FEBS Letters 508 (2001) 16–22 FEBS 25403

Involvement of CCAAT/enhancer-binding protein in regulation of the rat serine:pyruvate/alanine:glyoxylate aminotransferase gene expression

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Received 6 August 2001; revised 8 October 2001; accepted 8 October 2001

First published online 22 October 2001

Edited by Ned Mantei

Abstract In the rat liver, transcription of the serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT) gene occurs from two sites, +1 and +66, in exon 1, resulting in the formation of two mRNAs, one for a precursor of mitochondrial SPT/AGT and the other for peroxisomal SPT/AGT, respectively. In this study, we attempted to characterize the downstream promoter responsible for generation of peroxisomal SPT/AGT. The minimal downstream promoter was confined to the +21–+90 region. We demonstrated that C/EBPα and C/EBPβ bound around the downstream start site (+66) contribute to the promoter activity. The downstream promoter activity is also regulated positively by a short inverted repeat, located 20–30 bp upstream of the downstream start site, through a protein factor(s) bound to this region. On the other hand, the sequence just downstream of the start site may negatively regulate the promoter activity. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Serine:pyruvate aminotransferase; CCAAT/enhancer-binding protein; Two transcription start sites; Downstream promoter

1. Introduction

Serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT), which is expressed specifically in the liver, is a unique enzyme with dual species-specific and food habit-dependent organelle distributions and with dual functions. As for its organelle distribution, this enzyme is entirely peroxisomal in humans and herbivores and is largely mitochondrial in carnivores [1–4]. In the rat liver, this enzyme is located in both mitochondria and peroxisomes, and only the mitochondrial enzyme is greatly induced by administration of glucagon [5,6]. The marmoset, a New World monkey, also has SPT/AGT in both mitochondria and peroxisomes in the liver [4]. As for its function, SPT/AGT is involved in both the metabolism of L-serine and glyoxylate, and this enzyme plays an important role in serine metabolism irrespective of its mitochondrial or peroxisomal localization [7]. With respect to the glyoxylate metabolism, on the other hand, the major pathways of hepatic glyoxylate production in herbivores and carnivores appear to be oxidation of glycolate in peroxisomes and metabolism of hydroxyproline in mitochondria, respectively. Thus, the proper organelle destination of SPT/AGT may be important for the respective animal species to efficiently metabolize glyoxylate, because glyoxylate can otherwise be oxidized to oxalate, a useless and even toxic end-product of metabolism [8].

We have previously shown that transcription of the rat SPT/AGT gene occurs from two start sites in exon 1 [9]. Transcription from the upstream start site (+1) generates an mRNA for a 45-kDa precursor for mitochondrial SPT/AGT containing a mitochondrial targeting signal (MTS) of 22 N-terminal amino acids. The precursor is translocated into mitochondria and converted to the mature size (43 kDa) by processing. On the other hand, transcription from the downstream start site (+66) generates an mRNA that encodes a product of mature size. The product is then directed to peroxisomes by an intramolecular peroxisomal targeting signal (PTS) [9–11]. When glucagon is injected into rats, only the upstream site-derived mRNA is greatly induced by transcription activation through cAMP and protein kinase A [12,13]. The downstream site-derived mRNA appears to be constitutive, although its amount is very low. Thus, the most outstanding feature of this gene is that alternative usage of the two promoters eventually determines organelle localization of the expression product. Danpure et al. showed that transcription of the SPT/AGT gene in the cat occurs almost entirely from a single site corresponding to the upstream start site in the rat SPT/AGT gene, consistent with the largely mitochondrial localization of SPT/AGT [14]. Similar start sites were also found in the case of rabbits and humans, but the upstream AUG codon for translation of the N-terminal MTS sequence in these species had been mutated to ACA or AUA [15,16]. Therefore, the first methionine codon encountered in translation should be the downstream AUG corresponding to N-terminal methionine of mature SPT/AGT, consistent with the entirely peroxisomal localization of the enzyme. In the marmoset, however, two SPT/AGT mRNAs were found, suggesting that transcription was initiated from two different sites, as in the case of the rat [16]. It appears that the downstream transcription start site has been used to distribute SPT/AGT to peroxisomes in addition to mitochondria. We have been interested in how the downstream transcription start site was constructed in the rat and marmoset during evolution.

Transcription initiation is a complex process requiring assembly of many transcriptional factors on the promoter. The most common core promoter elements, which can act inde-

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0014-5793/01/$20.00
PII: S0014-5793(01)03010-1

pendently or in concert to determine the transcription start site, are the TATA box located about 30 bp upstream of the start site and an initiator element (consensus sequence: Py-PyANTPyPy) at the start site. The initiator element is capable of functioning in concert with either or both a TATA box and multiple upstream binding sites for some activators such as GC box for the Sp1 activator [17]. In a promoter containing a TATA box and strong activators, such as Sp1 and a CCAAT-binding protein, initiator activity does not contribute greatly to the core promoter, whereas in the absence of a TATA box, the initiator plays a major role in the promoter activity, which can be abolished completely when some mutations are introduced into the initiator sequence [18,19]. Within the consensus initiator sequence, PyA(+1)NT/A is the most critical for the strength of an initiator, and pyrimidines must surround the sequence [18]. Our previous studies indicated that the downstream promoter of the rat SPT/AGT gene is located between +21 and +106 [20]. However, this region contains neither a TATA box nor a consensus initiator sequence. In this study, we found that C/EBPα and C/EBPβ are positively implicated in the downstream promoter activity of the rat SPT/AGT gene. The results also suggested that the sequence just downstream of the +66 start site might negatively regulate the promoter activity through its binding to unidentified factor(s).

2. Materials and methods

2.1. Plasmid constructs

The origins and methods for construction of basal promoterless CAT plasmids, pSV001CAT and pSV001-mt, and test CAT plasmids containing rat SPT/AGT 5′-sequences of various lengths, pHR1362CAT (−1256–+106), pDR85CAT (+21–+106) and pNR69CAT (+37–+106), were described previously [20]. pDB70CAT (+21–+90) and pEnR212CAT (−102–+106) were constructed by inserting the *Dde*I (+21) – *Bst*NI (+90) fragment and *Hin*dIII (−102) – *Rsa*I (+106) PCR fragment of the SPT/AGT gene into the *Nru*I–*Eco*RV site and the *Hin*dIII–*Eco*RV site of pSV001CAT, respectively. pEnB196CAT (−102–+90) was constructed by double ligation of the *Hin*dIII (−102) – *Nhe*I (+37) and *Nhe*I (+37)–*Bst*NI (+90) PCR fragments into the *Hin*dIII–*Eco*RV site of the pSV001CAT. Site-directed mutagenesis was performed using mismatched primers and 320pUC18 containing the *Pvu*II (−191)–*Pvu*II (+128) fragment of the SPT/AGT gene as a template. Oligonucleotides are as follows: m1 (AGGCCTCGC CTCTGcagTCAGCCCAGAGCTA), m2 (TCTGAGTTCAGCtgAGAGCTAGCTGGGAAA), m3 (TTCAGCCCAGAGagAGCTGGGAAAT), m4 (TTCAGCCCAGAGCTctCTGGGAAATGTT), m5 (TTCAGCCCAGAGCTAGagGGGAAATGTT), m6 (AGCTAGCTGGGAccTGTTCCGGAT), m7 (TTCCGGATGTTGGCCAtGGCCAGTGTGACG), m8 (TGTTCGGATGTTGaCCAtGGCCAGTGTGA), m9 (GATGTTGGCCAAcagCAGTGTGACGC TG) and m10 (TGGGAAATGTTCCtGcTGTTGGCCAA) were used as mismatched primers. The PCR products, *Hin*dIII (−102)–*Rsa*I (+106) and *Dde*I (+21)–*Rsa*I (+106) fragments containing various mutations, were then ligated to the *Hin*dIII–*Eco*RV site and *Nru*I–*Eco*RV site of pSV001CAT, respectively. All of the SPT gene fragments described above were fused to the 5′-end of CAT cDNA to make ATG triplets within the SPT/AGT gene and the CAT cDNA out-of-frame with each other, so that mRNAs derived from the upstream start site would not be responsible for CAT activities in this study. Plasmids containing C/EBPα cDNA (MSV/EBPα) or C/EBPβ cDNA (MSV/EBPβ) were kind gifts from Dr. Steven L. McKnight (University of Texas Southwestern Medical Center, TX, USA). A C/EBPα expression vector, pcDNA3.1-C/EBPα, and a C/EBPβ expression vector, pcDNA3.1-C/EBPβ, were then constructed by inserting the *Bam*HI–*Hin*dIII fragment of MSV/EBPα and the *Eco*RI–*Xho*I fragment of MSV/EBPβ into the *Bam*HI–*Eco*RV site and the *Eco*RI–*Xho*I site of pcDNA3.1 (Invitrogen), respectively. An expression vector of a dominant-negative inhibitor of C/EBP, CMV500-4hep-C/EBP [21,22], was a generous gift from Dr. Charles R. Vinson (National Institute of Health, MA, USA).

2.2. Cell culture and transient transfection

Human hepatoma-derived HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum to 40–60% confluence in 6-well plates, and they were then transfected by the calcium phosphate precipitation method using a total of 1.6–1.9 μg of a DNA mixture containing 1.0–1.3 μg of test plasmid and 0.6 μg of SV40-β-gal as a transfection control. To determine the effects of C/EBPs on the reporter gene expression, 0.5–1.0 μg of pcDNA3.1-C/EBPα or pcDNA3.1-C/EBPβ was co-transfected with 0–0.5 μg of CMV500-4hep-C/EBP, 1.0 μg of a reporter plasmid (pDR85CAT), and 0.05 μg of RSV-β-gal as an internal control. Cells were washed with phosphate-buffered saline 6 h after the transfection, and then the culture was continued for an additional 42 h. Cell extracts were prepared 48 h after the transfection by freeze–thawing, and the CAT activity was measured as described by Gorman et al. [23]. All transfections were performed in duplicate and repeated at least three times.

2.3. Preparation of probes and competitors

An annealed synthetic complementary oligonucleotide corresponding to +21–+50 and that corresponding to +50–+76 of the rat SPT/AGT gene were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and used as probes for electrophoretic mobility shift assays (EMSAs). Double-stranded oligonucleotides corresponding to various *cis*-elements or mutated SPT/AGT gene were used as competitors. The sequences of the competitors were as follows: C/EBP, 5′-TGCAGATTGCGCAATCTG CA-3′; C/EBP mutant, 5′-TGCAGAGACTAGTCTCTGC A-3′. Mutated competitors (M2, M5, M7, M8, M9 and M10) are shown in Figs. 2A and 4A.

2.4. EMSA

Nuclear extracts were prepared from HepG2 cells as described previously [24]. In competitive binding experiments, nuclear extracts (5 μg protein) were preincubated with 3 μg of poly[d(I-C)] and a 50-or 100-fold molar excess of unlabeled competitors for 15 min on ice in a binding buffer (50 mM Tris–HCl, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 50 mM KCl, 5% (v/v) glycerol), followed by the addition of 10 fmol of an end-labeled probe. For a supershift assay, nuclear extracts were preincubated for 15 min on ice with 2 μl of an antibody against C/EBPα, C/EBPβ or C/EBPδ (Santa Cruz Biotechnology, CA, USA) or with preimmune serum as a control, prior to the addition of 10 fmol of an end-labeled probe. Then samples were incubated with the probe for 30 min on ice, applied to 5% polyacrylamide gel in 0.25×Tris borate/EDTA buffer, and subjected to electrophoresis at 12.5 mA at 4°C.

3. Results

3.1. The minimal downstream promoter is located within the region from +21 to +90

To identify the region essential for the promoter activity for transcription from the downstream start site, various deletion mutants of the putative downstream promoter region of the rat SPT/AGT gene were linked to the CAT gene and each of them was transiently expressed in HepG2 cells (Fig. 1A). For the following reasons, we consider that CAT activity measured in this study reflects only the downstream promoter activity. First, since the ATG triplet within the SPT/AGT gene at +114 to +116, which is supposed to be the N-terminal Met of SPTp, was eliminated in these constructs [20], the first Met codon encountered in translation of the downstream site-derived mRNA should be the initiation AUG (the third asterisk in Fig. 1A) for CAT, resulting in the expression of native CAT. In translation of the upstream site-derived mRNA, on the other hand, the translation begins from the AUG at +48 to +50 (the first asterisk), which corresponds to N-terminal Met of the SPTm-mRNA, but the CAT gene-derived AUG codon is expected to be out-of-frame, resulting in production

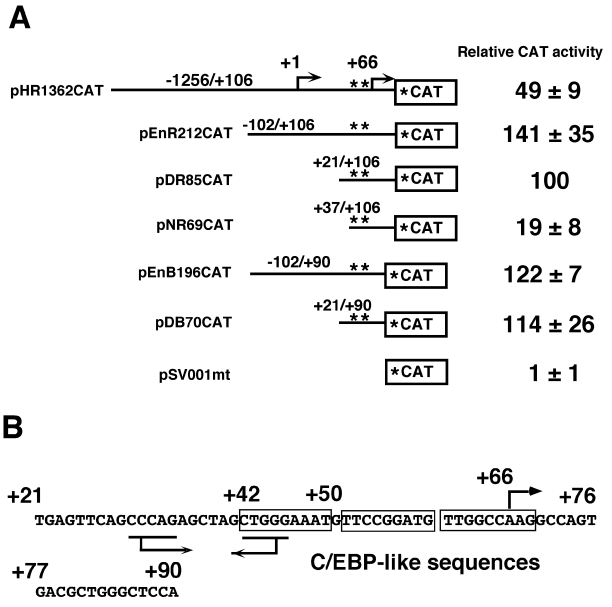

Fig. 1. Functional analysis of rat SPT/AGT promoter deletion mutants in HepG2 cells (A). In the schematic representation of rat SPT/AGT deletion mutants fused to the CAT reporter gene, the upstream and downstream transcription start sites are shown by hook-shaped arrows. Horizontal bars represent the SPT/AGT 5′-sequences with the nucleotide positions of the 5′- and 3′-ends relative to the upstream transcription start site. ATG triplets are indicated by asterisks. Note that the translation product of mRNA synthesized from the +1 site is out-of-frame of the CAT-coding gene. HepG2 cells were transfected transiently with each reporter plasmid and SV40-β-gal as a control for transfection efficiency. CAT activities are expressed relative to the activity obtained with pDR85CAT. Values are means ± S.D. of three separate experiments. B: Nucleotide sequence of the downstream promoter region of the rat SPT/AGT gene. The predetermined downstream transcription start site (+66) is shown by a hook-shaped arrow. A pair of opposite arrows denotes the short inverted repeat. Open squares indicate the putative C/EBP binding sequences. Consensus sequences for the C/EBP site: T(G/T)NNGNAA(G/T).

of inactive SPT-CAT fusion protein. Second, promoter activity of the region −102 to +36 containing neither the downstream start site nor the initiation ATG was less than 7–10% of that of the region −102 to +106 in which the ATG triplet was out-of-frame of the CAT-coding sequence. In addition, the region −102 to +106 containing 'in-frame'-ATG showed almost the same activity as that of the same region containing 'out-of-frame'-ATG, suggesting that the region −102 to +106 contributes greatly to promotion of the transcription from the downstream start site (data not shown, manuscript in preparation). In fact, as we showed by RNase protection analysis in our previous study [13], the basal level of endogenous mitochondrial SPT mRNA in the rat liver or in primary rat hepatocytes is considerably lower than that of peroxisomal SPT mRNA.

Deletion of the 5′ region from −1256 to −103 increased the transcriptional activity by about 3-fold, suggesting the presence of a negative element. Further deletion from −102 to +20 resulted in only a slight decrease. Upon subsequent deletion from +21 to +36, the promoter activity was further decreased by 80%. No change in the promoter activity was observed when the 3′ region was deleted from +91 to +106. These results suggest that the minimal downstream promoter is located in the +21–+90 region. The nucleotide sequence of

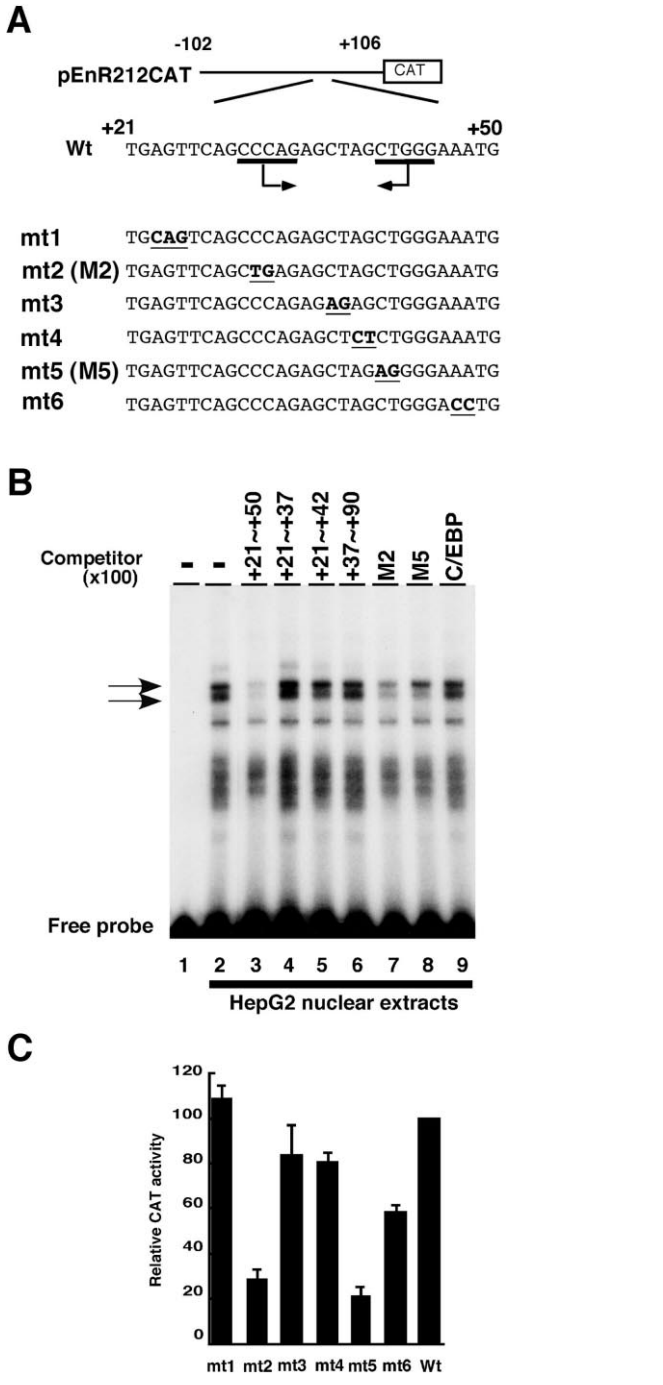

Fig. 2. Involvement of the short inverted repeat in the downstream promoter activity. A: Schematic representation of wild-type pEnR212CAT (wt) and its mutated CAT reporter plasmids (mt1 to mt6) that harbor mutations in or around the inverted repeat. The nucleotide sequence corresponding to positions +21 to +50 in the SPT/AGT downstream promoter region is shown, and mutated nucleotides are indicated by bold underlines. A pair of opposite arrows denotes the short inverted repeat. In EMSA, +21–+50 fragments containing mutations in one side of the short inverted repeat were used as competitors (M2 and M5). B: EMSA was performed with 10 fmol of a ^{32}P-labeled +21–+50 fragment of the SPT/AGT gene as a probe and 5 μg of nuclear extracts from HepG2 cells, as described in Section 2. Arrows show DNA–protein complexes. (C) Relative CAT activities. HepG2 cells were transfected transiently with each promoter reporter plasmid described in (A). CAT activities are expressed relative to the activity obtained with wild-type pEnR212CAT. All values are means ± S.D. of three separate experiments. Wt, wild-type.

the minimal promoter region is shown in Fig. 1B. The transcription start site for generation of mRNA for peroxisomal SPT/AGT had been determined to be at +66 [9]. Although no TATA-like sequence is present, there is a short inverted repeat (CCCAGAGCTAGCTGGG) at 20–35 bp upstream of the downstream start site. Three C/EBP binding sites are also located close to or overlapping the start site.

3.2. The short inverted repeat is involved in transcriptional regulation

The results showing that the +21–+36 region was requisite for the promoter activity prompted us to examine nuclear factor binding to this region by an EMSA using nuclear extracts from HepG2 cells. As shown in Fig. 2B, two bands were detected when the +21–+50 fragment was used as a probe, suggesting that some protein–DNA complexes had been formed. A complex was not detected in the presence of a 100-fold molar excess of an unlabeled self-competitor (Fig. 2B, lane 3). However, no competition was observed with the same molar excess of a competitor that covered only the 5′- or 3′ portion of the probe (fragments +21–+37, +21–+42 and +37–+90) or with the consensus C/EBP site (Fig. 2B, lanes 4–6 and 9). When the competitor contained mutations in one side of the short inverted repeat (M2 and M5, Fig. 2A), the competition was only partial (Fig. 2B, lanes 7 and 8). These results suggest that the short inverted repeat is the most likely binding site of factors that might be involved in the downstream promoter activity.

To examine whether the short inverted repeat is actually involved in transcriptional regulation, −102–+106 fragments containing the same mutations as those used in the gel shift assay were linked to the CAT gene (Fig. 2A), and each of them was transiently expressed in HepG2 cells (Fig. 2C). Mutations in either side of the inverted repeat (mt2, mt5) caused an approximately 80% decrease in the promoter activity, whereas mutations outside or in the intervening portion of the inverted repeat (mt1, mt3, mt4 and mt6) resulted in only a slight decrease. These findings further suggest that the short inverted repeat is involved in the promoter activity. The protein factors that bind to this region remain to be identified.

3.3. C/EBP binds to the downstream promoter of the SPT/AGT gene

To examine whether C/EBP binds to the putative C/EBP binding sites located close to or overlapping the downstream transcription start site, EMSA was performed using the +50–+76 fragment as a probe and nuclear extracts from untransfected HepG2 cells or C/EBP-transfected cells. In our preliminary experiments, the mobility shift pattern observed with the +50–+76 fragment as a probe was essentially the same as that obtained with the +50–+90 fragment. As judged from the competition by a 50-fold molar excess of an unlabeled self-competitor, the nuclear extract from untransfected cells gave two specific protein–DNA complexes (I and II, Fig. 3A,B, lane 2). The formation of the upper complex (I) was inhibited by a 50-fold molar excess of a fragment corresponding to the C/EBP site, but no competition was observed by the same molar excess of the mutated C/EBP site (Fig. 3A, lanes 4 and 5). In addition, complex (I) disappeared almost completely when nuclear extracts from untransfected cells were preincubated with an antibody against C/EBPα. An antibody against C/EBPβ also caused partial disappearance of complex (I) but that against C/EBPδ had no effect (Fig. 3A, lanes 6–8). The disappearance seemed to be caused by supershift of complex (I), since a weak and smeared band was observed near the origin of the gel when exposed for a longer time (data not shown). It has been reported that C/EBPs are expressed at low levels in the hepatoma cell line HepG2 [25], although C/EBPα

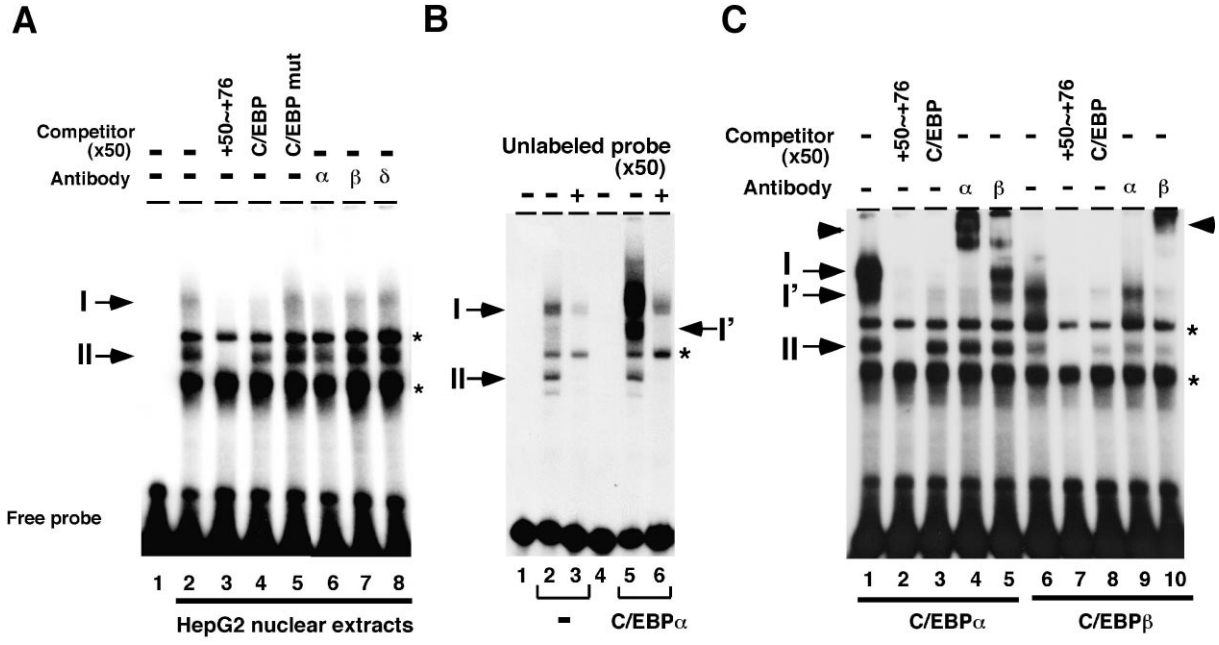

Fig. 3. Binding of C/EBP to the downstream promoter region of the SPT/AGT gene. EMSA was performed with a +50–+76 fragment as a probe and nuclear extracts from untransfected HepG2 cells (A) or HepG2 cells transiently transfected with a C/EBPα or C/EBPβ expression plasmid (B, C). The sequences of the competitor oligonucleotides used, C/EBP, and C/EBP mutant (C/EBP mut) are given in Section 2. The procedures for the supershift assay are also described in Section 2. Arrows indicate specific complexes (I, I′, II), and arrowheads indicate the supershifted bands. Asterisks represent non-specific bands. The lower non-specific band did not appear in (B) despite the fact that the same extracts as those used in (A) and (C) were used.

and C/EBPβ are highly expressed in the adult liver [26]. The amount of C/EBP in untransfected HepG2 cells was probably too low to be detected significantly as a supershifted band in these experimental conditions. To determine the interaction with C/EBP more clearly, we performed EMSA with nuclear extracts from HepG2 cells transiently transfected with a C/EBPα or C/EBPβ expression plasmid. In the case of the extracts from cells transfected with the C/EBPα expression plasmid, complex (I′) was observed in addition to complexes (I) and (II). Formation of protein complexes (I) and (I′) were effectively inhibited by the unlabeled +50–+76 fragment (Fig. 3B, lanes 5–6, and Fig. 3C, lanes 1–2) and the consensus C/EBP site (Fig. 3C, lane 3). We assume that the large complex of (I) and (I′) consists mainly of αα-heterodimers with some αβ-heterodimers of C/EBP. Assuming, based on the results of a supershift assay shown in Fig. 3A, that C/EBPα is a major contributor to complex (I) formation and that C/EBPβ is induced by C/EBPα as in the case of the *Xenopus* C/EBP [27], formation of αα- and αβ-complexes and their binding to the SPT gene would occur to a greater degree. This may be the reason why the anti-C/EBPα antibody perfectly supershifted both complexes and the anti-C/EBPβ antibody was partially effective as well (Fig. 3C, lanes 4 and 5). It is not clear at present, however, which complex of (I) and (I′) corresponds to αα- or αβ-dimers. When nuclear extracts from C/EBPβ-overexpressing cells were used, a specific protein–DNA complex that showed a similar pattern of migration to that of complex (I′) was formed. The formation of this complex was completely inhibited by a fragment of the consensus C/EBP site, and the complex was supershifted by anti-C/EBPβ antibody (Fig. 3C, lanes 6–10). Taken together, these results indicate that C/EBPα and C/EBPβ are able to bind to the downstream promoter around the transcription start site. On the other hand, complex (II) showed competition with a 50-fold molar excess of an unlabeled self-competitor but not with a fragment of the C/EBP site. Competitors corresponding to the DNA recognition sites of known initiator binding proteins, including YY1, USF1 and TFII-I, were also without effect (data not shown). It remains unclear why the signal of complex (II) was weak in the assay with C/EBPβ-overexpressing nuclear extracts.

3.4. The putative C/EBP binding sites are involved in the downstream promoter activity

To determine the function of the putative C/EBP binding sites, various mutations were introduced into the vicinity of the downstream start site in the context of pEnR212CAT (Fig. 4A), and CAT assays were carried out. As shown in Fig. 4B, introduction of mutations into either of the two putative C/EBP sites, one located just upstream of the +66 start site and the other partially overlapping the start site, resulted in a 40–60% decrease in the promoter activity (mt7, mt8 and mt10). On the other hand, introduction of a mutation into a downstream region of the start site (mt9) gave rise to a modest increase (2-fold) in the promoter activity. In EMSA, both complex (I) and complex (II) showed partial competition with a 50-fold molar excess of two unlabeled mutated competitors (M7 and M8, Fig. 4C, lanes 4 and 5). A mutated competitor, M10, competed partially for complex (I) and competed perfectly for complex (II) (Fig. 4C, lane 7). Interestingly, a mutated competitor, M9, competed for complex (I) but not for complex (II) (Fig. 4C, lane 6). Taken together, these results

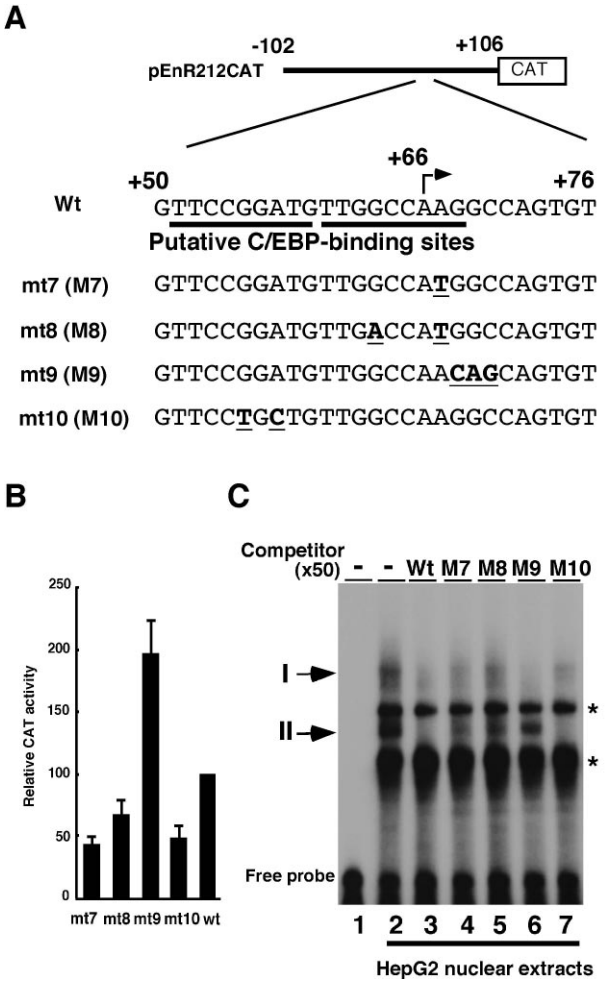

Fig. 4. Involvement of the putative C/EBP binding sites in the downstream promoter activity. A: Schematic representation of wild-type pEnR212CAT (Wt) and its mutated CAT reporter plasmids that harbor mutations in the C/EBP binding sites (m7, m8 and m10) or downstream of the start site (m9). The nucleotide sequence corresponding to positions +50–+76 bp in the SPT/AGT promoter region is shown, and mutated nucleotides are indicated by bold underlines. The hook-shaped arrow shows the predetermined downstream transcription initiation site (+66). In EMSA, +50–+76 fragments containing various mutations, described above, were used as competitors (M7–M10). B: Relative CAT activity. HepG2 cells were transfected transiently with each promoter reporter plasmid described in (A). CAT activities are expressed relative to the activity obtained with wild-type pEnR212CAT. All values are means ± S.D. of three separate experiments. C: EMSA was performed with 10 fmol of a ^{32}P-labeled +50–+76 fragment of the SPT/AGT gene as a probe and 5 μg of nuclear extracts from HepG2 cells, as described in Section 2. Arrows indicate two specific complexes (I, II). Asterisks represent non-specific bands. Wt, wild-type.

suggest that the two C/EBP sites are involved in the binding of C/EBP and activate transcription from the downstream start site. The results also suggest that an unknown factor that is responsible for the formation of the complex (II) may bind to the region overlapping or just downstream of the initiation site. Inhibition by the mutations of this factor's binding to this region might be involved in the increase in M9 promoter activity.

3.5. C/EBPα and C/EBPβ activate the minimal promoter of the SPT/AGT gene in HepG2 cells

To examine further whether C/EBPs function in the expres-

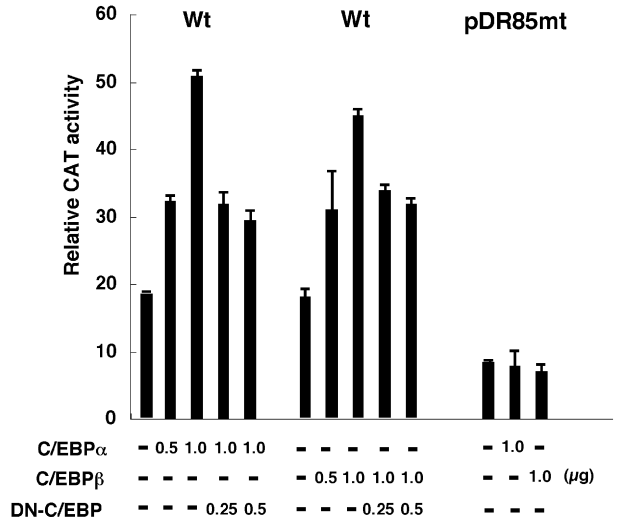

Fig. 5. Activation by C/EBPα and C/EBPβ of the downstream promoter activity. A C/EBPα expression vector (C/EBPα), pcDNA3.1-C/EBPα, or a C/EBPβ expression vector (C/EBPβ), pcDNA3.1-C/EBPβ, was co-transfected with wild-type pDR85CAT (Wt) or a mutated CAT reporter plasmid (pDR85mt) containing the same mutated C/EBP binding site as the mt8 vector. A dominant-negative inhibitor of C/EBP (DN-C/EBP) was co-expressed with C/EBPα or C/EBPβ. CAT activities are expressed relative to the activity obtained with wild-type pDR85CAT. All values are means ± S.D. of three separate experiments.

sion of the SPT/AGT gene, an expression vector of C/EBPα or C/EBPβ was co-transfected with pDR85CAT (Wt) into HepG2 cells (Fig. 5). Overexpression of both C/EBPα and C/EBPβ resulted in an approximately 3-fold increase in the basal expression of pDR85CAT. The enhancement of the expression by C/EBPs was reduced by co-expression of a dominant-negative inhibitor of C/EBP (DN-C/EBP) that forms an inactive heterodimeric complex with C/EBP [21,22]. Transfection of 1 μg of the DN-C/EBP plasmid alone caused an approximately 40% decrease in the promoter activity (data not shown). DN-CEBP did not completely block the promoter activity, possibly because of the unidentified factor(s) that binds to the promoter region (see Fig. 2), where it may play an additional important role in the regulation of the downstream promoter. Co-transfection of a C/EBPα or C/EBPβ expression vector with pDR85mt, which contains the same mutated C/EBP binding site as the mt8 vector depicted in Fig. 4A, had no effect. These results demonstrate that C/EBPα and C/EBPβ transactivate the downstream promoter of the SPT/AGT gene through the C/EBP binding sites.

4. Discussion

SPT/AGT is a liver-specific enzyme with unique features of dual species-specific and food habit-dependent organelle distributions. It is located largely in mitochondria in carnivores and entirely in peroxisomes in herbivores. In the rat, SPT/AGT is distributed in both mitochondria and peroxisomes, and the transcription of the rat SPT/AGT gene occurs from two start sites in exon 1 [9]. As described in Section 1, the downstream transcription initiation appears to be used as a means to distribute SPT/AGT to peroxisomes in addition to mitochondria. In the present study, we identified several *cis*-acting regulatory elements, including a short inverted repeat and C/EBP binding sites in the downstream promoter region. The short inverted repeat was located 20–35 bp upstream of the downstream start site and was found to be involved in transcriptional regulation. We found that the downstream promoter activity was controlled by C/EBP binding sites located around the downstream start site. Our results also indicated the possibility that an unknown factor might bind to the initiation site overlapping a C/EBP binding site to regulate the promoter.

Many cellular genes contain a wide variety of core promoter elements. Some genes contain only a TATA box, while others contain an initiator element of PyPyA(+1)NT/APyPy [18] in addition to or instead of a TATA box, or neither a TATA box nor an initiator. Furthermore, some genes exhibit a single transcriptional start site, whereas others exhibit multiple start sites. The downstream promoter region of the rat SPT/AGT gene contains no such *cis*-elements that would specify the site of transcription initiation, except for the initiator-like sequence of CCA(+1)AGGC. This sequence seems to be quite different from the known strong initiators such as the adenovirus major late promoter, terminal deoxynucleotidyltransferase gene promoter, and dihydrofolate reductase gene promoter [28], because it has been demonstrated that CA(+1)NT/A is the most important core sequence and that substitution of T/A(+3) with C/G, or following pyrimidines with purines, resulted in a significant reduction of promoter activity [18,19]. Besides, we did not find any specific contributions of the known initiator-binding factors such as YY-1, E2F/HIP1, TFII-I and USF-I to the formation of a protein complex with the SPT promoter. Moreover, neither the TATA box, GC box, nor CCAAT box, which serve as major regulatory elements within core promoters of many genes, was found. Therefore, we have been interested in a unique mechanism underlying the regulation of the TATA-less promoter of the SPT/AGT gene.

Sequence analysis of the downstream promoter revealed the presence of a short inverted repeat 20–35 bp upstream of the predetermined downstream start site. This short inverted repeat was subsequently shown to play an important role in the promoter activity by EMSA and transient CAT reporter analysis with a series of 5′-SPT/AGT gene constructs containing various mutations. Inverted repeat DNA sequences have the potential to form a cruciform structure through intra-strand base pairing, and the cruciform-binding proteins may be involved in the regulation of DNA transcription and replication [29]. In this study, however, it was not determined whether the short inverted repeat in the rat SPT/AGT gene could form a cruciform structure or whether the structure is involved in transcription regulation.

C/EBPs form a family of transcriptional factors that are crucial for cellular differentiation and functions in a variety of tissues. At least six members [30], C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (also known as CHOP), have been identified. Each of these proteins consists of an activation domain, a DNA-binding basic region, and a leucine-rich dimerization domain. Dimerization through the leucine zipper leads to the formation of homo- and heterodimers, which bind with their DNA-binding basic regions to a common palindromic DNA sequence of TT/GNNGNAAT/G in the promoter–enhancer region of a variety of genes [31].

In this study, HepG2 cells were used for functional analysis of the SPT/AGT downstream promoter. In neoplastic hepa-

tocytes, including HepG2 cells, basal expression of C/EBPα is known to be very low [25], unlike in mature hepatocytes, where C/EBPα is strongly and constitutively expressed [26]. The C/EBPβ expression level in these cells is higher than that of the α isoform but still lower than that in the adult liver. Therefore, the contribution, if any, of C/EBP to the basal promoter activity may be low in HepG2 cells because of its low content. However, when HepG2 cells were forced to overexpress C/EBPα or C/EBPβ, much stronger signals of C/EBP-specific bands were observed in EMSA (Fig. 3B,C), and the promoter activity was increased by about 3-fold in the CAT assay (Fig. 5), while the increase was suppressed by co-expression of dominant-negative inhibitor of C/EBP. Unfortunately, we could not achieve a complete suppression by the dominant-negative inhibitor to below the basal level of the downstream promoter activity, because the lower level of expression of the wild-type C/EBP reduced the promoter activation and the higher level of expression of the dominant-negative inhibitor caused cell death. Nevertheless, our results strongly suggest that C/EBP binds to and activates the downstream SPT/AGT promoter. Since it is known that C/EBPα and C/EBPβ play important roles in transcription regulation of liver-specific genes such as phosphoenolpyruvate carbooxykinase [32–34], it is reasonable to expect that the SPT/AGT gene is one of the targets of these factors under physiological conditions. The binding site(s) seems to partly overlap the transcription initiation site, indicating that C/EBP may be involved directly or indirectly in the machinery of initiator-binding proteins. However, we have not yet determined whether the initiator-like sequence is a functional element, and further study is therefore needed to elucidate the precise mechanism by which C/EBP and the inverted repeat sequence contribute to an initiation complex.

Our results also indicated the possibility that one of the C/EBP binding sites overlaps a binding site for a different kind of protein (Fig. 4C, complex II). When the 3′-region (+68–+70) from the start site was mutated, an unknown factor did not bind to the downstream promoter, whereas the binding of C/EBP to the promoter was not affected. In addition, this mutation increased the SPT/AGT promoter activity. These results suggest that a novel factor may function as a transcriptional repressor, or that the mutations unintentionally produced a stronger initiator and the behavior of complex (II) is not directly involved in the promoter activation. However, the latter seems to be less likely, because the sequence around the start site became much less homologous to the consensus initiator sequence by the mutations. Further study is needed to identify the transcription factor so that its role will be fully understood.

Acknowledgements: We thank Dr. Steven L. McKnight and Dr. Charles R. Vinson for providing us with C/EBP expression vectors and an expression vector of a dominant-negative inhibitor of C/EBP, respectively. This work was supported by a Grant-in-aid for scientific research (No. 11770059) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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