The Role of Sp1 and AP-2 in Basal and Protein Kinase A-induced Expression of Mitochondrial Serine:Pyruvate Aminotransferase in Hepatocytes*

Serine:pyruvate aminotransferase (SPT1; alternatively named alanine:glyoxylate aminotransferase-1) is a liver-specific enzyme, and a bipartite transcription initiation mechanism is involved in its expression in the rat liver. Use of the upstream transcription start site (+1) results in production of mitochondrial SPT mRNA (SPTm-mRNA) in response to a rise in intracellular cAMP level, whereas the downstream transcription start site (+66) is responsible for continuous generation of peroxisomal SPT mRNA (SPTp-mRNA) without notable induction by stimuli (1–3). These two mRNAs differ from each other in the lengths of 5’-terminal sequences (2, 4).

SPTm-mRNA is 65 bases longer than SPTp-mRNA and has an extra 5’ sequence that encodes an N-terminal mitochondria-targeting signal, whereas SPTp-mRNA lacks this sequence and the translated product is translocated into peroxisomes, presumably being directed by an intramolecular peroxisomal targeting sequence (5–9). Thus, the unique feature of this gene is that the alternative usage of two transcription start sites eventually determines organelle localization of the expression product.

The 5’-sequences of the SPT genes are well conserved up to 25–28 bp upstream from the downstream start sites, but the upper region is relatively diverse among different animal species (10–12). Our previous studies showed that SPT is involved in both serine and glyoxylate metabolism (13, 14). The role of SPT in glyoxylate metabolism is important, because glyoxylate can be oxidized to oxalate, a useless and toxic end product of metabolism, unless it is efficiently metabolized to glycine. The major pathways of hepatic glyoxylate production in herbivores and carnivores seem to be oxidation of glycolate in peroxisomes and metabolism of hydroxyproline in mitochondria, respectively (15). Therefore, it is important that the organelle localization of SPT is food habit-dependent. The marmoset, like the rat (16), has SPT in both of the organelles in the liver (12). Carnivores, such as cats and dogs, have the enzyme largely in mitochondria, whereas it is located entirely in peroxisomes in herbivores and man (10, 17). As for cat SPT, Lumb et al. (10) showed that transcription of the gene occurred almost entirely from a site corresponding to the upstream start site in the rat SPT gene, consistent with mitochondrial localization. In the human and rabbit SPT genes, the transcription start site is also a site corresponding to the upstream site in the rat gene, but the first ATG codon for translation of the N-terminal mitochondria-targeting signal in these species is mutated to ACA or AU(A) (11–12). Therefore, the methionine codon first encountered in translation should be the downstream AUG that corresponds to the N-terminal methionine of SPT (18). In the case of the rat and marmoset, it seems that the downstream start site had been prepared to distribute SPT to peroxisomes in addition to mitochondria to adapt their metabolism to their omnivorous food habits. Thus, regulation of transcription of the SPT gene is closely linked to its specific organelle localization and proper function of the enzyme in the liver. We have been particularly interested in how the two transcription start sites are controlled in response to physiological stimuli in the rat. To elucidate the mechanism of the transcriptional regulation responsible for the alternative subcellular destinations of rat SPT, we recently investigated the downstream promoter and possible activators including C/EBP α/β that regulates transcription from the downstream start site (19, 20). In this paper,
we report that Sp1 and AP-2 activate the upstream promoter responsible for the generation of rat SPTm-mRNA.

MATERIALS AND METHODS

Expression Vectors—Various fragments of the rat SPT gene were prepared by complete or partial enzymatic digestion of cDNA clone of rat SPT and inserted into the HindIII or XhoI-Nhel site of pGv-B (Nippongene), a luciferase reporter plasmid without promoters and enhancers. Nucleotide positions of the 5’-ends (the upstream transcription start site is numbered +1) of the digested fragments inserted into corresponding reporter constructs are as follows: −5500 (BamHI) for pGBBN550, −4900 (NcoI) for pGBBN270, −2400 (PsI) for pGBPN240, −1256 (HindIII) for pGBBN129, −386 (Nhel) for pGBBN42, −278 (SmaI) for pGBBSN1, −191 (PvuII) for pGBBN23, −102 (EcoNI) for pGBBN13, and −213 (BamHI) for pGBBN9. The 3’-ends of the SPT gene in these constructs are all +36 (Nhel). The pTA-Spl-Luc, pTA-AP-2/Luc, and pTA-Spl-AP-2/Luc plasmids were prepared by insertion of the −125 to −89 region, the −14 to +10 region, and both of these regions of the SPT gene into pTA-Luc plasmid (Clontech Laboratories, Inc.), respectively. Site-directed mutagenesis for construction of reporter plasmids containing mutant SPT promoters, M1 and M2, was performed by PCR-mediated splicing after overlap extension. In the initial steps, the left arm of the PCR product was generated from the wild-type SPT gene encompassing the −386 to +36 region using a sense-SPT primer with a HindIII site at the 5’-end and an antisense primer containing the desired mutations. Similarly, the right arm of the PCR product was generated using an antisense-SPT primer with an Nhel site at the 3’-end and an antisense primer containing the desired mutations. Amplified DNAs were gel-purified and subjected to the second step of PCR using the sense-SPT primer and the antisense-SPT primer. The amplified products were then digested with HindIII and Nhel and were subcloned into the HindIII-Nhel site of pGv-B. Other site-directed alterations (for M3–M9) were made in wild-type pGBNN42 by the LA-PCR method (21) using two complementary oligonucleotides with the desired mutations, followed by DNA purification.

A wild-type AP-2β expression vector, pCMX-AP-2β, was kindly donated by Dr. Reinhard Buettner, University Hospital RWTH, Germany (22), and Dr. Hitoshi Okazawa, University of Tokyo, Japan (23), and a wild-type Sp1 expression vector, RSV-Sp1, was a generous gift from Dr. Naoko Tanese, New York University Medical Center. To make a wild-type SPT expression vector, RSV-SPT, was a generous gift from Dr. Robert Tjian, University of California, Science, Tokyo, Japan (24). All PCR-mediated constructs were subcloned into the SP(RSV) vector. For preparation of an Sp1-Lan expression vector, an Sp1 cDNA fragment corresponding to the DNA-binding domain at amino acids 516 to 696 was amplified by PCR and subcloned into the pcDNA3.1-FLAG, which was a generous gift by Dr. Reinhard Buettner, University Hospital RWTH, Germany (22). Only 0.5 μl of the suspension was mixed with equal moles of a reporter construct (15–25 μg total amount of DNA being equalized to 25 μg by the addition of the pGV-B plasmid), 10 μg of RSV-β-galactosidase (an expression vector for β-galactosidase), and 50 μg of salmon testis DNA in a Gene Pulser cuvette with a 0.4-cm electrode (Bio-Rad) and incubated on ice for 15 min. After suspending the mixture gently, hepatocytes were exposed to 280 V/960 microfarads at 250 V/cm for 45 μs for gene delivery. Cells were then transferred carefully into 7.2 ml of an ice-cold maintenance medium (Williams E medium with 10% fetal bovine serum, 25 μg/ml kanamycin, 25 μg/ml Cefamezine, 1 mU insulin, and 25 μg/ml epidermal growth factor), suspended very gently, seeded into 4 wells of a 6-well dish, and incubated in 5% CO2, 30% O2 at 37°C for 8 h. The medium was then changed to a fresh maintenance medium with (8-Br-cAMP) or without (control) 0.1 mM 8-Br-cAMP, and the culture was continued for another 24 h, followed by measurement of the luciferase activity.

For transfection into HepG2 cells, cells grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum were seeded at 5 × 104 cells/ml in a 6-well dish and cultured overnight with plasmids by the calcium phosphate method (29) or with FuGENE6 Transfection Reagent (Roche Molecular Diagnostics). The RSV-β-galactosidase plasmid (0.05 μg) and an expression plasmid (0.2 μg) for a catalytic subunit of wild-type PKA (wt-PKA) or a mutant catalytic subunit of PKA (mut-PKA) were included in the transfection medium in our experiments with HepG2 cells. Where indicated, other plasmids were also used. Total amounts of plasmids were equalized by adding the pGV-B plasmid backbone vector plasmids. Cells were cultured for another 24 h after transfection and then harvested for measurement of luciferase activity.

The luciferase activity in the cell extracts was measured using Plkka gene plasmid as a reporter (NIPPON GENE Co., Ltd., Japan) or luciferase assay system (Promega BioSciences, Inc.) and normalized with β-galactosidase activity. The relative luciferase activity represents the relative fold value versus that of pGv-B (Fig. 1) or versus that of pGBNN42 (Figs. 2, 4, 6, 7) when transfected with the mutant PKA vector. The
mutant PKA vector expresses the catalytic subunit of PKAs lacking ATP binding ability but does not act in a dominant negative manner. All transfections were performed in duplicate and repeated at least three times.

RESULTS

Determination of the Proximal Promoter Region for Upstream Transcription Initiation in the Rat SPT Gene—To identify the region that controls the upstream promoter activity, we performed reporter gene assays using various deletion mutants of the rat SPT gene fused to the luciferase gene. These constructs carry only the upstream transcription start site (+1) as illustrated in Fig. 1A. In our preliminary experiments with primary rat hepatocytes, low transfection efficiency and weak promoter activity of the SPT gene made it difficult to obtain reproducible data. Therefore, we tested transfections into three cell lines, human hepatoma HepG2 and rat hepatoma H4IIE and FAA3T3 cells, to select cells in which the SPT promoter activity can be detected more efficiently with a similar pattern to that in primary rat hepatocytes. In HepG2 cells, all of the 5′-deletion constructs from −5500 to +36 and −1256 to +36 (Fig. 1A) showed higher basal and cAMP/PKA-induced promoter activities with a similar pattern to that in primary hepatocytes (Fig. 1B). In the other two cell lines, however, transcription efficiencies were as low as that in primary hepatocytes (data not shown). Based on these observations, we decided to use HepG2 cells in all of the following experiments.

Because the region from −1256 to +36 (pGBNN42 plasmid) maintained a significant PKA responsiveness with 10–12-fold induction, we examined the effect of further deletion to determine the proximal region involved in the upstream promoter activity of the SPT gene (Fig. 2). Deletions to position −387 slightly increased the PKA-induced promoter activity, suggesting the presence of a weak inhibitory element. Deletions to −277 resulted in about a 30% decrease in the activity, but the −191 to +36 region (pGBPNN23) maintained almost the same level of activity as that of the −276 to +36 region. Further deletions up to −103 caused 1⁄3-1⁄4-fold reductions. On the other hand, the basal promoter activity was almost unchanged until the 5′-end of the gene was shortened to −50; i.e. the −50 to +36 region (pGBBN9) showed only a very low activity in both control and PKA-transfected cells. Similar results were obtained when cells were stimulated with 0.5 mM 8-bromo-cAMP instead of the introduction of PKA (data not shown). These data indicate that the −191 to −51 region contains elements that play important roles in the upstream promoter activity of the SPT gene.

AP-2β Binds to the Region Overlapping the Transcription Start Site and Activates the Basal Promoter Activity—To characterize cis-elements regulating the SPT promoter activity, we performed DNase I footprinting analysis using HepG2 nuclear extracts and various SPT gene fragments, including the −386 to −277 region and the −191 to +36 region, as probes. Three protected regions were detected, two of which (I and II, Fig. 3A) were located close to or over the transcription start site, and the other was at −125 to −89 (cf. Fig. 5A). No protected region was observed within the −386 to −277 region (data not shown), although deletion of this region caused a
small but obvious reduction in the luciferase activity as shown in Fig. 2.

To determine the transcription factors that bind to the I–II region overlapping the transcription start site, we next performed gel mobility shift assays. Because several probes containing either region I or II failed to form any complex with HepG2 nuclear proteins, a DNA fragment of the −14 to +10 region was also tested as a probe, and eventually this region was found to form a specific protein-DNA complex that was detected as a single band (Fig. 3A, right, lane 2). As the −14 to +10 region contained putative binding sites for AP-1 and AP-2 (cf. Fig. 7A), we then used those consensus sites as competitors. Effective competition for the protein-DNA binding was observed by addition of a 50-fold molar excess of an unlabeled self-fragment and the consensus AP-2 site, whereas the consensus AP-1 site only slightly weakened the signal of the band (Fig. 3A, right, lanes 3–5). Other competitors, including the adenovirus major late initiator and the consensus binding sites for C/EBP, Sp1, and initiator-binding factors such as YY-1, USF-1, and CTF/NF1, did not show any effects on the complex formation. Indeed, the DNA sequence of 5′-TCACCCAAAAC-3′ at position −1 to +9 in the SPT gene had a high homology with the consensus AP-2 site (Fig. 3C) found in the human metallothionein IIα gene (29). As shown in Fig. 3B, preincubation of nuclear extracts with an anti-AP-2β antibody prevented the formation of protein-DNA complexes, suggesting that AP-2β is capable of binding to the sequence. An antibody against AP-2α also inhibited the complex formation but the effect was more partial. On the other hand, inhibition by an anti-AP-2γ antibody was not significant under the experimental conditions used. An antibody against c-Fos had no effect (data not shown).

Similar results were obtained when the consensus AP-2 site was used as a probe (Fig. 3B, right). These results suggest that AP-2β binds to the −14 to +10 region of the SPT gene in vitro.

We next investigated the possible function involvement of AP-2β in the regulation of SPT gene expression. For this purpose, AP-2β was overexpressed in a transient reporter assay using pGBNN42 (Fig. 4). Expression of wild-type AP-2β caused a 6–8-fold induction of basal promoter activity of the SPT gene in a dose-dependent manner. PKA-induced activity was also enhanced by wild-type AP-2β but, unexpectedly, only to about the same level as that of the AP-2β-induced basal activity or even to a lower level, depending on the amount of AP-2β transfected. No cAMP/PKA-induced increase in the level of AP-2β was detected under the experimental conditions used (data not shown). Therefore, the above results suggest that AP-2β can activate basal transcription without being involved in the PKA-induced expression of mitochondrial SPT. Western blot analysis showed that the AP-2β level in PKA- or cAMP-stimulated HepG2 cells transfected with a large amount of an AP-2β expression plasmid was slightly lower than that in nonstimulated cells transfected with the same amount of the plasmid (data not shown). This may be a reason why the PKA-induced activity was lower than the basal activity when a higher amount of AP-2β was expressed (Fig. 4, center column). Ectopic expression of AP-2α also enhanced the basal promoter activity (data not shown), suggesting that there is no subtype specificity within AP-2 transcription factors. AP-2 has been shown to bind to DNA as a dimer, with the binding and dimerization domains located within the C-terminal half of the protein (31). An N-terminal-truncated AP-2α region, ΔN278, containing only the dimerization domain is unable to bind to DNA but retains the ability to dimerize with the wild-type protein, thereby preventing binding of the latter to DNA (32). As the dimerization domain was highly conserved among the AP-2 subtypes, we expected that the homologous domain of AP-2β (amino acids 292 to the C-terminal end) might serve as a dominant negative factor. Indeed, coexpression of AP-2ΔN291 inhibited the AP-2β-induced promoter activity regardless of whether the cells had been co-transfected with PKA or not (Fig. 4). Single transfection of the AP-2ΔN291 expression plasmid without wild-type AP-2β caused only a slight reduction in the basal promoter activity (data not shown), probably because the endogenous AP-2β level in HepG2 cells was very low (30, 33). Based on these data, we conclude that AP-2β has the ability to elevate SPT expression by interaction with the region overlapping the transcription start site and that the function of AP-2 is independent of PKA activation.

Sp1 Is Involved in Both Basal and PKA-induced Promoter Activities—Another possible region responsible for regulation of the upstream promoter activity as determined by DNase I footprint analysis was the region covering positions −125 to −89, as shown in Fig. 5A (left panel). Fig. 5C shows a C-rich motif in this region that is homologous to the consensus Sp1 site (34, 35). In the gel mobility shift assay, specific protein binding to the −125 to −89 region (Fig. 5, A, right, lanes 2 and 7) was diminished by a 50-fold molar excess of a self-competitor and a DNA fragment containing the Sp1 site (Fig. 5A, right, lanes 3, 4, and 8) but not by binding sites for C/EBP, AP-1, hepatocyte nuclear factor 4, and AP-2 (Fig. 5A, right, lanes 5, 6, 9, and 10) or a DNA fragment of a mutant Sp1 site (Fig. 5B, lane 5). The DNA-protein complex was supershifted by the addition of an anti-Sp1 antibody (Fig. 5B, lane 6). Similarly, the consensus Sp1 site used as a probe also formed a specific...
complex with HepG2 nuclear extracts, and the complex was supershifted by the addition of an anti-Sp1 antibody (Fig. 5B, right). All these data indicate that the −125 to −89 region contains an Sp1-binding site.

Functional involvement of Sp1 in the regulation of SPT gene transcription was then demonstrated by the results of a transient reporter gene assay (Fig. 6A). Enforced expression of wild-type Sp1 enhanced basal promoter activity of SPT to produce marked elevation of PKA-induced activity in a dose-dependent manner, whereas a truncated Sp1 lacking the N-terminal activation domain and possessing only the DNA-binding domain (36) prevented the promoter activation as a dominant negative factor. The -fold induction by PKA relative to basal activity was almost unchanged, although a slight increase was observed in some experiments. These results suggest that Sp1 is involved in both basal and PKA-induced expression of the SPT gene mainly by activating the basal promoter.

**CBP Augments the PKA-induced and Sp1-enhanced SPT Promoter Activity as a Coactivator**—Recently, Sp1 has been shown to be associated with CBP, a general coactivator for a number of transcription factors, to display its regulation of the target gene expression (37, 38). Indeed, introduction of CBP enhanced PKA-induced promoter activity of SPT (Fig. 6B).
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Besides, coexpression of CBP with Sp1 was more effective in the enhancement than expression of CBP or Sp1 alone. On the other hand, CBP did not affect AP-2β-induced promoter activity at all (Fig. 7C), consistent with the fact that no direct interaction between AP-2β and CBP has been reported at present. Ectopic expression of wild-type E1A severely repressed the PKA-induced promoter activity (Fig. 6C), and CBP overcame the repression by E1A (Fig. 6D). This result indicated the participation of CBP, because E1A has been shown to inhibit CBP-dependent transcription because of its high affinity for CBP, thus preventing CBP-RNA polymerase II interaction (26). CBP-dependent transcription because of its high affinity for CBP, thus preventing CBP-RNA polymerase II interaction (26). CBP could not exert its coactivator function irrespective of Sp1 expression. These results indicate that the −125 to −89 region is necessary for both the binding of and the positive regulation of the PKA-induced promoter activity by Sp1.

Mutations within the putative AP-2β site resulted in a substantial decrease in both basal and PKA-induced promoter activities of the SPT gene (M7 and M8, Fig. 7A). In the case of M7, which has mutations at positions +1 and +2 in the SPT gene, the repression of the promoter activity seems to be due to inhibition of the formation of basal transcriptional machinery, because AP-2β binding to the −14 to +10 region was inhibited by an oligonucleotide (−14/+10m7) carrying the same mutation as that of M7 as strongly as by that by the wild-type self-competitor (−14/+10wt) (Fig. 7B). In the case of M8, it is likely that the decrease is caused by inhibition of the binding of AP-2β to DNA, considering that mutated oligonucleotide, −14/+10m8, was unable to compete for the binding (Fig. 7B, lane 8). Because the AP-2-binding site overlaps the transcription start site, AP-2 and its target element may contribute to the initiator complex so that mutations in this site might cause severe damage to the promoter context. That might be a reason why both basal and PKA-responsive activities were greatly reduced. In support of our tentative conclusion that AP-2β acts without having any correlation with the Sp1 binding to the −125 to −89 region, enforced expression of AP-2β elevated the basal activity of the M3 construct to the same degree as that achieved with the wild-type construct (Fig. 7C).

Mutations in other regions in the SPT gene had no effect. CRE-, AP-1-, and TATA-like sites are not significantly involved in the upstream promoter activity of SPT in this reporter assay system. Indeed, overexpression of CREB, AP-1, and TBP (TATA-box binding protein) was not effective on the promoter activation (data not shown). Also, the C/EBP-like site (−20 to −12) does not seem to regulate SPTm expression although C/EBP α/β binds to the region overlapping the downstream start site to mediate the production of SPTp-mRNA (20).

The finding that AP-2 and Sp1 serve as positive regulators in the activation of the SPT promoter through their binding to the respective specific cis-elements prompted us to ask whether Sp1 and AP-2 can also function as cAMP/PKA-responsive factors for a neutral promoter. We next constructed luciferase plasmids in which the Sp1 and/or AP-2 site of the SPT gene was fused with the minimal promoter of the herpes simplex virus-thymidine kinase gene containing the TATA-box but not other regulatory elements (the resulting plasmids were denoted pTA-Sp1-Luc, pTA-AP-2-Luc, and pTA-Sp1AP-2-Luc), and transfected these constructs into HepG2 cells. Introduction of the Sp1 or AP-2 site resulted in 2–3-fold induction of the basal activity of the minimal promoter. Also, introduction of both of these sites enhanced the basal activity by 16–17-fold (Fig. 8). However, unlike the results obtained with luciferase plasmids containing the SPT promoter, stimulation by 8-bromo-cAMP did not affect the luciferase activities when neither Sp1 nor AP-2 plasmid was cotransfected. Overexpression of Sp1 or AP-2β elevated the basal activity a further 1.5–2-fold, and Sp1 caused moderate cAMP responsiveness only with the pTA-Sp1-Luc plasmid. Thus, the Sp1 and AP-2 sites of the SPT gene introduced upstream of the TATA-box within the herpes simplex virus-thymidine kinase minimal promoter do not function as CAMP-responsive elements effectively, although these sites play roles in the enhancement of the basal activity of the
minimal promoter. These results indicate that the core promoter context of the SPT gene is important for its full cAMP responsiveness or that other unidentified cis-element(s) might be required.

**DISCUSSION**

In this study, we demonstrated the roles of specific cis-acting sequences and transcription factors in the regulation of the upstream promoter activity for generation of rat SPTm-mRNA. We identified two transcription factors, AP-2 and Sp1, which are involved only in activation of basal promoter and in both basal and PKA-induced promoter activity, respectively.

Some notable features of AP-2 should be noted before considering its role in regulation of SPT gene expression. First, AP-2 enhanced only the basal promoter activity and no further activation was observed upon expression of a catalytic subunit of PKA. Second, the binding site of AP-2 is in a region overlapping the upstream transcription start site. It has been shown that AP-2 transcription factors play important roles in the regulation of gene expression responsible for vertebrate embryonic development, differentiation of epidermal cell lineages (40, 41), tumorigenicity of various cancer cells (42), and cell cycle control (43). To respond to various signals for the regulation of the above mentioned cell functions, the AP-2 activity is modulated through different signal transduction pathways. Phorbol ester along with cyclic AMP induces AP-2 activity to enhance transcription of the human metallothionein
IIA gene without de novo protein synthesis (30). Park et al. (44) showed that AP-2 was phosphorylated by protein kinase A to mediate insulin induction of the acetyl-CoA carboxylase gene expression. It is also reported that retinoic acid induces AP-2 activity by increasing AP-2 mRNA levels in human teratocarcinoma cells (45). Based on the above information, we first expected that AP-2 might mediate the PKA responsiveness of the upstream promoter activity of the SPT gene. However, only the basal promoter activity was enhanced by overexpression of AP-2β. AP-2β may serve as an initiator-like factor, such as YY-1, USF1, or TFII-I, in functional association with other general transcription factors, but independent of the cAMP/PKA-signaling pathway.

Another point to be discussed is AP-2 in hepatic cells. Although our data clearly showed positive regulation by AP-2β of SPT gene expression, AP-2 is known to be primarily expressed in neural crest cells and related cells but not significantly in endodermally derived tissues such as the liver or in hepatoma cells, including HepG2 cells. This may provide at least a partial explanation for the low basal level of SPTm-mRNA in vivo (1).

Both basal and PKA-induced activities of the upstream promoter were elevated by overexpression of Sp1, suggesting that Sp1 is one of the mediators of the cAMP/PKA signaling pathway in SPT expression. Sp1 was originally identified as a specific factor required for simian virus 40 transcription (46) and has been characterized as a ubiquitous transcription factor that serves to maintain the basal level of transcriptions for constitutive expression of many other genes, including the adenosine deaminase gene (47), herpes simplex virus-thymidine kinase gene (48), epidermal growth factor receptor gene (49), and DNA polymerase β gene (50). Interaction of Sp1 with TBP (51), TAFII130 (52), and RNA polymerase II (53) has been demonstrated to be involved in the regulation of basal transcription by Sp1. The activation function of Sp1 has been mapped to its N terminus, which contains glutamine- and serine/threonine-rich domains (36). Although Sp1 is a constitutive transcription factor, several research groups have recently reported that Sp1 function might also be important in tissue-specific and developmental regulation of gene expression (54). For this regulation, Sp1 has often been shown to mediate cAMP-induced activation of gene expression. For example, Sp1 has been shown to confer cAMP responsiveness on the bovine CYP11A promoter (55). Rohlf et al. (56) showed that modulation of Sp1 by PKA causes an increase in the DNA binding activity and trans-activating properties of Sp1. Thus, it is likely that Sp1 has the ability to regulate the PKA-induced activation of SPT gene expression.

As in the case of many transcription activators/enhancers, Sp1 has been shown to be associated with CBP in response to hormonal stimuli via activation of other transcription factors (36, 37). It has been demonstrated that CBP recruits RNA polymerase II on the transcription start site of target genes by
bridging interaction between activator/enhancer and RNA polymerase II (25, 26) and that adenovirus E1A prevents the interaction by competitive binding to CBP through its N-terminal CBP-binding domain (27, 39, 57). Our data in this study support these reports and suggest that Sp1 is one of the major regulators of the upstream promoter activity for the generation of SPTm-mRNA.

The mechanism by which Sp1 mediates cAMP-induced expression of SPT is still unclear. Hormonal regulation of Sp1 expression could be one of the possible mechanisms. Alternatively, DNA binding activity may be modulated by phosphorylation by PKA (56). However, the expression level of Sp1 in HepG2 cells determined by Western blotting was hardly affected by 8-Br-cAMP. In addition, if the cAMP/PKA-induced expression of SPT were mediated merely by Sp1 expression, PKA would not cause further enhancement of SPT in the presence of sufficient Sp1, but this was not the case. Furthermore, the signal of DNA-protein complex in the gel shift assay was not increased when nuclear extracts from cAMP-stimulated or PKA-overexpressed HepG2 cells were used (data not shown), suggesting that neither the expression nor the DNA binding activity of Sp1 was increased by cAMP/PKA. Another possible explanation is that PKA regulates interactions of Sp1 with other factors. Sp1 can interact with several other transcription factors, such as AP-2 and CBP, which may be involved in the regulation of SPT expression.

Fig. 7. Involvement of the binding sites for AP-2 and Sp1 in the upstream promoter activity. A, effects of various mutations in the −386 to +36 region on promoter activity. Schematic diagram (bottom) represents putative cis-elements found in the −386 to +36 region and various mutants of the pGBNN42 plasmid. Wild-type (WT) or a mutant (M1-M9) pGBNN42 plasmid (1.5 μg each) was transfected into HepG2 cells, and the reporter gene assay was performed as described under “Materials and Methods.” Relative LUC activity represents the relative -fold value versus the control activity of wild-type pGBNN42 (−). B, effects of mutations within the putative Sp1 or AP-2 site on protein-DNA binding. Gel mobility shift assays were performed with HepG2 nuclear extracts and the −125 to −89 region (−125/−89wt) or the −14 to +10 (−14/+10) region of the SPT gene as a probe. For the competition assay (lanes 3, 4, and 7–9), the nuclear extracts were preincubated with a 50-fold molar excess of unlabeled annealed oligonucleotides of the wild-type sequence (−125/−89wt, −14/+/10wt) or mutant sequences having mutations shown in A (−125/−89m3, −14/+/10m7, and −14/+/10m8) prior to the addition of the probe. C, functional involvement of Sp1 in the promoter activity through binding to the −125 to −89 region. One μg of wild-type or mutant (M3) pGBNN42 was transiently transfected with an expression plasmid for Sp1 (0.25 μg), AP-2β (0.125 μg), CBP (0.3 μg), or CBP in combination with the Sp1 or AP-2β plasmids. The reporter gene assay was performed as described under “Materials and Methods.” Relative LUC activities represent the relative fold value versus the control activity of wild-type pGBNN42 (−).
The results of the reporter analysis revealed distinct activity of the binding regions for Sp1 and AP-2 in the SPT gene when these regions were introduced upstream of a minimal promoter of the herpes simplex virus-thymidine kinase gene, suggesting that specific promoter context and cross-talk of other transcription factors through additional regulatory regions may be necessary for Sp1- and AP-2-mediated promotion activation of the SPT gene. We had expected to find positive involvement of CREB, AP-1, and TBP, because there is one CRE-like element, two AP-1-like elements, and one TATA-like element within the −386 to +36 region of the SPT gene (Fig. 7). CREB and/or AP-1 have been shown to be mediators of cAMP/PKA-dependent transcription (62, 63). The TATA-like element (AATAAAA) is located about 30 bp upstream of the start site (+1) within the SPT gene, and a minor mutation (the first T to A) has been reported to give little effects on a TATA-dependent promoter activity (64). In this study, however, we found that these putative elements did not act for the SPT promoter. Thus, the rat SPT gene may possess a unique promoter from which SPTm-mRNA synthesis is TATA-independent despite the existence of this sequence and no canonical initiator factor contributes to its regulation. Further investigation will be necessary to rule out the possibility that the truncated promoter fused to a heterologous reporter gene is regulated differently from the endogenous SPT promoter.

As described in the Introduction, SPT has the unique feature that its transcriptional regulation is closely linked to its specific organelle localization depending on the feeding habits of the animal species. In the rat SPT gene, the downstream transcription start site is used for maintaining a steady level of SPT in peroxisomes to metabolize hepatic glyoxylate, whereas transcription from the upstream site is greatly activated in response to an increase of intracellular cAMP to produce mitochondrial SPT when a high protein diet causes a robust release of glucagon. Generation of SPTm-mRNA from the downstream transcription start site is dominant in a steady state condition and does not significantly respond to cAMP/PKA signaling. We have recently reported that C/EBP αβ binds to the region overlapping the downstream transcription start site to activate the transcription of SPTm-mRNA (20). However, the C/EBP-like site within the upstream promoter region did not contribute to transcription from the upstream start site. In light of these facts, we think it likely that C/EBP plays a dominant role as a key factor for liver-specific transcription in regulating the downstream promoter, so that regulatory elements for the upstream promoter cannot function. Conformational change of the promoter architecture by cAMP/PKA-signaling might switch the upstream site to a major transcription start site under control by the Sp1-CBP interaction. It would be interesting to determine whether Sp1 activates or inactivates the transcription from the downstream site. In our previous study, Northern blot analysis and a nuclear run-on assay with primary rat hepatocytes indicated that induction of SPTm-mRNA via a cAMP/PKA signaling pathway required on-going protein synthesis (1). Because the expression levels of Sp1 and AP-2 did not significantly increase in HepG2 cells stimulated with 8-Br-cAMP, unidentified factor(s) may also be involved in PKA-induced generation of SPTm-mRNA in vivo. Further studies will focus on molecular details of how the different transcription initiations from the two separate sites are regulated for proper arrangement of the gene products in cells.

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