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Promoter and 11-Kilobase Upstream Enhancer Elements Responsible for Hepatoma Cell-Specific Expression of the Rat Ornithine Transcarbamylase Gene

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The gene for ornithine transcarbamylase (OTC; EC 2.1.3.3), a urea cycle enzyme, is expressed almost exclusively in the liver and small intestine. To identify DNA elements regulating transcription of the OTC gene in the liver, transient expression analysis was carried out by using hepatoma (HepG2) and nonhepatic (CHO) cell lines. The 1.3-kilobase 5′-flanking region of the rat OTC gene directed expression of the fused chloramphenicol acetyltransferase gene in HepG2 cells much more efficiently than in CHO cells. Analysis of deletion mutants of the 5′-flanking region in HepG2 cells revealed that there are at least one negative and two positive regulatory elements within the about 220-base-pair immediate 5′-flanking region. DNase I footprint analysis showed the presence of factors binding to these regulatory elements in nuclear extracts of rat liver and brain, and footprint profiles at the two positive elements exhibited liver-specific features. Transient expression analysis also revealed the existence of an enhancer region located 11 kilobases upstream of the transcription start site. The OTC enhancer was able to activate both its own and heterologous promoters in HepG2 but not in CHO cells. The enhancer was delimited to an about 230-base-pair region, and footprint analysis of this region revealed four protected areas. Footprint profiles at two of the four areas exhibited liver-specific features, and gel shift competition analysis showed that a factor(s) binding to the two liver-specific sites is related to C/EBP. These results suggest that both liver-specific promoter and enhancer elements regulate expression of the OTC gene through interaction with liver-specific factors binding to these elements.

The liver exhibits various tissue-specific functions such as gluconeogenesis, urea biosynthesis, drug detoxication, and secretion of plasma proteins. These functions are performed by cooperation of multiple liver-specific gene products. Examination of the regulation of liver-specific genes should provide insight into the coordinated expression of tissue-specific genes and also into the mechanism of underlying cell differentiation. *cis*-Acting elements and *trans*-acting factors regulating liver-specific transcription have been characterized for genes of plasma proteins, including serum albumin (8, 9, 25, 31, 33, 42, 50), α-fetoprotein (21, 22, 26, 48, 55, 64), α_1-antitrypsin (11, 40, 46), fibrinogens (3, 16), and transthyretin (14). A liver-specific transcription factor, HNF1 (also designated LF-B1), that activates these genes has been purified (15, 41) and cloned (18). Another liver-enriched putative transcription factor, C/EBP, that binds to the *cis* elements of these genes has also been characterized (4, 19, 38, 67) following purification (35) and cloning (37).

Mammalian ornithine transcarbamylase (OTC; carbamoylphosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3) catalyzes the second step of urea biosynthesis and is encoded on the X chromosome (43). The OTC gene is expressed mainly in the liver and to a lesser extent in the small intestine (52, 66). mRNA of this enzyme begins to accumulate late in the fetal period and continues to increase postnatally to the level seen in adults (47, 53). We reported that the rat OTC gene is 75 kilobases (kb) long and is split into 10 exons (59). The promoter region of the gene lacks TATA and CCAAT elements situated at typical positions. The mouse (56) and human (27) OTC genes are highly homologous to the rat gene.

Recently, we generated transgenic mice that carry a chimeric gene bearing the 1.3-kb 5′-flanking region of the rat OTC gene (49a). The introduced gene was expressed exclusively in the liver and small intestine, thereby indicating that the 1.3-kb 5′-flanking region is capable of directing liver- and small intestine-specific transcription. However, expression of the introduced gene in the liver was much lower than that of the endogenous mouse OTC gene. Therefore, this region is apparently not sufficient for a high level of expression in the liver, and the presence of enhancerlike elements outside the 1.3-kb 5′-flanking region was suggested.

In this study, in order to identify *cis*-acting DNA elements in the promoter region, and in search of enhancerlike elements, hybrid genes containing portions of the OTC gene ligated to the reporter chloramphenicol acetyltransferase (CAT) gene were introduced into hepatic and nonhepatic cell lines. We found that there are at least one negative and two positive elements in the 5′-flanking region and that there exists a cell type-specific enhancer region located 11 kb upstream of the transcription start site. In addition, DNase I footprinting analysis using nuclear extracts of the rat liver and brain was carried out to identify factors binding to these *cis* regulatory elements. Footprints with liver-specific features were detected in the two positive promoter elements and in the enhancer region, showing the presence of DNA-binding factors that are candidates for liver-specific *trans*-acting factors.

MATERIALS AND METHODS

Plasmid constructions. The basal promoterless CAT plasmid pSV00CAT, a derivative of pSV0CAT (24), was described previously (1). This plasmid was devised to decrease

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the background CAT activity derived from nonspecific transcripts that start at the procaryotic vector region and bears a transcription stop signal of simian virus 40 (SV40) situated just upstream of the *Hin*dIII site, where relevant promoter sequences are inserted. To construct 5′-deletion mutants of the rat OTC promoter region, we first excised restriction fragments of the cloned OTC gene (59), all ending at the *Xba*I site of position +53 base pairs (bp) relative to the transcription start site and extending up to −3.1 kb. After construction of *Hin*dIII ends on both sides by using a synthetic linker, the fragments were inserted into the *Hin*dIII site of pSV00CAT. The resulting plasmids bear 3.1 kb, 1.3 kb, 446 bp, and 222 bp of the 5′-flanking sequences of the OTC gene, and 5′ endpoints of the sequences are *Hin*dIII, *Pvu*II, *Nco*I, and *Hin*cII sites, respectively. Further truncation of the 5′ end was done as follows. The *Hin*dIII linker-attached *Hin*cII (−220 bp)-*Xba*I (+53 bp) fragment of the OTC gene was inserted into pUC18. The resulting plasmid was opened at the polylinker *Bam*HI site situated adjacent to the *Hin*cII end of the OTC gene, digested with the BAL 31 nuclease for variable periods, blunt ended with Klenow fragment, and ligated with the *Hin*dIII linker. From the resulting plasmids, the OTC gene inserts with different 5′ ends were excised with *Hin*dIII and inserted into the *Hin*dIII site of pSV00CAT, generating plasmids bearing 162, 112, 85, 42, and 15 bp of the 5′-flanking sequences of the OTC gene. The precise 5′ endpoints of these deletion mutants were determined by sequencing the double-stranded plasmids (28) by the dideoxynucleotide chain termination method (54).

To detect enhancer activity, various restriction fragments spanning the region from −13.8 to +1.6 kb of the OTC gene were examined. After construction of *Bam*HI ends on both sides by using the *Bam*HI or *Bgl*II linker, the fragments were inserted into the *Bam*HI site of pOC1.3kCAT, which bears the 1.3-kb 5′-flanking region of the OTC gene as a promoter.

To examine the promoter specificity of the enhancer element, the relevant fragments of the OTC gene were inserted into the *Bam*HI site of ptkCAT (30; provided by H. Kondoh, Nagoya University) and $pA_{10}CAT_2$ (36; provided by S. Ishii, Institute of Physical and Chemical Research), which bear, respectively, the promoter region (−197 to +56 bp relative to the transcription start site) of the herpes simplex virus (HSV) thymidine kinase (*tk*) gene and the enhancerless promoter region of the SV40 early gene. As a positive control of enhancer activity, we used the SV40 enhancer region including 72-bp repeats, inserting the *Fok*I-*Pvu*II (positions 98 to 272; numbering according to reference 6) fragment (provided by J. Miyazaki, this institute) into the *Bam*HI site of pOC1.3kCAT, ptkCAT, and $pA_{10}CAT_2$.

The plasmids were prepared from *Escherichia coli* MC1061 $RecA^-$ by the method of Birnboim (5) and further purified by centrifugation to equilibrium in ethidium bromide-CsCl gradients. Purities of the plasmids were checked by agarose gel electrophoresis, confirming that intensities of plasmid bands were proportional to amounts of nucleic acids determined by spectrophotometry.

Cell culture and transient expression assays. Human hepatoma HepG2 cells (provided by K. Araki, this institute) and Chinese hamster ovary (CHO) cells were grown, respectively, in Dulbecco modified Eagle medium and F-12 medium, supplemented with 10% fetal calf serum. Before transfection, the cells were plated in 100-mm-diameter culture dishes at approximately 10% confluence and cultured for 24 h (HepG2) or 36 h (CHO). Transfection was carried out by the calcium phosphate precipitation method, using a total of 15 μg of DNA mixture containing 10 μg of the CAT gene recombinant plasmid and 5 μg of an internal standard plasmid bearing the β-galactosidase gene under the control of the chicken β-actin promoter (44; provided by J. Miyazaki). The cells were shocked 6 h later for 2 min with 12.5 and 15% glycerol for HepG2 and CHO, respectively. The cells were harvested 48 h after the transfection and broken by three cycles of freezing and thawing, followed by sonication. After centrifugation, supernatants were assayed for CAT and β-galactosidase activities. The CAT assay was carried out as described by Gorman (23). The reaction was allowed to proceed for 60 min in 75 μl of a mixture containing 300 to 400 μg of protein, 0.25 μCi of [^{14}C]chloramphenicol (specific activity, 60 mCi/mmol), and 0.5 mM acetyl coenzyme A. Quantification of CAT activity was done by scintillation counting of spots cut from the chromatograms. The β-galactosidase assay was performed as described previously (32), using 200 to 300 μg of protein and 0.6 mM *o*-nitrophenyl-β-D-galactopyranoside in 700 μl of the reaction mixture. CAT activity was normalized for transfection efficiency, dividing CAT activity by β-galactosidase activity.

DNase I footprint analysis. Nuclear extracts from rat liver and brain were prepared as described by Gorski et al. (25). The binding reaction was carried out for 15 min on ice in 50 μl of a mixture containing 25 mM Tris hydrochloride (pH 7.6), 50 mM KCl, 1 mM EDTA, 0.5 mM spermidine, 0.6 mM dithiothreitol, 12% glycerol, 0.3 μg of poly(dI-dC), 10 fmol of the ^{32}P-labeled DNA fragment (about 10^5 dpm), and 35 μg of nuclear extract protein. After addition of 50 μl of a solution containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ to the mixture, freshly diluted DNase I of an empirically adjusted amount was added, and digestion was allowed to proceed for 60 s at room temperature. The reaction was stopped by addition of 100 μl of 1% sodium dodecyl sulfate containing 20 mM EDTA and 200 mM NaCl. After addition of 20 μg of yeast tRNA as a carrier, nucleic acids were extracted three times with phenol-chloroform and collected by ethanol precipitation. Electrophoresis was carried out in a 5% polyacrylamide–7 M urea gel.

Gel shift assays. Double-stranded oligonucleotide probes were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The binding reaction was carried out in 20 μl of a mixture containing 25 mM Tris hydrochloride (pH 7.6), 50 mM KCl, 1 mM EDTA, 0.5 mM spermidine, 0.6 mM dithiothreitol, 12% glycerol, 5 μg of poly(dI-dC), 1 fmol of the ^{32}P-labeled oligonucleotide probe (about 2×10^4 dpm), 1 pmol of the oligonucleotide competitor, and 5 μg of liver nuclear extract protein. The competitor and the probe were mixed before addition of the extracts. After 30 min on ice, 5 μl of 20% Ficoll was added, and the samples were loaded onto a 5% polyacrylamide gel made in 0.25× TBE buffer (1× TBE consists of 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA). Electrophoresis was performed at 10 V/cm for 2 h at room temperature.

RESULTS

Identification of *cis*-acting regulatory elements in the promoter region. In previous work on transgenic mice, we noted that the 1.3-kb 5′-flanking region of the rat OTC gene can drive liver- and small intestine-specific transcription (49a). To identify *cis*-acting elements responsible for transcriptional regulation in the liver, we linked the rat OTC 5′-flanking sequences of various lengths to the CAT gene. These fused genes were transiently transfected into the

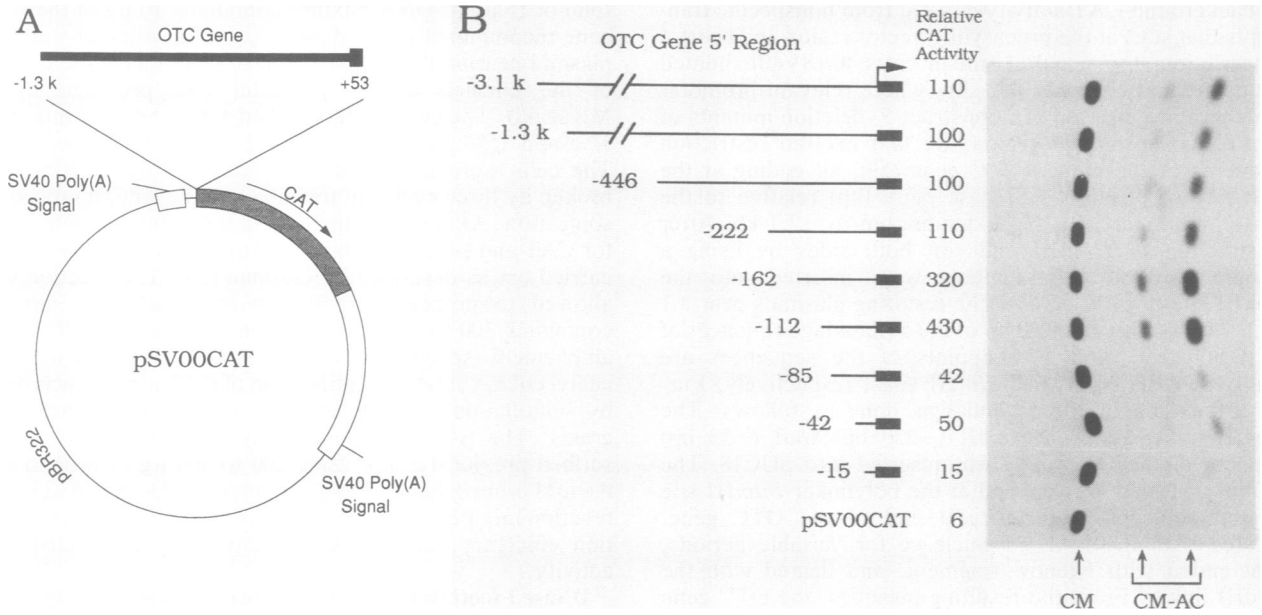


FIG. 1. Transient expression analysis of 5'-deletion mutants of the OTC promoter region by CAT assay with HepG2 cells. (A) Structures of plasmid pSV00CAT and its derivative pOC1.3kCAT, bearing the rat OTC gene 5' region from −1.3 kb to +53 bp inserted into the *Hin*dIII site of pSV00CAT. The thick line and solid box in the OTC gene insert represent the 5'-flanking region and exon 1, respectively. (B) Analysis of 5'-deletion mutants. A series of plasmids bearing the OTC 5'-flanking sequences of various lengths was constructed and subjected to CAT assay as described in Materials and Methods. CAT activities are expressed relative to the activity obtained with the construct bearing the 1.3-kb 5'-flanking region (underlined) and represent averages of at least three separate experiments. An autoradiogram of a representative CAT assay is shown on the right. Positions of the substrate chloramphenicol (CM) and acetylated products (CM-AC) are indicated.

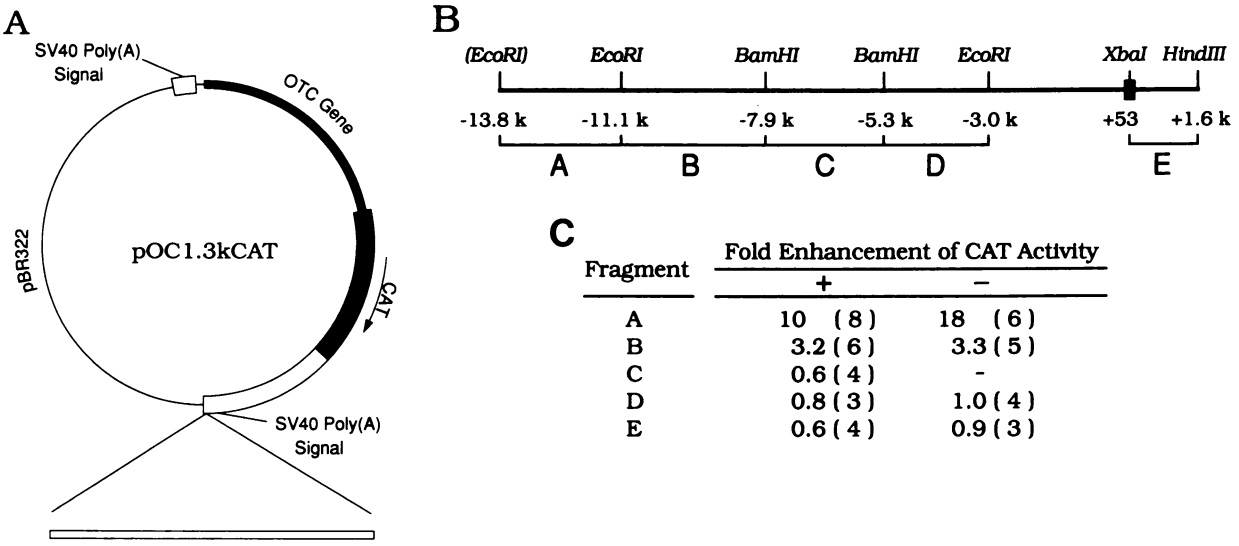
human hepatoma cell line HepG2, and CAT enzyme activities were measured (Fig. 1). To correct for differences in DNA uptake, the cells were cotransfected with a plasmid carrying the β-galactosidase gene under the control of the chicken β-actin promoter. CAT activity was normalized to β-galactosidase activity.

When plasmid pOC1.3kCAT, which bears the OTC gene 5' region from −1.3 kb to +53 bp, was transfected into HepG2 cells, the cell extracts gave CAT activity that was about 17-fold higher than that obtained with the backbone promoterless plasmid pSVOOCAT. Elongation of the 5'-flanking sequences to −3.1 kb from −1.3 kb led to no remarkable change in CAT activity. Deletion of the sequences from −1.3 kb to −446 bp and −222 bp also did not significantly affect activity. Deletion from −222 to −162 bp caused an about threefold increase in activity. Deletion from −112 to −85 bp resulted in an about 10-fold decrease in activity. Reduction of activity was also caused by deletion from −42 to −15 bp. The reduction was calculated as about fivefold, taking the CAT activity obtained with promoterless plasmid pSV00CAT as background and subtracting this value from activities obtained with the constructs carrying the OTC promoter regions. Although the background activity was relatively high in this case, we repeatedly observed reduction ranging from 4.3- to 5.2-fold in four separate experiments. These results suggest that there are at least one negative *cis*-acting regulatory element located within the region from −222 to −162 bp and at least two positive elements located within the regions from −112 to −85 bp and −42 to −15 bp.

Identification of the enhancer region located far upstream. To search for enhancer elements that might exist far from the promoter elements, we cleaved the region from −13.8 to +1.6 kb of the OTC gene into 1.7- to 3.2-kb segments with restriction enzymes and inserted them into the *Bam*HI site of plasmid pOC1.3kCAT, which carries the 1.3-kb 5'-flanking region of the OTC gene as a promoter of the CAT transcription unit. The constructs were tested for CAT activity by transient transfection into HepG2 cells (Fig. 2). Significant increases in CAT activity were observed when the constructs containing the 2.7-kb segment located in the region from −13.8 to −11.1 kb (fragment A) were transfected. The extents of the increases were 10- and 18-fold, respectively, when the 2.7-kb segment was inserted in the same and opposite orientations relative to the CAT transcription unit. Thus, a DNA element(s) located in the region from −13.8 to −11.1 kb activates the OTC promoter from a distance in an orientation-independent manner, exhibiting the properties of the enhancer. We refer to the putative element(s) as the OTC enhancer.

The constructs containing the segment in the region from −11.1 to −7.9 kb (fragment B) showed moderately increased CAT activities (about threefold); the role of this region in transcriptional regulation remains to be examined. Insertion of segments located in the region from −7.9 to +1.6 kb (fragments C, D, and E) led to no obvious change in CAT activity.

Promoter specificity of the OTC enhancer. To examine whether the OTC enhancer can activate the transcription from heterologous promoters, the 2.7-kb enhancer region was inserted into the *Bam*HI site of plasmids ptkCAT and $pA_{10}CAT_2$, in which the CAT gene is under the control of the HSV *tk* promoter and the SV40 early promoter, respectively. As a positive control of enhancer activity, the SV40 enhancer including 72-bp repeats was used and inserted into the corresponding site of each plasmid instead of the OTC enhancer. These constructs were tested for CAT activity in HepG2 cells (Fig. 3A). In all series of the constructs bearing



C

Fragment	Fold Enhancement of CAT Activity +	−
A	10 (8)	18 (6)
B	3.2 (6)	3.3 (5)
C	0.6 (4)	-
D	0.8 (3)	1.0 (4)
E	0.6 (4)	0.9 (3)

FIG. 2. Examination of various regions of the OTC gene for enhancer activity. (A) Structures of plasmids. The basic plasmid pOC1.3kCAT (see Materials and Methods and Fig. 1) bears the 1.3-kb 5′-flanking region of the OTC gene as a promoter that drives the CAT gene. DNA fragments to be tested for enhancer activity were inserted into the *Bam*HI site. (B) Location of the DNA fragments examined. The 5′ region of the OTC gene is drawn on the top. Exon 1 is indicated by the solid box; the 5′-flanking region and intron 1 are shown as thick lines. Restriction sites used to excise the test fragments are shown with their locations relative to the transcription start site. The *Eco*RI site indicated in parentheses is derived from the multiple cloning site of EMBL4. (C) Effects of fragments A to E on CAT expression in HepG2 cells. Except for fragment C, both constructs bearing the fragments inserted in the same (+) and opposite (−) directions relative to the CAT transcription unit were examined. CAT activities are expressed as fold enhancement relative to the activity obtained with pOC1.3kCAT and represent averages of the number of experiments indicated in parentheses.

the OTC, HSV *tk*, or SV40 early promoter, the OTC enhancer caused marked increases in CAT activity (Fig. 3A, lanes 2, 5, and 8) compared with the enhancerless constructs (lanes 1, 4, and 7). The extent of the increases by the OTC enhancer was comparable to that seen with the SV40 enhancer (lanes 3, 6, and 9). These results indicate that the OTC enhancer can activate heterologous promoters as well as its own promoter.

Cell type specificity of the OTC promoter and enhancer. The constructs used in Fig. 3A were tested for expression in a nonhepatic cell line, CHO cells (Fig. 3B). As for cell type specificity of the OTC promoter, plasmid pOC1.3kCAT bearing the 1.3-kb 5′-flanking region of the OTC gene as a promoter (Fig. 3B, lane 10) showed much lower CAT activity than did those bearing the HSV *tk* (lane 13) and SV40 early (lane 16) promoters. This is in sharp contrast to the results in HepG2 cells, where the activity of the OTC promoter (Fig. 3A, lane 1) was comparable to those of the HSV *tk* (lane 4) and SV40 early (lane 7) promoters. Thus, the 1.3-kb 5′-flanking region of the rat OTC gene exhibited much lower promoter activity in CHO than in HepG2 cells. This result is consistent with the observation of Veres et al. (61) that the 800-bp 5′-flanking region of the mouse OTC gene directs expression of the fused CAT gene in HepG2 cells but not in mouse fibroblast NIH 3T3 cells. These results are also consistent with our previous observation in transgenic mice that the 1.3-kb 5′-flanking region can drive liver- and small intestine-specific transcription (49a).

With regard to the cell type specificity of the enhancer, the constructs bearing the OTC enhancer region (Fig. 3B, lanes 11, 14, and 17), in the context of any promoter, showed much the same CAT activity with the enhancerless constructs (lanes 10, 13, and 16) in CHO cells, whereas the SV40 enhancer (lanes 12, 15, and 18) led to an increase in activity (even in the context of the OTC promoter, a low CAT activity was detected [lane 12]). These results indicate that the OTC enhancer is inactive in CHO cells. Thus, the enhancer activity of the region from −13.8 to −11.1 kb of the rat OTC gene is cell type specific.

Delimitation of the enhancer region. To identify the sequences responsible for enhancer activity, we examined various parts of the region from −13.8 to −11.1 kb and the adjacent downstream region from −11.1 to −7.9 kb. Excised restriction fragments were inserted into the *Bam*HI site of pOC1.3kCAT, and effects on CAT gene expression were tested in HepG2 cells (Fig. 4). Significant increases in CAT activity to the extent of more than 20-fold were caused by fragments sharing the *Hinc*II-*Eco*RI region of about 230 bp located just upstream of the *Eco*RI site at −11.1 kb (Fig. 4, lines 4, 5, 7, and 9). The *Hinc*II-*Eco*RI fragment by itself caused a 40-fold increase in activity (line 9). On the other hand, in CHO cells, the *Hinc*II-*Eco*RI fragment failed to augment expression of the CAT gene under the control of the OTC or SV40 early promoter (data not shown). Thus, the OTC enhancer region was delimited to the 230-bp region located at about −11 kb, preserving the cell type specificity.

DNase I footprint analysis of factors binding to the OTC promoter region. To identify DNA-binding factors that might be involved in transcriptional regulation, we carried out DNase I footprint analysis of the DNA fragment spanning the promoter region (Fig. 5; summarized in Fig. 7A). Nuclear extracts were prepared from rat liver and also from rat brain, in which OTC enzyme activity is not detectable. An equivalent amount of protein of each extract was used for the binding assay. With the liver nuclear extracts, three distinct protected regions (regions A, B, and C) were detected upstream of the transcription start site, and one protected region (region D) was detected just downstream. A partially protected region (region E) was also detected further downstream. With the brain nuclear extracts, almost the same ranges were protected for regions A and D,

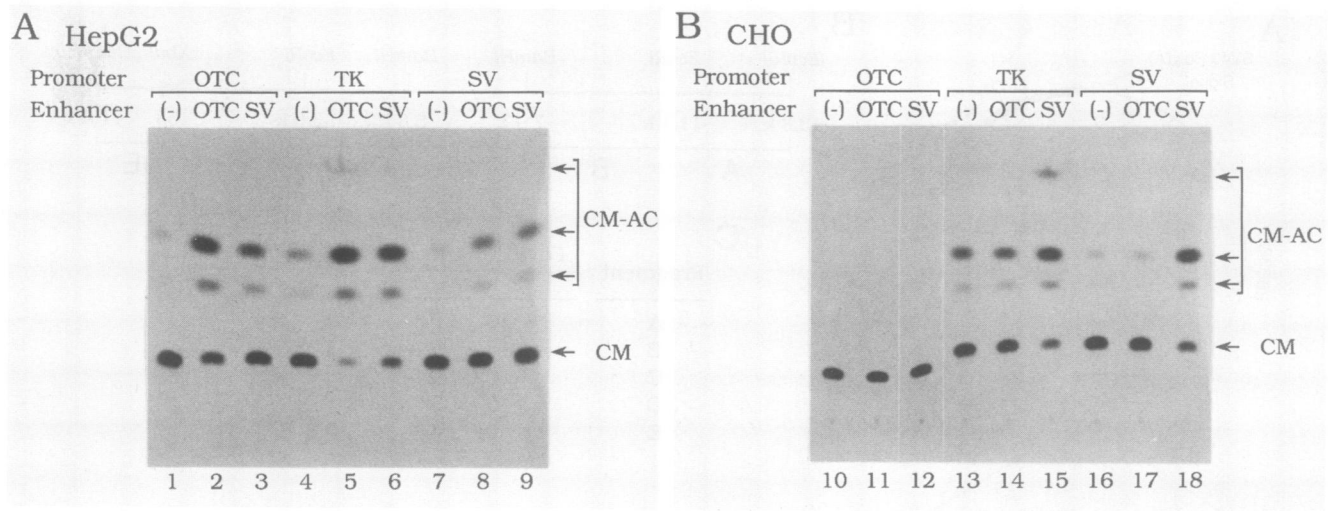


FIG. 3. Effects of the OTC and SV40 enhancers on expression of the CAT gene driven by the OTC, HSV *tk* (TK), and SV40 early (SV) promoters in HepG2 (A) and CHO (B) cells. The 2.7-kb OTC enhancer region (−13.8 to −11.1 kb) or the SV40 enhancer region (98 to 272; numbering according to reference 6) was inserted into the *Bam*HI site of plasmids pOC1.3kCAT, ptkCAT, and $pA_{10}CAT_2$, bearing the OTC, HSV *tk*, and SV40 early promoters, respectively. Resulting constructs were subjected to CAT assay. Orientations of the OTC enhancer inserts were the same as those of the CAT transcription units; orientations of the SV40 enhancer inserts were not determined. Positions of the substrate chloramphenicol (CM) and acetylated products (CM-AC) are indicated.

whereas for regions B and C more restricted ranges were protected than with the liver nuclear extracts.

Region A at positions −250 to −169 bp overlaps with the negative *cis*-acting regulatory sequence −222 to −162 bp. Thus, some of the factors binding to this relatively long range of region A might be negative *trans*-acting transcription factors, and they may be ubiquitous, since the protection pattern of this region is indistinguishable between the liver nuclear extracts and the brain extracts.

Region B at positions −112 to −92 bp overlaps with the positive *cis*-acting sequence at positions −112 to −85 bp, and region C at −37 to +2 bp overlaps with the positive sequence at positions −42 to −15 bp. Thus, factors that are present in the liver and bind to these regions might be positive transcription factors. Ranges of the two regions protected with the brain nuclear extracts were more restricted than ranges of regions protected with the liver extracts. Although the differences between the liver and brain were slight, similar differences were observed with a probe labeled at another restriction site on the upper strand (data not shown). The binding factors in the brain probably differ from those in the liver with respect to molecular characteristics or intracellular concentrations. These putative differences in binding factors may be responsible for the tissue-specific activation of the OTC promoter, considering the possibility that the binding factors in the brain are either negative or neutral to transcriptional activation.

Region D is situated at a potentially important location, that is, just downstream of the transcription start site, and functional analysis remains to be performed.

Analysis of factors binding to the enhancer region. The about 230-bp enhancer region was also subjected to DNase I footprint analysis (Fig. 6; summarized in Fig. 7B). Obvious protections were detected at four regions with the liver nuclear extracts (regions I to IV). Footprint profiles of regions I and IV were indistinguishable between the liver

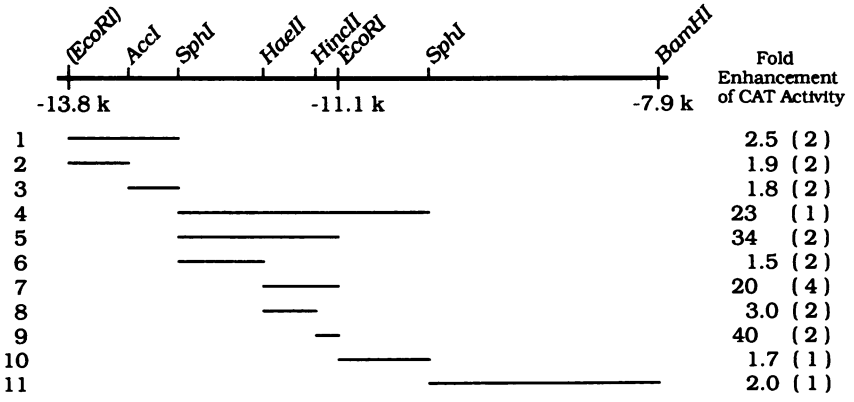


FIG. 4. Delimitation of the enhancer region. Restriction sites used to excise the test fragments and positions of some sites relative to the transcription start site are shown at the top. The *Eco*RI site shown in parentheses is derived from the multiple cloning site of EMBL4. Fragments indicated by horizontal bars (numbered 1 to 11) were inserted into the *Bam*HI site of pOC1.3kCAT, and resulting constructs (orientation of the inserts was not determined) were transfected into HepG2 cells. CAT activities are expressed as fold enhancement relative to the activity obtained with pOC1.3kCAT and represent averages of the number of experiments indicated in parentheses.

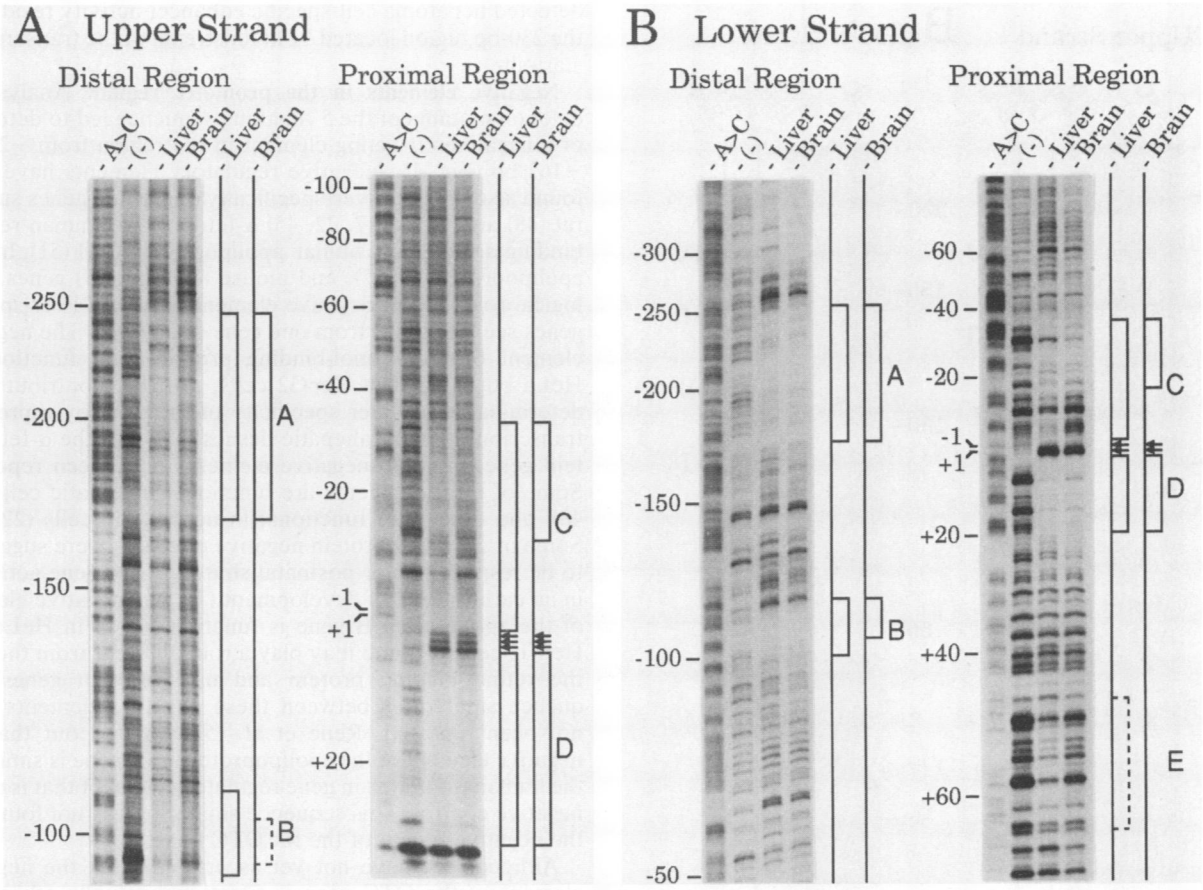


FIG. 5. Footprint analysis of the upper (A) and lower (B) strands of the OTC promoter region. Probes were the *Pst*I (−842 bp)-*Xba*I (+53 bp) fragment labeled at the 3′ end of the *Xba*I site with [α-^{32}P]dCTP and avian myeloblastosis virus reverse transcriptase (A) and the *Pst*I (−842 bp)-*Bam*HI (+108 bp) fragment labeled at the 5′ end of the *Bam*HI site with [γ-^{32}P]ATP and T4 polynucleotide kinase (B). Binding reactions with rat liver and brain nuclear extracts, followed by partial DNase I digestion, were carried out as described in Materials and Methods. Lanes marked (−) show the control digestion pattern obtained in the absence of added nuclear extracts; lanes A>C, chemical cleavage of the probes as sequence markers. Numbers refer to positions relative to the transcription start site. The boxes alongside the autoradiograms represent regions protected from DNase I digestion; the dashed boxes represent weakly protected regions. Arrows indicate DNase I-hypersensitive sites.

nuclear extracts and the brain extracts, suggesting that the factors binding to these regions are ubiquitous. No clear footprint corresponding to region II was detected with the brain extracts. The footprint of region III obtained with the brain extracts was less intense than that obtained with the liver extracts; therefore, the nature or amount of the factor(s) binding to this region is assumed to differ between the liver and brain. Thus, the OTC enhancer seems to bind multiple factors, some liver specific and others ubiquitous.

Relationship of the binding factors to previously characterized factors that participate in liver-specific transcription. Since it has been shown that a liver-specific transcription factor, HNF1, and a liver-enriched putative transcription factor, C/EBP, bind to *cis*-acting regions of multiple liver-specific genes, we carried out gel shift competition analysis to examine whether the factors binding to the regulatory regions of the OTC gene are related to these previously characterized factors.

C/EBP-DNA complexes were formed by incubating an oligonucleotide probe containing the C/EBP-binding site of the hepatitis B virus (HBV) enhancer (37, 58) with the liver nuclear extracts (Fig. 8A, lane 2). Multiple shifted bands were detected, as has been reported for C/EBP-binding sites of other genes (13, 34). Then we examined whether oligonucleotides each covering footprint regions I to IV in the OTC enhancer region, or each covering footprint region B or C overlapping with positive promoter elements, could compete for the complex formations (lanes 4 to 9). Competitions were observed with oligonucleotides covering footprint regions II and III (lanes 5 and 6) but not with others (lanes 4, 7, 8, and 9). Inversely, complex formations with the oligonucleotides covering regions II and III used as probes, but not with other oligonucleotides, were prevented by the unlabeled oligonucleotide containing the HBV C/EBP-binding site (data not shown). These results indicate that a factor(s) binding to regions II and III of the OTC enhancer is related to C/EBP.

The same set of oligonucleotides covering the OTC regulatory regions were tested for the ability to compete for HNF1 binding, using a probe containing the HNF1-binding site of the rat β-fibrinogen promoter (16) (Fig. 8B). No obvious competition was observed with any oligonucleotide. In addition, complex formations with the oligonucleotides covering the OTC regulatory regions were not prevented by the oligonucleotide containing the β-fibrinogen HNF1-bind-

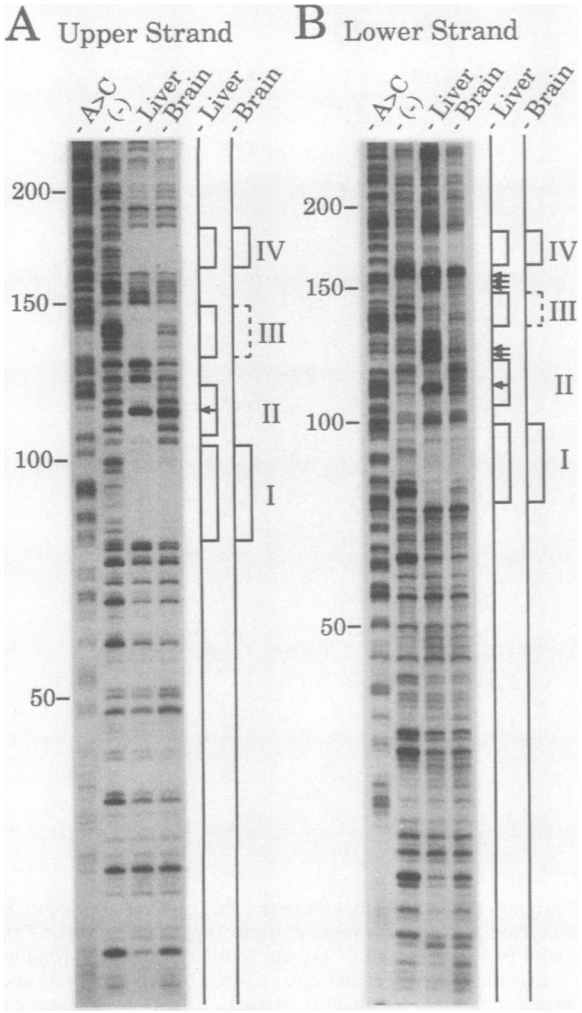


FIG. 6. Footprint analysis of the upper (A) and lower (B) strands of the OTC enhancer region. For preparation of the probes, the about 230-bp *Hin*cII-*Eco*RI enhancer segment with *Bam*HI linkers on both ends was inserted into the *Bam*HI site of pUC19. From the resulting plasmid, the about 370-bp *Hin*dIII (situated in pUC19, adjacent to the *Hin*cII end of the enhancer segment)-*Pvu*II (situated in pUC19) fragment including the enhancer segment was excised and used as the probe after labeling of the upper strand at the 5′ end of the *Hin*dIII site (A) and the lower strand at the 3′ end of the *Hin*dIII site (B) as described in the legend to Fig. 5. Numbers refer to positions relative to the most upstream nucleotide (no. 1) of the enhancer segment. Other symbols are as in Fig. 5.

ing site (data not shown). Thus, factors binding to these OTC regulatory regions differ from HNF1.

DISCUSSION

We investigated promoter and enhancer elements of the rat OTC gene in an attempt to understand how this gene is transcribed tissue specifically. Our previous work on transgenic mice indicated that the 1.3-kb 5′-flanking region of the rat OTC gene can drive liver- and small intestine-specific transcription (49a). In this study, we analyzed the promoter elements with a transient expression system, using the hepatoma cell line HepG2. One negative and two positive *cis*-acting sequences were detected within the 5′ region up to position −222 bp. In a further search for an enhancer, we detected hepatoma cell-specific enhancer activity residing in the 230-bp region located 11 kb upstream of the transcription start site.

Negative elements in the promoter region. Analysis of deletion mutants of the 5′-flanking sequence led to detection of a negative *cis*-acting element in the region from −222 to −162 bp (Fig. 1). Negative regulatory elements have been found also in other liver-specifically expressed genes such as rat (48) and mouse (7, 22, 45) α-fetoprotein, human retinol-binding protein (12), human apolipoprotein CIII (51), human apolipoprotein B (17), and mouse albumin (33) genes. Biological roles of the negative elements of these liver-specific genes seem to differ from one gene to another. The negative element of the retinol-binding protein gene functions in HeLa but not in the HepG2 cells, possibly contributing to determination of liver specificity of the gene by repressing transcription in extrahepatic tissues (12). For the α-fetoprotein gene, multiple negative elements have been reported. Some of those elements are functional in hepatic cells (45, 48), and others are functional in nonhepatic cells (22, 45). Some of the α-fetoprotein negative elements were suggested to be responsible for postnatal shutoff of the gene activated in an earlier stage of development (7). The negative element of the apoliprotein B gene is functional both in HeLa and HepG2 cells (17) and may play a role different from those of the retinol-binding protein and α-fetoprotein genes. Sequence similarities between these negative elements have not been reported. Reue et al. (51) pointed out that the negative element of the apolipoprotein CIII gene is similar to the human β-interferon gene regulatory element that is under negative control. This sequence similarity was not found for the negative region of the rat OTC gene.

Although we have not yet ascertained that the negative element of the OTC gene is functional in cells other than HepG2, our preliminary results showed that in CHO cells, elimination of the negative region brings about derepression of CAT gene expression (data not shown), which is otherwise almost completely repressed (Fig. 3). The presence of similar factors binding to the negative region in the liver and brain nuclear extracts (Fig. 5) also supports the idea that the negative element can function in various tissues. The negative element might be mainly responsible for repression of the OTC gene in tissues other than the liver and small intestine or in early developmental stages when the gene is not expressed. Alternatively, the negative element might be involved in precise transcriptional regulation of the gene in the liver and small intestine, interacting with positive elements.

Positive elements in the promoter region. Two positive regulatory elements were detected in the 5′-flanking region (Fig. 1). The more upstream element is situated in the region from −112 to −85 bp. The liver nuclear extracts contain a factor(s) binding to this positive region, and its footprint profile differs from that obtained with the brain nuclear extracts (Fig. 5), suggesting that a liver-specific *trans*-acting factor binding to this region might mediate transcriptional activation. This protected area (region B) overlaps with a sequence CTTaAAgTTcAAG (uppercase letters represent the palindromic region) at positions −110 to −98 bp. The A+T-rich palindromic feature of this sequence is reminiscent of the consensus sequence GTTAATnATTAAC for the binding sites of the liver-specific transcription factor HNF1 (15), which is identical or similar to factors named APF (8), LF-B1 (18, 46), HP1-recognizing factor (57), and AFP1 (55). However, gel shift competition analysis showed that the factor binding to region B is not related to HNF1 (Fig. 8).

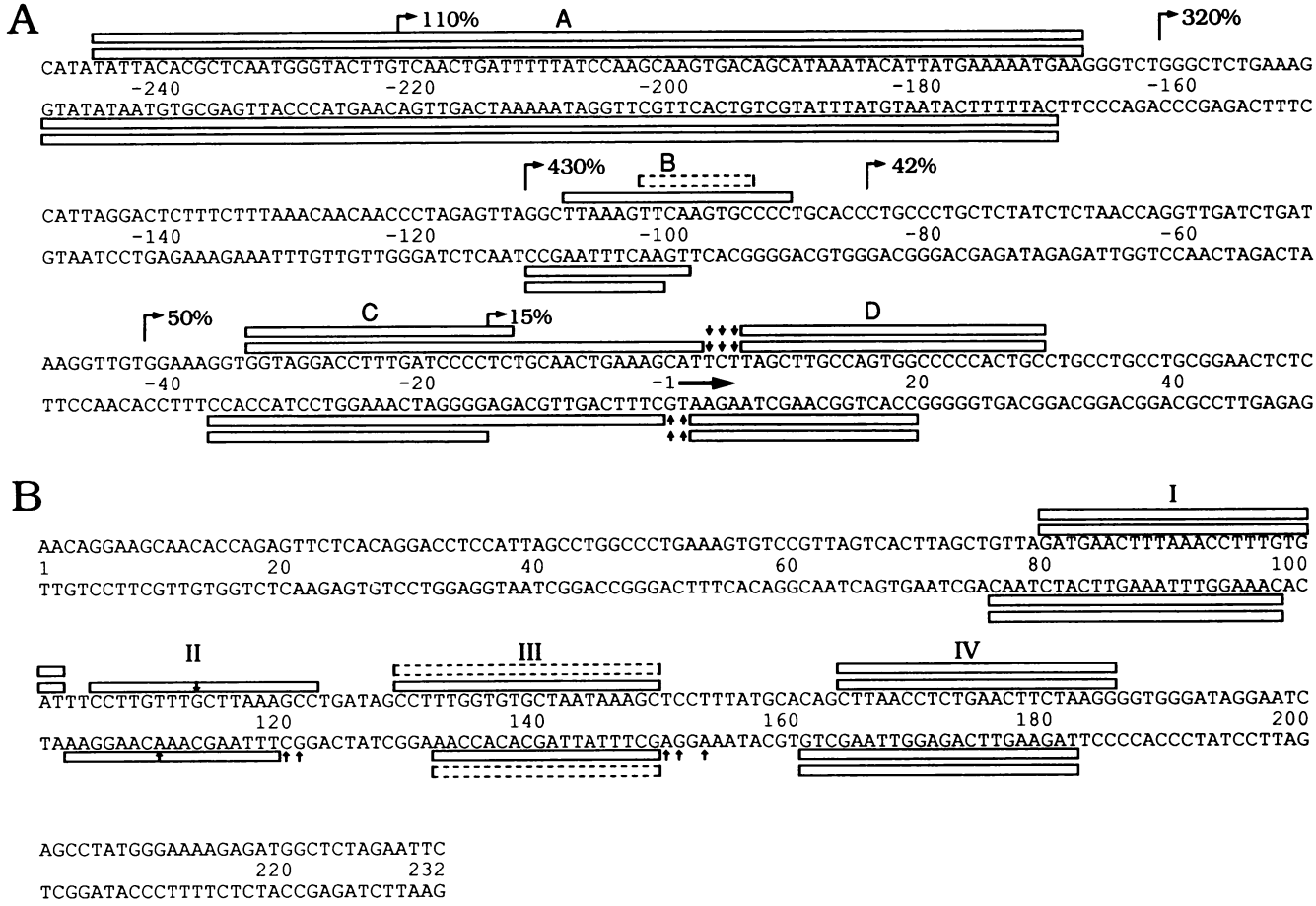


FIG. 7. Summary of analyses of the promoter (A) and enhancer (B) regions of the rat OTC gene. (A) The nucleotide sequence is from Takiguchi et al. (59). Numbers refer to positions relative to the transcription start site shown by the thick horizontal arrow. The hooked arrows indicate endpoints of the 5'-deletion mutants examined for promoter activities by CAT assay; the CAT activity of each mutant is shown by the value relative to that obtained with pOC1.3kCAT (see Fig. 1). Boxes adjacent to and apart from the sequence refer to footprint regions detected with nuclear extracts from rat liver and brain, respectively; the dashed box represents the weakly protected region. The letters A to D correspond to the regions shown in Fig. 5 (region E is now shown). The small vertical arrows indicate hypersensitive sites. (B) The nucleotide sequence was determined by sequencing a double-stranded plasmid by the dideoxynucleotide chain termination method after subcloning the about 230-bp *Hin*cII-*Eco*RI enhancer segment into pUC19. Numbers refer to positions relative to the most upstream nucleotide (no. 1) of the enhancer segment. Boxes and small vertical arrows are as in panel A. The letters I to IV correspond to the regions shown in Fig. 6.

Further experiments are required to clarify the nature of this binding factor.

The more downstream positive *cis*-acting sequence is situated at positions −42 to −15 bp. The TATA box usually found around position −30 bp in various eucaryotic genes is not present in the OTC gene. As we reported (59), there is a sequence GTGGAAAG at positions −44 to −37 bp that coincides completely with the enhancer core sequence (63). In the study described here, we did not detect any obvious footprint of this sequence. A footprint was detected at positions −37 to +2 bp with the liver extracts. Protection of a more restricted area at positions −37 to −14 bp was observed also with the brain extracts. This region from −37 to −14 bp, common to both liver and brain extracts, contains a sequence AGGACCTTTGATCCC that is highly similar to the sequence AGGACCTTTGACCCC present in the promoter region of the chicken apo very-low-density lipoprotein II (apoVLDL II) gene (65). (Table 1). The apoVLDL II sequence element was speculated to bind a factor related to the COUP transcription factor (60) on the basis of similarity in guanosine contact points. The COUP transcription factor was originally identified as a factor that stimulates transcription via binding to the chicken ovalbumin gene upstream promoter (COUP) sequence (60). Among the OTC, apoVLDL II, and ovalbumin sequence elements, the sequence GACCTTTGA is identical (Table 1); therefore, the factor binding to the OTC element might be related to the COUP factor. The COUP factor seems to be ubiquitous, since it has been purified from the chicken oviduct (2) and HeLa cells (62), and the apoVLDL II sequence element binds the factor present in chicken liver (65). In addition to the COUP factor, another transcription factor, designated S300-II, is required for efficient transcription of the ovalbumin gene (60). S300-II is a non-DNA-binding factor and appears to function by stabilizing the DNA-COUP factor complex. Longer-range protection of region C of the OTC gene with the liver extracts than with the brain extracts might reflect the complex formation between the COUP-related factor and another liver-specific factor(s) in this region.

The 11-kb upstream enhancer region and its binding factors. The enhancer region of the OTC gene is situated 11 kb

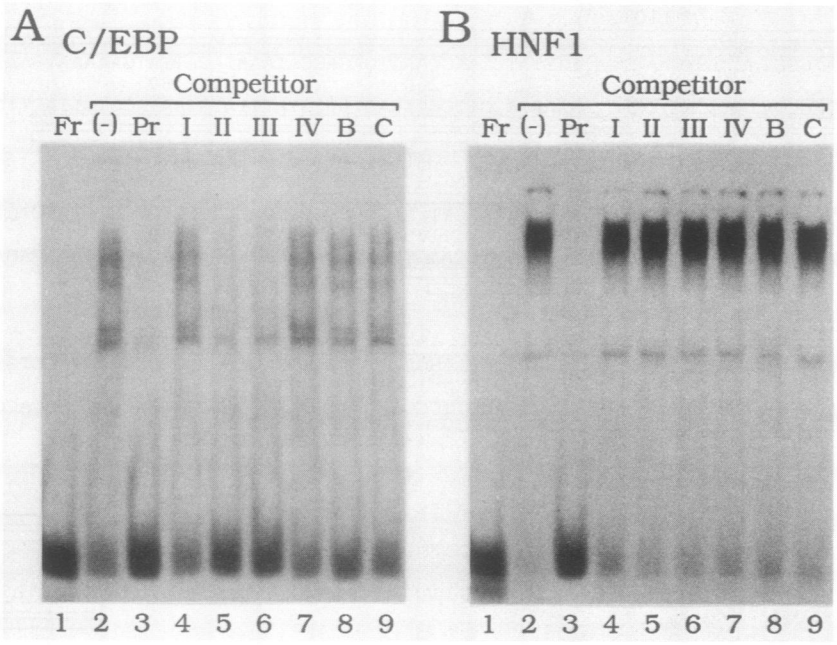


FIG. 8. Gel shift competition analysis for the binding of C/EBP (A) and HNF1 (B) with oligonucleotides covering OTC enhancer and promoter elements. Probes used were synthetic double-stranded oligonucleotides extending (A) from 1181 to 1212 of the HBV enhancer (37, 58) (A) and from −102 to −74 of the rat β-fibrinogen promoter (16) (B). The gel shift assay was performed with liver nuclear extracts (lanes 2 to 9) as described in Materials and Methods. Lanes 1, Free probes. Competitors of 1,000-fold molar excess were mixed in the binding reactions as follows: none (lanes 2); unlabeled oligonucleotides identical to probes (lanes 3); oligonucleotides extending from 73 to 103 of the OTC enhancer footprint region I (lanes 4), from 98 to 125 of region II (lanes 5), from 124 to 152 of region III (lanes 6), and from 162 to 187 of region IV (lanes 7); and oligonucleotides extending from −115 to −77 of the OTC promoter footprint region B (lanes 8) and from −41 to −14 of region C (lanes 9). The oligonucleotides had additional sequences for restriction ends on both sides.

upstream, far from the transcription start site. Far upstream enhancers have also been identified in other liver-specific genes such as the α-fetoprotein (up to −7 kb [21, 22, 26, 48, 55, 64]) and serum albumin (−10 kb [33, 50]) genes. Mullins et al. (49) reported that the CpG sequences located approximately 12 kb upstream of the mouse OTC gene were methylated in the kidney but hypomethylated in the liver, thereby exhibiting a correlation with the tissue specificity of OTC gene expression. The mouse OTC gene enhancer may be located about 12 kb upstream.

The hepatoma cell-specific OTC enhancer seems to bind multiple factors, some of which are liver specific and others of which are ubiquitous (Fig. 5). This feature of the OTC enhancer is reminiscent of the well-characterized mouse immunoglobulin heavy-chain enhancer, which functions in a B-lymphocyte-specific manner and binds both tissue-specific and ubiquitous factors (reviewed in reference 29). Several lines of evidence suggest that multiple B-lymphocyte-specific and ubiquitous binding factors can function as transcriptional activators, whereas the B-lymphocyte specificity is conferred by tissue-specific factors such as NF-A2 (also designated OTF-2 and Oct-2) binding to the octamer sequence of the heavy-chain enhancer (20, 39). The tissue-specific and ubiquitous factors binding to the OTC enhancer might play a role similar to that of the immunoglobulin enhancer-binding factors. Binding of both liver-specific and ubiquitous factors has also been reported for the mouse transthyretin (14) and mouse albumin (33) enhancers.

TABLE 1. Comparison of sequence elements of the OTC promoter and enhancer with those of regulatory regions of other genes

Origin of sequence	Sequence and location[a]	Reference
Rat OTC promoter	-31 AGGACCTTTGATCCC -17 ********** ***	
Chicken apoVLDL II promoter	-61 AGGACCTTTGACCCC -47 *********	65
Chicken ovalbumin promoter	-74 GACCTTTGA -82	60
HBV enhancer E site	1190 TGTTTGCT 1197 ********	58
Rat OTC enhancer region II	107 TTGTTTGCTTAAA 119 * ** **** * *	
Rat OTC enhancer region III	133 TGGTGTGCTAATA 145 *** ******	
Mouse transthyretin enhancer	34 TGTCCTAATA 43	14

[a] Identical nucleotides are indicated by asterisks.

Footprints bearing liver-specific features were detected in two sites (regions II and III) of the OTC enhancer (Fig. 6). Gel shift competition analysis (Fig. 8) showed that a factor(s) binding to these sites is related to a liver-enriched putative transcription factor, C/EBP, that was initially identified as the CCAAT homology- and enhancer core homology-binding protein (37). Comparison of regions II and III revealed sequence elements in which 9 of 13 nucleotides are identical (Table 1). Each of the sequence elements is, in turn, similar to a previously characterized C/EBP-binding site of the HBV enhancer (37; the E site in reference 58) or of the mouse transthyretin enhancer (oligomer 2 in reference 14; site 2 in reference 13) (Table 1). C/EBP was purified from the rat liver (previously named EBP20 [35]), and sequence analysis after gene cloning showed that the DNA-binding domain of C/EBP bears similarity to products of the *myc* and *fos* oncogenes (37), leading to the prediction of a structural motif, the leucine zipper (38). C/EBP binds to *cis* regulatory elements of liver-specific genes such as the serum albumin (13, 42), α_1-antitrypsin (13), and carbamyl phosphate synthetase I (34) genes, in addition to the HBV and transthyretin enhancer. A C/EBP expression vector transfected into cultured cells activates the cotransfected albumin promoter in a C/EBP-binding site-dependent manner, strongly suggesting that C/EBP is really a transcription factor (19). High levels of C/EBP mRNA were observed not only in liver but also in other tissues, including fat, intestine, and lung (4, 67). In detailed analysis of liver and fat cells, it was also shown that C/EBP expression is limited to terminally differentiated cells (4, 10, 19). A C/EBP-related factor(s) binding to the OTC enhancer may play a role in activation of the gene in fully differentiated hepatocytes.

Precise roles of the DNA elements of the enhancer and their binding factors, as well as the promoter elements and their binding factors, in determination of the overall liver specificity of OTC gene transcription remain to be elucidated. Analysis of the effects of each DNA element on transcriptional activation in hepatic and nonhepatic cells, along with characterization of the binding factors, should lead to a better understanding of the regulation of expression of the OTC gene.

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