Characterization of cis-Acting Elements of the Gene for Macrophage-stimulating Protein from the Human

THE INVOLVEMENT OF POSITIVE AND NEGATIVE REGULATORY ELEMENTS*

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To analyze the promoter region of the human macrophage-stimulating protein (MSP) gene, the 5′-flanking region of this gene was cloned. The major initiation site was determined at T located 49 base pairs upstream of the translation initiation site by primer extension with mRNA from HepG2 and Hep3B cells. There was no TATA sequence in this region. Transient transfection assay with 5′-deletion constructs showed that the transcription of this gene was regulated by positive and negative regulatory elements (PRE and NRE). The PRE (−34 to +2) was essential for the maximal transcription of this gene, and the NRE (−141 to −34) appeared to be responsible for the tissue-specific expression of the gene. The PRE contained the CCAAT sequence and a mutation from CCAAT to CTGAT resulted in a significant loss of transcriptional activity. Electrophoretic mobility shift assay suggested that two different proteins bound to the PRE (MSP-PRE-binding protein-1 (MSP-PREB1) and 2). MSP-PREB1 and 2 were detected in various cell types, and the CCAAT sequence was involved in these bindings. These findings indicate that MSP-PREB1 and 2 are positive regulators. Further characterization also revealed that MSP-PREB2 was identical to CCAAT-binding factor, also known as NF-Y.

Macrophage-stimulating protein (MSP)1 was originally purified as a human serum protein that made mouse resident peritoneal macrophages capable of responding to the chemottractant C5a (1). MSP is a heterodimeric protein that consists of a 53-kDa α-chain and a 25-kDa β-chain. The cloning of the MSP cDNA revealed that MSP was synthesized as a biologically active single chain pro-MSP (2). It can be converted to the biologically active disulfide-linked heterodimer by serine proteases (3, 4). MSP has high amino acid sequence similarity to hepatocyte growth factor/scatter factor (HGF/SF) (2, 5). The genomic sequence for MSP was independently cloned by Han et al. (6). Recently, the proteins Ron (7) and STK (8) were identified as the receptors for human and mouse MSP, respectively (9–12). MSP receptor has high amino acid sequence similarities to the c-met HGF/SF receptor and a member of receptor tyrosine kinase family. The concentration of pro-MSP in human plasma is optimal for biological activity, which is expressed when pro-MSP is cleaved to the MSP heterodimer. Therefore, it is likely that the expression of biological activity is controlled by the activation of pro-MSP and also by the expression of MSP receptors on the surface of target cells. Consistent with this view is the fact that the plasma concentration of pro-MSP is stable and does not increase like acute phase proteins of which concentrations increase in response to noxious stimuli (12).

We previously reported that the MSP mRNA was strongly expressed in the liver and weakly in the kidney and pancreas (2). The cloning of the MSP cDNA from human hepatoma cell line, HepG2, indicated that MSP was produced by hepatocytes. MSP mRNA was also detected at a lower level in the lung, adrenal, placenta and diaphragm during development in maternal rats in addition to the liver (13). Recently, transient expression of MSP was detected in the neural tube during development in the chicken (14). These findings suggest that the expression of MSP mRNA is controlled by factors expressed predominantly by hepatocytes or at the particular stages of the development. Recent studies also indicated that the MSP expression was up-regulated during liver regeneration after partial hepatectomy and hepatitis (15) and down-regulated during flumun pact hepatic failure (16).

The transcriptional mechanisms of the proteins produced specifically or predominantly by hepatocytes have been investigated (reviewed in Ref. 17). The transcription of these genes for albumin (18), transthyretin (19), and α1-antitrypsin (19, 20) are regulated by positive regulators such as CCAAT/enhancer-binding protein-α (C/EBP-α), or hepatocye nuclear factor (HNF) -1, HNF-3, or HNF-4. In contrast, the involvement of negative regulation is reported for apolipoproteins genes that are specifically expressed in the liver (21). The purpose of this study was to investigate the mechanisms that regulate the transcription of the human MSP gene. We characterized the cis-elements of the human MSP gene and found that the transcription of the human MSP gene was controlled by positive and negative regulatory elements.

EXPERIMENTAL PROCEDURES

Cell Lines—Hela, HOS (osteosarcoma), SK-R29 (renal cell carcinoma), and A172 cells (glioblastoma), were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% heat-inactivated fetal calf serum (FCS; Hydclone, Logan, UT). HepG2 hepatoma cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) with 10% FCS, and Hep3B hepatoma cells were cultured in minimum essential
medium (Life Technologies, Inc.) with 10% FCS. All cell lines were maintained at 37°C in 5% CO2.

RNA Analysis—Total RNA was extracted by TRIzol Reagent (Life Technologies, Inc.). Ten micrograms of total RNA were electrophoresed and blotted onto a Hybond-N+ nylon membrane (Amersham Corp.). The blots were hybridized with 32P-labeled MSP 9.13B cDNA probe (2) or β-actin cDNA probe. After hybridization, blots were washed, then exposed to XAR-5 x-ray films (Eastman Kodak) with an intensifying screen at –80°C.

Cloning of the 5′-Flanking Region of the Human MSP Gene—A human fibroblast genomic library was purchased from Stratagene (La Jolla, CA). One million clones were screened with the 32P-labeled MSP 9.13B cDNA probe (2). Sixteen positive clones after the second screening were amplified, purified, and analyzed for restriction enzyme mapping with HindIII (Life Technologies, Inc.). One of the clones, clone 8b, contained the 5.3-kb MSP 5′-flanking region. This 5.3-kb fragment was subcloned into the HindIII site of Bluescript II, SK(−) (Stratagene), termed pBlue5.3k, and used for DNA sequencing and preparation of chloramphenical acetyltransferase (CAT) constructs.

Primer Extension—An oligonucleotide (5′-CAGGGACCCCTAAG-CATTGAGTCAGAAC-3′), which was complementary to the region from +72 to +101 of the human MSP cDNA (2) (Operon, Alameda, CA), was used as the T4 polynucleotide kinase (Life Technologies, Inc.), with γ-32P ATP (6000 Ci/mmol, DuPont NEN). Aliquots of mRNA (5 μg) isolated from HepG2, Hep3B, and Hela cells were denatured at 65°C, incubated in a reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 20 mM dithiothreitol) containing 0.5 ng of the labeled oligonucleotide. Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and deoxynucleotide triphosphates (1 mM) at 37°C for 1 h. The synthesized cDNAs were denatured at 65°C and analyzed by electrophoresis on 6% acrylamide gels. The same primer was used for the DNA sequence ladder run on the same gels.

Construction of CAT Plasmids—The pCAT basic (Promega, Madison, WI) was used to construct MSP-CAT hybrids. To obtain pMD223, the DNA fragment covering the region from −223 to −59 of the 5′-flanking sequence of the gene was amplified by polymerase chain reaction (PCR) with oligonucleotide primers that corresponded to nucleotides from −223 to −204 of the sense strand (5′-GGCTGCAGCCCTTGCAACTGACCTA-3′) and from +40 to +59 of the antisense strand (5′-GGTCTAGACCTTCTGTGAGGCTCAG-3′). A Xbal linker was incorporated in the 5′-end of the antisense primer. The pBlue5.3k was used as the PCR template. The 280-bp PCR product digested with XbaI was cloned into the PstI site of pMD223. The pMD1.5k and pMD1.0k were constructed from the pMD5.3k by removing fragments with appropriate restriction enzymes. The pMD1.5k and pMD1.0k were used as the PCR template. The 280-bp PCR product digested with XbaI was cloned into the PstI site of pMD223. For primer extension experiment, transcriptional initiation site. The thymidine (T) located 49 bp downstream from the 5′-end of the antisense primer was used as the primer for primer extension with T4 polynucleotide kinase, 1 μg of poly(dI·dC), and 5 μg of each nuclear extract. For the competition assay, 10-, 30-, or 50-fold excess amount of the appropriate unlabeled probe was added to the binding reaction mixture. For antibody inhibition assay, nuclear extracts were preincubated with an antisera against NF-YB (kindly provided by Drs. D. Mathis, C. Benoist, and R. Mantovani) (24) or preimmune serum at 20°C for 20 min. Gel electrophoresis was carried out at 200 V for 2 h after a prerun at 200 V for 30 min.

RESULTS

Expression of MSP mRNA in Tumor Cell Lines of Different Tissue Origins—Tissue-specific expression of MSP mRNA was previously reported (2, 6, 13). Constitutive expression of MSP mRNA was detected in the liver and human MSP cDNA was consequently cloned from a human hepatoma cell line, HepG2 (2). In the present study, the expression of MSP mRNA was investigated by Northern blot analysis in six human tumor cell lines of different tissue origins. As shown in Fig. 1, marked expression of MSP mRNA was detected in HepG2 and Hep3B cells (lanes 1 and 2), but not in Hela (lane 3), HOS (lane 4), A172 (lane 5), or SK-RC29 cells (lane 6). Thus, MSP mRNA was detected in only hepatic cell lines.

Cloning and Characterization of the 5′-Flanking Region of the MSP Gene—After screening approximately one million individual clones from a human fibroblast genomic library, 40 positive clones were obtained. Sixteen clones were amplified and purified for further characterization. Restriction enzyme mapping with HindIII revealed that clones 8b and 14a contained the MSP gene (2, 6, 25). The other clones were from MSP pseudogenes located on chromosome 12 (2, 25). The 5.3-kb MSP 5′-flanking region was excised by HindIII from the clone 8b phage DNA and subcloned into the HindIII site of the Bluescript II, SK(−). A detailed restriction map of the 5′-flanking region of the MSP gene is shown in Fig. 2A. Primer extension experiments were performed to identify the transcriptional initiation site. The thymidine (T) located 49 bp downstream from the 5′-end of the antisense primer was used as the primer for primer extension with T4 polynucleotide kinase, 1 μg of poly(dI·dC), and 5 μg of each nuclear extract. For the competition assay, 10-, 30-, or 50-fold excess amount of the appropriate unlabeled probe was added to the binding reaction mixture. For antibody inhibition assay, nuclear extracts were preincubated with an antisera against NF-YB (kindly provided by Drs. D. Mathis, C. Benoist, and R. Mantovani) (24) or preimmune serum at 20°C for 20 min. Gel electrophoresis was carried out at 200 V for 2 h after a prerun at 200 V for 30 min.
upstream from the ATG translation initiation codon appeared to be the major transcription initiation site of the human MSP gene (Fig. 3).

Identification of the MSP Transcriptional Regulatory Region in the 5'-Flanking Sequence—To investigate the regions responsible for the MSP gene transcriptional regulation, Hep3B and HOS cells were transiently transfected with CAT constructs containing different lengths of the 5'-flanking region of the MSP gene obtained by progressive deletion of the 5'-end (Fig. 2B), and then CAT activities in the cell extracts were compared (Fig. 2C). High CAT activity was detected in the extracts of Hep3B cells transfected with pMD5.3k. A deletion of the 5'-flanking region from −5.3k to −141 had little effect on the CAT activity. An additional deletion to −34 resulted in a 1.5-fold increase. The removal of an additional 36-bp sequence resulted in a significant loss of the CAT activity. In HOS cells, no significant CAT activity was found in the cells transfected with pMD5.3k or other constructs with progressive deletions up to −141. However, the deletion to −34 resulted in a significant increase in CAT activity and the removal of an additional 36-bp sequence resulted in a loss of the CAT activity. These results suggest that the transcription of MSP gene is regulated by two different regulatory elements: a negative regulatory element (NRE) located between −141 and −34, and a positive regulatory element (PRE) located between −34 and +2. The binding of suppressor protein(s) to the NRE of the 5'-flanking region of the MSP gene may negatively regulate the expression of MSP mRNA in HOS cells.

The CCAAT Element Is Essential for the Transcription of the MSP Gene—Further experiments were performed to characterize the PRE. Several putative binding sites for trans-activators were found after sequencing the proximal 5'-flanking region (Fig. 4A). As described above, the region between bp −34 and +2 was responsible for positive regulation of the MSP gene transcription in Hep3B cells. In this region, we found a CCAAT motif involved in the binding of a small group of transcription factors such as CCAAT-binding factor (26–31). To investigate whether the CCAAT sequence was responsible for transcription of the MSP gene, six different mutations were introduced into the CAT construct as shown in Fig. 4B. The pMD34M2, which carried two mutated base pairs in the CCAAT sequence of the pMD34 vector, caused a significant decrease of the CAT activity in the cell extracts of Hep3B cells transfected with this construct. Other mutations had no effect on the CAT activity. These results indicate that the CCAAT sequence is the PRE of the MSP gene and is responsible for the positive regulation of human MSP gene transcription.
Protected extracts of A172 and SK-Rc29 cells. The mutation also caused decreases of the CAT activity in these cell lines. Thus, the PRE ("CCAAT") positively regulates transcription of the MSP gene in various types of cells and therefore is not responsible for the tissue-specific expression of the human MSP gene.

MSP-PREB1 and 2 Bind to the CCAAT Element of the MSP Gene—To examine the binding of positive regulators to the CCAAT element, EMSA was performed. In the assay, an annealed 29-mer oligonucleotide (PRE probe) coding from -34 to -6 of the 5'-flanking region including the CCAAT sequence was used (Table I). As shown in Fig. 5, four shifted bands (DNA-protein complexes) were found when the nuclear extracts of Hep3B cells and 32P-labeled PRE probe were incubated (lane 1). Addition of excess unlabeled PRE probe did not inhibit appearance of the complex 4, indicating that the complex 4 was nonspecific (lanes 2 and 3). Addition of a small amount of salmon sperm DNA caused disappearance of the complex 3 (lanes 4 and 5), indicating that the complex 3 was also nonspecific. In contrast, addition of excess unlabeled PRE but not salmon sperm DNA caused the disappearance of complexes 1 and 2, indicating that these complexes were specific. Thus, the proteins (MSP-PREB1 and 2) in the Hep3B nuclear extracts appeared to bind specifically to the PRE and form the complexes. MSP-PREB1 and 2 did not bind to the 20-mer probe that contained the CCAAT sequence but not the 9-bp of the 3'-end of the PRE probe (see Fig. 7B, lanes 4–6), indicating that the 3'-sequence following the CCAAT sequence is also important for the protein binding.

To locate the sequence essential for the binding, a competition assay was performed with mutated PREs. The mutated PREs (M1, M2, M3, M4, M5, and M6) were cut out from the CAT constructs described above (pMD34M1, pMD34M2, pMD34M3, pMD34M4, pMD34M5, and pMD34M6, respectively).

Table I

| Probe | Sequence | Reference |
|-------|----------|-----------|
| PRE   | 5'-'AGCCACCACACATGGGACAGGTTTC TGCGTTGGTTAGGGCATCCCTGCAAG-5' |            |
| 20-mer| 5'-'GAGCCACCACATGGGGACAGGTAGG GACAGGGTGTTGACGCCCTGCAAG-5' |            |
| CBF   | 5'-'CAGTCTCCACATGGGAGGGCTGGG CAGAAGGGTGGTACCCCGGCAACCGG-5' | 25, 31     |
| CEBP/α| 5'-'TGCAAGATGGGCAATCGGA-5' | 29         |
| NF-1  | 5'-'CCTTGGCATGTGTCGCCCTAAT-5' | 34         |
| Sp1   | 5'-'ATTCCATGGGGGCGCGGCGGACAGC TAAATGCCCCTGCGCGCGGTGCG-5' |            |

Transcriptional Regulation of the Human MSP Gene

Fig. 4. Characterization of the PRE of the MSP gene. A, the nucleotide sequence from -223 to -49 is shown. Putative cis-elements are underlined. The PstI site is also underlined. Arrows indicate the first base pair included within each primer. The major (closed triangle) and minor (open triangle) transcriptional initiation sites were detected by primer extension (see Fig. 3). The boxed region shows similarity to the consensus sequence for the Inr. Mutated PRE probes are shown on the lower panel, and mutated bases are indicated by lower case letters.

B, CAT assays were performed with the cell extracts of Hep3B or HOS cells transfected with the mutated PRE constructs. Results are expressed as the ratio of the CAT activity in each cell extract relative to the CAT activity in Hep3B cells transfected with pMD34. Data are presented as means ± S.D. from three independent experiments.

C, CAT assays were performed with the cell extracts of Hep3B, Hela, HOS, A172, and SK-Rc29 cells transfected with the pMD34 or pMD34M2. Results are expressed as the ratio of the CAT activity in each cell extract relative to the CAT activity in the cell extract from Hep3B cells transfected with the pMD34.
The high affinity binding of the proteins. The results obtained by CAT assay and EMSA indicated the ubiquitous expression of the proteins. EMSA was also performed with nuclear extracts of HepG2, Hep3B, Hela, HOS, A172, and SK-RC 29 cells (Fig. 7A). Binding of MSP-PREB1 and 2 to the PRE probe was observed with all of the nuclear extracts, indicating the ubiquitous expression of the proteins. The results obtained by CAT assay and EMSA strongly suggest that MSP-PREB1 and/or 2 are the positive regulatory protein(s) for MSP gene transcription.

MSP-PREB2 Is Identical to the CCAAT-Binding Factor, NF-Y/CBF—To compare MSP-PREB1 and 2 with known CCAAT-binding proteins such as NF-Y/CBF (26), C/EBPα (30), and NF-I (31), EMSA was performed with oligonucleotide probes carrying the sequences of the specific binding elements for each protein (Table I). Among the probes, an excess of the nonlabeled CBF probe which carried the CCAAT sequence of the α2(I) collagen gene strongly reduced the binding of PREB2, but only weakly reduced the binding of PREB1 (Fig. 7B, lanes 7-9). EMSA was also performed with nuclear extracts of Hep3B cells and the 32P-labeled CBF probe. As shown in Fig. 7C, lanes 1-3, a shifted band that competed with the excess amount of unlabeled CBF probe was observed, indicating that the band was a complex of NF-Y/CBF binding to the CBF probe. This band also competed with excess amount of the PRE probe (Fig. 7C, lanes 4-6). A weak band was also detected just above the

**DISCUSSION**

In the present study, we investigated the transcriptional mechanisms of the human MSP gene. The 5′-flanking region of the human MSP gene was cloned, and the transcription initiation sites were determined by primer extension. As shown in Fig. 3, multiple initiation sites were detected. However, the major extension product started at T located 49 bp upstream of the ATG translation initiation site. Multiple initiation sites are commonly observed in TATA-less genes (33). In fact, a TATA box was not found in the 5′-flanking region of the MSP gene (Fig. 4A). These results suggest that transcription of the human MSP gene is regulated by TATA-less promoters.

We next investigated the cis-acting elements regulating the MSP gene transcription by using CAT constructs ligated with progressively deleted 5′-flanking sequences, and found positive and negative regulatory elements. The PRE was located in the region from −34 to +2, and is essential for the maximal transcriptional activity. The NRE was located in the region from −141 to −34, and the hepatocyte-specific transcription of the
The positive regulatory element contained the CCAAT sequence. Mutation in this sequence resulted in significant decreases of the transcriptional activity in Hep3B, HOS, and Hela cells. EMSA suggested that two different proteins, MSP-PREB1 and 2, bound to the positive regulatory element. The CCAAT sequence is characterized as the binding site for several transcription-activators. One of the CCAAT-binding transcription factors, C/EBPα, is known as a hepatocyte-specific transcriptional factor, and recognizes two cis-regulatory motifs, “TGTGG(A/T)(A/T)(A/T)G” and “CCAAT” (30). C/EBPα is reported to regulate transcription of genes specifically or predominantly expressed in the liver including albumin (18), factor VIII (34), and α1-antitrypsin (19, 20). However, the present study suggests that C/EBPα is not the major factor that regulates the liver-specific expression of the MSP gene because the C/EBPα probe did not compete with the binding of either MSP-PREB1 or 2 to the PRE probe in EMSA.

CCAAT-binding transcription factor (CTF)/nuclear factor-I (NF-I) was originally identified as a cellular factor that was essential for the efficient replication of adenovirus DNA (31, 35). However, recent studies revealed that CTF/NF-I recognized the sequence “TGG(N)6GCCAA” instead of the CCAAT sequence (36). Since the “TGG(N)6” sequence was not conserved on the 5′-flanking region of the MSP gene and the CTF/NF-1 probe did not compete the binding of MSP-PREB1 and 2 to the PRE probe, CTF/NF-1 does not appear to regulate the transcription of the MSP gene.

Other CCAAT-binding factors such as NF-Y/CBF (26, 32), CP1 (28, 29), CCAAT binding protein for the TK gene (CBP/tk) (37), or a 114-kDa CBF for the hsp70 gene (38) were candidates for the transcription-activators of the MSP gene. NF-Y and CBF were originally identified as a binding protein to the Y box of the major histocompatibility complex (MHC) class II gene (27) and α1(I) collagen gene promoter (26), respectively. This factor consists of 32k-, 40k-, and 40k-subunits (A, B, and C subunits, respectively) (32, 39). cDNA cloning of NF-Y and CBF subunits revealed that these factors were identical (39–42). Recently it was reported that CP1, which was characterized as the binding protein of the α-globin promoter (28, 29), was also identical. As shown in Figs. 7 and 8, our study indicated that the MSP-PREB2 was identical to NF-Y/CBF. MSP-PREB1 also bound to the CCAAT element of the MSP gene and the α2(I) collagen gene, but MSP-PREB1 showed high affinity only to the CCAAT element of the MSP gene. This observation suggests that MSP-

**Fig. 7.** Characterization of MSP-PREB1 and 2 binding to the CCAAT elements of the MSP gene and α2(I) collagen gene. A, EMSA was performed with the nuclear extracts from HepG2 (lane 1), Hep3B (lane 2), Hela (lane 3), HOS (lane 4), A172 (lane 5), and SK-RC29 cells (lane 6). B, competition assay with a 10- or 50-fold excess amount of the PRE probe (lanes 1-3), PRE (20-mer) (lanes 4-6), CBF (lanes 7-9), C/EBPα (lanes 10-12), NF-I (lanes 13-15), and Sp1 (lanes 16-18). The sequences of the probes are shown in Table I. C, NF-Y/CBF binding (arrow) to the CBF probe (lanes 1 and 4). This binding was inhibited by a 10- or 50-fold excess of nonlabeled CBF probe (lanes 2 and 3) or a 10- or 50-fold excess of nonlabeled PRE probe (lanes 5-7). Weak binding was detected with Hep3B nuclear extracts (arrowhead). D, the effect of EDTA on the binding of NF-Y/CBF and MSP-PREB1 and 2 to the CCAAT elements. The binding buffer contained 8 mM Ca2+ (lanes 1 and 6), 4 mM Ca2+ (lanes 2 and 7), 2 mM Ca2+ (lanes 3 and 8), 2 mM EDTA (lanes 4 and 9), or 5 mM EDTA (lanes 5 and 10).

**Fig. 8.** Antibody inhibition assay with an antiserum against the B subunit of NF-Y (24). Nuclear extracts of Hep3B cells were preincubated with 0.2 μl (lane 2), 0.4 μl (lane 3), 0.6 μl (lane 4), 0.8 μl (lane 5), or 1.0 μl (lane 6) of the antiserum or 1.0 μl of control rabbit serum (lane 7).

MSP gene appears to be regulated by this element.

The positive regulatory element contained the CCAAT sequence. Mutation in this sequence resulted in significant decreases of the transcriptional activity in Hep3B, HOS, and Hela cells. EMSA suggested that two different proteins, MSP-PREB1 and 2, bound to the positive regulatory element. The CCAAT sequence is characterized as the binding site for several trans-activators. One of the CCAAT-binding trans-activators, C/EBPα, is known as a hepatocyte-specific transcriptional factor, and recognizes two cis-regulatory motifs, “TGTGG(A/T)(A/T)(A/T)G” and “CCAAT” (30). C/EBPα is reported to regulate transcription of genes specifically or predominantly expressed in the liver including albumin (18), factor VIII (34), and α1-antitrypsin (19, 20). However, the present study suggests that C/EBPα is not the major factor that regulates the liver-specific expression of the MSP gene because the C/EBPα probe did not compete with the binding of either MSP-PREB1 or 2 to the PRE probe in EMSA.

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PREB1 may be different from NF-Y/CBF. However, there were a few similarities between MSP-PREB1 and 2. The mobilities on the gels were similar. The CCAAT sequence was essential for the binding of the proteins. EDTA enhanced the bindings of the proteins to the PRE probe. The MSP-PREB1 binding was also weakly inhibited by an antisemirum against the B subunit of NF-Y/CBF. These facts suggested that MSP-PREB1 might be a NF-Y/CBF-related protein.

Although the CCAAT motif is essential for the NF-Y/CBF binding to DNA, the upstream and downstream sequences of the CCAAT element also affect on the binding affinity. Recently, Roy and Lee (43) reported that the G-rich sequence, which was conserved downstream of the CCAAT sequence of the α(2l) collagen gene and GRP78 Bip gene, was required for the maximal DNA-binding affinity of NF-Y/CBF. This G-rich sequence, however, is not conserved on the Y box of the MHC class II gene, the α-globin promoter, or the PRE of the MSP gene. In our study, the affinity of the CBF binding to the α(2l) collagen promoter sequence appeared to be higher than that to the PRE of the MSP gene (Fig. 7, B and C). The CCAAT sequence was also essential for the MSP-PREB1 binding to the PRE, but MSP-PREB1 did not bind to the CCAAT element of the α(2l) collagen promoter. Therefore, an additional sequence, which is located in either upstream or downstream of the CCAAT sequence of the MSP gene, may be required for the MSP-PREB1 binding. We attempted to locate the additional sequence for MSP-PREB1 binding by both CAT assay and EMSA with mutations, but we failed to find the sequence. To identify the binding motif required for the maximal binding of MSP-PREB1, further analyses will be necessary.

Several questions remained unanswered. CAT assay indicated the most potent enhancer activity was in the CCAAT element, but some enhancer activity was still detectable after the deletion of this sequence. Although EMSA indicated the ubiquitous expression of MSP-PREB1 and 2, CAT assay with mutated constructs showed higher activities in Hep3B, HOS, and Hela cells than in A172 or SK-RK29 cells. Therefore, another positive regulator might be involved in the regulation of the MSP gene transcription. In some TATA-less promoters, the initiator (Inr) element is critical in positioning RNA polymerase II, and sufficient to direct basal levels of transcription (44). We performed CAT assay with additional CAT constructs carrying different deletions. In the pMD141/D6, the sequence between –5 and +49 was deleted from the pMD141. A significant decrease (1/20) of the CAT activity was observed in the cell extracts of Hep3B cells transfected with pMD141/D6. In contrast, no significant decrease was detected with the pMD141/36 that lacked the sequence between +37 and +49. As shown in Fig. 4A, a sequence similar to the consensus sequence for the Inr (–3YYCAYYYYY–10) was found within +23 and +31. However, substitutions of base pairs GG (+18, +19) with TT, CA (+25, +26) with AG, or CA (+32, +33) with TT did not affect on the transcriptional activity (data not shown). These results indicated that the region between –5 and +49 was important for the transcription of this gene but the sequence between +23 and +31 was not responsible for the transcriptional activity. Further investigation of this region will lead us to a better understanding of the positive regulation of the MSP gene.

As described above, the negative regulation of the genes specifically expressed in the liver has been investigated. In the case of apoB gene, the binding of positive regulators, such as C/EBPα, HNF-3, and HNF-4 to each regulatory element is essential for the maximal transcriptional activity of this gene, and this activity is negatively regulated by repressors that bind to these positive regulatory elements. For example, nuclear proteins, COUP-TFs, are capable of binding to the HNF-4 binding site of the apoB gene, and competitively down-regulate the transcription of this gene.

Our study also suggested the role of the NRE in the hepatocyte-specific expression of the MSP gene. However, the mechanisms for this negative regulation are not well known at present. In this region, two putative binding consensus sequences for MyoD (47, 48) and AP-2 (49–51) were found. MyoD is a nuclear protein expressed in skeletal cells and contains a region homologous to the proteins of c-myc family. MyoD regulates the transcription of genes specifically expressed in skeletal muscle by binding to the promoter sequences of the genes. Since MSP expression was detected in diaphragm during development in maternal rats (13), it will be interesting to investigate whether MyoD is involved in the MSP gene transcription in skeletal muscle. AP-2 was detected in several kinds of cells such as Hela cells but not in HepG2 cells (49). Therefore, AP-2 might be involved in the negative regulation of the MSP gene. Further investigation of the NRE is necessary to understand the tissue-specific expression of the MSP gene.

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