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Characterization of Human Serum Mannan-Binding Protein Promoter[1]

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Serum mannan binding protein (MBP), a mannose/*N*-acetylglusosamine-specific lectin, is important in innate immunity. In order to elucidate the mechanism underlying the wide intra- and interracial variety in the MBP serum level, we have studied the transcriptional regulation of human MBP. Rapid amplification of cDNA ends (5′ RACE) analysis of Hep G2 RNA indicated the presence of a novel exon, designated as "exon 0," upstream of previously identified exon 1 [Taylor, M.E. *et al.* (1989) *Biochem. J.* 262, 763-771]. Two MBP mRNAs with different sized 5′-noncoding regions were detected: the longer transcript starts at exon 0 and the shorter one at exon 1. Promoter analysis involving a luciferase assay vector revealed that the transcript starting from exon 1 predominates over that starting from exon 0. In addition, a hepatocyto-specific nuclear factor, (HNF)-3, which is known to control the expression of hepatocyto-specific genes, up-regulates the transcription of human MBP from exon 1, while a glucocorticoid, which is known to up-regulate acute phase proteins, markedly suppresses MBP transcription. Recently, polymorphisms were found to occur in the promoter region at two positions [Madsen, H.O. *et al.* (1995) *J. Immunol.* 155, 3013-3020]. Functional promoter analysis indicated that three haplotype variants as to these positions, HY, LY, and LX, exhibit high, medium and low promoter activity, respectively, in accordance with the results of a previous population study.

Key words: gene expression, HepG2, mannan-binding protein, promoter.

Mannan-binding protein (MBP) is a liver-derived serum lectin, which has been isolated from various mammals and characterized (*1-4*). Human MBP is a homooligomer of approximately ~650 kDa consisting of ~18 identical subunits of 31 kDa. Each subunit has a carbohydrate-recognition domain (CRD) at the COOH terminus and a collagen-like domain at the NH_2 terminus (*5*). MBP shows structural similarity to C1q, which is the first component of the classical complement pathway. This enables MBP to activate complement in a C1q-independent manner (*6, 7*). When MBP binds to carbohydrate ligands on the surface of microorganisms, it activates the complement pathway, *i.e.*, the lectin pathway, and prevents infections (*8-10*). Recently, a single nucleotide mutation in the collagen-like domain of human MBP was found to be associated with the opsonin defect, which causes recurrent infections in infants (*11-13*). Thus, human MBP is an innate immune factor and functions in the first-line host defense until the acquired immune system is fully developed.

The structure of the human MBP gene was first described by Taylor *et al.* (*14*). They showed that the gene consists of 4 exons (exons 1 to 4) separated by 3 introns, the exons encoding a N-terminal cysteine-rich region, a collagen-like domain, a neck region and a carbohydrate recognition domain (CRD), respectively. They also revealed the presence of several consensus sequences in the promoter region, which might be involved in the control of the expression of the gene. More recently, Madsen *et al.* (*15*) indicated that genetic polymorphisms exist at two positions in the promoter region, giving rise to H/L and X/Y variants as to the respective positions, and the promoter haplotypes of HY, LY, and LX are associated with high, medium and low serum levels of MBP, respectively. Furthermore, the expression level of human MBP was reported to be elevated in acute phase liver (*16*). On the other hand, population studies indicated that the serum level of MBP varies very widely even in healthy individuals of the same race. These results led us to assume that there might be characteristic mechanisms that regulate the expression of this important molecule.

To date, however, there has been very little information on the nature of the transcriptional control mechanism for MBP. In this paper, we wish to report functional characterization of the MBP gene promoter to elucidate its biological significance with respect to gene expression.

[1] The sequence reported in this paper has been deposited in the DDBJ database (accession No. AB025350).

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Abbreviations: MBP, mannan-binding protein; GRE, glucocorticoid-responsive element; HNF, hepatocyto-specific nuclear factor; PCR, polymerase chain reaction.

MATERIALS AND METHODS

Materials—A radionucleotide, [α-^{32}P]dCTP (111 TBq/mmol), was purchased from ICN Biomedical (Costa Mesa, CA). The oligonucleotides used as a probes and primers were custom synthesized by Cruachem (Kyoto). Restriction enzymes and other enzymes for DNA manipulations were purchased from Takara Shuzo (Kyoto), Toyobo (Osaka), Nippon Gene (Tokyo), Amersham Pharmacia Biotech, UK (Bucks, UK), and GIBCO BRL Life Technologies (Rockville, MD). A human placenta genomic DNA library (EMBL3 SP6/T7) and a human multiple tissue Northern blot were purchased from Clontech Laboratories (Palo Alto, CA). The PGV-B luciferase assay vector was obtained from Toyo Ink (Tokyo). HepG2 (ATCC CRL 1651), HeLa (ATCC CCL2), and CHO (ATCC CCL61) cells were obtained from ATCC.

5′ Rapid Amplification of cDNA Ends Strategy (5′ RACE)—cDNAs encoding the 5′ termini of human MBP transcripts were prepared from HepG2 total RNA. The first strand cDNA was synthesized using an antisense primer located in exon 4 [5′-AACTTGACACACAAGGCCT-3′ (546–527 in Fig. 4 of Ref. *14*)] in a programmed incubator (ATTO ZYMOREACTOR II, MODEL AB-1820; ATTO, Tokyo). After polyadenylation to the 5′ termini, amplification was performed by means of polymerase chain reaction (PCR) with Taq polymerase (Perkin-Elmer, Foster City, CA) according to the manufacturer's protocol using an oligo dT primer and an MBP antisense primer [5′-GATGGCACCAAGGGAGA-3′ (+1364–+1381)], which is located in exon 1. The primary PCR product was used for the second PCR with an oligo dT primer and a nested MBP antisense primer [5′-TGTGAGGATGCCCAA-3′ (+1281–+1295)]. The second PCR product was used for the third PCR with an oligo dT primer and a nested MBP antisense primer [5′-TCCTTCTCCTGAGTA-3′ (+1234–+1248)]. The amplified PCR products were then subcloned into the pCRII vector (Invitrogen, Carlsbad, CA) and characterized by DNA sequencing with a DNA sequencer (Model 373A; Perkin Elmer, Applied Biosystem Division, CA).

Library Screening and Sequencing of the Human MBP Gene—A human placenta genomic DNA library was hybridized at 42°C overnight with a radiolabeled human MBP DNA fragment spanning from exon 0 to intron 0, which had been generated by PCR with the amplification primers, 5′-TCCTCCCTCACTCTGAGGCATCTGCCA-3′ (+20–+47) and 5′-TACTACGTTGGCCCTGGCAGAGTGCTA-3′ (+551–+524), and then radiolabeled with [α-^{32}P]dCTP by the random primer method. The filter was washed 3 times with 2×SSC, 0.1% SDS at 65°C for 20 min, twice with 0.2×SSC, 0.1% SDS at 65°C for 20 min, and then twice with 5×SSC at room temperature for 5 min. Among the 4.9×10^5 plaques screened, 4 positive clones were identified, which comprised two kinds. LA-PCR was performed with purified phages as templates, and the longest PCR product was directly sequenced and characterized.

Northern Blotting—A human multiple tissue Northern blot containing approximately 2 μg of pure polyA$^+$RNA from specific tissues was purchased from Clontech. The Northern blot was probed with a radiolabeled human MBP cDNA exon 0 or exon 1 fragment. These probes had been amplified by PCR as described above except that 150 mCi of [α-^{32}P]dCTP was included. The amplification primers used were 5′-CTACATTGCTGAGCCCAG-3′ (+1–+18) and 5′-GTGTTTCTGCAGAGCAGG-3′ (+105–+88) for the exon 0 probe, and 5′-CATGTCCCTGTTTCCAT-3′ (+1208–+1224) and 5′-CACTGCAGGGCAGGTCT-3′ (+1313–+1297) for the exon 1 probe. Hybridization was performed at 42°C overnight. The filter was washed twice with 2×SSC, 1% SDS at 65°C for 10 min and 3 times with 0.2×SSC, 0.1% SDS at 65°C for 10 min, and then analyzed with a BAS2000 image analyzer (Fuji Film, Tokyo).

Luciferase Reporter Gene Constructs—Plasmid DNA constructs were prepared from DNA fragments, which had been amplified by PCR as described above using the genomic DNA of human liver as a template. A series of DNA fragments of the intron 1 region were prepared using the following 5′ end primers, 5′-AATGGTACCCTGATGGCTTTAGGCATGTG-3′, 5′-AATGGTACCCTGCCATTACCCTGACCCAG-3′ and 5′-AATGGTACCGATGGGTGTGTGCGTGCATG-3′, and the 3′ end primer, 5′-AATAAGCTTGGGCTCTGGCAGATGCCTCA-3′ (+53–+34), subclones PGV-1707, PGV-1143, and PGV-325, being generated respectively. A series of DNA fragments of intron 0 were prepared using the following 5′ end primers, 5′-TATGGTACCCTGCAGTGGAGACTGTCTTT-3′, 5′-AATGGTACCGTCACGCAGTGTCA-3′, 5′-AATGGTACCCTTTTCCAAATCCCC-3′, 5′-AATGGTACCCCAGCTAGAGGCCA-3′, 5′-AATGGTACCTTCTATATAGCCTGC-3′, 5′-ATTGGTACCCCTGCACCCAGATT-3′ and 5′-GTCGGTACCGTGTTCATTAACTGAGAT-3′, and the 3′ end primer, 5′-GATAAGCTTGTCCTCACCTTGGTGTGAGA-3′ primer, 5′-GATAAGCTTGTCCTCACCTTGGTGTGAGA-3′ (+1207–1188), subclones PGV+297 (equal to PGV-LY), PGV+1030, PGV+1057, PGV+1070, PGV+1100, PGV+1110, and PGV+1153 being generated, respectively. *Kpn*I sites are indicated by double underlines and *Hin*dIII sites by single underlines. The PCR product was gel purified, digested with *Kpn*I and *Hin*dIII, and then ligated into the PGV-B luciferase assay vector at the *Kpn*I and *Hin*dIII sites. The DNA sequences of the resulting clones were determined by DNA sequence analysis. PGV+495, PGV+704, PGV+833, and PGV+995 were generated by means of an EXOIII/Mung bean nuclease deletion system (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The LX and HY promoter haplotypes were generated from the LY haplotype (PGV+297) by nucleotide-directed mutagenesis with a Transformer™ Site-Directed Mutagenesis Kit (Clontech). The DNA constructs were designated as PGV-LX and PGV-HY.

Promoter Assay—Plasmids for transfection were purified on Qiagen (Qiagen Gmbh, Germany) columns. HepG2, HeLa, and CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM), Eagle's MEM, and Ham's F12 medium supplemented with 10% fetal calf serum, respectively. Semiconfluent cells in 3.5-cm dishes were cotransfected with 10 μg of a sample plasmid and 5 μg of a pCH110 β-galactosidase assay vector (Amersham Pharmacia Biotech, Uppsala, Sweden) by the calcium phosphate method. After 45 h, cell lysates were assayed for luciferase and β-galactosidase activities. The results are expressed as the means for three experiments.

Stimulation of the Promoter in HepG2 Cells—Reporter gene constructs were transfected into HepG2 cells by the calcium phosphate method as described above. After 42 h,

the cells were treated with 1×10^{-7} M dexamethasone and 25 ng/ml IL-6. After 6 h exposure to these agents, luciferase activity was measured as described above. The results are expressed as the means for three experiments.

Nuclear Extract and Gel Shift Assay—A nuclear extract was prepared from HepG2 cells according to the procedure of Dignam *et al.* (*17*). Two kinds of ^{32}P-labeled DNA fragments were prepared: one contained the HNF-3 recognition site in intron 0 of human MBP (designated as MBP-HNF-3), and the other contained a strong-affinity HNF-3 site derived from the transthyretin (TTR) promoter (*18*) (designated as HNF-3). These DNA fragments were radiolabeled by means of polymerase chain reaction (PCR), as described above. The oligonucleotide template used for PCR for MBP-HNF-3 was 5′-ACGCAGTGTCACAGGGAATGTTTACTTTTCCAAATCCCCAGC-3′ (+1033–+1074), and the amplification primers were 5′-ACGCAGTGTCACAAG-3′ and 5′-GCTGGGGATTTGGAA-3′. The template oligonucleotide for HNF-3 was 5′-GTGAAGCTTGCACGTGATTATTGACTTCGAGCGTCTAGACTC-3′, and the primers were 5′-GTGAAGCTTGCACGT-3′ and 5′-GAGTCTGACGCTGC-3′. The core binding site recognized by HNF-3 is single underlined. Gel shift assays were performed according to the procedure of Ausubel *et al.* (*19*) with slight modifications. The binding reaction was performed in 18 μl of 1 × gel shift buffer (10 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM DTT, and 1 mM EDTA) containing 10,000 cpm of ^{32}P-labeled DNA, 2 μg of poly(dI-dC)·poly(dI-dC), and 9 μg of the nuclear extract. The binding reactions for the competitor lanes included a 50, 200, or 500-fold molar excess of DNA, as indicated. After 30 min incubation at room temperature, the resulting complexes were subjected to electrophoresis in 6% acrylamide gels with 1 × Tris/borate/EDTA buffer. The gels were dried and then analyzed with a BAS2000.

RESULTS

Sequence and Intron/Exon Organization of the MBP Gene—In order to understand the transcriptional regulation of human MBP, we first determined the 5′-terminus of the mRNA transcript of MBP using the 5′-rapid amplification of cDNA ends (5′RACE) method. 5′RACE was performed with total RNA prepared from a hepatoma cell line, HepG2. The PCR products were subcloned and sequenced. Most of the 12 subclones analyzed had the 5′ cap structure of mRNA. Among these subclones, 6 were assigned as being derived from exon 1, as previously reported by Taylor *et al.* (*14*), and another 2 started 58 bp downstream from the 5′-end of exon 1, but we also found that the remaining 4 clones contained an unidentified DNA sequence of 107 bp upstream of exon 1. As it seemed that this sequence should be a new exon (now designated as exon 0), we screened a human genomic library to determine the genomic organization and to examine the putative promoter regions of the MBP gene (Fig. 1).

The 5′ upstream region of exon 0, designated conventionally as intron −1 in the following text, contained several consensus motifs. One TATA box was found in the sequence of −60 to −55, one type 1 IL-6 responsive element (TT/GNNGNAAT/G) (*20, 21*) at −185, and two type 2 IL-6 responsive elements (CTGGGAA) (*22, 23*) at −736 and −75. These two types of IL-6 responsive elements have been found in the promoter regions of many kinds of acute phase proteins and are often involved in the induction of their mRNAs. Sequences similar to the binding element for HNF-3, which is involved in liver-specific expression, were found in the sequences at −1251, −1015, −870, and −461.

In the 1,027 bp of intron 0, in addition to the previously described glucocorticoid-responsive element (GGTACAnnnTGTTCT) (−895), CAAT box (+1063), and TATA box (+1103) (*14*), one type 1 IL-6 responsive element (+549) and one HNF-3 binding element (+1049) were present.

By analyzing the genomic sequence of human MBP utilizing the BLAST sequence similarity search program, we found that it shared many scattered homologous regions with the mouse liver MBP gene (*24*) and a few homologous regions with the rat liver MBP gene (*25, 26*). Figure 2 shows that exons 0 and 1 of human MBP correspond to exons 1 and 3 of rodent liver MBP, respectively, and an exon equivalent to exon 2 of rodent liver MBP is missing in the human MBP gene, although the length of intron 0 of human MBP is almost the same as the total length of intron 1, exon 2 and intron 1 of rodent liver MBP. Human MBP exhibits higher homology to rodent serum MBP in complement-activating activity and the formation of higher oligomers (*27*), but human MBP seems to be evolutionary more related to liver MBP.

In the approximately 60 bp upstream regions of exon 0 of human MBP and exon 1 of mouse liver MBP, there is a consensus TATA box (TATTAA), which is probably functionally active in transcription (Fig. 2). Human MBP contains another consensus TATA box (TATATA) at −23 to −27 bp upstream of exon 1. This TATA box is conserved in intron 2 of mouse liver MBP. However, the sequence, TATATA, in human MBP has changed to TACAAT in mouse liver MBP at 86 to 81 bp upstream of exon 3, which appears to have no functional activity. We also found a TATA box about 15 bp downstream from the 5′-end of exon 1 of human MBP, which probably contributed to the generation of 2 subclones containing a little shorter transcript at the 5′-terminus of exon 1 on 5′RACE.

Expression of the mRNA Transcript Starting from Exon 0—In order to confirm that the longer mRNA containing exon 0 was actually transcribed in the human liver, Northern blot analysis was performed on various human tissues using probes specific to exons 0 and 1, respectively. As shown in Fig. 3, several bands were only detected for the liver with both probes. The major band corresponded to a molecular size of about 4.0 kb. The radiolabeled probe for exon 1 hybridized to the transcripts starting from both exon 0 and exon 1, in contrast, the probe for exon 0 only recognized the former. The intensity of each band did not exactly reflect the level of transcription, because the lengths and specific radioactivities of the two probes may be different from each other. The transcript starting from exon 0 should be about 100 bp longer than that starting from exon 1, and both transcripts should be included in each band in Fig. 3B, but such a small base pair difference between them could not be detected under the conditions used. These results indicated clearly that the longer MBP mRNA starting from exon 0 is expressed in the liver, and suggested that the transcripts starting from both exon 0 and exon 1 may exhibit no significant difference in their posttranscriptional modification or polyadenylation.

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-1707 ctgatggctttaggcatgtggctctgtaccatcctggacatttttccaggcacagcagacaggtcagcaggaagtaaggaagccagtccctccacaagcc
-1607 aagaagacttcaattggaagggacctggaaatgcatcttactcacatttcttttggtgtttggaggcctccatagtacccccaccacatggccccgcagc
-1507 ctctgaacttttctggatgggctccccaacaccccaagctacatggcatttttctgaaagccatatcagtaatgtagttgagaatcaaatgaactatgtt
-1407 cgtaaaagtattcttccatcccttctagattgtccccattaaccacaggaagatggttcaagccattaacacacacgatacccatgatcttatagtgcca
-1307 agtgctgttcttttgcttcttatctcagctgagtggcccctagatagtcaagtcataaatgtgtgtgcccatgaaaacttcttattatcactcactattc
                                                                     HNF-3
-1207 tagctaattctcaagtgcaatcagtacctcctgtttctcaagtgtgcctaccttcttaatctagctgccattaccctgacccaggccactgtcttctgct
-1107 gcttagatcatggcaagcacttccttttagatctgcttttctccagtcctgttcccctaaacatcatagccagaacaagccttgcaaaattcaaatgtga
                                                                                                  HNF-3
-1007 tcacctctccactattgcagtccctctagtgccccctggaaggaaacccaaagtctttaacacagtttacaattcttgttatgaggatgaaacttgaaga
 -907 cattgtgctaagtgaaataagccagatacaaggggctattgtatgatttcacatatatgaggtacctaggatagatgaattcagagagacagaaagtaa
                                              HNF-3
 -807 aacagaagttaccagtggctgtgggtaggggtaaatggagaattctagtttaatgagttgagagtttcagtttgggatgatgaaaatcatcccatgatgt
                                                                            type2 IL-6 RE
 -707 ggtgatgatagcacaacagtgtgaatgtacttaatgctactgagttgtgtgcttaataataatggtcaaaatggtaaattttatgtctattttaccacaa
 -607 tgaaaaactggaaaagtaaagacaatgactataatatcataaagtgtaagtaacatgagagaaaataattcaggctaagaggatagagactgctaggtag
 -507 gactattttagaataagtgatcaaggtactgatttctctgaaattggaatatttgaatagaagagaaagagctagctggcgatgcccgtaaggggtccag
                                                   HNF-3
 -407 gcatggagacatcaagtgaagagtctgaggcaggaatggccttaatgcattaatggcagcaagaggtctatgtggtttcatggatgggtgtgtgcgtgca
 -307 tgcacgtgtctgtgtgtatagttggtgtgcatgagcatgtgtatgcatttgtgagtatctttgttttctgttggaaacaaggatgtcaacaggactaac
 -207 taggtgaacctagctgtggtagttcagcaatgagatttgccaaaatggatggcaattgaaagaggaattgtgctgtattaaagcagacagctgccgagca
                            type1 IL-6 RE    CAAT box
 -107 ctgtattggaggctcctaaatttctcatagttctgagaagagctgtttattaatctggagttgaaggccaggcccaggatatccttgttcacaaggttga
               exon 0                type2 IL-6 RE    TATA box
   -7 ttgggccCTACATTGCTGAGCCCAGCCTCCTCCCTCACTCTGAGGCATCTGCCAGAGCCCCAGGCTAGAGGGCCAGCGTCCTTGTCACTGAGTCCCTGCT
  +94 CTGCAGAAACACCAgtaagtcatttctggtgaataaatctgggtttccggatcaagggtttggggagctgacatgaccctggaggcatggaggaggcttt
 +194 ctaggggtgggggggagtgggatggaaagcagaaatcaggttgaggatgtctctttttctgcttgtgctggagcatctgtgactttcctccattatctgtt
 +294 catctgcagtggagactgtctttgttttcaaagggaaacttggaggcttagacctatggggctaggctgctgaggtttcttagggggcaatagctggaag
 +394 aaagctctgaagaacaaatgaaaggttaatactgagaaatgggaggaggattcaaggcaagttttctaattgccagtggtttttgactcacagaacatgg
 +494 ggaattcctgccagaaagtagagaggtatttagcactctgccagggccaacgtagtaagaaatttccagagaaaatgcttacccaggcaagcctgtctaa
                                                        type1 IL-6 RE                        L haplotype
 +594 aacaccaaggggaagcaaactccagttaattctgggctgggttggtgactaaggttgaggttgatctgaggttgagaccttcctctttggatcaccagct
 +694 ttcagctcagggcctgccaatgagtaaatgatagttaacaggtcctggaggggaatcagctgcccagatacaaagatgggattcaggtggcagatggacc
 +794 cgaagaggacatggagagaaagaggaagctcctacagacacctgggtttccactcattctcattccctaagctaacaggcataagccagctggcaatgca
 +894 cggtcccatttgttctcactgccaccgaaagcatgtttatagtcttccagcagcaacgccaggtgtctaggcacagatgaacccctccttaggatcccca
      Glucocorticoid Responsive Element   Y haplotype
 +994 ctgctcatcatagtgcctacctttgttaaagtactagtcacgcagtgtcacaaggaatgtttactttccaaatccccagctagaggccagggatgggtc
                                             exon 1      HNF-3       CAAT box
+1094 atctatttctatatagcctgcacccagattgtaggacagagGGCATGCTCGGTAAATATGTGTTCATTAACTGAGATTAACCTTCCCTGAGTTTTCTCAC
          TATA box                                   TATA box
+1194 ACCAAGGTGAGGACCATGTCCCTGTTTCCATCACTCCCTCTCCTTCTCCTGAGTATGGTGGCAGCGTCTTACTCAGAAACTGTGACCTGTGAGGATGCCC
+1294 AAAAGACCTGCCCTGCAGTGATTGCCTGTAGCTCTCCAGGCATCAACGGCTTCCCAGGCAAAGATGGGCGTGATGGCACCAAGGGAGAAAAGGGGGaacc
+1394 ag
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Fig. 1. **DNA sequence of the 5′ flanking region of the human MBP gene.** The upper case letters denote the exon sequences (exons 0 and 1), and the lower case ones the intron sequences (introns −1 and 0). Nucleotide numbers, the first nucleotide of exon 0 being numbered +1, are indicated on the left of the lanes. TATA boxes, CAAT boxes, HNF-3 recognition sites, type 1 and 2 IL-6 responsive elements and a glucocorticoid-responsive element are underlined. Circles indicate the sites of the H/L and X/Y haplotypes. Arrows denote the starting sites of exons 0 and 1, respectively.

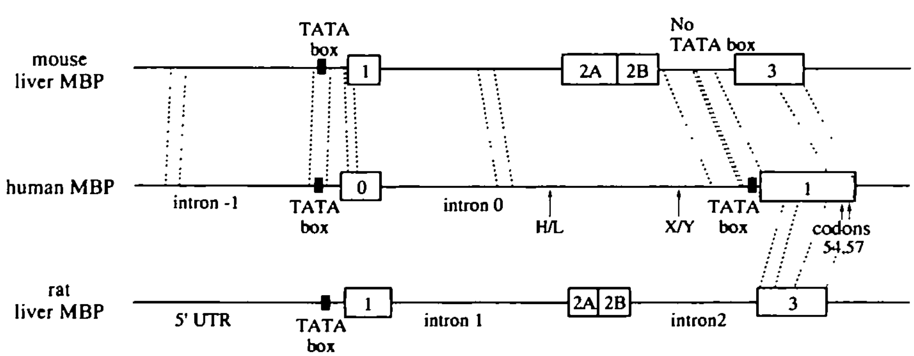

Fig. 2. **Genomic organization alignment of the promoter regions of the human MBP, mouse liver MBP, and rat liver MBP genes.** Open boxes represent exons, closed boxes TATA boxes, and lines introns. The regions joined by dotted lines exhibit high sequence similarity between human MBP and rodent liver MBPs. Arrows denote the positions of the polymorphic sites.

Promoter Activity of Introns −1 and 0—To determine whether or not introns −1 and 0 of the human MBP gene have the ability to regulate promoter activity, these DNA fragments were subcloned into a promoterless, enhancerless luciferase reporter gene. The resulting constructs were transfected into a human hepatoma cell line, HepG2 cells, which was shown by RT-PCR to express endogenous MBP mRNA (data not shown). As shown in Fig. 4, intron −1 (PGV-1707) exhibited relatively low promoter activity compared with that of intron 0 (PGV+297). On the basis of

these results, it is speculated that MBP mRNA is, for the most part, transcribed from exon 1. This notion may explain the low frequency of exon 0-containing subclones on 5′RACE and the fact that exon 0 was not identified in the previous study (*14*).

To determine the region of the human MBP promoter responsible for transcription, a series of 5′ deletion mutants was prepared and transfected into HepG2 cells. As shown in Fig. 4, the deletion of most of intron −1 (from −1707 to −325) had no detectable effect on the transcriptional activity, although the region contained several sequences similar to the HNF-3 recognition site and the IL-6 responsive element. On the other hand, the deletion of the sequences from +704 to +833 and +995 to +1030 in intron 0 enhanced the transcription of the luciferase reporter gene, suggesting the presence of negatively controlling cis-acting elements in these regions. The deletion of the region from +1030 to +1057, in which the HNF-3 recognition site is present, resulted in an approximately 50% decrease in the luciferase activity, suggesting that HNF-3 (*28*), which is known to be associated with the transcription of liver-specific genes, is involved in the liver-specific expression of MBP. The deletion of the sequence from +1110 to +1153, in which a TATA box sequence is present from +1146 to +1151, abolished the transcriptional activity almost completely.

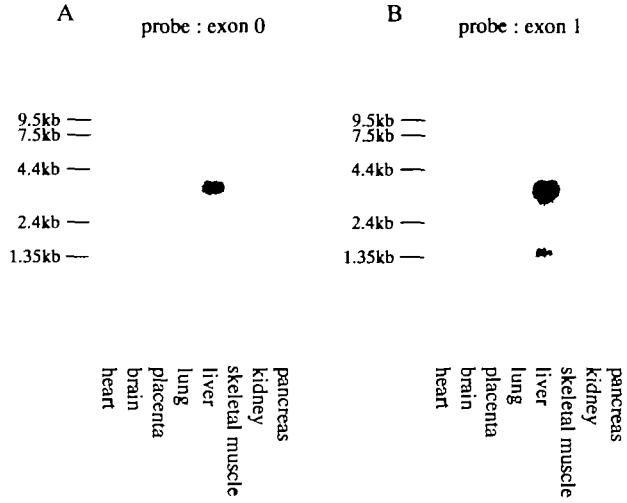

Fig. 3. **Expression of human MBP mRNA in adult human tissues.** The Northern blot was purchased from Clontech. In each lane, 2 mg of polyA⁺RNA from a human tissue was loaded. The blot was hybridized with radiolabeled human MBP exon 0 and exon 1, as described under "MATERIALS AND METHODS."

In order to confirm the notion that the transcription of mRNA from exon 1 is controlled mainly by HNF-3, we next measured the promoter activities of PGV+297, PGV+1030, and PGV+1057 in HeLa and CHO cells as well as HepG2 cells. The highest promoter activity was detected in HepG2 cells for all three constructs, each of which showed up- and down-regulation, as shown in Fig. 4. The luciferase activities of PGV+297, PGV+1030, and PGV+1057 in HeLa and CHO cells showed no significant differences as to the promoters used, and they corresponded to one-eighth (PGV+1030) to one half (PGV+1057) those in Hep G2 cells (data not shown). These results suggest that the up- and down-regulation of the luciferase activity by these constructs is a cell-specific event, and that the transcription of MBP is most probably up-regulated by the HNF-3 recognition site between +1030 and +1057.

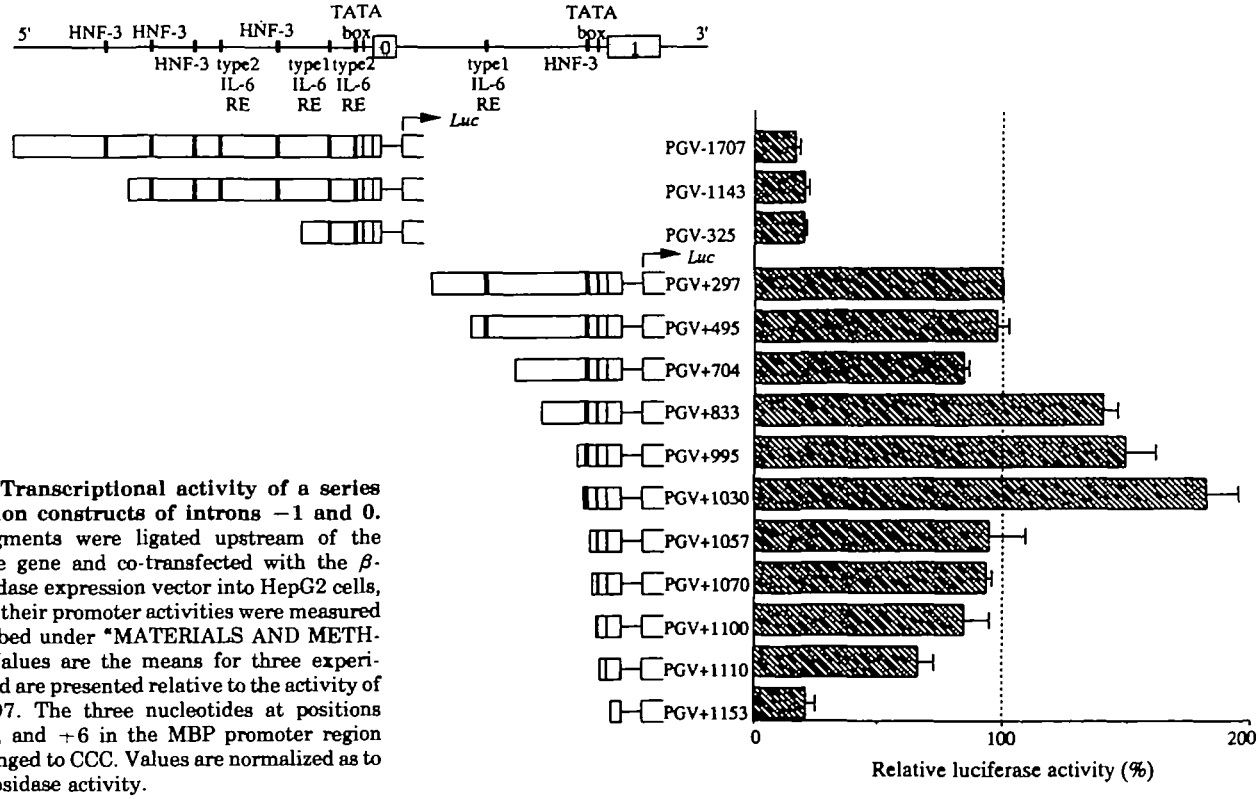

Fig. 4. **Transcriptional activity of a series of deletion constructs of introns −1 and 0.** The fragments were ligated upstream of the luciferase gene and co-transfected with the β-galactosidase expression vector into HepG2 cells, and then their promoter activities were measured as described under "MATERIALS AND METHODS." Values are the means for three experiments and are presented relative to the activity of PGV+297. The three nucleotides at positions +4, +5, and +6 in the MBP promoter region were changed to CCC. Values are normalized as to β-galactosidase activity.

Gel Mobility Shift Assay for the HNF-3 Recognition Site of the MBP Gene—In order to confirm that the transcription of the MBP gene from exon 1 is mediated by the binding of HNF-3 proteins, gel shift assays were performed. As shown in Fig. 5A, a nuclear extract of HepG2 cells binds to the HNF-3 recognition site (+1049 to +1059) in intron 0 (designated as the MBP-HNF-3 site). These DNA-protein complexes seemed to contain either HNF-3α, β, or γ (*29, 30*). The formation of these complexes decreased in the presence of increasing concentrations of the unlabeled oligonucleotide probe added. The binding of HNF-3 proteins to the HNF-3 site was confirmed by cross competition between an authentic TTR promoter (*19*) (designated as the HNF-3 site) and the MBP-HNF-3 site in a gel shift assay. Figure 5B shows that the formation of oligonucleotide-protein complexes was competed for to the same extent by either competitor, indicating that transcription factor HNF-3α, β, or γ binds to the promoter of MBP as strongly as to the high affinity HNF-3 site, which resulted in the hepatocyte-specific expression of MBP.

Effects of Inflammatory Cytokines and Dexamethasone—The sequences of introns −1 and 0 contain several type 1 and type 2 IL-6 responsive elements. In order to determine the effect of IL-6 upon the transcription of the MBP gene, the luciferase activities of PGV-1707 and PGV+297 were assayed in the presence of IL-6. No significant change due to IL-6 was observed for either construct. In addition, neither TNF-α nor INF-γ affected the transcriptional activity (data not shown). We also examined the effect of dexamethasone on the transcription of PGV+297, which includes a glucocorticoid-responsive element. As shown in Fig. 6, the promoter activity was decreased by more than 60% on exposure to dexamethasone (*31*), revealing that glucocorticoids down-regulate the MBP expression, in contrast to most other acute phase proteins, which are up-regulated by glucocorticoids. In order to define the region responsive to dexamethasone, PGV+297, PGV+833, and PGV+995 were transiently transfected into HepG2 cells, and then their luciferase activities were measured with or without exposure to dexamethasone. The promoter activity of PGV+833 decreased by approximately 60% upon treatment with dexamethasone as in the case of PGV+297, but that of PGV+995 was not affected by dexamethasone, indicating that the response to dexamethasone is mediated by a region between +833 to +995. These results suggested that, in contrast to the previous hypothesis that the glucocorticoid-responsive element at +895 is involved in positive responses to inflammatory stimuli (*14*), this glucocorticoid-responsive element-like structure appears to function as a negative regulatory element.

Promoter Activity of Promoter Variants HY, LY, and LX—Recently, Madsen *et al.* reported that polymorphisms exist at positions +588 (H/L variant) and +919 (X/Y variant) of the human MBP gene, and that the promoter haplotypes of HY, LY, and LX are associated with high,

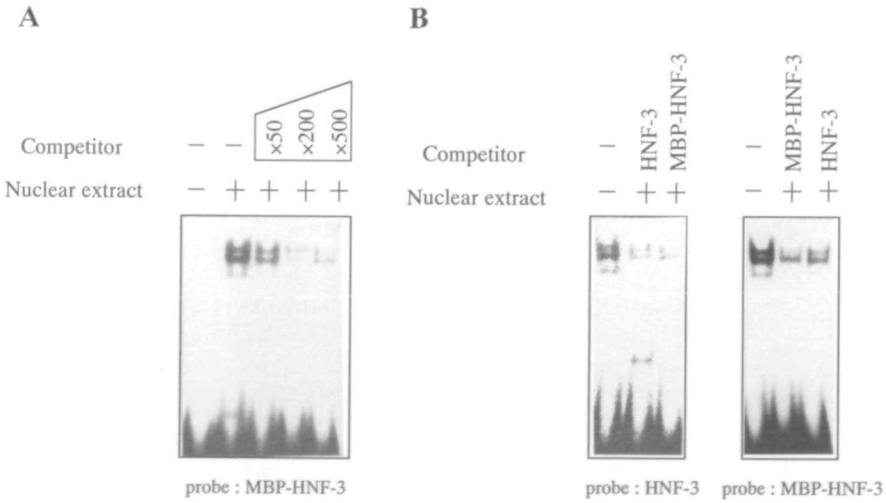

Fig. 5. **Gel shift assay for the HNF-3 recognition site of the MBP promoter region.** A: The gel shift assay was performed with a double-stranded oligonucleotide corresponding to the HNF-3 recognition site of the MBP promoter. The preparation of a nuclear extract, hybridization reactions with the ^{32}P-labeled oligonucleotide, and electrophoresis were performed as described under "MATERIALS AND METHODS." Nine micrograms of the nuclear extract and a molar excess (x-fold) of the unlabeled competitor oligonucleotide DNA were used in the reaction. B: A cross competition study was performed with the ^{32}P-labeled HNF-3 recognition site of the MBP promoter (MBP-HNF-3) and the strong affinity HNF-3 binding site of the TTR promoter (HNF-3). The competition lanes included a 250-fold molar excess of unlabeled MBP-HNF-3 or HNF-3.

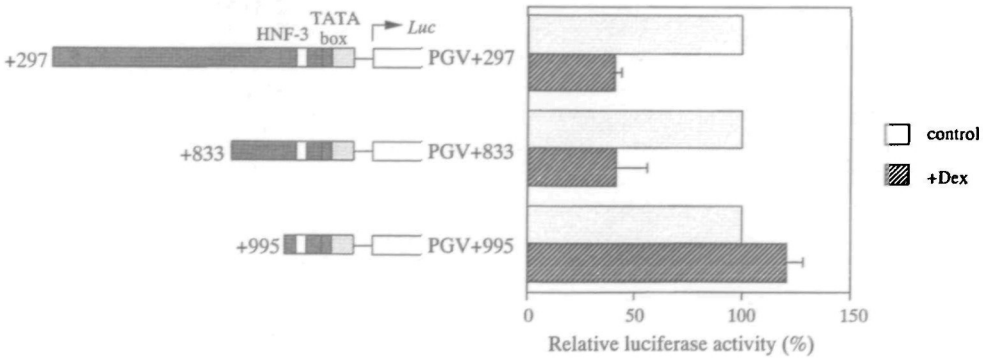

Fig. 6. **Suppression of MBP gene expression by dexamethasone.** PGV+297, PGV+833, and PGV+995 were transfected into HepG2 cells. Six hours after exposure to 1×10^{7} M dexamethasone, luciferase activity was measured. Values are the means for three experiments and are presented as the fold increase, relative to the control value in the absence of dexamethasone (taken as 1.0).

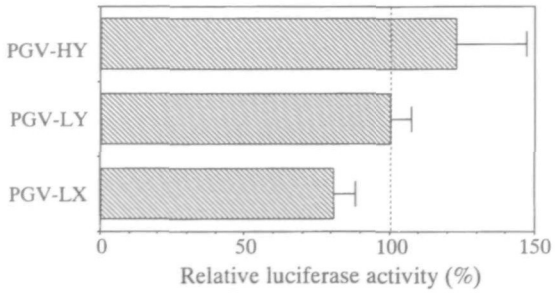

Fig. 7. **Transcriptional activity of HY, LY, and LX variants.** HY, LY, and LX variants were ligated upstream of the luciferase gene, co-transfected with the β-galactosidase expression vector into HepG2 cells, and then assayed for luciferase activity. Values are the means for three experiments relative to the activity of PGV-LY (taken as 100%), and normalized as to β-galactosidase activity. The LX variant differed significantly from the LY variant ($p=0.031$), and the LY variant did not differ very significantly from the HY variant ($p=0.193$).

medium and low serum levels of MBP, respectively (*15*) (see Fig. 2). In order to determine the promoter activities of HY, LY, and LX variants, PGV-HY, PGV-LY, and PGV-LX were transfected into HepG2 cells, and then their promoter activities were measured. As shown in Fig. 7, the HY, LY, and LX variants exhibited high, medium, and low promoter activity, respectively, in agreement with the results of a population study. It is interesting to note that positions +588 and +919 are not included in any previously described transcription factor binding sites. As shown in Fig. 4, deletion of the sequence containing position +588 (from +495 to +704) slightly decreased the activity, and deletion of the sequence containing position +919 (from +833 to +995) increased the activity, suggesting the presence of positively-regulating element(s) around +588 and negatively-regulating element(s) around +919 in the LY haplotype. This promoter polymorphism may significantly contribute to the large variation in the promoter activity of the MBP gene.

DISCUSSION

The major purpose of this study was to elucidate the mechanism underlying the transcriptional regulation of the human MBP gene. Such information may provide a new insight into the liver-specific expression and the wide variation in the serum concentration of MBP.

First, we identified a new exon, designated as exon 0, upstream of the previously described exon 1 and characterized the genomic structure of the MBP gene. The genomic organization and the DNA sequence of human MBP were similar to those of rodent liver MBP, except that human MBP did not contain an exon equivalent to exon 2 in rodent MBP, although exons 0 and 1 of human MBP appeared to correspond to exons 1 and 3 of rodent liver MBP, respectively. We showed that human MBP has two transcriptional initiation sites, one located in the 5′ flanking region of exon 0, and the other in the 5′ flanking region of exon 1. Each mRNA transcript appeared to be controlled by its own promoter containing a TATA box. Rodent liver MBP does not have a functional TATA box upstream of exon 3, and consequently transcription does not start at exon 3. The presence of a TATA box in intron 0 of human MBP may have occurred by chance through mutational changes in the TATA-like sequence in a common ancestral gene.

We next tried to elucidate the functional promoter sequence of MBP by constructing a series of 5′ deletion mutants and examining their ability to stimulate transcription. Human MBP has two promoters for transcription. Intron −1 contains a TATA box for basic transcription, but failed to exhibit any significant promoter activity under the conditions examined. In contrast, the full length intron 0 exhibited high promoter activity. In this region, however, there are negatively-acting and positively-acting elements, indicating that the transcription of MBP is regulated through a complex system that responds to various stimuli. The results also provided evidence that the nuclear factor, HNF-3, plays an important role in the expression of MBP. HNF-3 was first discovered during functional analysis of the transthyretin (TTR) promoter, and was subsequently shown to participate in the expression of several other liver-derived genes, such as those of albumin, transthyletin (TTR), and α1-antitrypsin (*28-30*). The liver is the exclusive site of MBP synthesis (*1-4*), and deletion of the HNF-3 recognition site in intron 0 results in a more than 50% decrease in transcriptional activity in HepG2 cells, which express HNF-3. These results indicated that liver-specific expression of MBP is primarily dependent on HNF-3.

IL-6 is an inducer of various typical acute phase proteins, such as fibrinogen, C-reactive protein, serum amyloid A protein, *etc.* (*31*). In our experiments, treatment with IL-6 did not have any significant effect on the transcriptional activity of PGV+297. On the other hand, treatment with a glucocorticoid decreased the promoter activity by approximately 60%. Two mechanisms of glucocorticoid receptor (GR)-mediated repression have been described: (i) direct interaction between GR and a negative glucocorticoid-response element (nGRE), as seen in the bovine prolactin (*32*) and rat pro-opiomelanocortin (POMC) genes (*33, 34*), and (ii) protein-protein interactions, as seen in the AP-1 (*35, 36*) and NF-kB (*37*) responsive genes, in which GR is assumed to form complexes with AP-1 and the resulting complex shows no AP-1 *trans*-activating function. The former may be the case with glucocorticoid-mediated suppression of the MBP gene. The consensus motif of positive GRE has been defined as a 15 bp sequence of GGTACAnnnTGTTCT. The nGRE sequence was shown to contain conserved features of positive GREs (*34*). However, a major difference between the nGRE and GRE sequences is the absence of the conserved CA dinucleotide at positions 5 and 6 (*34, 38*). nGRE appears to contain three GR molecules, whereas GR binds GREs as heterodimers (*34*). The GER-like structure of the MBP promoter region comprises GGTCCCATTTGTTCT, in which the three nucleotides at positions 4, 5, and 6 have changed to CCC. In addition, judging from the results of the promoter deletion study (Fig. 3), no other *trans*-activating transcription factors appear to function around this region. These results suggest that alteration of these nucleotides may cause the repressive transcription in response to glucocorticoid, although the detailed mechanism of this repression remains to be elucidated.

It is well known that the serum levels of MBP show wide intra- and interracial variety. Population analysis revealed

that the serum level is closely associated with point mutations at codon 54 or 57, which disrupt the characteristic tandem repeat of the Gly-Xaa-Yaa triplet structure in the collagen-like domain (see Fig. 2). Our recent observation that a mutant MBP, in which Gly at codon 54 was converted to Asp, showed a much more rapid turnover rate in plasma compared to the wild type explains, at least in part, the lower serum concentration of the mutant MBP (*39*).

In addition, promoter variants have recently been shown to exist at +588 (H/L) and +919 (X/Y). Haplotypes HY, LY, and LX exhibit correlation with high, medium and low serum concentrations in man. We examined the transcriptional activities of the HY, LY, and LX sequences *in vitro*, and confirmed the results of a previous population study at the molecular level. According to the report of Medsen *et al.*, the average serum levels of MBP in Caucasoids were 2.3 mg/liter for the HY/HY haplotype, 1.9 mg/liter for the LY/LY haplotype, and 0.35 mg/liter for the LX/LX haplotype, the ratio being about 1.2:1.0:0.18. The ratio of the promoter activities estimated in this study was 1.1:1.0: 0.81, suggesting that the relative promoter activity of PGV-LX is higher than the relative serum level in the LX/LX haplotype. This disaccord might be explained by the difference between the internal environment of HepG2 cells and that of hepatocytes in the liver of individuals. As the MBP promoter regions contain several negatively- and positively-acting elements, it is possible that the transcription level varies from individual to individual, depending on their situations, such as infectious stimulation, stress and age.

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