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The Lipopolysaccharide-Binding Protein Is a Secretory Class 1 Acute-Phase Protein Whose Gene Is Transcriptionally Activated by APRF/STAT-3 and Other Cytokine-Inducible Nuclear Proteins

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Acute-phase reactants (APRs) are proteins synthesized in the liver following induction by interleukin-1 (IL-1), IL-6, and glucocorticoids, involving transcriptional gene activation. Lipopolysaccharide-binding protein (LBP) is a recently identified hepatic secretory protein potentially involved in the pathogenesis of sepsis, capable of binding the bacterial cell wall product endotoxin and directing it to its cellular receptor, CD14. In order to examine the transcriptional induction mechanisms by which the LBP gene is activated, we have investigated the regulation of expression of its mRNA in vitro and in vivo as well as the organization of 5′ upstream regulatory DNA sequences. We show that induction of LBP expression is transcriptionally regulated and is dependent on stimulation with IL-1β, IL-6, and dexamethasone. By definition, LBP thus has to be viewed as a class 1 acute-phase protein and represents the first APR identified which is capable of detecting pathogenic bacteria. Furthermore, cloning of the LBP promoter revealed the presence of regulatory elements, including the common APR promoter motif APRE/STAT-3 (acute-phase response element/signal transducer and activator of transcription 3). Luciferase reporter gene assays utilizing LBP promoter truncation and point mutation variants indicated that transcriptional activation of the LBP gene required a functional APRE/STAT-3 binding site downstream of the transcription start site, as well as an AP-1 and a C/EBP (CCAAT enhancer-binding protein) binding site. Gel retardation and supershift assays confirmed that upon cytokine stimulation APRF/STAT-3 binds to its recognition site, leading to strong activation of the LBP gene. Unraveling of the mechanism of transcriptional activation of the LBP gene, involving three known transcription factors, may contribute to our understanding of the acute-phase response and the pathophysiology of sepsis and septic shock.

The acute-phase response results from a complex series of reactions aimed at reconstituting the homeostatic state of the organism following injury, trauma, or infection (6). Upon disturbance of homeostasis, the local release of mediators, secreted predominantly by tissue macrophages, appears to be the first step that leads to a systemic reaction of the body (28). Macrophage-derived cytokines activate the vascular endothelium and tissue-resident stroma cells to release chemotactic factors that spur the accumulation of inflammatory cells, which then act as additional sources of proinflammatory cytokines (35). Besides the brain, in which the temperature setpoint in the hypothalamus is adjusted, and the adrenal-pituitary axis, which is activated for production of corticosteroids, the liver is the central organ regulating the acute-phase response by releasing specific acute-phase reactants (APRs) (17). Activation of hepatocytes for release of APRs has been recently examined at the levels of ligand-receptor interaction and signal transduction (55). Interleukin-1 (IL-1) and IL-6 were shown to be the major inducers of APRs in hepatocytes by acting either alone or in synergy with dexamethasone or tumor necrosis factor alpha (TNF-α) (21, 42).

So far, two types of acute-phase proteins have been identified and classified according to their predominant inducers: class 1 APRs, induced by IL-1 in synergy with IL-6, include C-reactive protein (CRP), serum amyloids A and P (SAA and SAP), α_1-acid glycoprotein, haptoglobin, and hemopexin; and class 2 APRs, induced by IL-6 only, include the three chains of fibrinogen, α2-macroglobulin, and various antiproteases. Similarly, expression of APRs can be induced by stimulating hepatocytes with leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and IL-11 (22, 54), which share with the IL-6 receptor a gp130 subunit and thus exhibit a spectrum of biological activities similar to that of IL-6 (31, 52).

Over the past few years, it has been shown that expression of APRs during induction of the acute phase is transcriptionally regulated (1, 47). This notion was substantiated by the finding that the promoters of acute-phase protein genes contained common regulatory elements capable of activating transcription of APR genes upon binding of the respective transcription factors. A regulatory element that was found to be crucial for almost all acute-phase protein genes is the APRE/STAT-3 (acute-phase response element/signal transducer and activator of transcription 3) binding site (36, 63). The structure of the transcription factor recognizing this site, termed acute-phase response factor (APRF) or STAT-3, was recently identified as a member of the family of signal transducers and activators of transcription (STAT family) (4, 65). Like other members of this protein family, APRF/STAT-3 was shown to be induced

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via activation of Janus kinases (13, 38, 51). Another class of regulatory elements found in all APR promoters is the CCAAT enhancer-binding protein (C/EBP) family of transcription factors. This group of transcription factors, which includes NF-IL6 (LAP, IL-6DBP, AGP/EBP, C/EBPβ, or CRP-2) and C/EBPα, -γ, and -δ, can confer activation by IL-1 and IL-6 via heterodimerization through the b-ZIP region and subsequent binding to a consensus sequence in acute-phase promoters (2, 30, 32). Also, glucocorticoid-responsive elements (GRE) were found to be involved in the regulation of APR genes (7, 39).

The functions of APRs such as α1-antitrypsin, fibrinogen, or haptoglobin have been identified and include tissue repair, modulation of coagulation, and metal binding. However, the role of other major APRs, i.e., CRP, SAP, and SAA, is still unclear (53). Bacterial challenge of the organism is a major cause for induction of an acute-phase response. It is therefore believed that APRs act to fight infection. A possible mechanism whereby APRs may counteract bacteremia should involve their direct interaction with pathogenic bacteria. So far, only CRP has been shown to bind bacteria; however, this binding may be nonspecific because of the ability of this APR to bind many other substances. The reason for release of APRs in the context of bacteremia has thus remained enigmatic.

The structure and function of a serum protein synthesized in the liver that is involved in recognition, binding, and transport of the bacterial cell wall compound lipopolysaccharide (LPS), or endotoxin, termed LPS-binding protein (LBP), have recently been described (50, 56, 59). This protein binds gram-negative bacteria via the lipid A part of LPS, which has been viewed as the causative principle for the development of the gram-negative sepsis syndrome in humans (23, 44, 46). LBP delivers endotoxin to its cellular receptor, the CD14 molecule (26, 43, 48, 64), and enhances LPS-mediated cytokine induction. Therefore, LBP is a protein that appears to be directly involved in the recognition of pathogenic bacteria and may also be involved in the pathogenesis of sepsis, as it appears to enhance the systemic release of cytokines and activate the cascade of events leading to septic shock.

In order to examine whether LBP meets the criteria of an APR, we have examined its transcriptional induction pattern, promoter organization, and regulation of expression of its gene. From these experiments, we deduce that LBP is an acute-phase protein of the class 1 type specifically involved in the recognition of products of pathogenic bacteria. Furthermore, by promoter studies and reporter gene and gel shift assays, we delineate the mechanism of transcriptional activation of LBP and the involvement of certain transcription factors and regulatory elements.

MATERIALS AND METHODS

Culture of HUH-7 cells and RNA extraction. HUH-7 hepatoma cells (kindly provided by J. Raynes, School of Hygiene and Tropical Medicine, London, United Kingdom) were kept in Dulbecco's modified Eagle's medium supplemented with L-glutamine, antibiotics, and 5% fetal calf serum. Cells used for experiments were kept in six-well plates and grown to 40 to 70% confluency. The wells were stimulated with various cytokines for 24 h before cell-free supernatants were collected. The cells were then washed with phosphate-buffered saline (PBS), and RNA was extracted and processed as follows. A 150-μl volume of GITC buffer containing 4 M guanidinium isothiocyanate, 0.5% *N*-laurosylsarcosine, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol (pH 7.0), and 15 μl of 3 M sodium acetate (pH 4.0) was added, and the lysed cell material was scraped off the plate and collected in microtubes. At this time, samples were frozen or were directly prepared by an acid-phenol extraction followed by an isopropanol precipitation. The RNA was washed twice with 80% ethanol, dried, and dissolved in 20 μl of sterile water. The optical densities at 260 and 280 nm were measured, and the integrity of the RNA was assessed on an agarose gel. Northern (RNA) blot experiments were then performed as described below.

Animals, detection of LBP levels in serum, RNA preparation, and Northern blot analysis. New Zealand White rabbits were subcutaneously injected with 1 ml of a 0.5% $AgNO_3$ solution. Serum was collected at various times, and animals were sacrificed. LBP levels in serum were determined as described in detail elsewhere (56). Briefly, serum was placed on LPS-coated 96-well plates. Bound LBP was detected by use of a goat-anti-rabbit LBP serum followed by incubation with a secondary enzyme-coupled antibody and a colorimetric detection procedure. Tissues of various organs were processed immediately and snap-frozen in liquid nitrogen. RNA was prepared by homogenizing frozen tissues in a tissue homogenizer in the presence of guanidinium isothiocyanate as described below. Twenty micrograms of the resulting RNA was run on an agarose gel as described elsewhere (37) and then transferred to nylon membranes by capillary blotting overnight in 10× SSC (1.5 M NaCl, 150 mM sodium citrate; pH 7.0). RNA was fixed by being subjected to UV cross-linking (120 s) and baked for 2 h at 80°C. Prehybridization was performed at 65°C for 1 h with a buffer containing 2× SSC, 2% Denhardt's solution, 2.5% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 0.1% $Na_2H_2P_2O_7$, 2 mM EDTA, and 30 μg of salmon sperm DNA per ml. The rabbit LBP probe was an ~500-bp cDNA fragment cut with *Eco*RI and *Eco*RV. Radioactive labeling of the probe was performed by random priming: 5 μl of ^{32}P-labeled dCTP (Amersham; 3,000 μCi/mmol, 10 μCi/μl) was incubated with 50 ng of the cDNA and 10 μl of reagent mix, including hexameric primers and 1 μl of Klenow enzyme. The probe was isolated with a Sephadex G-50 column. The resulting probes had an activity of approximately 2×10^6 cpm/ml. Hybridization was performed overnight at 65°C in 50 ml of the buffer described above. After one wash each with 2× SSC–0.1% SDS–0.05% $Na_2H_2P_2O_7$–1 mM EDTA, 1× SSC–0.1% SDS, and 0.4× SSC–0.1% SDS, the filters were exposed to X-ray films for 1 or 2 days at −80°C with intensifying screens.

Nonradioactive nuclear run-on transcription assay. Approximately 10^8 HUH-7 cells were stimulated at the times indicated below, washed repeatedly in cold PBS, and lysed in lysis buffer containing 10 mM Tris HCl (pH 7.4), 10 nM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5% Nonidet P-40. After centrifugation at 500 × *g* and repeated washings, the nuclei were resuspended in glycerol buffer containing 50 mM Tris HCl (pH 8.3), 40% glycerol, and 5 mM $MgCl_2$ and stored at −20°C. For the run-on reaction, nuclei were mixed with a half-volume of reaction buffer (10 mM Tris HCl [pH 8.0], 5 mM $MgCl_2$, 0.3 M KCl, 5 mM DTT, 40 U of RNasin per ml, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 1% bovine serum albumin [BSA], 0.3 mM $MgCl_2$, 0.5 mM ATP, CTP, and GTP, and 0.2 mM digoxigenin [DIG]-UTP), and the mixture was incubated for 30 min at 26°C. The run-on reaction was terminated by addition of DNase I buffer, containing RNasin and tRNA, and an incubation for 15 min at 28°C was carried out. Next, proteinase K was added in the presence of 1% SDS, and samples were incubated at 37°C for 30 min and then subjected to phenol-chloroform extraction and two ethanol precipitations. The purified RNA was then hybridized to filters containing 10 μg of slot blotted plasmid DNA containing the LBP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inserts or no insert as a negative control. The filters were prehybridized for 1 h at 50°C and separately hybridized with each run-on isolated overnight at 50°C in a hybridization buffer containing 50% formamide, 5× SSC, 2% blocking reagent (Boehringer, Mannheim, Germany), 0.1% *N*-lauroylsarcosine, and 0.02% SDS. The filters were then repeatedly washed for 5 min at room temperature in 2× SSC–0.1% SDS and then repeatedly washed at 68°C in 0.1× SSC–0.1% SDS. DIG-UTP was detected by an alkaline phosphatase immunoassay and chemiluminescence as described in the manufacturer's instructions.

Subcloning and sequencing of the LBP promoter. A human foreskin fibroblast P1 genomic library (Du Pont Merck Pharmaceutical Company) (DMPC-HFF no. 1) was screened by a PCR-based method with two primers designed according to the 5′ 118 bp of the LBP cDNA. The sequences of the primers were ATG GGG GCC TTG GCC AGA GC and TGC AGT CCC TTG TCG GTG ATC. The resulting positive clone has accession no. 2672 864B1. This clone, approximately 85 kb in size, was purified and cut with several restriction enzymes for Southern blot analysis using a radiolabeled 5′ LBP cDNA probe. A *Bam*HI fragment giving rise to a signal in the Southern blot analysis was subcloned into a Bluescript-derived vector and sequenced by cycle sequencing (Sanger method). The primer used was designed according to the 5′-terminal region of the cDNA sequence of LBP in the 3′-to-5′ direction, with the sequence GCA GCA ATG CCA GCA GTA TG (P1). The subsequent sequences were obtained by using primers according to the new sequences obtained (primer walking) and compared with transcription factor databases.

Identification of the transcription start site. RNA from stimulated HUH-7 cells was prepared by the method described above, and 5 μg of RNA was annealed with 20 ng of two different 6-FAM-labeled oligonucleotides (Perkin-Elmer/Applied Biosystems, Weiterstadt, Germany). These primers were designed to be complementary to two regions of the 5′ region of the LBP cDNA, 46 bp downstream (P1, sequence given above) and 14 bp upstream of the ATG site, with the sequence TGC AGT GGG CCA GGA CTG TC. The RNA was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (10 U) and then subjected to a phenol-chloroform extraction and an overnight ethanol precipitation. The cDNA product was run on a denaturing 6% polyacrylamide–6 M urea sequencing gel, with a Genescan-500 ROX 35- to 500-bp standard in the same lane (Applied Biosystems). The reactions gave rise to two sharp and clearly distinguishable single bands that both corresponded to a site 110 bp upstream of the ATG site as determined by computer analysis.

Luciferase reporter gene assays. Truncated promoter fragments were constructed by PCR amplifications using the forward primers indicated in Fig. 3 and a reverse primer complementary to the sequence directly upstream of the ATG site (CCT AGA TTC CCA GTG CAG TG). In addition, a *Bam*HI site and an *Xho*I restriction site were introduced. These sites were used to subclone the fragments into a luciferase vector, kindly provided by Heike Pahl, University of Freiburg, Freiburg, Germany. For detailed information, see reference 40. The STAT-3, AP-1, and C/EBP mutations were introduced by PCR amplification using forward primers introducing the mutations and the reverse primer mentioned above. All resulting clones were confirmed by sequencing. Each well of a 12-well plate was seeded with 1.5×10^5 HUH-7 cells, and the cells were grown overnight. Cotransfection of the cells with the luciferase vectors containing the LBP promoter fragments and the cytomegalovirus (CMV) plasmids with β-galactosidase plasmids was performed after repeated washings of the cells with Lipofectamine reagent (Gibco Life Technologies, Eggenstein, Germany) according to the manufacturer's instructions. Briefly, 0.8 μg of DNA diluted in 40 μl of serum- and antibiotic-free medium was gently mixed with 8 μl of Lipofectamine reagent, diluted in 40 μl of medium, and incubated for 30 min at room temperature. A 320-μl volume of medium was added to the mixture, which was then incubated with the cells for each transfection after repeated washings. The cells were incubated for 6 h before the medium was removed and a serum-containing medium was added. After an additional 24 h, the cells were stimulated for the periods indicated below, washed, and lysed with 140 μl of a lysis buffer containing 100 mM K_2PO_4 (pH 7.8), 0.2% Triton X-100, and 1 mM DTT. After incubation for 10 min at room temperature, lysed cells were rinsed off and transferred to a microtube, spun down briefly, and incubated with luciferase reaction buffer. A 50-μl volume of lysate was mixed with 180 μl of this buffer, containing 25 mM K_2PO_4 (pH 7.8), 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (pH 8.0), 15 mM $MgSO_4$, 1 mM DTT, and 1 mM ATP. Measurement was done for 10 s in a luminometer (Berthold, Bad Wildbach, Germany) after injection of 50 μl of 10 μM luciferin (Sigma, Deisenhofen, Germany). Simultaneous transfections of separate cell cultures were performed with the luciferase vector containing a CMV promoter or an empty plasmid, as indicated. Transfection efficacy was normalized by comparison with values obtained by measurement of β-galactosidase activity (Galacto-light; Tropix Inc., Bradford, Mass.) after cotransfection with a plasmid containing the β-galactosidase gene under control of a CMV promoter. Inducibility of the cells transfected with the promoter constructs was evaluated by comparison of the luciferase activity of cytokine-stimulated cells with that of nonstimulated cells transfected with the same construct within one experiment. This resulted in the determination of fold induction. At least three independent experiments carried out in duplicate were performed.

Electrophoretic mobility shift assays (EMSA) and supershift assays. Nuclear proteins for the STAT-3 gel shift experiments were prepared 15 min after stimulation with 500 U of IL-6 per ml according to a published protocol (62). Extracts for AP-1 and C/EBP gel shifts were prepared as follows. Forty to 70% confluent HUH-7 cells (approximately 10^9 cells) were stimulated with cytokines as described above for 24 h, after the cells were washed once with PBS and scraped off the plates. After an additional washing, the pellet was carefully resuspended in 6 ml of a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.3 mM sucrose, 0.5 mM PMSF, and 0.1 mM EGTA. After 10 to 20 min of incubation on ice, 2 ml of the buffer described above was added to the suspension, which was then mixed and transferred to a prechilled homogenizer. After 50 to 100 strokes, the suspension was transferred to microtubes and centrifuged for 2 min at 14,000 rpm in a Microfuge at 4°C. The supernatant containing the cytosolic proteins was removed, and the pellet was resuspended in 3 ml of a buffer containing 20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 0.1 mM EGTA. This solution was gently rocked for 30 min at 4°C and then centrifuged in a Microfuge at 14,000 rpm for 30 min at 4°C. The supernatant was dialyzed for a minimum of 6 h with at least one change of buffer against a buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Finally, the solution was centrifuged for 20 min at 14,000 rpm in a Microfuge at 4°C, and the supernatant was snap-frozen in aliquots of 20 to 50 μl and stored at −70°C.

Gel retardation assays (or EMSA) were performed as described previously (62). Briefly, after 10 min of preincubation and addition of competitor DNA, if required, 5,000 cpm (10 fmol) of a ^{32}P-labeled, double-stranded synthesized DNA oligonucleotide probe (see Fig. 8) was added to 2 to 5 μg of the nuclear protein sample, and the mixture was incubated for another 10 to 30 min at room temperature. As a control, labeled oligonucleotides were tested for intrinsic gel shift activity by incubation without nuclear proteins. The oligonucleotides used for gel shift assays, if not stated differently, were synthesized on a gene assembler (Pharmacia, Freiburg, Germany), annealed, and gel purified. The sequences of the upper strands of the oligonucleotides used were as follows: AP-1 (position −532 of the LBP promoter), T TTA CTG GCA CAC TGA CTC AAT TAT GTA TT; C/EBP (position −446), TGC CAA TTG CCT TCC AGA AAA TTT CAC CA; and STAT-3 (position +98), GGC CCA CTG CAC TGG GAA TCT AGG ATG GG. The competitor oligonucleotide for STAT-3 was the binding motif from the rat α2-macroglobulin gene (kindly provided by U. Wegenka, Max Delbrück Center for Molecular Medicine, Berlin, Germany) with the sequence GAT CCT TCT GGG AAT TCC TA. The double-stranded oligonucleotide was also labeled and used for additional control experiments (see Fig. 8B), demonstrating specificity of the STAT-3 shift. The competitor oligonucleotide for AP-1 (obtained from Stratagene, Inc., Heidelberg, Germany) had the sequence CTA GTG ATG AGT CAG CCG GAT C. For C/EBP, the oligonucleotide described above was used for competition. The oligonucleotide used for nonspecific competition had the sequence GAT CGA ATG CAA ATC ACT AGC T.

The samples were mixed with a sample buffer containing 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 5 mM DTT, 0.7 mM PMSF, 1 mg of BSA per ml, and 1 mg of poly(dI-dC) per ml and run on a nondenaturing 3% polyacrylamide gel in TEAE buffer (40 mM Tris-HCl, 1 mM EDTA, 5 mM sodium acetate, and 32 mM formic acid) for 1.5 h at 200 V, transferred to Whatman paper, dried, and subjected to autoradiography for 1 to 24 h. For the STAT-3 supershift analysis, the supershift antibody TransCruz Gel supershift reagent sc-482X, obtained from Santa Cruz, Inc., Santa Cruz, Calif., was used. The antibody was added to the nuclear proteins after addition of the labeled oligonucleotides. The solution was incubated for 1 h at 4°C before gel loading and electrophoresis.

Chemicals and culture reagents. Sodium pyruvate and fetal calf serum were purchased from Seromed, Biochrom, Berlin, Germany. L-Glutamine, penicillin, streptomycin, amphotericin, and Lipofectamine were from Gibco Life Technologies. Guanidinium isothiocyanate and formamide were obtained from Fluka, Neu-Ulm, Germany. Phenol and *Taq* polymerase were from Appligene, Heidelberg, Germany. DIG-UTP, the DIG detection kit, and poly(dI-dC) were from Boehringer. Chloroform, isoamyl alcohol, isopropanol, sodium acetate, glycerine, EDTA, and formaldehyde were purchased from Merck, Darmstadt, Germany. [^{32}P]dCTP and Hybond-N membranes were purchased from Amersham-Buchler, Braunschweig, Germany. Twelve-well plates were obtained from Nunc, Roskilde, Denmark. MMLV reverse transcriptase and restriction enzymes were from New England Biolabs. 6-FAM-labeled primer and 500-bp Genescan-500 ROX-labeled size marker were purchased from ABI. All other chemicals, including Dulbecco's modified Eagle's medium, were from Sigma.

Nucleotide sequence accession number. The nucleotide sequence of the human LBP gene has been submitted to the EMBL database under accession no. X84745.

RESULTS

Induction of LBP protein and transcript synthesis in vivo and in vitro. For experimental in vivo acute-phase induction, New Zealand White rabbits were injected with 1 ml of 0.5% $AgNO_3$. Rabbit serum was collected at various times thereafter and assessed for LBP levels. The results (Fig. 1A) indicate that the concentrations of LBP rose approximately 100-fold upon $AgNO_3$ challenge, with the highest levels seen at 24 h. Within the next 2 days, LBP concentrations declined, remaining, however, above starting levels at the last time measured (72 h). Next, Northern blot experiments were performed with RNAs from different rabbit tissues obtained from animals sacrificed at different times after acute-phase induction. As shown in Fig. 1B, RNA prepared from rabbit liver gave a very weak constitutive LBP signal that could be significantly enhanced by $AgNO_3$ challenge, with a maximum after 24 h. LBP transcripts were not detected in other tissues, including thymus, lung, spleen, kidney, adrenal gland, gut, testis, and brain tissues, bone marrow, and blood leukocytes (not shown). The lower band (2.0 kb) seen in the Northern blot obtained from rabbit tissue was comparable in size to a band also seen for primary human liver cells (not shown) and hepatoma cell lines (see Fig. 3 and 4). This band corresponds exactly to the predicted size of the LBP transcript according to cDNA sequencing and determination of the transcription start site (see below). A high concentration of IL-6 (5,000 U/ml) in combination with IL-1β gave rise to a faint second band (3.5 kb) in hepatoma cell lines (Fig. 1C) that was also detectable in rabbit tissues (Fig. 1B). Exact identification of the nature of this 3.5-kb band requires further experimentation, which is under way.

To examine the spectrum of cytokines inducing LBP transcript synthesis in vitro, the hepatoma cell line HUH-7 was instrumental. A combination of proinflammatory cytokines known to induce APRs was used, and LBP transcripts were analyzed by Northern blotting. To examine whether LBP rep-

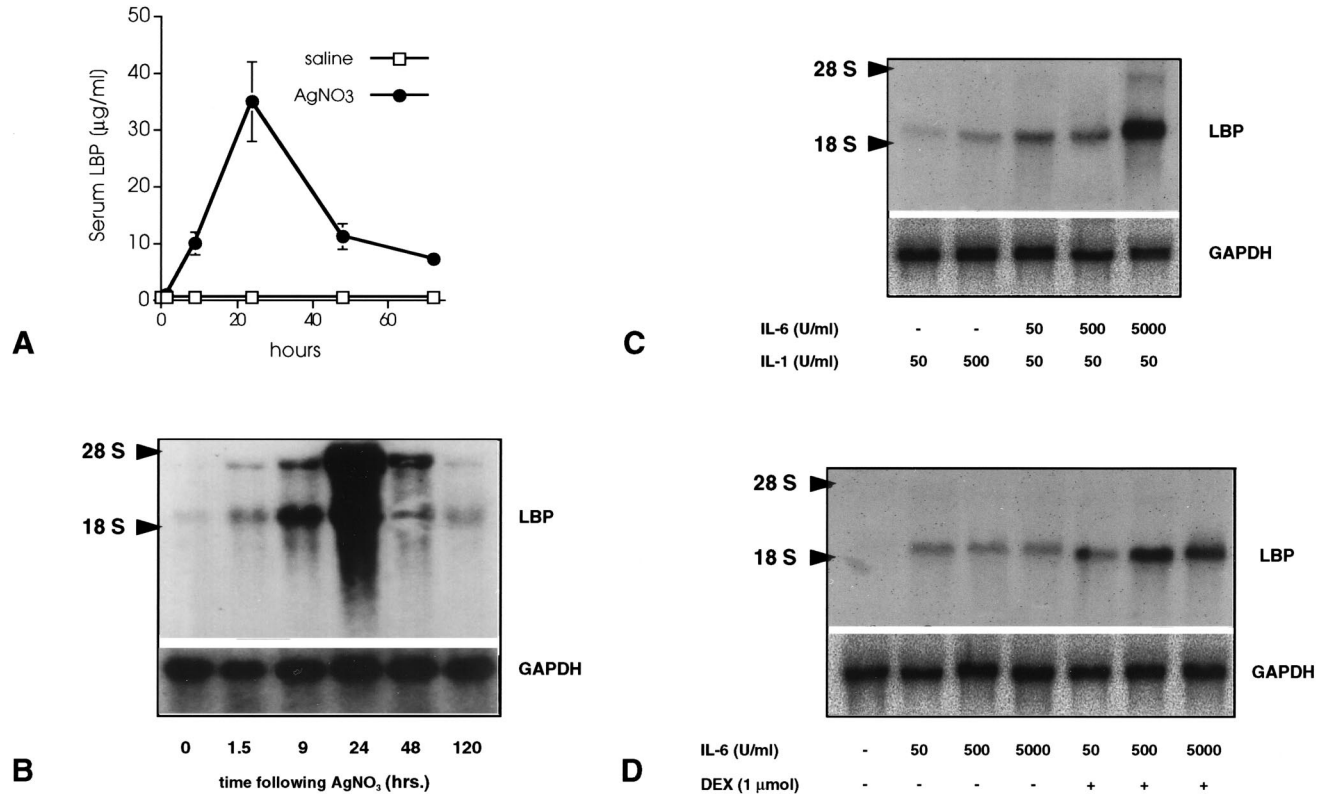

FIG. 1. LBP protein and transcript levels during the acute phase in vivo and in vitro. (A) LBP levels in serum were assessed in New Zealand White rabbits injected with 1 ml of a 0.5% $AgNO_3$ solution at the times indicated. LPS binding was assayed as described in Materials and Methods. Shown are mean values of four experiments ± standard deviations. (B) Animals were sacrificed at the times indicated below the gel, and liver tissues (and other tissues [results not shown here]) were prepared and immediately frozen in liquid nitrogen. RNA was isolated as described in Materials and Methods, and hybridization was performed with a 200-bp rabbit LBP probe. Shown is a time course of in vivo LBP RNA accumulation during the acute-phase response of rabbits. (C) HUH-7 cells were stimulated in vitro with cytokines as indicated for 24 h. RNA was prepared, and Northern blot experiments were carried out with a 1.4-kb human LBP probe. LBP mRNA accumulation in cells, stimulated in vitro by IL-1β either alone or combined with IL-6, is shown. Nonstimulated cells showed low, if any, levels of LBP transcripts (see also panel D). (D) HUH-7 cells were stimulated with IL-6 and dexamethasone (DEX) as described in the text, and hybridization was performed. The GAPDH cDNA probe controls for RNA integrity and comparable loading of RNA in single lanes.

resents a class 1 APR, HUH-7 cells were stimulated with increasing concentrations of recombinant IL-1β either alone or in combination with IL-6. Figure 1C shows that LBP transcripts could be induced by as little as 50 U of IL-1β per ml and that this effect was strongly enhanced by addition of IL-6 in a way typical for class 1 acute-phase proteins. IL-6 also induced LBP transcript accumulation in HUH-7 cells, and addition of dexamethasone at a concentration of 1 μM enhanced this effect (Fig. 1D). Enhancement of IL-6-mediated APR-induction by dexamethasone is characteristic for acute-phase proteins and is most likely due to upregulation of the IL-6 receptor. TNF-α and IL-1β were also acting synergistically with IL-6 in inducing LBP transcription. Reference acute-phase protein genes (encoding albumin, haptoglobin, and C3) were included in our analysis as well, confirming that the pattern of LBP induction was similar to that of a class 1-type APR (not shown). Overall, although IL-6 alone is able to induce LBP, the inducibility by IL-1 alone and the synergistic activity of IL-1β and IL-6 indicate that LBP behaves like a class 1 APR.

Nuclear run-on transcription assay. In order to examine whether the increase in LBP transcript levels was the result of an enhanced rate of gene transcription, a nonradioactive nuclear run-on technique with DIG-labeled nucleotides was employed. Nuclear extracts of HUH-7 cells stimulated with IL-6, IL-1β, and dexamethasone were prepared and incubated for 30 min in the presence of DIG-labeled UTP. RNA was then isolated and hybridized to DNA containing the LBP cDNA. Controls were performed with GAPDH cDNA and vector DNA lacking an insert. Detection of the hybrids was carried out with DIG-labeled antibodies and an enzymatic color reaction. The results, shown in Fig. 2, indicate that LBP transcription was induced approximately 3.7-fold at 24 h. The kinetics correspond to that seen in the Northern blot experiments. This transcriptional activation, however, appeared to be weaker than the induction seen in Northern blot experiments, so that accumulation of LBP transcripts apparently occurred as a result of both transcriptional and posttranscriptional mechanisms. Posttranscriptional and transcriptional expression control has also been shown for other acute-phase proteins of the liver (6).

Cloning of the LBP promoter and identification of the transcription start site. After it was shown that the increase in LBP transcripts occurred at least in part as a result of transcriptional gene activation, the 5′-flanking region of the LBP gene was isolated for promoter analysis. A fragment of 4.5 kb corresponding to the 5′ untranslated region of the LBP gene was isolated from an approximately 85-kDa genomic clone and subcloned, and ~1.1 kb was sequenced. Figure 3A shows the 1-kb 5′-flanking region of the LBP gene with a total of 11 (C)CAAT or reverse (C)CAAT boxes [ATTG(G)], the transcription start site, and the ATG site of the coding region. A canonical TATA box, however, was not found within the re-

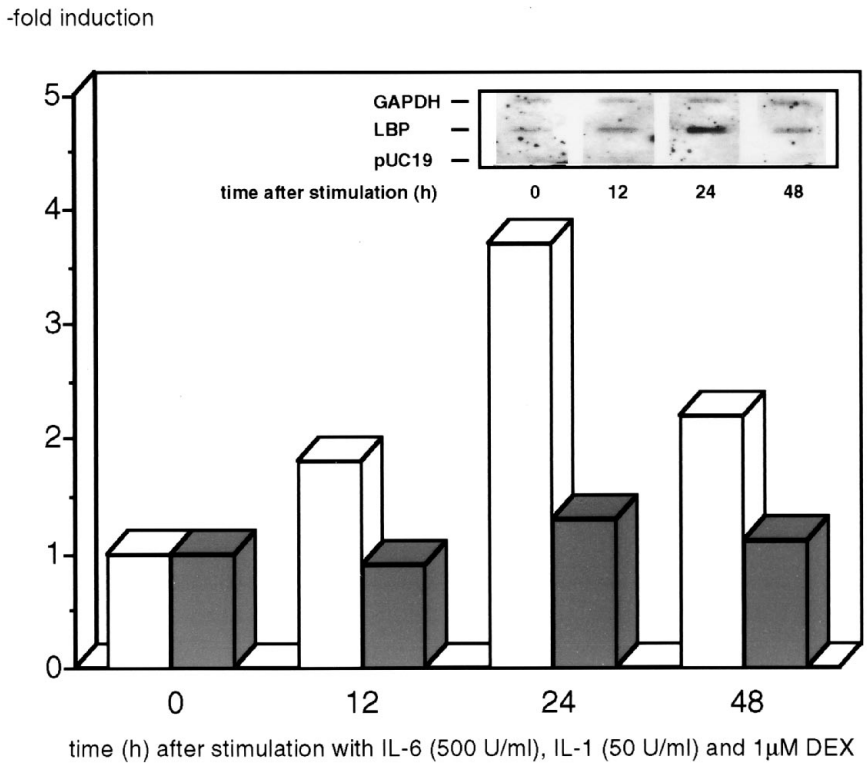

FIG. 2. Nuclear run-on transcription assay showing transcriptional control of LBP expression in HUH-7 cells. RNA obtained from HUH-7 cells, stimulated with IL-6, IL-1β, and dexamethasone (DEX) for 24 h, was in vitro transcribed in the presence of DIG-UTP and hybridized to slot blotted DNA containing the cDNA of LBP (white bars) or GAPDH (gray bars), respectively, in a pUC19 vector and to vector lacking the insert. Hybridization was carried out overnight, and a DIG-labeled-antibody-based colorimetric detection system was employed. Results obtained by laser densitometric scanning of the LBP and GAPDH bands are shown.

gion 30 bp upstream of the transcription start site. A number of putative transcription factor binding sites were identified, some of general nature and some typical for acute-phase promoters. Table 1 gives a survey of the putative transcription factor binding sites identified in the LBP promoter along with their consensus sequences. The organization of the 5′-flanking region of LBP with the transcription start site and the location of several key transcription factor binding sites are depicted in Fig. 3B. The presence of two APRE/STAT-3 sites, three C/EBP sites, and one GRE site is in line with the Northern blotting and nuclear run-on data.

The transcription start site was determined by the use of a primer extension technique employing reverse transcriptase and a 6-FAM-labeled primer. The resulting reaction products were compared with size markers run on the same polyacrylamide gel and analyzed by using automatic sequencing and computer software. This experiment, also confirmed with different primers, gave rise to a sharp peak indicating the presence of the transcription start site 155 bp upstream of the primer and 110 bp upstream of the ATG (Fig. 4). A second, smaller peak representing a larger transcript was also detectable, which could potentially represent the larger transcript seen in the Northern blot experiments. However, this is speculative, and proof will require additional experimentation, which is under way.

Luciferase reporter gene assays. (i) Inducibility and time course. To identify sites within the LBP 5′-flanking region conferring gene-regulatory activities, functional analysis of the LBP promoter was performed by a luciferase reporter gene assay. To this end, the approximately 1,100-bp LBP promoter was first cloned in front of the firefly luciferase gene and used to transfect HUH-7 hepatoma cells by lipofection. Cells were also transfected with an empty vector as a negative control (mock transfection) as well as with one containing the CMV promoter. Levels of luciferase activity were assessed after stimulation with different cytokine combinations. High concentrations of IL-6 and IL-1β (500 and 50 U/ml, respectively) and a combination of low concentrations of IL-6 and IL-1β (50 and 5 U/ml, respectively) with 100 U of TNF-α per ml (proven to be a strong stimulatory concentration in the Northern blot experiments) were used in the presence of 1 μM dexamethasone. As shown in Fig. 5A, 15- and 20-fold induction could be observed with the cytokine-stimulated LBP promoter. The promoter activity was equivalent to almost 2% of that of a CMV promoter, which represents an intermediate to strong promoter activity of the LBP gene. As expected, very little induction of the constitutively active CMV promoter and no effect on the LBP promoter were observed with the mock transfections.

In order to investigate the time course of inducibility of the LBP promoter, luciferase activity was examined at different times after cytokine induction of cells transfected with the LBP promoter-luciferase construct. As shown in Fig. 5B, a 5-fold induction of the LBP promoter was seen after 2 h, increasing to a maximum of almost 30-fold after 48 h with the higher IL-6 and IL-1β concentrations. Stimulation with the IL-6–IL-1β–TNF-α combination led to a maximum of 18-fold induction of luciferase at 48 h. After 5 days, activation of the LBP promoter was still detectable, with 12- and 3-fold inductions with the two different activation protocols. Loss of activity may also be due to reduced expression over time in the experimental system. The maximum inducibility after 24 to 48 h, as seen for LBP, is

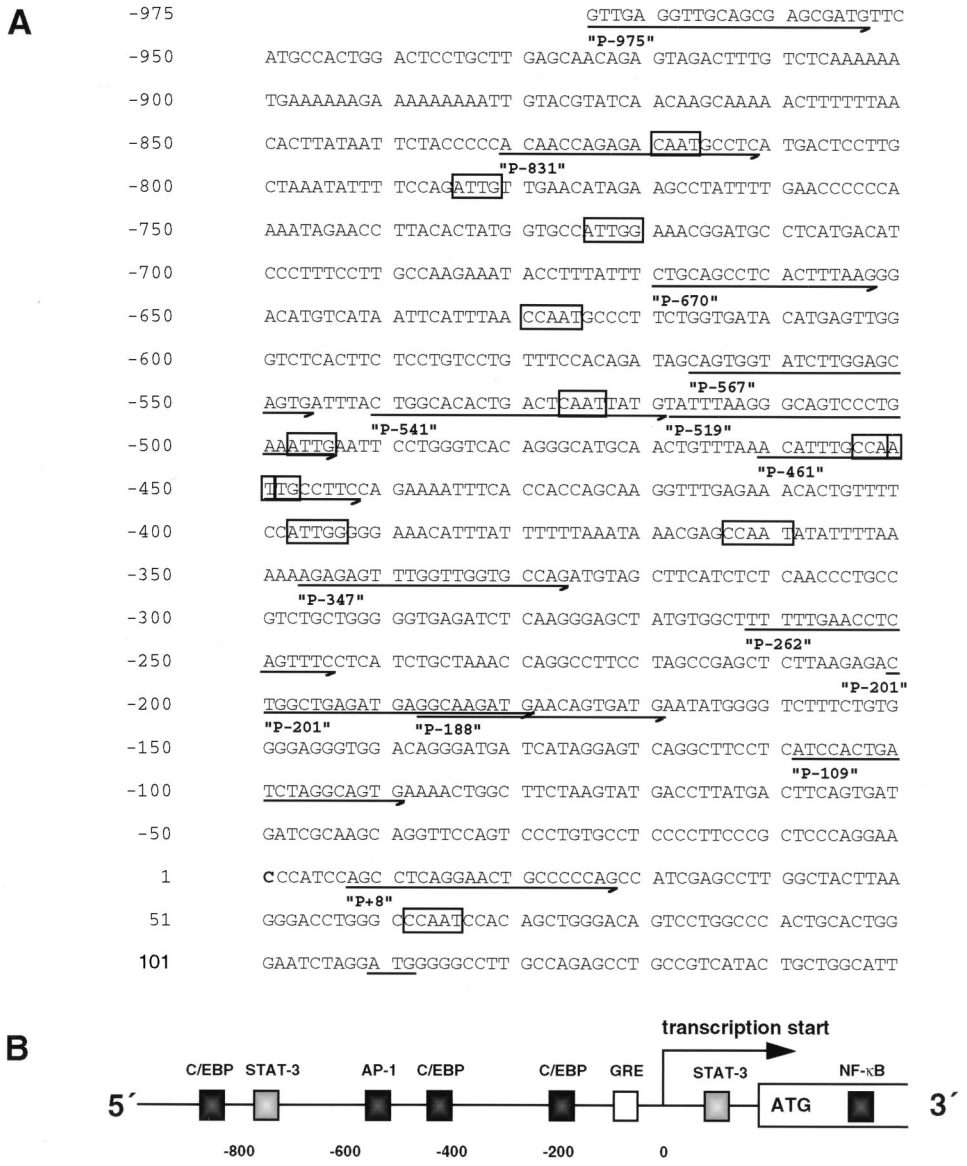

FIG. 3. Nucleotide sequence and organization of the human LBP promoter. (A) Complete nucleotide sequence of approximately 1 kb of the 5′-flanking region of the LBP gene. The transcription start site (boldface), the ATG site (underlined), the 12 (C)CAAT or reverse (C)CAAT [ATTG(G)] boxes (boxed), and the primer-annealing sites of the primers used for truncation design (underlined) are indicated. (B) A selection of prominent and acute-phase relevant putative regulatory elements within the LBP promoter shown in relation to the coding area. ■, IL-6- or IL-1-dependent regulatory elements; ▨, APRE/STAT-3; □, GRE.

well in line with the induction patterns reported for several other APRs (6).

(ii) Transcriptional activity of truncation mutants of the LBP promoter. To study the functional activity of certain regions of the LBP promoter, truncated versions of the 1.1-kb promoter in <100-bp steps were cloned into the pLuc vector in front of the gene encoding the firefly luciferase. HUH-7 cells were transiently transfected with these constructs and stimulated for 24 h with different cytokines, and induction of the luciferase gene was determined. To further confirm that LBP is a class 1 acute-phase protein, inducible by IL-1β alone, cells were stimulated with 50 U of IL-1β per ml and luciferase activity was measured, as shown in Fig. 6A. It can be seen that a three- to fivefold activation of these constructs could be achieved by stimulation with IL-1β alone. A truncated promoter lacking the transcription start site failed to be inducible.

Next, cells transfected with the truncation constructs were stimulated with 500 U of IL-6 per ml or with a combination of IL-6 and IL-1β (500 and 50 U/ml, respectively) to confirm the synergistic action of these cytokines, also typical for class 1 acute-phase promoters. As seen in Fig. 6B, IL-6 was able to strongly enhance activity of the luciferase gene, and this effect could be further increased by the addition of IL-1β. Even a 100-bp fragment (fragment 109) of the LBP promoter confers as much as almost 10-fold inducibility. The presence of the area from positions −567 to −461 of the LBP promoter was able to enhance additional IL-6-mediated activation of the luciferase gene from an 18-fold to a 35-fold induction. An area between positions 519 and 541, containing an AP-1 site, conferred the strongest increase in inducibility. This site was consequently studied by point mutation and gel retardation analyses. A truncated promoter containing the area from positions

TABLE 1. Putative regulatory elements found in the LBP promoter

Factor (synonym)	Consensus sequence	Location(s) (bp)	Inducer	Activity[a]
APRF (STAT-3)	CTGGRAA	−792, 97	IL-6	−, +
C/EBP	TKNNGYAAK	−863, −446, −191	IL-1 or IL-6	−, +, (+)
MGF (STAT-5)	ANTTCTTGGNA	−691	Prolactin	−
AP-1	TGANTMA	−532	IL-6 or IL-1	+
GCN4	GAGTCA	−810, −124	General (TPA)[b]	−, (+)
AP-3	TGTGGWWW	−580, 64	General	(+), −
AP-4	CAGCTGTGG	−40, 67	General	(+), −
GRE	AGAWCAGW	−105	Glucocorticoids	(+)

[a] + and (+), activity of the regulatory elements evaluated by point mutation experiments or by truncation mutation experiments only, respectively; −, no detectable activity.
[b] TPA, tetradecanoyl phorbol acetate.

−670 to −567 confers a 60-fold inducibility of the promoter, approximately three times as strong as that of the largest promoter fragment (fragment 975). Thus, the area from −670 to −975 apparently contains silencer sequences. To obtain formal proof for the importance of transcription factor binding sites within the promoter likely to be involved in transcriptional activation, we next introduced point mutations carrying changes in the recognition sites and investigated the inducibility of these mutated promoter variants in the luciferase reporter gene assay.

(iii) Promoter mutants carrying point mutations of regulatory elements. In order to examine the activity of the APRF/STAT-3 binding site, we inserted a 2-bp point mutation to change the core consensus sequence by the use of a PCR-based method. The APRF/STAT-3 site, located downstream of the transcription start site, CTGGGAA, was mutated into **G**TGG**AT**, resulting in a significant loss of activity (Fig. 7A). Inducibility of the LBP promoter fragment at bp −461, which in its wild-type form exhibited a 16-fold inducibility after cytokine stimulation, decreased to 6-fold (25%) after 12 h, and inducibility was completely lost at later times investigated. Similar results were obtained by using the fragment at bp −109 and a STAT-3 mutation (not shown). Although APRF was shown by others to bind very rapidly to the APRE/STAT-3 site, a similar loss of the 24-h inducibility by a STAT-3 point mutation has also been observed for other acute-phase proteins, with a maximum of inducibility at 24 or 48 h (6). This result indicates that the APRF site, although at an unusual location downstream of

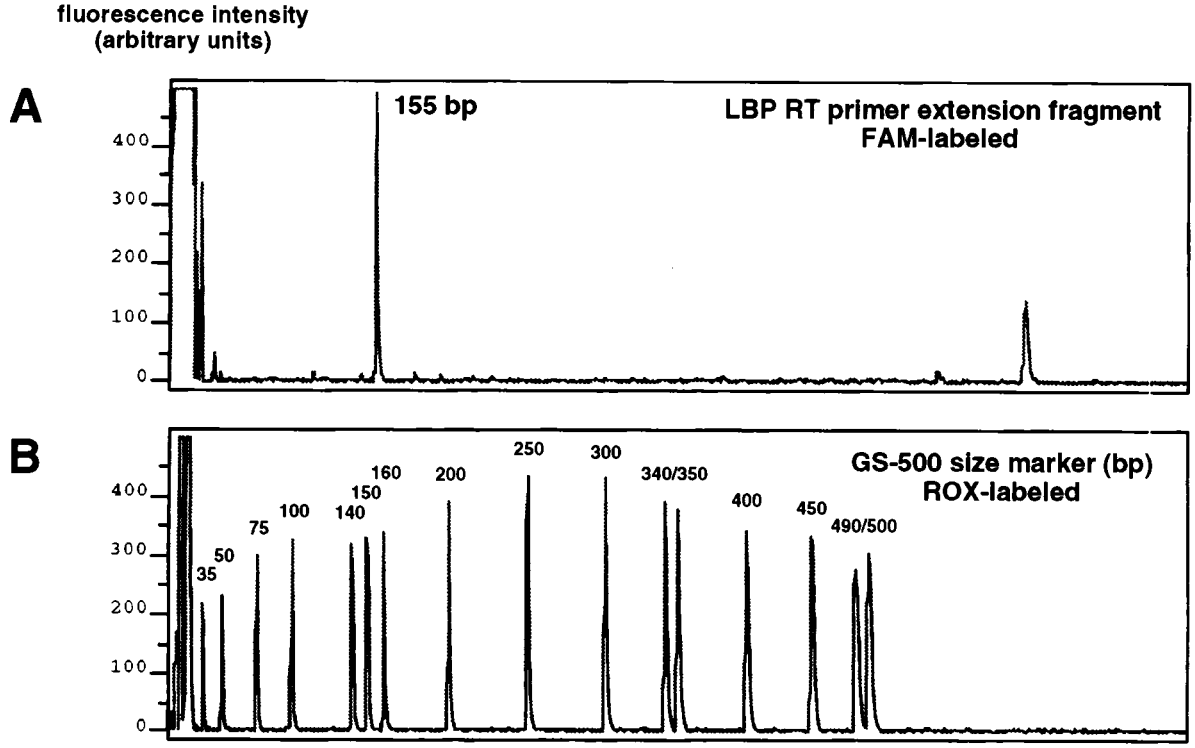

FIG. 4. Identification of the transcription start site by use of reverse transcriptase primer extension of a 6-FAM-labeled primer and an ABI sequencer. A 5-μg sample of stimulated HUH-7 RNA was annealed with 200 ng of two different 6-FAM-labeled oligonucleotides, designed to be complementary to two 5′ regions of the LBP gene. RNA was reverse transcribed with MMLV reverse transcriptase (RT), purified, and run on a sequencing gel in parallel to a ROX-labeled size standard. (A) The resulting single band was analyzed by ABI sequencing software. Shown is one representative result of a total of six experiments, using two different primers. (B) The Genescan-500 (GS-500) ROX 35- to 500-bp size standards show that the single band in panel A migrates at between 150 and 160 bp. Computer analysis revealed a size of the reverse transcribed fragment of 155 bp, leading to a transcription start site 110 bp upstream of the ATG site.

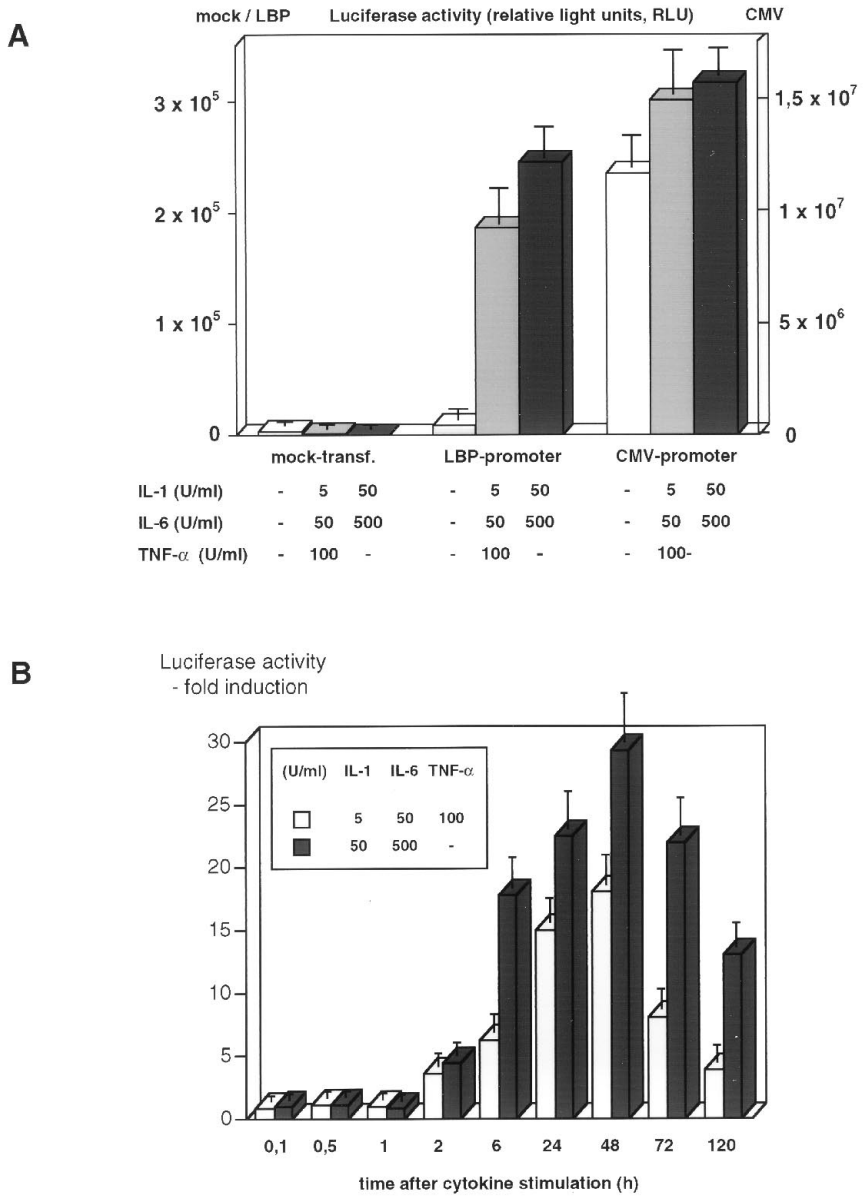

FIG. 5. Functional characterization of the LBP promoter. (A) Results of reporter gene assays showing inducibility of the LBP promoter by IL-1β, IL-6, dexamethasone, and TNF-α. A total of 1,100 bp of the LBP promoter were cloned in front of the firefly luciferase gene, and HUH-7 cells were transfected with this construct by lipofection. The cells were then stimulated with the indicated two combinations of cytokines and were lysed after 24 h. Luciferase activity was examined with a luminometer; relative light units are shown for stimulated and nonstimulated cells. As controls, results for mock-transfected cells and a transfection with the CMV promoter are shown. (B) Luciferase activity was measured at different times after stimulation. Shown is the fold induction of induced cells compared with that of noninduced cells (mean values ± standard deviations of a total of six independent experiments).

the transcription start site, seems to be essentially involved in the transcriptional induction of LBP and that complete integrity of this common acute-phase regulatory element is required for transcriptional activation of the LBP gene to occur.

Two other potential transcription factor binding sites, AP-1 and C/EBP, were also mutated by us, and the resulting promoter constructs were assessed for their inducibility by cytokines. The results of the luciferase reporter gene assay are shown in Fig. 7B. In this case, a more severe mutation was introduced, changing the entire binding region. The transfected cells were induced with either IL-1β or IL-6 alone. This strategy was chosen in order to discriminate between IL-1β and IL-6 effects, because the C/EBP site is known to be utilized by a transcription factor family, induced mainly by IL-6. A significant reduction of luciferase activity was observed when the mutants were used. However, it was not as strong as that seen for the STAT-3 mutation. AP-1 mutants showed a stronger reduction with regard to IL-1β-mediated activation, whereas the C/EBP-mutation appeared to be involved in IL-6-mediated induction only. A second potential C/EBP binding site, identified by us, was also mutated, and the resulting construct was analyzed in the luciferase reporter gene assay. This site, located at bp −191, is apparently not utilized at all, as a mutation did not change LBP promoter activity (data not shown).

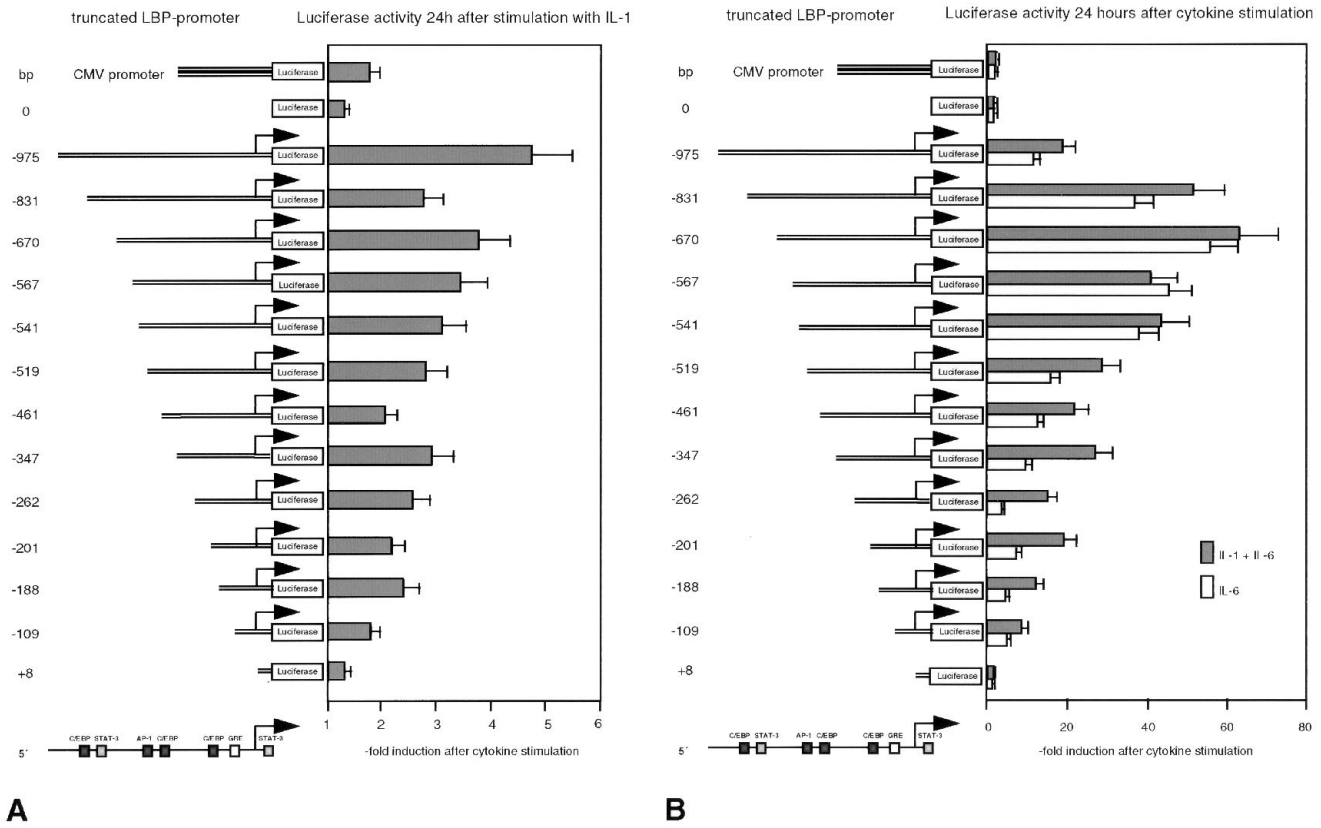

FIG. 6. Functional activity of truncation mutants of the LBP promoter stimulated by cytokines, examined with the luciferase reporter gene assay. (A) The LBP promoter was truncated at the sites indicated by a PCR-based cloning technique. The truncated mutants of the LBP promoter were cloned in front of the luciferase gene, and HUH-7 cells were transfected and stimulated with 50 U of IL-1β per ml. Twenty-four hours after stimulation, the cells were lysed and light emission was measured in a luminometer following addition of luciferin. Mean values ± standard deviations are shown. (B) Cells were stimulated with 500 U of IL-6 per ml with and without 50 U of IL-1β per ml to determine synergistic action of these cytokines. Fold induction with the cytokines indicated for stimulated and nonstimulated cells of a total of four experiments (means ± standard deviations) is shown. The luciferase vector with and without the CMV promoter served as a control.

Taken together, the reporter gene assays revealed by truncation experiments that the region from positions −831 to −461, containing a C/EBP site, an APRE/STAT-3 site, and an AP-1 site, is necessary for LBP activation. Point mutation analysis furthermore confirmed that integrity of the common acute-phase promoter motif APRE/STAT-3, located at an unusual position downstream of the transcription start site, as well as of the AP-1 site and one C/EBP site is required to confer complete transcriptional activation to the LBP gene.

EMSA using oligonucleotides designed according to the STAT-3, AP-1, and C/EBP sites found within the LBP promoter. According to the results obtained from the reporter gene assays, we performed gel retardation assays with nuclear proteins in order to show a protein-DNA interaction at the transcription factor binding sites APRE/STAT-3, AP-1, and C/EBP found to be present within the LBP promoter. Nuclear proteins of nonstimulated and cytokine-stimulated HUH-7 cells were incubated with radiolabeled oligonucleotides designed according to the binding site sequence, and the complexes formed were visualized by electrophoresis, transfer, and autoradiography. As can be seen in Fig. 8A, nuclear proteins obtained from cells stimulated with cytokines bind to all three double-stranded oligonucleotides, resulting in the formation of complexes of the expected sizes. Furthermore, the complex formations could be inhibited by competition with specific nonlabeled (cold) oligonucleotides designed according to commonly accepted transcription factor binding sites (APRF/STAT-3 and AP-1) or the nonlabeled form of the oligonucleotide designed according to the sequence found within the LBP promoter (C/EBP), shown in Fig. 8A, lanes 3. Nonspecific competition with unrelated oligonucleotides failed to reduce complex formation (Fig. 8A, lanes 2). Other nonspecific oligonucleotides were also used and failed to inhibit complex formation (not shown). Nonstimulated cells did not give rise to any complexes (Fig. 8A, lanes 4), and labeled oligonucleotides without nuclear proteins added also failed to exhibit a shift signal (not shown). C/EBP and AP-1 shifts were performed with nuclear extracts from cells lysed 24 h after stimulation with IL-1β, IL-6, and dexamethasone, whereas the nuclear proteins used for STAT-3 shifts were collected as early as 15 min after stimulation with IL-6 alone, as it is known that STAT-3 binds early to its target DNA sequence before it rapidly dissociates (62). As an additional control for STAT-3–DNA interaction, gel shift experiments were performed using a double-stranded labeled rat α2-macroglobulin STAT-3 oligonucleotide and nuclear extracts from stimulated cells, resulting in complex formation which could be inhibited by addition of cold oligonucleotides designed according to the sequence found within the LBP promoter (data not shown).

The exact nature of the proteins binding to the C/EBP site and the AP-1 site is not known. These proteins could potentially include any of the dimers of the C/EBP and Fos/Jun family members in homo- or heterodimer configuration. Supershift analyses, which, because of the large group of proteins,

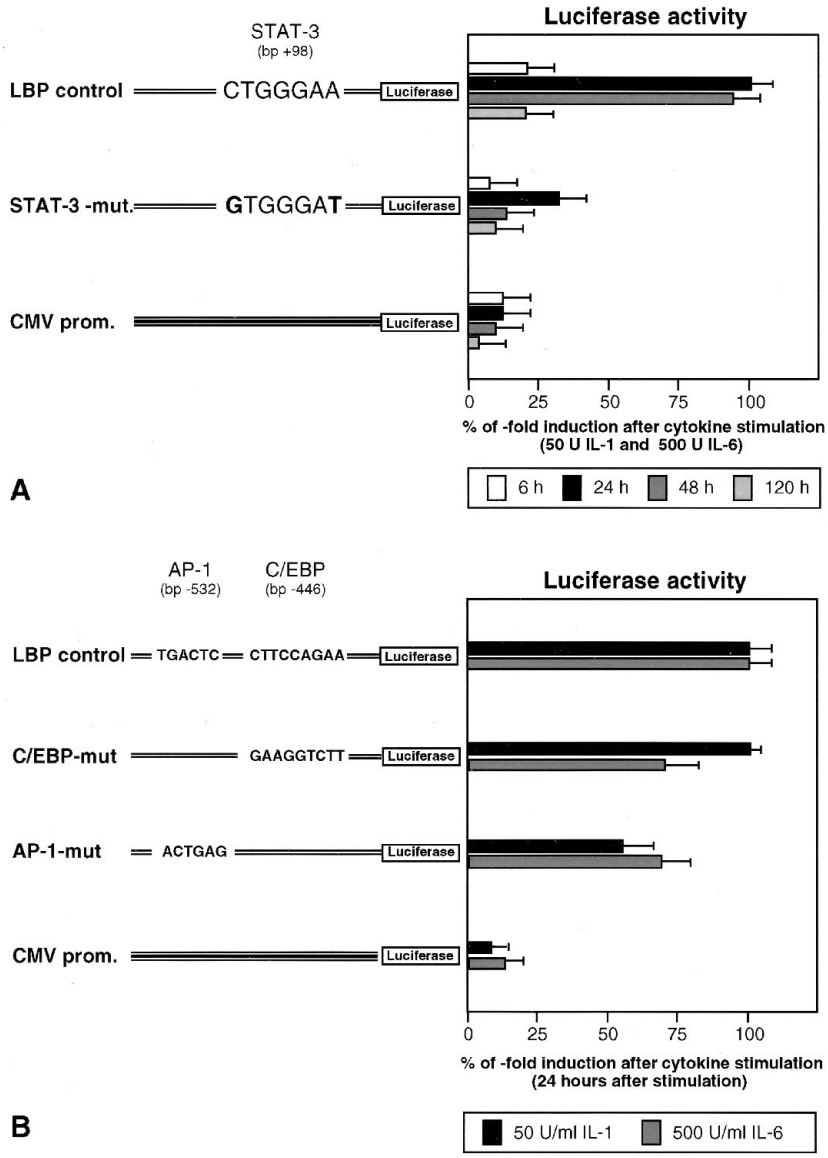

FIG. 7. Point mutations of transcription factor binding sites reduce inducibility of the LBP promoter. (A) A 461-bp promoter fragment was mutated at the putative APRF/STAT-3 binding site by a 2-bp point mutation. This promoter mutant was cloned in front of the luciferase gene, and hepatoma cells were transfected and stimulated with a combination of IL-1β and IL-6, as described in the text. Shown is a time course of luciferase activity measured by chemiluminescence after cytokine stimulation. Values for the cytokine-stimulated cells were divided by the values for nonstimulated cells to obtain fold induction. The maximum inducibility of the control promoter was set as 100%, and the other values obtained are expressed relative to the control. (B) The putative AP-1 binding site, located at bp −582, and the putative binding site for proteins of the C/EBP family, located at bp −446, of the LBP promoter were also mutated, and the mutant constructs were cloned in front of the luciferase gene. Cells were stimulated with the cytokine for 24 h after transfection, before they were lysed and luciferase activity was measured. Mean values ± standard deviations of a total of three experiments are shown. mut, mutant; prom., promoter.

will require substantial additional work, are under way. The multiple bands seen for the AP-1 gel shift are typical for complex formation with different dimers of the family of transcription factors and may represent different forms of Jun/Fos, Jun/Jun, or Fos/Fos. Here, also, additional experiments will be needed to obtain formal proof that members of this group of proteins are binding to the AP-1 site of the LBP promoter.

APRF/STAT-3 supershift analysis. In order to prove that the APRF/STAT-3 protein binds to its recognition site, supershift assays were performed, using a supershift anti-STAT-3 antibody (TransCruz Gel supershift reagent sc-482X, purchased from Santa Cruz, Inc.) known to compete with DNA binding. First, to obtain further evidence that the complex formed is a STAT-3 complex, an oligonucleotide synthesized according to the STAT-3 site of the α2-macroglobulin promoter was examined for formation of a complex of the expected size. It can be seen in Fig. 8B that the complex formation with this control oligonucleotide could be eliminated successfully by the addition of cold LBP–STAT-3 oligonucleotide (lanes 1 and 2) and vice versa (lane 3). The supershift anti-STAT-3 antibody was added immediately after incubation of nuclear proteins with labeled oligonucleotides, and lanes 5 to 7 of Fig. 8B show that this antibody was able to inhibit STAT-3–DNA complex formation in a dose-dependent fashion, confirming binding of the transcription factor APRF/STAT-3 to the consensus site within the LBP promoter.

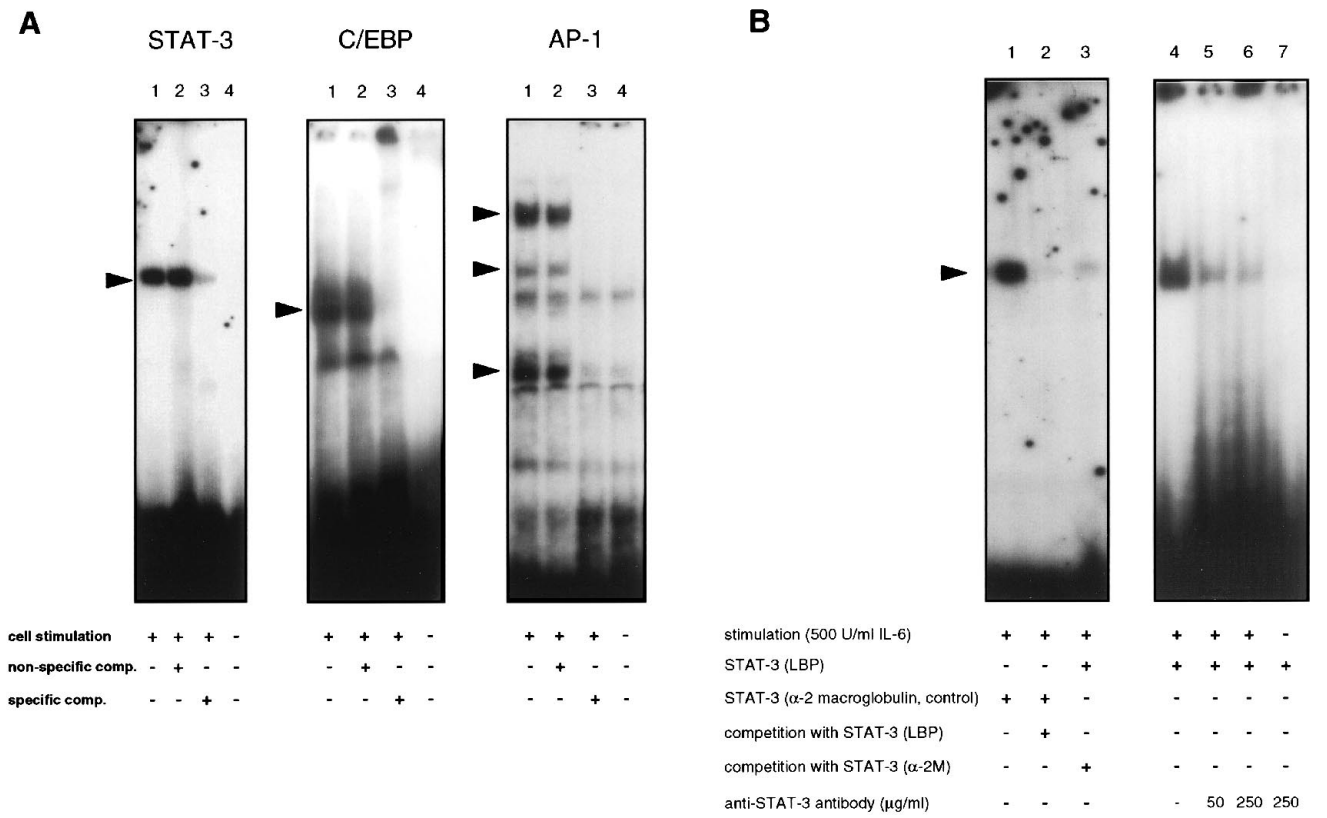

FIG. 8. Results of EMSA and supershift assays of double-stranded oligonucleotides representing STAT-3, C/EBP, and AP-1 consensus sites present in the human LBP promoter. (A) Gel retardation assays for nuclear proteins binding to the putative transcription factor binding sites STAT-3, C/EBP, and AP-1 found within the LBP promoter were performed according to a protocol described in detail in Materials and Methods. Nuclear proteins were prepared from cytokine-stimulated and -nonstimulated hepatoma cells as described in the text. Lanes 1, experiment using radiolabeled oligonucleotides, synthesized according to the sequence present within the LBP promoter, incubated with nuclear extracts of stimulated cells in the absence of competing molecules (one or several bands can be seen, indicating binding of nuclear proteins to the oligonucleotide); lanes 2, addition of a 20-fold molar excess of a nonspecific (cold) competitor, without any change in signal intensity; lanes 3, results obtained by addition of a specific competitor, leading to a significant blocking of signal; lanes 4, nuclear extracts of nonstimulated cells used as a control. For more-detailed information on the sequences, see Materials and Methods. The positions of specific complexes (arrowheads) are indicated. comp., competitor. (B) Supershift analysis of a double-stranded oligonucleotide representing the STAT-3 site in the human LBP promoter. An anti-STAT-3 antibody known to interfere with STAT-3–DNA binding was utilized to inhibit protein-DNA interaction at the STAT-3 binding site. Lane 1, radiolabeled STAT-3 oligonucleotide from the α2-macroglobulin (α-2M) promoter incubated with nuclear extracts of stimulated cells as a control; lane 2, reaction inhibited by the addition of cold LBP STAT-3 oligonucleotide; lane 3, result obtained with a labeled LBP STAT-3 oligonucleotide, inhibited by competition with cold α2-macroglobulin STAT-3 oligonucleotide; lane 4, the shift complex with nuclear extracts of stimulated human hepatoma cells and the radiolabeled LBP STAT-3 oligonucleotide alone. In lanes 2 and 3, antibody was added (1 and 5 μl, respectively; for details, see Materials and Methods). In lane 4, 5 μl of antibody solution was added in the presence of nuclear extracts from nonstimulated cells as a control. The results shown are from one representative experiment of a total of three experiments.

In summary, our data show the transcriptional inducibility of the LBP promoter by IL-1β and IL-6 which takes place in a way typical for a class 1 acute-phase protein. At least three transcription factor binding sites, APRF/STAT-3, AP-1, and C/EBP, are involved in activation of this novel acute-phase protein, leading to a strong activation of the LBP gene, as shown here by gel shift and reporter gene assays. We furthermore show that the APRF/STAT-3 transcription factor binds to a regulatory element located downstream of the transcription start site and that this interaction is apparently centrally involved in transcriptional activation of LBP.

DISCUSSION

LBP is a member of a structurally and functionally related family of proteins (14, 15, 24, 57). LBP shows the highest sequence homology with another protein capable of binding LPS found in the granules of neutrophils and referred to as bactericidal/permeability-increasing protein (BPI) (16, 41). LBP, however, is the only secretory protein besides the soluble CD14 receptor, present in high quantities in serum, which is known to specifically bind and transfer bacterial LPS (26, 49, 66). Therefore, characterization of the regulation of LBP synthesis during the onset and course of gram-negative sepsis deserves further attention.

The results demonstrating transcriptional activation of the LBP gene by IL-6, IL-1β, TNF-α, and dexamethasone are in line with the induction patterns seen for other acute-phase proteins (9, 10, 21, 27, 29). The central role of IL-1β and IL-6 in the transcriptional activation of LBP is also reflected by our promoter studies. Furthermore, our data also place LBP in the category of a class 1 acute-phase protein, as evidenced by its synergistic inducibility with IL-1β, IL-6, and TNF-α. Enhancement of IL-6-mediated APR transcription by dexamethasone is also a typical acute-phase feature (6, 39). The presence of a GRE in the LBP promoter suggests its interaction with the glucocorticoid receptor in the 5′-flanking region of the LBP gene, which could explain the effects exerted by dexamethasone seen in our study. However, it is also known that dexamethasone upregulates expression of the IL-6 receptor (8), which may also contribute to the synergy of IL-6 and dexa-

methasone in inducing LBP expression. We have also observed that leukemia inhibitory factor is capable of inducing LBP. This effect, however, was not enhanced by dexamethasone (59a), so upregulation of the IL-6 receptor gp80 subunit by dexamethasone appears to be more likely than upregulation of the commonly utilized gp130 chain.

Cloning of the LBP promoter, as done in this study, not only led to a descriptive characterization of the LBP gene but also gave insights into the mechanisms of LBP gene induction, involving a combination of transcription factors. We failed to detect a canonical TATA box 30 bp upstream of the transcription start site. However, the presence of a cap consensus sequence 7 bp downstream of the transcription start site (CAG CCT) confirms that the transcription start site is located at the position found by us. On the other hand, the presence of a TATA box and other transcription initiation sites located further upstream could also indicate the existence of an intron in the 5′ untranslated region, in a fashion similar to that described for the gene of the LBP-related phospholipid transfer protein (PLTP) (58). Additionally, we obtained evidence for a potentially unique dual transcription start site organization of the LBP gene which is utilized upon stimulation. This organization of the promoter could account for the additional, larger transcripts seen upon specific stimulation regimens and is the focus of ongoing studies (30b).

Regulation of APRs in the liver is based on the unique proximity of Kupffer cell macrophages to hepatocytes (28). Upon stimulation, the Kupffer cells release the major acute-phase-inducing cytokines of the IL-6 and the IL-1β family either directly or by activating stroma cells for cytokine release in a paracrine fashion. Proteins of both families may then stimulate the neighboring hepatocytes for acute-phase protein release (6, 18). Because hepatocytes have a limited capacity to store preformed proteins, the increase in APR biosynthesis results in most cases from increased gene transcription (53). This is mediated through *cis*-acting promoter elements that are binding sites for nuclear factors such as APRF (APRE/STAT-3), C/EBP, IL-6-responsive element-binding protein (IL-6 RE), GRE, Ets, or AP-1 (2, 6, 30, 63, 65). However, there is also evidence to suggest that posttranscriptional events may contribute to increased levels of APRs in plasma (6).

The discovery of a number of *cis*-acting promoter elements of the acute-phase type within the 5′-flanking region of the LBP gene further supports the view of LBP as an APR. The acute-phase-typical elements APRE/STAT-3, C/EBP, and GRE, which were found in the 5′-flanking region of LBP, are all well in line with the transcriptional induction pattern seen for LBP. In this study, we have shown that binding of the transcription factors STAT-3, C/EBP, and AP-1 at least contributes to transcriptional activation of LBP. The APRE/STAT-3 site, found at an unusual location 3′ of the transcription start site, appears to be essential for LBP promoter activity. This site is found in all acute-phase promoters, and our data agree with those of other studies, stressing the role of this site for IL-6-mediated gene activation (11, 12, 36). STAT-3 was shown to bind to its recognition site relatively early after cellular stimulation. However, it has been shown that the STAT-3 recognition element is also centrally involved in longer-ongoing acute-phase stimulation (34), which is confirmed by our luciferase point mutation studies. Our supershift assay provides strong evidence that APRF/STAT-3 binds to this site. However, to obtain more-detailed information on this interaction, the time kinetics of binding events at the APRE/STAT-3 promoter site are being analyzed by us, utilizing EMSA as well as in vitro and in vivo footprinting assays.

The second major regulatory element in APR promoters, the C/EBP site, was found in three places within the LBP promoter. One of the two sites studied by point mutation experiments by us, located at bp −446, has been shown to be active, whereas the one more downstream, at bp −191, seems not to be utilized. Binding of members of the C/EBP family of transcription factors was clearly shown to be important for IL-6-regulated reactions and also for some IL-1β-induced reactions (3, 5, 29). Which dimer of nuclear factors within the C/EBP family binds to this site is being investigated in our laboratory. The AP-1 regulatory element is also known to be important for IL-6- and IL-1β-related processes because it binds members of the transcription factor family consisting of dimers of the nuclear proteins c-Fos and c-Jun (32). Our results obtained by promoter point mutation and gel shift analyses indicate an active role of the AP-1 site for LBP induction, including binding of transcription factors most likely belonging to this family.

In addition, analysis of the LBP promoter revealed several transcription factor binding sites of a more general nature which are also of potential interest in acute-phase induction. The AP-1-related GCN-4 site was also shown by others to be important for IL-6-induced processes. The transcription factor MGF, which binds to the MGF site found within the LBP promoter, was recently shown to belong to the STAT family and named STAT-5b (60). Activity of these sites within the LBP promoter has yet to be proven, and so far we cannot provide any evidence for such activity.

In vitro experiments have suggested that hepatocytes are the major source of LBP (25, 45). In this study, we have confirmed by in vitro and in vivo experiments that transcripts of LBP can be induced in hepatocytes. In an animal model in which the acute-phase response is mimicked by $AgNO_3$, we observed synthesis of LBP exclusively in the liver. Furthermore, reporter gene assays showed that cells of nonhepatic origin transfected with the LBP promoter are not inducible by IL-6 and IL-1β (30a). These data, taken together with the finding that transcripts for LBP are not inducible in tissues other than the liver, indicate that LBP production is restricted to hepatocytes. Moreover, we show that the pattern of LBP induction by cytokines resembles that observed for other APRs in vitro. Induction of LBP transcripts in cell lines was weaker than that seen in vivo, which increased. However, preliminary studies using primary human hepatocytes show strong induction by IL-6 and IL-1β, similar to that seen in vivo (not shown).

A recent study has examined the induction of LBP in rats, measured by Northern blot analysis (61). These investigators, in contrast to us, failed to see a synergistic effect of dexamethasone and IL-6. An explanation for this difference can be found most likely in the different species used; regulation of acute-phase proteins is known to be quite different in rats, as can be seen in the opposite roles that CRP and α2-macroglobulin play in humans and in rats (33).

The pathophysiological ramification of our findings is that strong induction of LBP during the acute phase may lead to an autocrine loop in which the proinflammatory cytokines induced by LBP-LPS complexes stimulate the hepatocytes for enhanced LBP production. This view of an overstimulation of LBP as a cause of prolonged inflammatory reactions (as seen in septic shock) is supported by in vitro and in vivo results showing that blocking of LBP by antibodies could suppress proinflammatory cytokine production in vitro and resulted in increased survival of mice in a sepsis model (19, 20, 50). Knowledge of the transcriptional activation pattern of LBP could thus contribute to the design of novel experimental intervention strategies on the level of DNA or RNA (e.g., antisense, ribozyme, or triple-helix-forming oligonucleotides) to

lower LBP levels and thus cause a blockage of LPS-induced stimulation (or overstimulation) of cytokine production.

By examining tissue distribution, induction pattern, and functional organization of the promoter region of LBP, we have characterized this protein as a novel acute-phase protein that can be induced on the transcriptional level, with the involvement of a distinct group of regulatory elements. Portraying LBP in the context of the acute-phase response will help to better understand its function in host defense and endotoxin recognition. Further studies of this serum protein may help to elucidate the complex nature of the acute-phase reaction and will eventually point to novel therapeutic intervention strategies in gram-negative sepsis. Furthermore, the unusual localization of the STAT-3 binding site and the absolute requirement for activation may contribute to our understanding of transcriptional activation of cytokine-induced genes.

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