Interleukin-1-Mediated Enhancement of Mouse Factor B Gene Expression via NFKB-Like Hepatoma Nuclear Factor

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Complement factor B, a serine protease playing a pivotal role in alternative pathway activation, is an acute-phase plasma protein. Previous studies have revealed that interleukin-1 (IL-1) mediates, at least in part, the acute-phase induction of factor B expression and that the IL-1-responsive element resides in the region between −553 and −478 relative to the transcription initiation site of the mouse factor B gene. In this paper, we demonstrate a specific binding site for a nuclear factor of human hepatoma HepG2 cells in this region of the factor B gene, using gel shift and methylation interference analysis. The nucleotide sequence of the binding site is closely similar to the NFκB or H2TF1 binding motif. The binding activity of HepG2 showed very similar specificity to that of NFκB or H2TF1, as shown by a competition binding assay, and was induced by IL-1α treatment. A synthetic oligonucleotide corresponding to this binding site, as well as a similar sequence found in another class III complement C4 gene, conferred IL-1 responsiveness on the minimal factor B promoter. In contrast, a mutated oligonucleotide that could not bind to the HepG2 nuclear factor did not confer IL-1 responsiveness. These results suggest that IL-1 induces factor B expression via NFκB or a closely related factor in hepatocyte nuclei.

Factor B (Bf) is a serine protease member of the complement cascade which plays a pivotal role in activation of the alternative pathway (25). Genes encoding Bf, C2, and two C4 isotypes are located in the major histocompatibility complex in both humans and mice and are designated as major histocompatibility complex class III genes (4, 5). Bf is an acute-phase protein, and its concentration in plasma increases during acute-phase response together with C-reactive protein, serum amyloid A, and several other proteins in the clotting and complement system. Although the molecular mechanisms regulating Bf expression during the acute-phase response are not well understood, interleukin-1 (IL-1) and tumor necrosis factor (TNF), major cytokines that mediate acute-phase response, have been shown to increase Bf gene expression in primary hepatocyte culture (24), in hepatoma cell lines, and in L cells transfected with cosmid clones bearing the Bf gene (20, 21). In a previous study, we analyzed the promoter region of the mouse Bf gene by chloramphenicol acetyltransferase (CAT) assay and found that the region between −553 and −478 relative to the transcriptional start site is essential for IL-1 responsiveness (15). Since there are only 386 bases between the transcription start site of the mouse Bf gene and the polyadenylation site of the adjacent C2 gene, the IL-1-responsive region is located completely in the 3' untranslated region of the C2 gene. To elucidate the molecular basis for the IL-1-induced enhancement of Bf gene expression, we have analyzed this region and identified an IL-1-responsive element. We demonstrate the critical role of this element and the NFκB-like binding factor in human hepatoma nuclei during IL-1-induced expression of the Bf gene.

MATERIALS AND METHODS

Plasmid construction. Isolation and characterization of the 5'-flanking region of the mouse Bf gene, and construction of a d92 recombinant that contains a −92 to +10 fragment of the Bf gene inserted into the HindIII site of pSVOCAT, have been described previously (15). Oligonucleotides were synthesized using an ABI 391 synthesizer. Coding and noncoding strands of each oligonucleotide were designed to generate blunt ends after annealing. Among them, BfxB, C4xB, and C4xBmu oligonucleotides were phosphorylated by T4 polynucleotide kinase, ligated to XbaI linker, and introduced into the XbaI site of the d92 construct to test the possible functional role of these sequences in IL-1-induced expression of the Bf gene. The numbers and directions of the inserted oligonucleotides were determined by nucleotide sequence analysis, using the dideoxy-chain termination method (28). Similarly, BfxB and C4xB oligonucleotides were introduced into the BglII site of pA10CAT2 after ligation with BglII linker.

Gel shift assay. Nuclear extracts were prepared from HepG2 cells by using the rapid method (19). Nuclear extracts and 3'-end-labeled probes were incubated (29) and analyzed by polyacrylamide gel electrophoresis in TBE buffer (89 mM Tris-borate, 2.5 mM EDTA [pH 8.3]). Probes were prepared as follows: the Smal (−553)–AluI (+126) fragment of the Bf promoter was subcloned into the Smal site of pGEM4, generating pBf500. pBf500 was linearized with HindIII, 3' end labeled with a Klenow fragment, and digested with HpaII. The resultant probe representing the Smal (−553)–HpaII (−305) region (fragment A of Fig. 1) of the Bf promoter with radioactivity at the 3' end of the noncoding strand was gel purified. To make the probe with radioactivity on the coding strand, the HindIII–HpaII fragment of pBf500 was subcloned between the HindIII and AccI sites of pGEM4. This plasmid was linearized with AvaI, 3' end labeled with a Klenow fragment, and digested with HindIII. To make the probe or competitor representing fragment B of Fig. 1 (−504 to −478), this region of the d553 construct was amplified by a polymerase chain reaction, using primers with artificial BamHI sites at the 5' ends. Amplified products were digested with BamHI and subcloned into the BamHI site of pGEM4. Human IL-1α (recombinant; Dainippon Pharmaceutical Co. Ltd.) induc-
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FIG. 1. Schematic representation of a mouse DNA segment encompassing the 3' end of the C2 gene and the 5' end of the Bf gene. The last exon of the C2 gene and the first exon of the Bf gene are shown as boxes. Black and white portions correspond to the protein coding region and untranslated region, respectively. The IL-1-responsive region, delimited by the IL-1-responsive d553 construct and the IL-1-unresponsive d478 construct (15), is shown together with the nucleotide sequence. The position of the probes and competitors (A and B) used for gel shift and methylation interference analysis is shown. The xB-like sequence is underlined.

of HepG2 cells was performed in a serum-free Dulbecco modified Eagle medium.

Methylation interference assay. Methylation interference analysis was performed essentially as described previously (3), using probes prepared as described for gel shift assay.

FIG. 2. Binding of a HepG2 nuclear factor to the Smal(-553)-HpaII(-505) fragment. Gel shift assay was performed as described in Materials and Methods, using the probe A labeled at the 3' end of the noncoding strand. Lane 1, no extract; lanes 2 to 4, 10 μg of HepG2 nuclear extract; lane 3, 10 ng of the cold fragment A as a competitor (50-fold molar excess); lane 4, 50-fold molar excess fragment B. The dried gel was exposed for autoradiography for 15 h at -70°C.

Briefly, 10^5 cpm of partially methylated probe was mixed with 200 ng of HepG2 nuclear extract in the presence of poly(dI-dC). The mixture was electrophoresed through the 6% polyacrylamide gel as described for gel shift assay. Free and bound probes were located with autoradiography and purified, using DEAE paper. After cleavage with piperidine, the product was analyzed in an 8% denaturing polyacrylamide gel.

CAT assay. CAT assay was performed as described previously (8), using a transient transfection system with
HepG2 cells (17). A total of 5 × 10^5 cells were plated in a
60-mm-diameter dish, and 10 μg DNA was transfected by
the calcium phosphate coprecipitation method. Glycerol
(25%) shock was performed 4 h after transfection. The
next day, the medium was changed to serum-free Dulbecco
modified Eagle medium with or without IL-1. The assay
was repeated at least three times. Radioactive spots were
determined using Bio-image analyzer BA 100 (Fuji Photo
Film Co.).

RESULTS

Binding of the HepG2 nuclear factor to the IL-1-responsive
region of the mouse Bf gene. In a previous report, we
demonstrated that the −553 to −478 region of the mouse Bf
gene, which is completely within the 3′ untranslated region
of the adjacent C2 gene, is essential for full response of
the Bf promoter to IL-1 induction (15). To test the possible
presence of a binding factor to the −553 to −478 region,
HepG2 nuclear extracts were analyzed by gel shift assay.
When the 3′-end-labeled fragment extending from −553 to
−505 in the Bf promoter (Fig. 1, fragment A) was used as a
probe, HepG2 nuclear extracts formed three complex bands,
I, II, and III (Fig. 2, arrowheads in lane 2). The formation of
all three bands is sequence specific, since 20 ng of unlabeled
fragment A (100-fold molar excess) completely inhibited
complex formation (lane 3), whereas the same molar excess
of the adjacent fragment B showed almost no effect (lane 4).
There was no obvious additional binding site between −505
and −478 for a HepG2 nuclear factor, since no specific
complex band was detected when the fragment B was used
as a probe (data not shown).

Sequence of the binding site. To locate the binding site
more precisely, a methylation interference assay was per-
formed as shown in Fig. 3. Scaled-up gel shift analysis was
performed by using fragment A as a probe, and the gel was
exposed to an X-ray film for 3 h at 4°C. Only one complex
band considered to correspond to band II of Fig. 2 was
detected. When the 3′ end of the coding strand was labeled,
two bands, −525 and −524, were dramatically reduced in
the specific complex (lane B) compared with the free probe (lane
F). In addition, partial reduction of the −523 band was
observed in the specific complex. When the noncoding
strand of the same fragment was radiolabeled and used as a
probe, cleavage at three guanines at positions −516, −517,
and −519 was dramatically reduced in the specific complex
compared with the free probe. Again, partial reduction was
observed at the −518 position. No other difference in
the level of cleavage of guanines was detected on either DNA
strand over the −553 to −505 region. Therefore, the binding
site of the HepG2 nuclear factor in the −553 to −505
fragment was located in the −525 to −516 segment, GGG
AATCCCC. This sequence bears a close resemblance to
the binding motif of the transcription factors NFκB, H2TF1,
and PRDII-BF1 (3, 10, 30).

Competition experiments with κB-related sequences. We
tested the binding specificity of the HepG2 nuclear factor to
the κB-like binding motif in the Bf promoter using various
κB-related oligonucleotides as competitors. The oligonucleo-
tide corresponding to this motif of the Bf gene was synthe-
sized and designated BfκB (Fig. 4). Its mutant, BfκBmu,
was synthesized with nucleotide substitution at four positions
demonstrated to contact with the HepG2 nuclear factor by
methylation interference assay. As reported previously (35),
the NFκB or H2TF1 binding motif was found in the pro-
moter region of another class III complement gene, C4.
Although functional importance of this sequence in the C4
promoter is still to be demonstrated, the specific binding of a
HepG2 nuclear factor to this sequence has been described
previously (35). Therefore we have tested the ability of this
sequence, designated C4xB, and its mutated version, C4xBmu, to compete for binding to a HepG2 nuclear factor with the Bf fragment. Also tested were the NFkB binding site found in the mouse immunoglobulin κ chain gene (IgκB) (30) and H2TF1 binding site found in the H-2Kb gene promoter (3). BfκB, C4xB, IgκB, and H-2Kb oligonucleotides at 1,000-fold molar excess completely abolished the binding of the HepG2 nuclear factor to the Bf fragment. In contrast, BfκBmu and C4xBmu showed almost no competition. Since BfκBmu and C4xBmu have nucleotide substitutions at the positions reported to be essential for NFkB or H2TF1 binding, the binding specificity of a HepG2 nuclear factor to the Bf fragment seems to be similar or identical to that of NFkB or H2TF1.

**Induction of the binding activity by IL-1.** Using a gel shift assay, we tested whether the HepG2 nuclear factor that binds to the BfκB sequence was induced by IL-1α or not. Nuclear extracts were prepared from HepG2 cells treated with 0 to 30 ng of IL-1α per ml for 30 min. IL-1 induced the binding activity in a dose-dependent manner (Fig. 5A). All three bands, I, II, and III, were induced, and in addition, a few new bands were detected at high IL-1 concentrations between bands II and III and also above band III. Maximum induction was observed at 3 to 30 ng of IL-1α per ml and was estimated to be sixfold using a Bio-image analyzer BA 100. The specificity of the IL-1-induced binding activity could not be discriminated from that of unstimulated cells, since both binding activities were abolished by BfκB, whereas BfκBmu showed almost no effect. Figure 5B shows the time course of IL-1 induction at 20 ng/ml. Induction was very quick, and about fivefold induction was observed within 30 min of IL-1 addition. The induced activity declined gradually and reached about the twofold level at about 24 h after IL-1 addition.

**BfκB and C4xB are IL-1-responsive elements.** To test the possible role of this binding site in IL-1-mediated induction of Bf expression, we inserted BfκB, C4xB, and C4xBmu into the d92 construct. As has already been shown, the d92 construct contains only a 92-bp Bf proximal promoter region and does not respond to IL-1, although the basal level transcription from the normal initiation site has been demonstrated by RNase mapping analysis (15). Transient expression in HepG2 cells was assayed with and without IL-1 induction for 24 h at 20 ng/ml. Both BfκB and C4xB had no effect in IL-1-induced expression when they were inserted as a single copy (Fig. 6). However, three, four, or six copies of BfκB or four or nine copies of C4xB rendered the d92 construct responsive to IL-1 induction by 2.2- to 3.8-fold. In contrast, C4xBmu, which showed no binding activity by competition binding assay (Fig. 4), had no effect even at four or five copies, suggesting that the specific binding of the HepG2 nuclear factor plays an important role in IL-1-induced expression of the Bf gene. To test whether this binding site can confer the IL-1 responsiveness to a heterologous promoter or not, seven copies of BfκB or eight copies of C4xB were inserted into the BglII site of pA10CAT2, which contains simian virus 40 early promoter without enhancer. Both sequences rendered the simian virus 40 promoter responsive to IL-1 induction (Fig. 6).

**DISCUSSION**

In the present study, we have demonstrated that transcriptional activation of the Bf gene promoter by IL-1 in HepG2...
cells is mediated by a nuclear factor having the identical or nearly identical binding specificity to that of NFκB. NFκB or a closely related factor seems to be involved in IL-1-mediated induction of the immunoglobulin κ gene (33), IL-6 gene (32), angiotensinogen gene (26), and human immuno-deficiency virus enhancer (19). Moreover, transcriptional activation of a wide variety of genes by phorbol 12-myristate 13-acetate, lipopolysaccharide, cyclic AMP, TNFα, and double-stranded RNA have been reported to proceed via a κB-like binding motif (13). There are at least three different proteins, NFκB, H2TF1, and PRDII-BF1, to recognize the κB-like binding motif (3, 10, 30). Although PRDII-BF1 has been cloned recently (10), the other two factors are still to be cloned. Therefore we cannot decide which of these three factors, if any, is responsible for IL-1-induced expression of the Bf gene. However, the rapid increase of the binding activity upon IL-1 induction suggests that posttranslational modifications are involved in the activation process. This fits with the activation mechanism of NFκB, which is present in cytoplasm as an inactive complex with an inhibitor, IκB (2), and is released from an inhibitor and transferred into nuclei (31) when IκB is inactivated by phosphorylation (7). Therefore, we favor the idea that the data presented here provide additional evidence for the ubiquitous role of NFκB in transducing extracellular signals (13).

The liver is the major site of synthesis of plasma Bf protein (1) as well as other major acute-phase proteins, such as C-reactive protein, serum amyloid A, and α-1-antitrypsin. Therefore, it is important to understand the molecular events in the liver during acute-phase response. Here we have demonstrated that an NFκB-like factor in hepatoma nuclei is induced by IL-1 treatment. Moreover, mouse liver contains a protein that forms a DNA-protein complex with BfκB indistinguishable in size from that formed by the HepG2 nuclear factor (data not shown). Therefore we suggest that NFκB or a closely related factor plays a role in mediating the IL-1 signal in hepatocytes during the acute-phase reaction. Recently, Ron et al. (26) reported a similar result using another acute-phase protein, angiotensinogen. However, κB-like sites of the Bf and angiotensinogen genes detect different sets of binding proteins in a HepG2 nuclear extract in spite of the close similarity of their nucleotide sequences. While the BfκB site detects only IL-1-inducible bands, the angiotensinogen κB site detects both inducible and constitutive bands. Although the functional role of the constitutive factor is not clear, the inducible NFκB-like factor is suggested to mediate acute-phase expression of both the Bf and angiotensinogen genes. The NFκB-like factor has also been shown to mediate the phorbol ester signal in another hepatic acute-phase protein, the human serum amyloid A gene (6). These results suggest that NFκB, or a closely related factor, plays a general role in the transcriptional activation of many acute-phase proteins.

Although the major site for plasma Bf synthesis is the liver, extrahepatic expression of the Bf gene and its induction by IL-1 has been reported previously (9, 16, 27). Moreover, the Bf gene or Bf-CAT chimeric constructs transfected into mouse L cells respond to IL-1 induction (15). The wide distribution of NFκB or closely related factor among mammalian cells, including mouse L cells, suggests the possibility that an NFκB-like factor mediates the IL-1 signal during acute-phase reaction also in extrahepatic sites. Supporting this idea, BfκB and C4κB sequences act as IL-1-responsive elements in mouse L cells (M. Nonaka et al., unpublished data). Recently, IL-6, another cytokine that mediates acute-phase reactions, has been shown to induce human or rat hepatoma nuclear factors that bind to the IL-6-responsive element in the human haptoglobin (18), hemopexin (23), or rat α2-macroglobulin (11) genes. Therefore, it appears that IL-1 induces acute-phase protein synthesis in the liver through two different pathways, one direct and the other via IL-6, since IL-1 enhances IL-6 gene expression (34). Interestingly, at least two different regions of the IL-6 promoter have been reported as IL-1-responsive elements and one region shows close similarity to the κB sequence (12, 14, 32).

It is interesting to note that a single copy of the BfκB sequence does not confer IL-1 responsiveness to the d92 construct (Fig. 6), since a single copy of the κB-like sequence has been shown to be effective as an inducible enhancer by using various promoters such as the c-fos (22) or IL-6 gene (32). One possible explanation for this apparent discrepancy is that the c-fos and IL-6 promoters used have an additional element which coordinates with the κB and
such an element is missing from the d92 construct. The idea that the coordination between trans-acting factors is required for induction is further supported by the observation that multiple copies of BfKB sequences conferred IL-1 responsiveness to the d92 construct (Fig. 6). In this context, it is interesting to compare the promoters of two class III complement genes, Bf and C4, since both genes have a single copy of kB-like sequence (35), and only the Bf gene is responsive to IL-1 or TNF. C4 synthesis by human hepatoma cells is not affected by IL-1 or TNF (20, and the mouse C4 promoter-CAT construct containing a kB-like sequence does not respond to IL-1 induction (data not shown). The C4kB and BfKB sequences, however, inhibited each other with indistinguishable affinity upon competition binding assay, using a HepG2 nuclear extract. The mobilities of complexes formed by the BfKB and C4kB oligonucleotides are very similar (data not shown), suggesting that the same nuclear factor in HepG2 recognizes both the BfKB and C4kB sequences. Moreover, both the BfKB and C4kB sequences conferred IL-1 responsiveness upon the Bf and simian virus 40 early promoters when they were introduced in multiple copies (Fig. 6). All these results strongly suggest that the kB-like sequence can act as an IL-1-responsive element only in the certain context of the surrounding sequence. Therefore, it is probable that the Bf promoter has an additional element between −478 to −92 which coordinates with the BfKB for IL-1 induction and that the C4 promoter lacks it, although this is still to be demonstrated directly.

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LITERATURE CITED


