Autoregulatory Loop in the Regulation of the Mammalian ftz-f1 Gene

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The mammalian ftz-f1 (mftz-f1) gene encoding Ad4BP/SF-1 has been demonstrated to be essential for the development of adrenal and gonadal glands. In a previous study, we identified an E box as the transcriptional element in the 5′-upstream region of the rat mftz-f1 gene. In the present study, we found a steroidogenic cell-specific transcriptional element in the first intron of the gene. Gel mobility shift and DNase I footprint analyses clearly revealed that Ad4BP itself binds to the element (Ad4 site). This finding was further supported by the positive effect of an Ad4BP expression vector on the transcription and by the significant decrease in the transcription caused by nucleotide substitutions within the Ad4 site. Similar loss was also caused by substitutions in the E box, indicating that the two elements are essential for the full transcriptional activity of the gene. DNase I hypersensitivity assays revealed that the chromatin structure around the Ad4 site and the E box was “open up” in the adrenal glands and Y-1 cells, whereas “closed down” in the liver. These observations indicated that the mftz-f1 gene is controlled by the autoregulatory loop in the steroidogenic tissues. The autoregulatory mechanism seems to be necessary to keep the mftz-f1 gene activated and thus maintain the tissues differentiated.

Ad4BP (or SF-1) was identified as a tissue-specific transcription factor that regulates all the steroidogenic P-450 genes in the adrenal cortex and gonads (1, 2). Analyses of the cDNA clone encoding Ad4BP revealed that the factor is a member of the steroid/thyroid hormone receptor superfamily (3), in which all the members have a zinc finger motif as the DNA binding domain (4). A functional study using an expression vector for Ad4BP confirmed that the transcription factor governs the tissue-specific expression of the steroidal P-450 genes (5). Recently, wider functions of Ad4BP in addition to the regulation of the steroidogenic P-450 genes have been shown by the following investigations.

The expression of Ad4BP was examined immunohistochemically using the specific antisera (6) and in situ hybridization (7). Consistent with its role in the regulation of the steroidogenic P-450 genes, Ad4BP was confirmed to be expressed in the steroidogenic cells of the adrenal gland and gonads. In addition, Ad4BP was found to be expressed in the pituitary gonadotroph, which secretes follicle-stimulating hormone and luteinizing hormone, as well as in the ventromedial hypothalamic nucleus (8–10), which controls female sexual behavior (11). Considering the physiological functions of those tissues, it is clear that Ad4BP is one of the essential factors which control the reproductive function of animals, although the genes controlled by Ad4BP in the ventromedial hypothalamic nucleus still remain to be clarified.

Further investigation of the developing tissues revealed that Ad4BP is expressed even in the primordial cells of the adrenal gland and gonads of the fetuses (7, 12). In the fetal gonads, a significant amount of Ad4BP was expressed in the testes, whereas only a trace amount was expressed in the ovaries. Sexual differentiation of the gonads is known to be initiated by the transient expression of SRY in the somatic cells of the urogenital ridge (13). The sexually dimorphic expression of Ad4BP observed in the differentiating gonads suggested that the mammalian ftz-f1 (mftz-f1) gene encoding Ad4BP might be one of the genes located downstream to SRY. It is likely that the gonads express the steroidogenic P-450s and Müllerian inhibitory substance in a sex-dependent manner as a consequence of the dimorphic expression of Ad4BP (12, 14). In the adrenal glands, however, a significant amount of Ad4BP was continuously expressed throughout the life regardless of the sexes (12).

These postulated functions of Ad4BP have been supported by the targeted disruption of the mftz-f1 gene (10, 15–17). Ad4BP null mice showed a complete absence of the adrenal gland and gonads, indicating that Ad4BP governs the genes essential for the adrenal and gonadal differentiation. Therefore, information about the transcriptional mechanism of the mftz-f1 gene is essential for the elucidation of the molecular basis of adrenal and gonadal differentiation. In our previous report, an E box (18) responsible for the specific expression of the gene in the steroidogenic cells was identified in the 5′-upstream region (19). In the present study, we focused our attention on the contribution of the first intron to the gene expression and identified an Ad4 site as the transcriptional element. The function of the Ad4 site in the expression of the mftz-f1 gene clearly indicated that the gene is autoregulated by its own product, Ad4BP.

EXPERIMENTAL PROCEDURES

Plasmid Constructions for CAT Assay—As described previously (19), Ad4CAT2.0K and Ad4CAT0.8K contain the 2.0 and 0.8 kb upstream regions of the mftz-f1 gene.

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‡ The abbreviations used are: kb, kilobase; P-450, cytochrome P-450; CAT, chloramphenicol acetyltransferase; CYP11A, gene encoding side-chain cleavage P-450; CYP11B, gene encoding 11β-hydroxylase P-450 (40); bp, base pair(s).
regions from the EcoRI site in the first exon of the rat mftz-f1 gene, respectively, and CAT reporter gene (20). Ad4ECAT0.8K contains the 5.6-kb DNA fragment between the EcoRI site at 2.0-kb upstream region from the transcriptional initiation site and a Smal site generated by site-directed mutagenesis in the second exon at 5 bp upstream from the initiation methionine. Ad4ECAT2.0K was employed to generate deletion constructs by either digestion or partial digestion at the restriction enzyme sites as shown in Fig. 1. For the construction of Ad4ECAT0.8K, the BamHI site at 0.8 kb upstream from the transcription initiation site was used. Ad4ECAT0.8K, Ad4ECATΔ2.2K, Ad4ECATΔ1.8K, Ad4ECATΔ0.8K, and Ad4ECATΔ0.4K were constructed by using the XbaI site at 3.0 kb, BamHI site at 2.2 kb, HindIII site at 1.8 kb, SacII site at 0.8 kb, and XbaI site at 0.4 kb upstream from the splice acceptor site, respectively. Ad4ECATΔXX, Ad4ECATΔXX-ΔXX, Ad4ECATΔXX-ΔXX, and Ad4ECATΔXX-ΔXX-ΔXX were obtained by internal deletions of 1.4-kb HincII-XbaI, 1.8-kb BamHI-XbaI, 2.6-kb XbaI-OXbaI, and 3.2-kb PmacI-XbaI fragments, respectively. Ad4ECATΔXX-ΔXX-ΔXX, -ΔA, -A24, and -A0, and -B82, -B36, -B17, and -B1 were constructed from Ad4ECATΔXX by Bal31 nuclease digestion from the XbaI site to the upstream and downstream regions, respectively. The deletion end points were determined by DNA sequencing. The numbers preceded by A and B indicate the distances from the splice donor and splice acceptor sites, respectively. Ad4CAT0.8K-EF and -ER were generated by insertion of a 91-bp fragment of the genomic fragment downstream of the map of the gene. Ad4CAT2.0K carries the 2.0-kb upstream region from the EcoRI site in the first exon as described previously (19). All the other plasmids were constructed as described under “Experimental Procedures.” The arrows indicate the splice donor and acceptor sites. The CAT activities are shown in comparison with that of Ad4ECAT2.0K in the right panel.

Fig. 1. Identification of the regulatory region in the first intron of the rat mftz-f1 gene. Transient transfection assays using adenoviral Y-1 cells were performed with the deletion constructs indicated below on the map of the gene. Ad4CAT2.0K carries the 2.0-kb upstream region from the EcoRI site in the first exon as described previously (19). All the other plasmids were constructed as described under “Experimental Procedures.” The arrows indicate the splice donor and acceptor sites. The CAT activities are shown in comparison with that of Ad4ECAT2.0K in the right panel.

Ad4CAT0.8K and Ad4CAT0.8KM were generated by insertion of a 91-bp fragment of the genomic fragment (underlined) was used as the primer. The nucleotide substitution in dENC (underlined). An antiserum to Ad4BP was added after the reaction with the Klenow fragment in the presence of [32P]dCTP (110 mCi/mmol, Amersham, United Kingdom). All transfection experiments were performed at least 3 times.

DNase I Footprint Analysis—A DNA fragment spanning from +57 bp to +316 bp was obtained by polymerase chain reaction, inserted into the Smal site of pUC19, and digested at either the EcoRI or HindIII site of the polylinker. After end labeling with polynucleotide kinase in the presence of [γ-32P]ATP (222 TBq/mmol, Amersham), DNase I footprinting was performed with a nuclear extract prepared from Y-1 cells as described previously (22).

Gel Mobility Shift Assay—Double stranded oligonucleotides, dENC, containing the protected sequence from the DNase I digestion in the first intron (5′-GTAAGTGAAGGCCGGGGCCCAGGCCT-3′/3′-ATTCCCGCCCCGGGTCCCGAGA-5′) and dAd4, containing an authentic Ad4 site in the promoter region of the bovine CYP11B (5′-GAGCATATCCCAAGGTCCCTTTT-3′/3′-TGATATGGTTCCCAGGGAAAGG-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 a labeling reaction with the Klenow fragment in the presence of [γ-32P]dCTP (110 TBq/mmol, Amersham). For competition assays, a 50-fold molar excess amount of the nonradiolabeled double stranded oligonucleotides, dENC, dEM1 (5′-GACTGTGAAGCCGGGGCCCAGGCCT-3′/3′-TGACACTTCCCGCCCCGGGTCCCGAGA-5′), dEM2 (5′-GTAAGTGAAGCCGGGCCCAGGCCTTATGAGCTTTT-3′/3′-ATTCCCGCCCCGGGTCCCGAGA-5′), and dEM3 (5′-GTAAGTGAAGCCGGGCCCAGGCCTTATGAGCTTTT-3′/3′-ATTCCCGCCCCGGGTCCCGAGA-5′) were added prior to the addition of the probe. In the above oligonucleotides, dEM1-M6 have three sequential nucleotide substitution in dENC (underlined). An antiserum to Ad4BP was added after the addition of the probe. The procedure for the gel mobility shift assay
and the initiation methionine are indicated by the CAT gene is also indicated in the second exon. The E box, Ad4 site, constructs indicate the truncated positions. The connecting site with second exons. The addition to the 2.0 kb upstream from the Ad4ECAT2.0K which contains the 3.4-kb long first intron in the rat mftz-f1 gene—

The transcription element in the 5'-upstream region of the rat mftz-f1 gene has a pyrimidine-rich sequence at the 3'-splice junction and/or from the 5'-splice junction (Fig. 2). To locate the transcriptional element more precisely, the transcriptional activities of Ad4ECATΔXX-A24 and -A0. In the Ad4ECATΔXX-B series, on the other hand, a 3-fold increase of the CAT activity was observed when the deletion reached to 82 bp from the 3'-splice junction (Ad4ECATΔXX-B82), while a decrease of the CAT activity was observed when Ad4ECATΔXX-B17 to -B17. Further deletion to 1 bp (Ad4ECATΔXX-B1) abolished the CAT activity. It is widely accepted that the splice junction sequences for correct splicing require not only a GT-AG rule but a pyrimidine nucleotide cluster just upstream from the 3'-splice junction and the consensus several nucleotides at the 5'-splice junction (24–26).

FIG. 3. Transcriptional activity of the regulatory region in Y-1 cells. The genomic 91-bp fragment (+146/-236) was inserted into Ad4CAT0.8K or pCAT plasmid in forward (Ad4CAT0.8K-EF and pCAT-EF) and reverse (Ad4CAT0.8K-ER and pCAT-ER) directions as described under “Experimental Procedures.” The relative CAT activities in comparison to the original plasmids (Ad4CAT0.8K and pCAT) were averaged for three experiments (±S.E.) and are shown on the right of the figure.

the CAT plasmid in Y-1 cells by reverse transcriptase-polymerase chain reaction analyses. The amount of the ELP mRNA was significantly lower than that of Ad4BP, showing a good correlation with our previous observation (6) (data not shown). Therefore, the CAT activity of Ad4ECAT mostly reflects the Ad4BP promoter activity.

To identify the transcriptional element in the first intron, various deletion plasmids were constructed based on Ad4ECAT2.0K as shown in Fig. 1. A drastic decrease of the CAT activity was observed when the 5'-deletion reached to the XbaI site (5'-side) (Ad4ECATΔ3.0K). Further truncations to the BamHI, HindIII, SacI, and Xbal (3'-side) sites (Ad4ECATΔ2.2K,Δ1.8K,Δ0.8K, and Δ0.4K, respectively) abolished the remaining weak CAT activity. No remarkable change in the CAT activity was observed when Ad4ECATΔ3.0K. The decrease seems to be due to the lack of both the first exon and the E box element, the latter of which had been identified as a transcriptional element located from -82 to -77 bp (19). Judging from these observations, the transcriptional element in the first intron is likely to be located from the 5'-splice junction to the XbaI site (5'-side) and/or from the XbaI site (3'-side) to the 3'-splice junction. To locate the transcriptional element more precisely, the transcriptional activities of Ad4ECATΔXX-A and -B series of the CAT plasmids were investigated. In the case of the Ad4ECATΔXX-A series, a drastic decrease of the CAT activity was observed between Ad4ECATΔXX-A24 and -A0. In the Ad4ECATΔXX-B series, on the other hand, a 3-fold increase of the CAT activity was observed when the deletion reached to 82 bp from the 3'-splice junction (Ad4ECATΔXX-B82), while a decrease of the CAT activity was observed with the plasmids from Ad4ECATΔXX-B37 to -B17. Further deletion to 1 bp (Ad4ECATΔXX-B1) abolished the CAT activity.

RESULTS

Identification of the Transcriptional Element in the First Intron of the Rat mftz-f1 Gene—We previously identified the E box as the transcriptional element in the 5'-upstream region of the rat mftz-f1 gene (19). In the present study, a further investigation of the gene transcription was performed with the CAT reporter gene constructs indicated in Fig. 1. Interestingly, Ad4ECAT2.0K which contains the 3.4-kb long first intron in addition to the 2.0 kb upstream from the EcoRI in the first exon showed an approximately 200-fold stronger CAT activity than Ad4CAT2.0K which lacks the first intron (19). The CAT activity of Ad4CAT2.0K was examined in other steroidogenic I-10 cells. Moreover, in Y-1 cells, the activity shown by Ad4CAT2.0K showed an approximately 200-fold stronger CAT activity than Ad4ECAT2.0K as shown in Fig. 1. A drastic decrease of the transcriptional activity of Ad4ECAT2.0K was examined in other steroidogenic I-10 cells. The enhancement of the transcriptional activity of Ad4CAT2.0K showed an approximately 200-fold stronger CAT activity than Ad4ECAT2.0K as shown in Fig. 1. A drastic decrease of the transcriptional activity of Ad4ECAT2.0K was examined in other steroidogenic I-10 cells.

To identify the transcriptional element in the first intron, various deletion plasmids were constructed based on Ad4ECAT2.0K as shown in Fig. 1. A drastic decrease of the CAT activity was observed when the 5'-deletion reached to the XbaI site (5'-side) (Ad4ECATΔ3.0K). Further truncations to the BamHI, HindIII, SacI, and Xbal (3'-side) sites (Ad4ECATΔ2.2K,Δ1.8K,Δ0.8K, and Δ0.4K, respectively) abolished the remaining weak CAT activity. No remarkable change in the CAT activity was observed when Ad4ECATΔ3.0K. The decrease seems to be due to the lack of both the first exon and the E box element, the latter of which had been identified as a transcriptional element located from -82 to -77 bp (19). Judging from these observations, the transcriptional element in the first intron is likely to be located from the 5'-splice junction to the XbaI site (5'-side) and/or from the XbaI site (3'-side) to the 3'-splice junction. To locate the transcriptional element more precisely, the transcriptional activities of Ad4ECATΔXX-A and -B series of the CAT plasmids were investigated. In the case of the Ad4ECATΔXX-A series, a drastic decrease of the CAT activity was observed between Ad4ECATΔXX-A24 and -A0. In the Ad4ECATΔXX-B series, on the other hand, a 3-fold increase of the CAT activity was observed when the deletion reached to 82 bp from the 3'-splice junction (Ad4ECATΔXX-B82), while a decrease of the CAT activity was observed with the plasmids from Ad4ECATΔXX-B37 to -B17. Further deletion to 1 bp (Ad4ECATΔXX-B1) abolished the CAT activity.

The strong and steroidogenic cell-specific transcription of the Ad4BP promoter activity.

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It is widely accepted that the splice junction sequences for correct splicing require not only a GT-AG rule but a pyrimidine nucleotide cluster just upstream from the 3'-splice junction and the consensus several nucleotides at the 5'-splice junction (24–26). Indeed, the mftz-f1 gene has a pyrimidine-rich sequence at the 3'-splice junction, (T/C)₅ACAG/G, and the consensus sequence, CA/GTAAGT, at the 5'-splice junction (Fig. 2). Taking the rule into consideration, the decrease of the CAT activity between Ad4ECATΔXX-B17 and -B1 seems to be caused by the impairment of the consensus 3'-sequence. However, the CAT plasmid in Y-1 cells by reverse transcriptase-polymerase chain reaction analyses. The amount of the ELP mRNA was significantly lower than that of Ad4BP, showing a good correlation with our previous observation (6) (data not shown). Therefore, the CAT activity of Ad4ECAT mostly reflects the Ad4BP promoter activity.

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ever, since it was not clear whether the decrease between Ad4ECATΔXX-B37 and -B17 resulted from the deletion of a putative transcriptional element or a decrease in the splicing efficiency, we investigated the structure of the mRNA transcribed from Ad4ECATΔXX-B17. A significant portion of the mRNA was found to be unspliced (data not shown). Accordingly, it is likely that the decrease of the CAT activity mainly resulted from the decrease in the splicing efficiency. On the other hand, there was also a possibility that a putative transcriptional element is located in the 24-bp nucleotides from the 5’ splice junction (+154/+177). To examine the transcriptional activity of the region, Ad4CAT0.8K-EF and -ER were constructed by insertion of a 91-bp fragment (+146/+236) including the 24-bp nucleotides at the Eco81I site (–265) of Ad4CAT0.8K. The CAT activities in Y-1 cells increased by about 7-fold by the insertion of the fragment regardless of their orientations as shown in Fig. 3. The fragment also increased the CAT activities of the SV40 core promoter by about 5-fold (pCAT-EF and -ER) (Fig. 3).

Binding of Ad4BP to the Transcriptional Element of Its Own Gene—The binding factor(s) to the transcriptional element described above was investigated with the nuclear extract prepared from Y-1 cells. DNase I footprint analyses were performed using a DNA fragment from +57 to +316 containing the element. When the nuclear extract prepared from Y-1 cells was used, a single region was protected from DNase I digestion as shown in Fig. 4A. Interestingly, the protected region, 5’-TGAAGGCCGGGGCCCA-3’, contained a possible sequence (underlined) recognized by Ad4BP (Ad4 site) (1). To determine whether Ad4BP or other proteins were responsible for the observed binding, gel mobility shift assays were performed with labeled dENC containing the protected sequence from the DNase I digestion. For characterization of the binding sequence, competition experiments were also performed with the oligonucleotides carrying nucleotide substitutions (dEM1 to dEM6) (Fig. 4B). As shown in Fig. 4C (left panel), a single complex formation with dENC was observed and was completely inhibited by an excess amount of nonradiolabeled dENC. The oligonucleotides, dEM2 and M3, carrying disrupted Ad4 sites did not function as competitors, whereas the other oligonucleotides, dEM1, M4, M5, and M6, carrying the intact Ad4 site were able to function as the competitors. As shown in Fig. 4C (right panel), the complexes with dENC showed the same mobility on a polyacrylamide gel as that with dAd4, an authentic Ad4 site in the bovine CYP11B (22). The signals with the two distinct probes completely disappeared by the nonradiolabeled oligonucleotides each other. Finally, both complex formations were inhibited by the addition of an antiserum to Ad4BP (24). The same results were also obtained with a nuclear extract prepared from rat adrenal glands.

Autoregulatory Loop in the mftz-f1 Gene Transcription—To investigate the function of the Ad4 site directly, the GAAGGCCG sequence in Ad4ECAT0.8K was mutagenized to GAATATCG to generate Ad4ECAT0.8KA. The mutated sequence was confirmed to be incapable of binding to Ad4BP as shown in Fig. 4A. The DNA fragment from +57 to +316 bp carrying the region was end-labeled at +57 bp and used for footprint analysis. Increasing amounts of the nuclear extract prepared from Y-1 cells (from 0 to 25 µg) were used. To determine the protected region, G + A and T + C ladders were prepared by chemical cleavage of the probe (41). The shaded oval indicates the portion protected from the DNase I digestion. The corresponding nucleotide sequence is shown in bold letters. B, the nucleotide sequences of the probes used in the gel mobility shift assays. dENC contains the protected region from the DNase I digestion. Each of the underlined three nucleotides is substituted to make dEM1 to dEM6. The closed box indicates the Ad4 site. C, gel mobility shift assays with the nuclear extract prepared from Y-1 cells. The 32P-end labeled oligonucleotides, dENC or dAd4, were incubated with 5 µg of the nuclear extract. For the competition assays, a 50-fold molar excess of each nonradiolabeled oligonucleotide was added prior to the addition of the probes. The antiserum to Ad4BP (sAd4BP) was added after the addition of the probes. The incubation mixtures were then examined on a 4.5% polyacrylamide gel. The complexes with dENC and dAd4 showing the same mobility are indicated by an arrowhead.

**Fig. 4. Binding of Ad4BP to the regulatory region.** A, DNase I footprint analysis of the regulatory region. The DNA fragment from +57 to +316 bp carrying the region was end-labeled at +57 bp and used for footprint analysis. Increasing amounts of the nuclear extract prepared from Y-1 cells (from 0 to 25 µg) were used. To determine the protected region, G + A and T + C ladders were prepared by chemical cleavage of the probe (41). The shaded oval indicates the portion protected from the DNase I digestion. The corresponding nucleotide sequence is shown in bold letters. B, the nucleotide sequences of the probes used in the gel mobility shift assays. dENC contains the protected region from the DNase I digestion. Each of the underlined three nucleotides is substituted to make dEM1 to dEM6. The closed box indicates the Ad4 site. C, gel mobility shift assays with the nuclear extract prepared from Y-1 cells. The 32P-end labeled oligonucleotides, dENC or dAd4, were incubated with 5 µg of the nuclear extract. For the competition assays, a 50-fold molar excess of each nonradiolabeled oligonucleotide was added prior to the addition of the probes. The antiserum to Ad4BP (sAd4BP) was added after the addition of the probes. The incubation mixtures were then examined on a 4.5% polyacrylamide gel. The complexes with dENC and dAd4 showing the same mobility are indicated by an arrowhead.
Fig. 5. Functional analyses of the E box and the Ad4 site. The recombinant plasmids shown in the upper panel were constructed from Ad4ECAT0.8K by nucleotide substitution in either or both the E box and the Ad4 site. The crosses indicate the substitutions introduced. These constructs were transfected into Y-1 cells. The relative CAT activities to Ad4ECAT0.8K were averaged for three experiments (±S.E.) and are shown in the lower panel.

Fig. 6. Activation of the mftz-f1 gene promoter by the expression of Ad4BP. The expression vector for Ad4BP (Ad4BP) or the truncated form, Ad4BP207 (Δ207), was cotransfected with the CAT reporter plasmids, Ad4ECAT2.0K and Ad4ECAT2.0KA, into CV-1 cells. The CAT activities relative to that of Ad4ECAT2.0K with no effector (−) are shown. The closed and shaded bars indicate the CAT activities expressed by Ad4ECAT2.0K and Ad4ECAT2.0KA, respectively.

strong evidence for the autoregulatory loop involved in the transcriptional regulation. The significant transcriptional enhancement by the first intron gave us a clue to find the autoregulatory loop. The regulatory region was located near the splice donor site in the first intron and the presence of an Ad4 site in the region was confirmed by using the specific antisem to Ad4BP in the gel mobility shift analyses. In a previous paper (1), we described the purified Ad4BP bound to PuPuAGGTCA as well as PyCAAGGPyPyPu. Although the Ad4 site (GAAG-GCCG) identified in the present study satisfied neither of the two consensus sequences completely, it is likely to be an active derivative of the former sequence, where TCA at the 3' side are changed to CCG. Based on our previous observation that the former consensus sequence showed a weaker binding affinity than the latter (1), the Ad4 site identified in this study has relatively weak affinity in spite of its strong transcriptional activity. The inconsistency between the transcriptional activity and the binding affinity is probably explained by the following observation. In the mftz-f1 gene, the E box is the essential cis-element functioning cooperatively with the Ad4 site since the disruption of the E box caused a significant decrease of the transcriptional activity even in the presence of the intact Ad4 site. A similar decrease was also observed when only the Ad4 site was inactivated. These observations support the notion that the transcriptional activity of the Ad4 site is regulated by the function of the E box and thereby the Ad4 site shows the strong transcriptional activity in spite of the weak binding affinity.

The regulatory region including the Ad4 site was shown to activate both the original mftz-f1 gene promoter carrying the E box and the heterologous SV40 basal promoter. In both cases, however, the transcriptional activations by the region were insufficient to explain the significant transcriptional enhancement by the first intron. A possible reason for the discrepancy is as follow. It was reported in several genes that the splicing reaction itself seems to be critical for efficient production of the mRNA species (27, 28). It is possible that the pre-mRNA from Ad4ECAT0.8K was efficiently spliced to produce the mature
The E box and the Ad4 site are shown. The restriction enzyme sites in the region are identical between the rat and mouse. Hin-d digested nuclei were subjected to Southern blotting. The genomic DNAs purified from the digested nuclei were subjected to Hind III digestion and then to Southern blotting. The 1.0-kb fragment indicated in the map was used as the probe. The E box and the Ad4 site are shown. The restriction enzyme sites in the region are identical between the rat and mouse mtz-f1 genes. The numbers above the autoradiographs are the concentrations of the DNase I. Size markers are shown on the left of the panel. The arrowheads indicate the digested fragments.

mRNA, whereas those from Ad4CAT0.8K-ES/-ER and pCAT-ES/-ER were not. The function of the Ad4 site in the first intron of the mtz-f1 gene was further supported by the cotransfection assays with the expression vector for Ad4BP using CV-1 cells. The transcription was activated only when the CAT vector carrying the intact Ad4 site and the expression vector coding intact Ad4BP were used, although the activation by the coexpression of the expression vector coding intact Ad4BP was smaller than expected. Since the transcription of the CYP11A and CYP11B genes was activated by Ad4BP only in the presence of an expression vector for the catalytic subunit of protein kinase A (5), the effect of the protein kinase A was also examined. However, a further activation was not achieved with the mtz-f1 gene. Considering the cooperative function of the E box and the Ad4 site, the low activation might be due to the absence of the E box binding factor in CV-1 cells. In fact, when the nuclear extracts prepared from Y-1 cells and CV-1 cells were examined by the gel mobility shift analyses, the E box binding factor was detectable with the Y-1 but not with the CV-1 nuclear extract (data not shown).

Differentiated tissues originate from the primordial cells through successive events. Various sets of genes express their functions at the destined time points along the differentiation steps, and finally the tissues acquire their specific functions by expressing the final set of the tissue-specific genes. In the case of the adrenal cortex, the differentiated tissue is able to synthesize steroid hormones owing to the functions of the steroidogenic tissue-specific P-450s. When the differentiation process is considered, it seems reasonable to suppose that a gene regulatory cascade functions along the process, in which the genes encoding specific transcription factors are involved as the components. In the cascade required for adrenocortical differentiation, Ad4BP should be located upstream of the adrenocortical specific genes including the steroidogenic P-450 genes, while it is located downstream of other genes regulating the mtz-f1 gene transcription. It was clarified in the present study that the mtz-f1 gene is activated by the autoregulatory loop. Therefore, it is quite interesting to suppose that once the autoregulatory loop starts to function in the particular cell types, the upstream genes essential for the initial activation of the mtz-f1 gene transcription are no longer necessary thereafter to maintain the Ad4BP expression. Such the autoregulation was reported to function in the HOX4C (29), Hox 4.2 (30), MyoD1 (31), and pit-1 (32–34) genes in mammals, and the fushi tarazu (35), deformed (36), even-skipped (37), Ultrabithorax genes (38), and sex-lethal (39) genes in Drosophila. Interestingly, all the genes listed above have been reported to play significant roles in the differentiation of particular tissues or cell types. These observations, including the present one, seem to indicate that the autoregulatory mechanism is widely adopted as the transcriptional regulation of the key transcription factors during differentiation.

The chromatin structure of the mtz-f1 gene was also investigated by detecting the hypersensitive site to DNase I. The region containing the Ad4 site and the E box was observed to be open in the steroidogenic tissue but not in the non-steroidogenic tissue. The steroidogenic tissue-specific chromatin structure is probably essential to the mtz-f1 gene transcription by making the transcription factors such as Ad4BP and E box binding factor accessible to their binding sites. Accordingly, it is supposed that the tissue-specific transcription of the gene is also guaranteed by the tissue-specific chromatin structure in addition to the tissue-specific transcription factor.

The present study demonstrated that the rat mtz-f1 gene is autoregulated through the Ad4 site in the first intron and that the E box is essential for the function of the Ad4 site. These observations, however, were made with Y-1 cells which have differentiated features as the adrenocortical cells. Because of the crucial role of Ad4BP in adrenal and gonadal differentiation, it is of importance to study the transcriptional regulation of the gene during the differentiation processes of the animal tissues.

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