248 bp and −1,342 to −1,334 bp, contained Ad4s. The remaining portion located between the two Ad4 sites, tentatively named Ad7, was less protected than the two Ad4 sites. Comparison of the nucleotide sequence of Ad7 (Table I) with those of other known *cis*-elements revealed that Ad7 contains sequences showing homology with NF-IL6, *myc* or E4TF-1 binding sites (*33*) (Fig. 5a). Gel shift analysis was performed to determine whether or not the factor binding to Ad7 recognizes the homologous sequences. As shown in Fig. 5b, the retarded signal of Ad7 disappeared on the addition of unlabeled NF-IL6 oligonucleotide as well as Ad7. Since NF-IL6 is well characterized as a transcription factor enriched in the liver, the presence of the factor binding to Ad7 was examined with a nuclear extract prepared from rat liver. The retarded signal of Ad7 was also detected and competed by the unlabeled NF-IL6 oligonucleotide. Although the presence of NF-IL6 in the adrenal cortex was not examined, the above result indicated the presence of NF-IL6 or related factor(s) in the adrenal cortex. Determination of the primary structure of NF-IL6 revealed the presence of a basic region and a leucine-zipper (b-ZIP) structure, which is present in the CREB/ATF family in common, for DNA binding and dimer formation (*34–36*). The distal promoters of the CYP11A and CYP11B genes were revealed to have a similar structure, which contains a binding site for the b-ZIP protein between the two Ad4 sites. As described above, the distal promoters of the CYP11A and CYP11B genes contained CRE and NF-IL6 binding sites, respectively.

TABLE I. **Nucleotide sequences of the protected regions of the CYP11A and CYP11B distal promoters determined by footprint analysis.** The Ad4 sites and HSC-CRE site are indicated by shaded boxes. The locations of the protected regions are shown at the left of each nucleotide sequence. The tightly protected nucleotide in the Ad7 site is indicated by dots over the sequence.

CYP11A Distal Promoter	Ad-4 -1637/-1629	5'CAGGCTCAAGGTCATCATGG 3' 3'GTCCGAGTTCCAGTAGTACC 5'
	HSC-CRE -1653/-1646	5'TTGGCTGATGTCATTCCA 3' 3'AACCGACTACAGTAAGGT 5'
	Ad-4 -1719/-1727	5'GTCTTCCAAGGACTAGTTTT 3' 3'CAGAAGGTTCCTGATCAAAA 5'
CYP11B Distal Promoter	Ad-4 -1248/-1240	5'AACTGCCAAGGTCTCTCATC 3' 3'TTGACGGTTCCAGAGAGTAG 5'
	Ad-7 -1304/-1278	5'GAGGACAGCTCTTACGCCACCTGGGGC 3' 3'CTCCTGTCGAGAATGCGGTGGACCCCG 5'
	Ad-4 -1342/-1334	5'GCACCCCAAGGGCAGGCTGG 3' 3'CGTGGGGTTCCCGTCCGACC 5'

Although CRE is well known as a *cis*-element conferring cAMP-responsiveness (*18*), NF-IL6 is not involved in such a cAMP-dependent pathway (*34–36*). In our previous studies (*15, 20*), it was clarified that the Ad4 site located at −331 to −323 bp in the CYP11B gene promoter also contributes to the cAMP-responsive function. The Ad4 site in the distal promoters possibly functions as a cAMP-responsive sequence.

Tissue Specific Transcriptional Activity of the Distal Promoters—The steroidogenic cell-specific transcriptional activities of the CYP11A and CYP11B gene promoters were confirmed in the previous study (*14*). In this study, we examined whether or not the distal promoters of the two genes described above have steroidogenic cell-specific transcriptional activity. Two recombinant plasmids, 3×[S-RR]SV-CAT and 3×[B-RR]SV-CAT, which have SV40 core promoter and three tandem repeats of the distal promoter of the CYP11A gene, 3×[S-RR], or the CYP11B gene, 3×[B-RR], were transfected into steroidogenic Y-1 and I-10 cells. As shown in the upper two panels of Fig. 6,

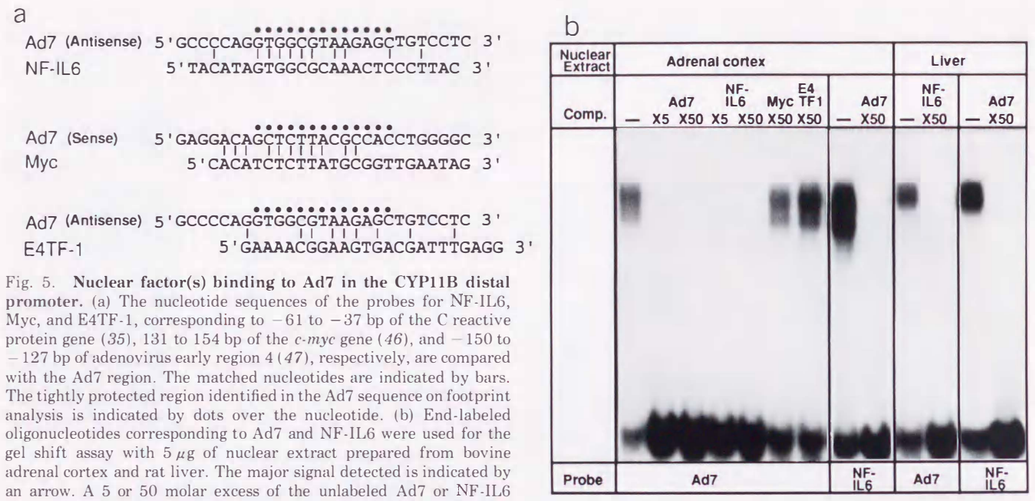


Fig. 5. **Nuclear factor(s) binding to Ad7 in the CYP11B distal promoter.** (a) The nucleotide sequences of the probes for NF-IL6, Myc, and E4TF-1, corresponding to −61 to −37 bp of the C reactive protein gene (*35*), 131 to 154 bp of the *c-myc* gene (*46*), and −150 to −127 bp of adenovirus early region 4 (*47*), respectively, are compared with the Ad7 region. The matched nucleotides are indicated by bars. The tightly protected region identified in the Ad7 sequence on footprint analysis is indicated by dots over the nucleotide. (b) End-labeled oligonucleotides corresponding to Ad7 and NF-IL6 were used for the gel shift assay with 5 μg of nuclear extract prepared from bovine adrenal cortex and rat liver. The major signal detected is indicated by an arrow. A 5 or 50 molar excess of the unlabeled Ad7 or NF-IL6 oligonucleotide, or a 50 molar excess of the unlabeled Myc or E4TF-1 oligonucleotide was added to the binding mixture as competitors.

the SV40 enhancer promoter (SV2-CAT) triggered the transcription of the CAT gene in the two cell lines without any response to cAMP. On the other hand, 3×[S-RR] and 3×[B-RR] enhanced the transcriptional activity of the SV40 core promoter 14- and 14-fold in Y-1 cells, and, 5- and 6-fold in I-10 cells, respectively, in the absence of cAMP. 3×[S-RR] and 3×[B-RR] also conferred cAMP responsiveness to the promoter in both Y-1 and I-10 cells. For investigation of the tissue-specific transcriptional activity of the region, non-steroidogenic CV-1 cells were used for further transfection experiments. An expression vector for the catalytic subunit of protein kinase A (PKA) was used to activate the PKA-dependent signaling pathway, since CV-1 cells do not fully respond to Bt_2cAMP (*37*). When transfected into CV-1 cells, 3×[S-RR] and 3×[B-RR] did not trigger transcription of the CAT gene. The transcriptional activities were still weak even in the presence of the PKA expression vector (lower panel of Fig. 6). These observations indicated that the distal promoters of the CYP11A and CYP11B genes are active only in the steroidogenic cells, even when connected to the heterologous promoter. The transcriptional activities of the distal promoters were further analyzed to clarify the role of Ad4 sites in the regions using an expression vector of the Ad4 site binding protein, Ad4BP, which is expressed only in steroidogenic tissues (*25, 29, 36, 39*) (lower panel of Fig. 6). As described above, 3×[S-RR] and 3×[B-RR] did not trigger transcription in the non-steroidogenic CV-1 cells. Since the inactivity of the regions seemed to be caused by the absence of Ad4BP in CV-1 cells, the Ad4BP expression vector was cotransfected. As expected, the CAT activities driven by the distal promoters, 3×[S-RR] and 3×[B-RR], were significantly induced by the expression of Ad4BP. The PKA expression vector further enhanced the activities by 2-fold. Likewise, the CAT activity of the pCAT plasmid was also enhanced on cotransfection of the PKA expression vector in CV-1 cells. The enhancement by PKA was likely to be dependent on the SV40 core promoter function in CV-1 cells. However, the CAT activities of 3×[S-RR]SV-CAT and 3×[B-RR]SV-CAT were enhanced by the addition of cAMP in Y-1 and I-10 cells, as shown in the upper panel of Fig. 6. Therefore, the distal promoters were considered to have a cAMP-responsive function. These observations indicated that the distal promoters of the CYP11A and CYP11B genes express their functions only in the presence of Ad4BP, showing a good correlation with the steroidogenic tissue specific expression of the CYP11A and CYP11B genes.

Functional Interaction between Distal and Basal Promoters—As indicated above, the two distal promoters had similar effects to the heterologous promoter although their structures are different, except for Ad4 site. To clarify the functional differences between the regions, we examined the functional interaction between the distal promoters and the basal promoters of the CYP11A and CYP11B genes. In the case of the CYP11B distal promoter, 125 bp fragment from the transcription initiation site, which contains two *cis*-elements, Ad1(CRE) and Ad2, was essential for the basal transcriptional activity of the CYP11B gene, as reported previously (*13, 20*). The recombinant plasmids, 3×[B-RR]B125-CAT and 3×[S-RR]B125-CAT, were constructed by combination of the basal promoter of the CYP11B gene and the three tandem repeats of each distal promoter (upper panel of Fig. 7). In the case of the CYP11A gene, Inoue *et al.* (*19*) reported that the region to −44 bp from the transcription initiation site was sufficient for expression of the basal transcriptional activity. Since their result was in good agreement with our finding that an Ad4 site is located at −45 to −37 bp (*27*), we used −45 bp fragment as a putative basal promoter of the CYP11A gene. Then, two additional recombinants, 3×[B-RR]S46-CAT and 3×[S-RR]S46-CAT, were constructed in the same

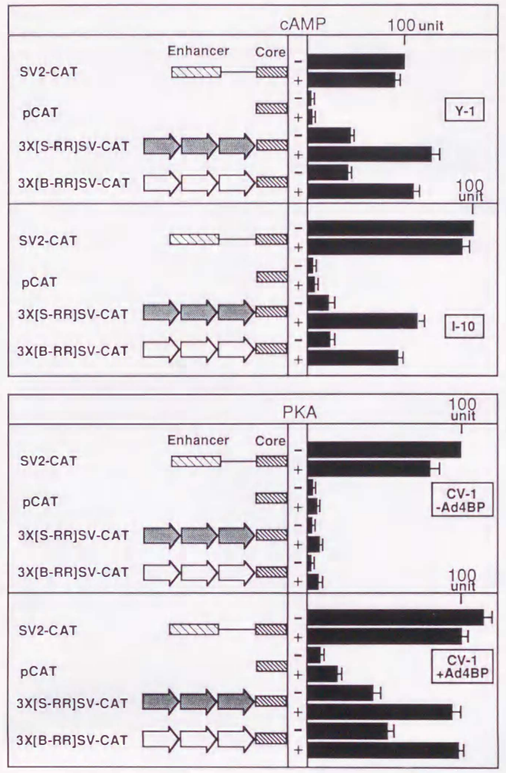


Fig. 6. **Tissue specific transcriptional activity of distal promoters and transcriptional activation on the expression of Ad4BP cDNA.** (Upper panel) 3×[S-RR]SV-CAT and 3×[B-RR]-SV-CAT were constructed by insertion of a three tandem repeat of the CYP11A distal promoter, 3×[S-RR] (shaded arrows), and the CYP11B distal promoter, 3×[B-RR] (open arrows), into pCAT, which contains SV40 core promoter, indicated by a thickly hatched box. These constructs were transfected into Y-1 and I-10 cells. pCAT and SV2-CAT, which contains SV40 enhancer promoter, indicated by a thinly hatched box, were used as controls. The CAT activities indicated by closed bars are the averages ± SEM for three experiments. The CAT activity of SV2-CAT without treatment with Bt_2cAMP was taken as one hundred units. (Lower panel) The 3×[S-RR]SV-CAT and 3×[B-RR]SV-CAT plasmids were transfected into CV-1 cells with or without Ad4BP and PKA expression plasmids, as described under "MATERIALS AND METHODS." pCAT and SV2-CAT were also transfected as controls. The CAT activities indicated by closed bars are the averages ± SEM for three experiments. The CAT activity of SV2-CAT without cotransfection of Ad4BP and PKA was taken as 100 units.

way with the putative basal promoter of the CYP11A gene (lower panel of Fig. 7). The CAT activities of the former two recombinant plasmids are summarized in the upper panel of Fig. 7 with those of p7β1.5H-CAT and p7β0.1S-CAT as controls. p7β0.1S-CAT, that contained the basal promoter alone, showed low level transcriptional activity in both Y-1 and I-10 cells. 3×[B-RR] and 3×[S-RR] when attached to p7β0.1S-CAT enhanced the basal activity 7- and 16-fold in Y-1 cells, and 5- and 50-fold in I-10 cells, respectively. 3×[B-RR] attached to pS46-CAT showed similar responses, *i.e.*, 9- and 5-fold enhancement of the basal activity in Y-1 and I-10 cells, respectively. All these constructs also responded to cAMP. On the contrary, 3×[S-RR] when attached to pS46-CAT did not show any enhancement of either basal transcriptional activity or cAMP responsiveness. Although the distal promoter of CYP11B was functional with both the CYP11A and CYP11B basal promoter regions, the CYP11A distal promoter was not functional with the CYP11A basal promoter, indicating that the two distal promoters require basal promoters with different structures. In contrast with the results described above (*19*), these observations indicated that the 46 bp fragment of the CYP11A promoter region was insufficient for the functional interaction with the distal promoter of CYP11A, although the reason for the discrepancy remains to be determined.

Although both of the CYP genes used in this study are expressed in steroidogenic tissues, the cell-type specificity of the expression in steroidogenic tissues differs. Namely, the CYP11A gene is expressed in many steroidogenic tissues, while the CYP11B gene is expressed only in the adrenal cortex. Comparison of the structures and functions of the two distal promoters was expected to reveal at least in part the mechanism which controls the cell-type specific transcription in steroidogenic tissues. A functional difference between the two distal promoters was observed in I-10 cells, when they were attached to the CYP11B basal promoter. Although both of the CYP genes are transcribed in the adrenal cortex, the CYP11B gene is inactive in testicular Leydig cells. 3×[S-RR] greatly enhanced the transcription, when compared with 3×[B-RR], in I-10 cells, showing a good correlation with the different cell-type specificity of the expression of the CYP11A and CYP11B genes. However, only the function of the CYP11B distal promoter might be insufficient for adrenal specific expression of the gene, because 3×[B-RR]B125-CAT and 3×[B-RR]S46-CAT were significantly active.

Characterization of the CYP11A Basal Promoter—Since the 46 bp fragment was insufficient for expression of the function of the distal promoter of the CYP11A gene, we constructed two additional recombinant plasmids, 3×[S-RR]S57-CAT and 3×[S-RR]S130-CAT, which have 57 and 130 bp of the CYP11A upstream region, respectively. As shown in Fig. 8, 3×[S-RR] attached to pS57-CAT acquired the transcriptional activity and the responsiveness to cAMP in both Y-1 and I-10 cells. 3×[S-RR] attached to the pS130-CAT showed almost the same response compared with 3×[S-RR]S57-CAT. The 11 nucleotides from −57 to −46 bp in the CYP11A upstream region seem to be necessary for the basal promoter function.

Since the CYP11A gene upstream regions of four animal species, man (*11*), cow (*9*), rat (*40*), and mouse (*41*), have been reported, we compared the nucleotide sequences of the basal promoter regions. As shown in Fig. 9a, three highly conserved regions are present in the regions up to −60 bp. In addition to TATA box and the Ad4 site (−37/−45) [or SF-1 (*38*)], a sequence, −58 to −50 bp, is conserved in all the animal species, suggesting the functional importance of region −58/−50. To investigate the factor binding to region −58/−50, gel shift analyses were

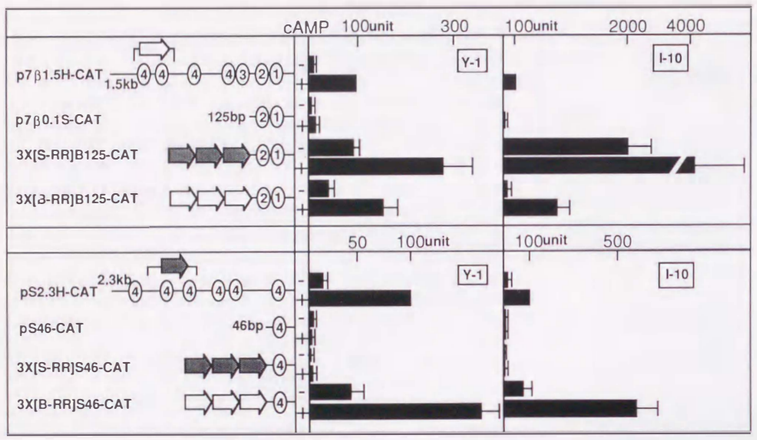


Fig. 7. **Functional interaction between distal and basal promoters.** The recombinant plasmids, 3×[S-RR]B125-CAT and 3×[B-RR]B125-CAT (upper panel), and 3×[S-RR]S46-CAT and 3×[B-RR]S46-CAT (lower panel), were constructed as described under "MATERIALS AND METHODS." These constructs were transfected into Y-1 and I-10 cells. The CAT activities indicated by closed bars are the averages±SEM for three experiments. The CAT activity of p7β1.5H-CAT or pS2.3H-CAT in the presence of Bt_2cAMP was taken as 100 units.

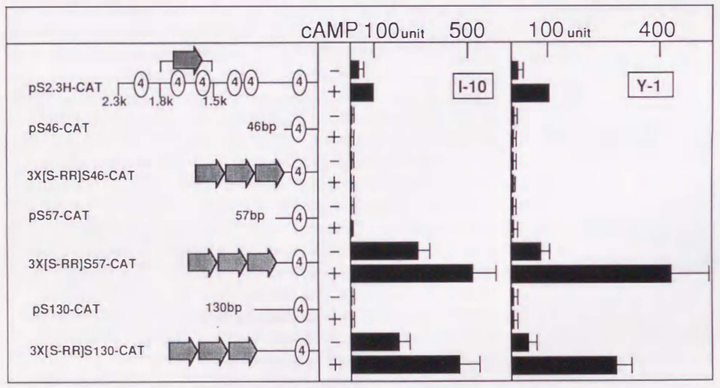
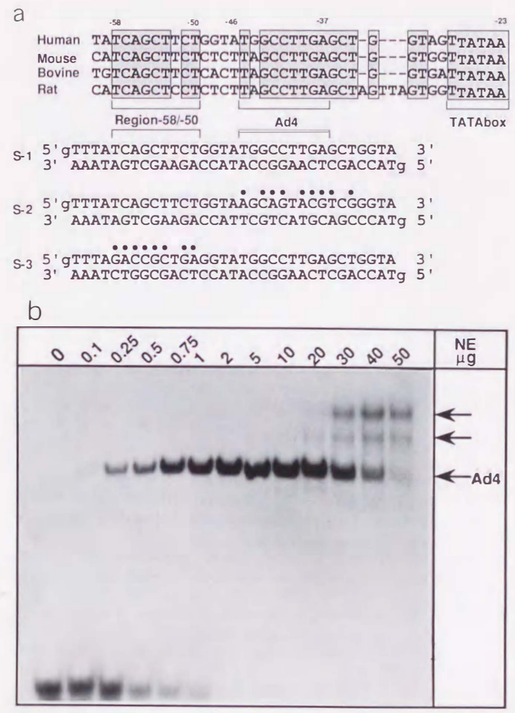


Fig. 8. **CYP11A basal promoter necessary for the function of the CYP11A distal promoter.** 3×[S-RR]-S57-CAT and 3×[S-RR]S130-CAT were constructed by the insertion of 3×[S-RR] at −57 bp of pS57-CAT and −130 bp of pS130-CAT, respectively. These constructs were transfected into Y-1 and I-10 cells. The CAT activities indicated by closed bars are the averages±SEM for three experiments. The CAT activity of pS2.3H-CAT in the presence of Bt_2cAMP was taken as 100 units.

performed with a synthetic oligonucleotide probe (S-1), corresponding to −62 to −30 bp of the CYP11A basal promoter region (Fig. 9a). Since preliminary gel shift analysis revealed faint signals in addition to that of Ad4, suggesting the formation of other complexes with the probe DNA (data not shown), we investigated the gel shift analysis conditions for detecting the complexes clearly. When increasing amounts of the nuclear extract, up to 50 μg, were used, the Ad4 signal showed maximum intensity with 2 to 20 μg of nuclear extract and gradually decreased with increasing amounts of the nuclear extract over 30 μg (Fig. 9b). In contrast, other signals (upper and middle signals in Fig. 9b) appeared with over 30 μg of the nuclear extract.

Under the conditions when the upper signal was clearly detected, other gel shift analyses were performed with three oligonucleotides, S-1, S-2, and S-3, to reveal which signals are derived from the two conserved regions (Fig. 9a). As shown in Fig. 9c, only the middle signal was detected with probe S-2, whereas an intense Ad4 signal and a faint signal corresponding to the middle signal were detected with probe S-3. The upper signal was competed out on the addition of a 50-molar excess of unlabeled S-2, in which mutations were introduced to the Ad4 site, whereas the Ad4 signal increased conversely. On the addition of a 100-molar excess of unlabeled S-3, in which nucleotides −58 to −50 bp were substituted, the Ad4 signal was competed out with a concomitant decrease in the upper signal. The middle signal remained, however, even in the presence of these competitors. These results obtained on the gel shift analyses revealed that the upper signal was formed by the combined participation of Ad4BP and another unknown protein(s). Although we cannot explain the presence of the middle signal in the competition assay with S-2, the nuclear extract seems to contain a factor binding to the −58/−50 region. As described above, it was suggested that the region from −57 to −46 bp of the CYP11A basal promoter has *cis*-element(s) necessary for the transcriptional activity of the distal promoter. The occurrence of the nuclear protein binding to the region (−57 to −40 bp) indicated by the gel shift analyses showed a good correlation with the results of the functional anal-



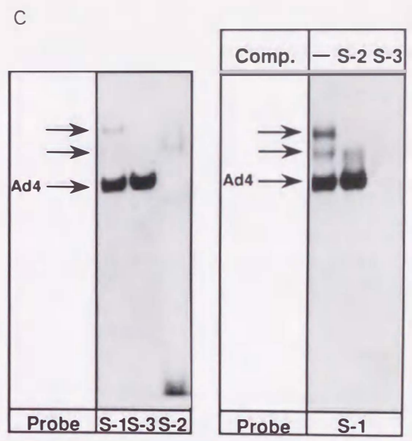


Fig. 9. **(a) Alignment of the 5′ upstream sequences of the human, mouse, bovine, and rat CYP11A genes, and synthetic oligonucleotide sequences for the gel shift assay.** The upstream regions of the human (*11*), mouse (*41*), bovine (*9*), and rat (*40*) genes are aligned. Gaps were introduced to maximize sequence identity. The bases identical in the four animals are indicated by shaded boxes. Three highly conserved regions (TATA box, Ad4, and Region −58/−50) are indicated by brackets. The numbers over the alignment correspond to the positions from the transcription initiation site of the human CYP11A gene. Under the alignment, the nucleotide sequences of the three synthetic oligonucleotides, S-1, S-2, and S-3, used for the gel shift assay are shown. Nucleotide S-1 corresponds to −62 to −30 bp of the human CYP11A gene promoter. The Ad4 site and Region −58/−50 are mutated in S-2 and S-3, respectively. The nucleotide substitutions indicated by dots were introduced into the S-1 oligonucleotide to make S-2 and S-3. **(b) Gel shift assay with a synthetic probe, S-1.** Increasing amounts (0 to 50 μg) of a nuclear extract prepared from bovine adrenal cortex were used for the gel shift assay. Three bands are indicated by arrows. The lower band corresponds to the Ad4 signal. **(c) Gel shift assay with probes S-1, S-2 and S-3.** (Left panel) The gel shift assay was performed with probes S-1, S-2, and S-3. Forty micrograms of nuclear extract was used to detect the three different complexes indicated by arrows. (Right panel) A competition assay was performed with S-2 and S-3 as competitors, S-1 being used as the probe. A 100 molar excesses of unlabeled nucleotide S-2 or S-3 was added to the binding reaction mixture.

yses. A protein complex on the basal promoter may be necessary for the interaction between the basal and distal promoters of the CYP11A gene.

Two *cis*-elements in the CYP11A basal promoter region, basic transcription element (BTE), originally identified in the rat CYP1A1 gene promoter (*42*), and cAMP responsive sequence (CRS) (*43*), were reported previously. A BTE homologue was reported to be located at −86 to −71 bp, whereas the transcriptional activity of the sequence was not detected in this study. CRS was also reported to be a cAMP responsive sequence located at −118 to −100 bp in the bovine CYP11A promoter region. Likewise, we did not find transcriptional activity in the CRS-containing region. The discrepancy between this study and the previous ones might have been caused by the different reporter plasmids used. For the functional analysis of CRS, OVEC, which has a minimal promoter of the β-globin gene, was used, whereas we used the native CYP11A and CYP11B promoter regions in this study.

Recently, Moore *et al.* (*44*) identified, in the human CYP11A gene, a powerful basal element between −152 and −142, a repressor element between −177 and −152, and a forskolin-responsive region between 108 and −89, which express their own functions specifically in JEG-3 choriocarcinoma cells. Our observations concerning the basal promoter elements agree with their report, which described the absence of the functions in the three regions in Y-1 cells. The CYP11A gene is transcribed in almost all steroidogenic cells, although the functions of the cells are different. Different *cis*-elements in the CYP11A gene seem to be functional in different cell types.

In this study, we identified two distal promoters in the human CYP11A and bovine CYP11B genes, which triggered the transcription of the genes in heterologous as well as homologous combinations with the basal promoters in both the presence and absence of cAMP. The distal promoters containing Ad4 sites expressed their functions only in steroidogenic cells, indicating the significant contributions of the Ad4 sites and Ad4BP to the tissue-specific expression of the CYP11A and CYP11B genes. On the other hand, these distal promoters showed different requirements for the basal promoters. The difference in the interactions between the distal promoters and the basal promoters is interesting in view of the expression of the CYP11A and CYP11B genes in different tissues.

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