Role of a Distal Enhancer Containing a Functional NF-κB-binding Site in Lipopolysaccharide-induced Expression of a Novel α1-Antitrypsin Gene*

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α1-Antitrypsin (α1-AT) is one of the major protease inhibitors in serum. Its primary physiological function is to inhibit neutrophil elastase activity in lung, but it also inhibits other serine proteases including trypsin, chymotrypsin, thrombin, and cathepsin. We have previously reported a novel α1-AT, S-2 isoform, from rabbit that is induced up to 100-fold in the liver during acute inflammatory condition (Ray, B. K., Gao, X., and Ray, A. (1994) J. Biol. Chem. 269, 22080–22086). Here, we present evidence that the expression of this α1-AT S-2 gene is also induced in lipopolysaccharide (LPS)-treated peripheral blood monocytes. From the cloned genomic DNA, we have identified a distal LPS-responsive enhancer located between −2438 and −1990 base pairs upstream of the transcription start site. In vitro DNA-binding studies demonstrated an interaction of an LPS-inducible NF-κB-like nuclear factor with a κB-element present in this enhancer region. Antibodies against p65 and p50 subunits of NF-κB supershifted the DNA-protein complex. A mutation of the NF-κB-binding element virtually abolished the LPS-responsive induction of the chimeric promoter in monocytic cells. Furthermore, overexpression of NF-κB induced the wild-type promoter activity. Taken together, these results demonstrated that during LPS-mediated inflammation, NF-κB/Rel family of transcription factors play a crucial role in the transcriptional induction of the inflammation responsive α1-AT gene.

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‡The abbreviations used are: α1-AT, α1-antitrypsin; bp, base pair(s); kb, kilobase pair(s); LPS, lipopolysaccharide; CAT, chloramphenicol acetyltransferase; EMSA, electromobility shift assay; IL, interleukin; ICE, IL-1β-converting enzyme.

α1-Antitrypsin (α1-AT), also known as α1-proteinase inhibitor, is one of the major protease inhibitors in plasma. α1-AT has a broad range of activities (Laurell and Jepsson, 1975) but primarily protects the elastic fibers in lung alveoli from excessive digestion by neutrophil elastase (Olsen et al., 1979). The importance of this particular function was first proposed by Laurell and Eriksson (1963), after observing that genetically α1-AT-deficient individuals often develop either a degenerative lung disease early in life (Eriksson, 1964) or a liver disease (Sharp et al., 1969). Genetic α1-AT deficiency seems to affect 1 in 2000 Europeans and is manifested by emphysema in adults and liver disease in children. The genetic variants associated with the deficiency of α1-AT are termed as P1, P2, and Pnull. Individuals homozygous for the P1 allele exhibit a deficiency level of 60% those homozygous for the P2 allele exhibit 10–15% deficiency; and Pnull individuals show almost no detectable amount of α1-AT in their serum (Gitlin and Gitlin, 1975; Allen et al., 1974; Muensch et al., 1986). Increased expression of α1-AT also have clinical importance in the case of individuals carrying P2 α1-AT mutant allele. A glutamate to lysine substitution at position 342 (Nukiwa et al., 1987; Sifers et al., 1988) results in aggregation of the mutant α1-AT protein within the rough endoplasmic reticulum (Lomas et al., 1992) of hepatocytes and forms insoluble intracellular inclusions which lead to the hepatocellular damage in these patients. There is now strong evidence that the liver disease of the P2 homoygotes is a direct consequence of α1-AT accumulation and degradation in the hepatocyte (Carlson et al., 1988). Since inflammatory condition increases the synthesis of α1-AT protein severalfold, prevention of inflammation and pyrexia could considerably lower the accumulation of the defective protein in the liver of a homoygous P2 patient.

α1-AT is primarily synthesized in the liver (Laurell and Jepsson, 1975), to a lesser extent in bronchialveolar and breast milk macrophages, and in blood monocytes (Perlmutter et al., 1985). It is also reportedly expressed at lower levels in submandibular glands (Chao et al., 1990), renal tubular epithelial cells, intestinal epithelial cells, nonparenchymal cells of gastric mucosa, and pancreatic islet cells (Carlson et al., 1987; Kelsey et al., 1987; Koopman et al., 1989). The plasma level of α1-AT in a variety of species is normally 2–4 mg/ml, but levels increase 3–4-fold under inflammatory conditions, pregnancy, and after administration of synthetic androgen danazol (Laurell and Rannevik, 1979).

Due to the clinical importance, molecular basis of the regulation of α1-AT gene expression has been intensively studied. The minimal promoter element required for liver specific basal expression of human α1-AT gene was reported to be confined within 261 nucleotides from the transcription start site (Ciliberto et al., 1985; DeSimone et al., 1987). But the mouse α1-AT gene which is structurally similar to the human gene requires 500 bases upstream of the transcription start site for its maximal basal expression (Grayson et al., 1988). The difference between the enhancers of these two genes is very surprising. Also, the presence of negative elements that are active in liver cells is reported for both human (DeSimone and Cortese, 1989) and mouse α1-AT genes (Montgomery et al., 1990). Despite numerous reports on α1-AT promoter and its liver-specific expression, very little is known regarding the mechanisms or regulatory elements that control inducible expression of α1-AT during inflammatory condition. It is only known that, in liver, α1-AT is induced by IL-6, whereas in monocytes and macrophages it is induced by both IL-6 and bacterial lipopolysaccha-
ride (Perlmutter et al., 1989; Barbeay-Morrel et al., 1987). During 1L-6-induced expression, human α1-AT promoter utilizes a distant transcriptional initiation site located about 2 kb upstream of the hepatocyte-specific transcription start site (Hafeez et al., 1992). A lack of reports on the mechanisms of the inducible expression of α1-AT is possibly due to its moderate level of regulation which makes it less conducive for such regulation of studies. Our recent studies have identified a highly inducible isoform of α1-AT in rabbit whose expression is increased about 100-fold in response to inflammatory signals (Ray et al., 1994). Rabbit genome contains multiple α1-AT genes that are significantly different in terms of their expression under inflammatory condition. In response to inflammation, the mRNA level of the two members of this family, F and S-1, increases nominally (1.5-fold), whereas expression of the S-2 isoform increases about 100-fold. Thus S-2 isoform becomes a major component of α1-AT in the rabbit under inflammatory conditions (Ray et al., 1994). We have therefore initiated a study to understand the regulation of the inducible expression of the rabbit gene as a prelude to the establishment of a rabbit model system to study the pathology associated with the overexpression and deficiency of α1-AT.

To identify the elements required for transcriptional induction of the rabbit α1-AT S-2 gene, we performed a detailed analysis of the S-2 promoter region. In this report, we show that a distal region located about 2.5 kb upstream of the transcription start site confers the LPS inducibility to the S-2 promoter. We have identified one NF-κB-like-binding element (Sen and Baltimore, 1986) at this region. Mutation of the NF-κB site virtually eliminates LPS responsiveness in a reporter construct containing the rabbit S-2 upstream promoter. Further characterization showed that several members of the NF-κB family can bind to this NF-κB enhancer motif. Also, cotransfection of NF-κB induces expression of the reporter CAT construct. These results provide strong evidence that NF-κB might be involved as a major regulator in the induction of α1-AT gene brought about by LPS-mediated inflammation.

MATERIALS AND METHODS

Preparation of Rabbit Monocyte Cells—Rabbit peripheral blood monocytes were isolated from citrated blood obtained from normal healthy male New Zealand white rabbits. The citrated blood was centrifuged at 300 × g for 20 min and 800 × g for another 20 min to sediment the cells. The buffy coat layer was removed and washed three times by centrifugation using RPMI 1640 medium supplemented with L-glutamine and gentamycin (50 μg/ml). Washed mononuclear cells were resuspended in RPMI 1640 medium plus 5% heat-inactivated serum and allowed to adhere at 37°C for 2 h. Next, the nonadherent cells were removed by vigorous washing with warm RPMI 1640 medium. The adherent cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated serum at 37°C, 5% CO2. For LPS stimulation, the cells were treated with 10 μg of LPS/ml of medium at 37°C, 5% CO2, for different times as indicated in the text and figure legends.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from uninduced and S-h LPS-induced blood monocytes as described by Chomczynski and Sacchi (1987). Fifty micrograms of total RNA were fractionated on a 1% agarose gel containing 2.2M formaldehyde and transferred onto a nylon membrane. Hybridization was carried out using a 32P-labeled rabbit α1-AT S-2 cDNA as probe. S1 Nuclease Protection Assay—Ten micrograms of total RNA isolated from uninduced and LPS-induced monocytes were incubated with a 2438 to 70 protein. Five independent positive clones were identified by hybridization analysis and were subsequently cloned. The DNA sequence of S-2 isoform in this region is unique and distinguishable from the other isoforms (Ray et al., 1994). From sequence analysis using a primer downstream of the reactive center region, the right genomic clone containing the S-2 gene sequence was identified. Next, a restriction map of the S-2 genomic clone was generated by single and double digests with various restriction enzymes and Southern hybridization.

DNA Sequence Analysis—Regions of phage DNA spanning the α1-AT gene were subcloned and sequenced by deoxyribonucleotide chain termination method (Sanger et al., 1977) using a Sequenase sequencing kit (U. S. Biochemical Corp.).

Computer Experiments, Oligonucleotides, and Electromobility Shift Assay (EMSA)—Nuclear extracts were prepared from uninduced and 4-h LPS (10 μg/ml)-induced blood monocytes by following the method of Dignam et al. (1983). Protein concentrations were measured by the method of Bradford (1976). DNA-binding assays were carried out following a standard protocol described earlier (Ray and Ray, 1994). The labeling of DNA probe, containing sequences from –2235 to –2250 of α1-AT S-2 gene, was performed by filling in the overhangs at the termini by Klenow enzyme and [α-32P]dATP. In some binding assays, competitor oligonucleotides were added in the reaction mixture prior to the addition of the radiolabeled probe. For antibody interaction studies, a mixture of anti-p50 and anti-p65 NF-κB proteins were added prior to the addition of the probe. The DNA-protein complexes were resolved in 6% native polyacrylamide gels.

A palindromic NF-κB oligonucleotide sequence used as a competitor in EMSA is 5'-GATCCATGGGGAATTCCCCATG-3'. For self-anneling, the oligonucleotide was heated at 95°C for 2 min in 50 mM Tris, pH 7.4, 60 mM NaCl, 1 mM EDTA and allowed to cool slowly to room temperature for 2–3 h. A mutant NF-κB oligonucleotide was also used as a competitor in EMSA and, its sequence is 5'-GATCCATGCGATC-TCGAGATG-3'. Underlined bases represent mutated sequences.

Plasmid Constructs, Cell Cultures, and Transfections—pAT-3.5 CAT plasmid was prepared by ligating the S-flanking and exon 1 sequence (from positions –3.5 kb to +70) of the rabbit α1-AT S-2 gene into a promoterless plasmid vector pBLCAT3 (Lucdow and Schutz, 1987). The sequence of α1-AT promoterless plasmid vector pBLCAT3 (Lucdow and Schutz, 1987) utilized for this report was cloned from the cDNA sequence of rabbit α1-AT S-2 gene, was performed by filling in the overhangs at the termini by Klenow enzyme and [α-32P]dATP. In some binding assays, competitor oligonucleotides were added in the reaction mixture prior to the addition of the radiolabeled probe. For antibody interaction studies, a mixture of anti-p50 and anti-p65 NF-κB proteins were added prior to the addition of the probe. The DNA-protein complexes were resolved in 6% native polyacrylamide gels.

A palindromic NF-κB oligonucleotide sequence used as a competitor in EMSA is 5'-GATCCATGGGGAATTCCCCATG-3'. For self-anneling, the oligonucleotide was heated at 95°C for 2 min in 50 mM Tris, pH 7.4, 60 mM NaCl, 1 mM EDTA and allowed to cool slowly to room temperature for 2–3 h. A mutant NF-κB oligonucleotide was also used as a competitor in EMSA, and its sequence is 5'-GATCCATGCGATC-TCGAGATG-3'. Underlined bases represent mutated sequences.

The BNL liver cell (BNL CL.2) used in the transfection assays (ATCC TIB 73) is a normal embryonic liver cell line. These cells were cultured in Dulbecco's modified Eagle medium containing a high level of glucose (4.5 g/liter) and supplemented with 10% fetal bovine serum. Ten μg of reporter plasmid were used in each transfection assay, with 2 μg of pSV-β-gal plasmid (Promega) as a control for measuring transfection efficiency. In cotransfection experiments, various amounts of CMV-p65 and CMV-p50 plasmid DNAs were added (indicated in figure legends) together with reporter plasmid DNA and carried out in 70% of the total amount of DNA in each transfection assay remained constant. Transfection of liver cells was carried out by the calcium phosphate method (Graham and Van der Eb, 1973) with minor modifications as described in Ray and Ray (1994). Monocytes used in transfection assays were prepared from rabbit peripheral blood as described above. Monocytes were transfected using the DEAE-dextran method (Sambrook et al., 1989). For LPS-stimulation of transfected monocytes, cells were incubated in the presence of 10 μg/ml Escherichia coli LPS (Sigma). As a measure of monitoring transfection efficiency, cells were cotransfected with pAT-3.5 CAT plasmid and pBS-CAT (Promega) as a control for measuring transfection efficiency. In cotransfection experiments, various amounts of CMV-p65 and CMV-p50 plasmid DNAs were added (indicated in figure legends) together with reporter plasmid DNA and carried out in 70% of the total amount of DNA in each transfection assay remained constant. Transfection of liver cells was carried out by the calcium phosphate method (Graham and Van der Eb, 1973) with minor modifications as described in Ray and Ray (1994). Monocytes used in transfection assays were prepared from rabbit peripheral blood as described above. Monocytes were transfected using the DEAE-dextran method (Sambrook et al., 1989). For LPS-stimulation of transfected monocytes, cells were incubated in the presence of 10 μg/ml Escherichia coli LPS (Sigma). As a measure of monitoring transfection efficiency, cells were cotransfected...
with pSV-β-galactosidase (Promega) plasmid. Chloramphenicol acetyltransferase (CAT) was assayed (Sambrook et al., 1989) using β-galactosidase-equivalent amounts of cell extracts which were heated at 60 °C for 10 min to inactivate endogenous acetylase. All transfections were repeated at least three times.

RESULTS

LPS Stimulates S-2 Gene Expression in Blood Monocytes—Earlier studies showed that the α1-AT S-2 gene is highly induced in acute phase liver (Ray et al., 1994). To examine, if expression of S-2 gene is also induced in monocyte cells, we used Northern blot analysis of total cellular RNA isolated from peripheral blood monocytes following 5 h of LPS stimulation. Results in Fig. 1A demonstrate that LPS mediates an increase in α1-AT gene expression in peripheral blood monocytes. To determine specifically the expression of the S-2 isoform of α1-AT genes, we performed S1 nuclease analysis (Fig. 1B). An antisense 18-mer oligonucleotide specific to the reactive center region of S-2 isoform was 32P-labeled at the 5′ end by [γ-32P]ATP and T4 polynucleotide kinase. Molar excess of this oligonucleotide was hybridized with total cellular RNA prepared from normal and LPS-induced blood monocytes. After hybridization, the RNA-oligonucleotide duplex was briefly digested with S1 nuclease and electrophoresed in a sequencing polyacrylamide gel in which the protected duplex fragment migrates slower than the digested oligonucleotide probe. It is evident that expression of the S-2 gene is significantly induced in monocyte cells in response to LPS treatment (compare lanes 3 and 4). The control reaction using yeast tRNA (lane 1) indicated absence of any nonspecific hybridization demonstrating the specificity of this assay.

Organization of the Rabbit α1-Antitrypsin S-2 Gene—To understand the mechanism of induction of this protein, we undertook the cloning and characterization of the rabbit α1-AT S-2 gene. Using a rabbit α1-AT cDNA as probe for screening of a λEMBL3 rabbit genomic library, five independent hybridizing clones were detected. Previous studies (Ray et al., 1994) indicated that rabbit at least three isoforms of α1-AT gene exist. In order to know which of these five genomic clones code for the highly inducible S-2 isoform, we used the following strategy. Although the overall cDNA sequences of the three α1-AT isoforms are 96% homologous, a striking mismatch is present at the reactive center region (Ray et al., 1994). Thus, we first sought to identify the DNA fragments carrying the reactive center region of the five genomic clones. This was accomplished by hybridization of the BamHI fragments of the genomic clones using a short DNA fragment containing sequences around the reactive center region as a probe. Next, these BamHI fragments were subcloned and sequenced for further characterization. On the basis of the sequence similarity at the reactive center region, one λ- phage clone was positively identified to be carrying the gene coding the S-2 isoform of the α1-AT protein. This phage clone contains an insert of about 19 kb in size. Southern blot hybridization and DNA sequence analyses with exon-specific oligonucleotides indicated that the rabbit S-2 gene is composed of five exons separated by four introns. These results are summarized in Fig. 2. The arrangements of exons and introns in the S-2 gene are very similar to those in the human and mouse α1-AT genes (Long et al., 1984; Krauter et al., 1986). Inspection of the sequences at splice junctions of the S-2 gene showed that they all contain the correct conserved sequence at the 5′-splice donor and at the 3′-splice acceptor sites (Sharp, 1987).

The S′-Flanking Region of α1-AT S-2 Gene Contains Potential Regulatory Elements—The sequences of the 5′-flanking promoter region of the S-2 gene is presented in Fig. 3. The transcription start site of this gene is known from a previous primer extension analysis (Ray et al., 1994). The sequence of the first 47 nucleotides of the 51-nucleotide 5′-untranslated region ob-

![Fig. 1. LPS stimulation of α1-AT S-2 gene expression in monocytes.](http://www.jbc.org/)

- A: Northern analysis of the total α1-AT mRNA isolated from rabbit peripheral blood monocytes grown in the absence (lane 1) or in the presence (lane 2) of LPS for 5 h. Total RNA samples were fractionated in a 1% agarose gel containing 2.2 M formaldehyde according to the method described by Ray et al. (1994) and hybridized to a 32P-labeled α1-AT cDNA probe. As a control, the membrane was reprobed with an actin cDNA probe that reveals the qualitative and quantitative estimation of the two samples.
- B: result of an S1 nuclease assay (described under "Materials and Methods") that determines the level of α1-AT S-2 transcripts in the mRNA preparations from monocytes grown in the absence (lane 3) or presence of LPS (lane 4). As a negative control yeast transfer RNA (lane 1) was used. Lane 2 contains untreated 32P-labeled S-2 isoform-specific antisense oligonucleotide only. The S1-protected fragments were separated on a 10% polyacrylamide, 8 M urea sequencing gel and visualized by autoradiography.

![Fig. 2. Physical map of the α1-AT S-2 gene of rabbit.](http://www.jbc.org/)

A genomic clone, λα1-AT12, containing a 19-kb insert encompass the S-2 gene. Its identity as S-2 isoform specific was determined by Southern hybridization using S-2 gene-specific probe. Location of the gene within a 10-kb region spanning BamHI and Sall restriction sites was determined by Southern hybridization analysis with the λα1-AT S-2 cDNA probe (Ray et al., 1994), and a restriction map was generated. The relative position and size of the exons, identified by Roman numerals, were determined by DNA sequence analysis of the exons and the surrounding intronic regions. Transcription start site is indicated by an arrow.
TATA box for the binding of transcription factor TFII D, several other transcription factor binding sites, including those for HNF1/LF-B1 (Frain et al., 1989; Costa et al., 1989), HNF3 (Grayson et al., 1988), HNF2/LF-A1 (Rangan and Das, 1990), and C/EBP (Johnson et al., 1987; Costa et al., 1989; Desimone et al., 1987) are located. Several Sp1 binding GC boxes 5'-GGCGG-3' (Kadanoga et al., 1988; Gidoni et al., 1984) are present in direct or inverse orientation at the proximal promoter (within −570 bp) region. At the distal region about 2400 bp upstream of the transcription start site, a potential NF-κB (Sen and Baltimore, 1986)-binding site is identified. Also, several APRF binding motifs (Wegenka et al., 1993) are seen at the distal region. The sequence of the 5'-flanking region and exon 1 of the rabbit S-2 gene was compared and aligned with the human (Long et al., 1984) sequence (Fig. 4). It is evident that a high level of homology (~67%) exists between these genes at the proximal region (sequences up to −200 from +1). However, further upstream of this region, at sequences between −550 and −200, the overall homology drops to less than 55%. Interestingly, the sequences identified as the DNA-binding elements of HNF1/LF-B1, HNF2/LF-A1, C/EBP, and HNF3 transcription factors, located within the proximal region, are highly conserved between the species, indicating the importance of these elements and the cognate transcription factors in controlling α1-AT gene expression. The divergence at the distal promoter region is quite intriguing.

The 5'-Flanking Region of the S-2 Gene Has Inducible Promoter Activity in Liver and Monocytic Cells—A 3.5-kb DNA fragment containing the upstream 5'-flanking region and the first exon, from nucleotides −3551 to +70 was cloned in the right orientation into the plasmid vector pBLCAT3 (Luckow and Schutz, 1987). This vector does not contain a functional promoter and is entirely dependent upon the functional promoter activity of the ligated heterologous gene sequence. The recombinant reporter gene (pAT-3.5 CAT) was transiently transfected into BNL liver and rabbit peripheral blood monocyte cells to analyze its promoter activity. As a control, we also used the parent plasmid pBLCAT3. Transfected liver cells were grown in the absence and presence of 25% conditioned medium of a source of active cytokines obtained from LPS-induced monocytes (DeSimone et al., 1993). Transfected liver cells were grown in the absence and presence of 25% conditioned medium, a source of active cytokines obtained from LPS-induced monocytes (Ray et al., 1993). Transfected monocytes were induced using 10 μg/ml Escherichia coli lipopolysaccharide (Sigma) in the incubation medium. Forty-eight hours after transfection, cells were harvested, and CAT activity was measured. As an internal control for transfection efficiency, cells were cotransfected with a second plasmid pSV-β galactosidase (Promega). Results presented in Fig. 5 show that pAT-3.5 CAT is expressed at a low level in both BNL liver and monocyte cells. However, the promoter activity is highly induced when BNL cells are treated with conditioned medium. A similar level of induction was seen in LPS-treated (10 μg/ml) monocytes. These data suggest that the DNA sequences present in the 5'-flanking region (~−3551/+70) of the rabbit α1-AT S-2 gene contain elements necessary for induction of reporter gene expression in both liver and monocyte cells under inflammatory conditions.

Identification of a Distal LPS-responsive Enhancer Region—To further define the regulatory regions required for LPS-mediated α1-AT gene induction, a series of deletion constructs containing variable lengths of upstream promoter sequences were made (Fig. 6). These plasmids were transiently transfected in rabbit monocyte cells, grown for 24 h with or without LPS, and CAT activity was measured. Results presented in Fig. 6 show that the construct containing a 3.5-kb upstream promoter sequence is highly responsive to LPS stimulation. Deletion of sequences up to −2438 has little effect on
the LPS responsiveness of the α1-AT S-2 promoter. However, removal of sequences up to 1990 bp resulted in a severe drop in the promoter inducibility by LPS. Further deletion of upstream sequence up to 2619 bp caused a modest increase in the basal transcription which is independent of LPS. This is possibly due to the removal of a negative element which might be located upstream of the 2619-bp position. Identity of this putative element is yet to be determined. Together, these data demonstrated that an LPS-dependent transcriptional enhancer element is located between the PstI sites at position 2243 and 2199.

The LPS-responsive Region of α1-ATS-2 Promoter Contains a Functional NF-κB-binding Site—To probe further into the nature of the regulatory elements present in the LPS-responsive region of the α1-AT S-2 promoter, the sequence of this region was examined. As shown in Fig. 3, a NF-κB-binding element is seen to be present in this region. To test whether this element is an active binding site for transcription factor NF-κB, we performed EMSA. Nuclear extracts prepared from unstimulated and LPS-stimulated monocyte cells (4 h) were analyzed by electromobility shift assay using a probe spanning the S-2 promoter from 2295 to 2250 which contains an NF-κB element. Two DNA-protein complexes were seen only with induced nuclear extract (Fig. 7A, lane 2). The complexes were competed by molar excess of an oligonucleotide containing NF-κB binding element (Fig. 7B, lane 2). Inability of a mutant oligonucleotide to compete for the complex formation (Fig. 7B, lane 3) indicates specificity of the NF-κB binding. To further verify that the factor binding to the S-2 promoter is indeed a member of the NF-κB family, we used specific antibodies to NF-κB proteins in EMSA. Anti-p65 and anti-p50 antibody effectively supershifted the DNA-protein complexes (Fig. 8). Activation and participation of p50 subunit of NF-κB is evident from the results in lanes 2 and 3. Likewise, involvement of p65 subunit of NF-κB is evident from the results in lanes 4 and 5. These data indicated that both the p50 and p65 subunit of the NF-κB family can interact with the NF-κB element of S-2 gene, located at position 2287 and 2276 in different combinations to form the complexes A and B.

Mutation of the NF-κB Site in S-2 Promoter Diminishes LPS

stream sequence up to 619 bp caused a modest increase in the basal transcription which is independent of LPS. This is possibly due to the removal of a negative element which might be located upstream of the 619-bp position. Identity of this putative element is yet to be determined. Together, these data demonstrated that an LPS-dependent transcriptional enhancer element is located between the PstI sites at position 2243 and 2199.

The LPS-responsive Region of α1-ATS-2 Promoter Contains a Functional NF-κB-binding Site—To probe further into the nature of the regulatory elements present in the LPS-responsive region of the α1-AT S-2 promoter, the sequence of this region was examined. As shown in Fig. 3, a NF-κB-binding element is seen to be present in this region. To test whether this element is an active binding site for transcription factor NF-κB, we performed EMSA. Nuclear extracts prepared from unstimulated and LPS-stimulated monocyte cells (4 h) were analyzed by electromobility shift assay using a probe spanning the S-2 promoter from –2295 to –2250 which contains an NF-κB element. Two DNA-protein complexes were seen only with induced nuclear extract (Fig. 7A, lane 2). The complexes were competed by molar excess of an oligonucleotide containing NF-κB element (Fig. 7B, lane 2). Inability of a mutant oligonucleotide to compete for the complex formation (Fig. 7B, lane 3) indicates specificity of the NF-κB binding. To further verify that the factor binding to the S-2 promoter is indeed a member of the NF-κB family, we used specific antibodies to NF-κB proteins in EMSA. Anti-p65 and anti-p50 antibody effectively supershifted the DNA-protein complexes (Fig. 8). Activation and participation of p50 subunit of NF-κB is evident from the results in lanes 2 and 3. Likewise, involvement of p65 subunit of NF-κB is evident from the results in lanes 4 and 5. These data indicated that both the p50 and p65 subunit of the NF-κB family can interact with the NF-κB element of S-2 gene, located at position –2287 and –2276 in different combinations to form the complexes A and B.

Mutation of the NF-κB Site in S-2 Promoter Diminishes LPS

FIG. 5. Functional analysis of the promoter region of α1-AT S-2 gene. A DNA fragment containing sequences from a BamHI (–3551) to an Apal (+70) of the α1-AT S-2 gene was subcloned in right orientation into plasmid vector pBLCAT3. The resultant plasmid, pAT-3.5 CAT, was used in transfection of liver and monocyte cells to assess the inducibility of the α1-AT S-2 gene promoter DNA. BNL CL.2 liver and peripheral blood monocyte cells were transfected separately with pBLCAT3 (as a control) and pAT-3.5 CAT plasmid DNA (10 μg of each) in the presence and absence of 25% conditioned medium or LPS (10 μg/ml). Details of the transfection and CAT assay are described under "Materials and Methods." CAT activity was measured as radioactivity (counts/min) in [3H]chloramphenicol acetate produced by an equivalent amount of transfected cell extracts. Transfections were normalized to the p-galactosidase control activity. Plotted values represent an average CAT activity relative to that of uninduced vector plasmid pBLCAT3 from three separate transfection assays, and the range represents the maximum values obtained in an individual assay.

FIG. 4. Comparison of the promoter region of rabbit α1-AT S-2 gene and human α1-AT gene. S-2 gene sequences from 1 to 964 were used for comparison. Symbols: ●, identical nucleotides; –, absence of nucleotides; R, rabbit, and H, human sequence. The position of the transcription start site is indicated by an arrow.

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Fig. 6. Analysis of the LPS responsive promoter of the α1-AT S-2 gene. Specific sequences of the α1-AT S-2 gene (horizontal bars) inserted upstream of the CAT gene of pBLCAT3 vector are aligned with a schematic locating specific restriction enzyme sites. Peripheral blood monocytes were transiently transfected with the constructs indicated (10 µg of DNAs were used for each plasmid). After transfection, cells were maintained in the medium in the absence of stimulation or treated with 10 µg/ml of LPS for an additional 24 h. The histogram represents uninduced (solid bars) and LPS-induced (hatched bars) CAT activity. Details of transfection and the CAT activity assay are described under “Materials and Methods.” Relative CAT activity was determined as described in Fig. 5.

Fig. 7. Distal promoter element of α1-AT S-2 gene interacts with the LPS-induced nuclear factors. EMSAs were performed with 32P-labeled α1-AT S-2 promoter DNA containing a NF-κB element (−2295 to −2250). Nuclear extracts (10 µg of protein), prepared from normal untreated (lane 1) and LPS-treated (lane 2) monocytes, were incubated with the 32P-labeled probe following the method described under “Materials and Methods,” and the resulting complexes were resolved in a 6% native polyacrylamide gel. The positions of two DNA-protein complexes A and B formed by the factors in the LPS-induced nuclear extract are shown. B, prior to the addition of 32P-labeled probe, the LPS-induced nuclear extract was incubated with competitor oligonucleotides containing either the wild-type (wt) (lane 2) or mutant (mt) (lane 3) sequence for the NF-κB element. The sequences of these two oligonucleotides are described under “Materials and Methods.” A control assay containing no competitor oligonucleotide is shown in lane 1.

Responsiveness of α1-AT S-2 Promoter—To determine whether the interaction of NF-κB is indeed responsible for the LPS-mediated transcripional induction of the α1-AT gene, we selectively mutagenized the transcriptional binding element of the S-2 promoter, and a mutated chimeric gene was constructed that contained this altered NF-κB-binding element. The sequence was changed from 5′-'GGGGCTTTCCCC-3′ to 5′-'GCTCCTTTTTC-3′. This mutated construct designated as pAT2.4mt NF-κB CAT reporter gene was then transfected into monocytes, and its response to LPS was compared with the reporter gene containing a wild-type sequence (Fig. 9). Mutation of the NF-κB element virtually eliminated the induction of the mutated reporter gene, while the wild-type promoter, in the presence of LPS, induced CAT activity more than 15-fold over background. Thus, the NF-κB-binding site appears to be a necessary regulatory element for activation of the α1-AT gene by LPS.

Overexpression of NF-κB Induces α1-AT Gene Expression—For further verification of the role of NF-κB in potentiating transcription of the α1-AT S-2 gene, NF-κB expression vectors were cotransfected into blood monocytes together with wild-type pAT2.4 CAT or pAT2.4 mt NF-κB CAT reporter plasmids. Overexpression of NF-κB resulted in strong transcription of the α1-AT promoter in a manner which was dependent on the integrity of the identified NF-κB-binding element (Fig. 10). Both p50 and p65 subunits of NF-κB induced transcription in a dose-dependent manner. These results clearly demonstrate the involvement of NF-κB in the transcriptional induction of the α1-AT S-2 gene.

DISCUSSION

Extremely high inducibility of the α1-AT S-2 gene under inflammatory conditions has prompted a detailed analysis of the regulatory elements of this gene. α1-AT is one of the major proteinase inhibitors in serum with a broad range of activities but mainly protects the elastic fibers in lungs from excessive digestion by neutrophil elastase. During the host response to inflammation/injury expression of α1-AT is induced. However, the molecular mechanisms of this induction process is still
element binds with NF-κB, and its mutation abolishes the LPS-mediated inducibility of α₁-AT promoter construct in monocytic cells. Overexpression of NF-κB in monocytes induced the α₁-AT expression. The data taken together strongly suggest that LPS-mediated α₁-AT gene induction is controlled by NF-κB. Involvement of NF-κB in the α₁-AT gene induction process has not been reported earlier.

What is the significance of the high level induction of the α₁-AT S-2 gene under inflammatory conditions? A logical explanation may be to down-regulate the proteolytic cascade associated with the cell death pathway activated during inflammation. Interleukin 1-β produced and activated in response to inflammation can activate many cellular events causing cell death. IL-1-β-converting enzyme (ICE), a mammalian proteinase processes pro-IL-1-β to the active form mature IL-1-β by proteolytic cleavage at aspartic residues (Black et al., 1988). ICE is also homologous to the ced-3 gene of Caenorhabditis elegans that is essential for apoptosis (Ellis et al., 1991). Recently it has been shown that ICE can be inhibited by a novel cysteine proteinase inhibitor CrmA originally identified in cowpox virus (Ray et al., 1992). Expression of crmA can prevent cell death mediated by ICE as well as quelling other IL-1-mediated inflammatory response. Overexpression of crmA can also prevent cell death mediated by TNF receptor associated death domain protein (Hsu et al., 1995), Fas associated death domain protein (Chinnaiyan et al., 1995), and a death protease Yama, a mammalian homolog of ced-3 (Tewari et al., 1995). In view of high inducibility of the α₁-AT S-2 gene under inflammatory conditions, it is possible that a rabbit genome has evolved and maintained this highly inducible proteinase inhibitor to maintain cellular homeostasis and cope with the influx of proteinases brought in by infectious agents upon wounding or infection. It is known that parasites such as Schistosoma mansoni (McKerrow et al., 1985) and bacteria such as Pseudomonas (Welb et al., 1982) synthesize serine proteinases that act as virulence factors to increase the efficiency of infection.

In an effort to elucidate the mechanism of transcriptional induction, we undertook the cloning and characterization, as well as sequencing, of the immediate upstream promoter region of the rabbit α₁-AT S-2 gene. This gene spans about a 10-kb region and consists of five exons. A translation initiator ATG codon is present at the beginning of the second exon, while the first exon codes for the 5'-untranslated region. The proximal promoter region, up to 200 bp upstream of the transcription start site (Fig. 4) of S-2 gene, shows a high degree of homology with the proximal region of human and mouse α₁-AT genes. This region contains a number of major transcription factor-binding elements including HNF1/LF-B1, HNF2/LF-A1, HNF3, and C/EBP which are known to be highly conserved. Such a high conservation of several transcription factor binding sequences argues for their importance in controlling the basal expression of α₁-AT across the species. These elements, however, are not sufficient for the inflammation-induced expression of the S-2 gene. Additional regulatory elements are crucial for its inducibility. This is evident from the detailed promoter analyses of this gene (Fig. 6).

Functional studies show that promoter of the S-2 isoform is highly inducible both in liver and monocyte cells in response to cytokines present in the conditioned medium or LPS (Fig. 5). Transient transfection of monocyte cells (Fig. 6) using a series of CAT gene reporter constructs, demonstrates that upstream sequences located between −2438 and −1990 (a 0.5-kb PstI fragment) are important for the induction response mediated by LPS. Analysis of the LPS-responsive region of S-2 promoter revealed the presence of a NF-κB-binding element in this region. Drastic reduction of LPS responsiveness, when the NF-
NF-kB-binding element is altered, clearly suggests that NF-kB plays a crucial role for efficient induction of the α1-AT S-2 gene by LPS in monocytic cells. The abilities of (i) inducible NF-kB-like factors to bind to the α1-AT promoter, (ii) anti-p65 and anti-p50 antibodies to supershift the LPS-inducible DNA-protein complex to the α1-AT promoter, and (iii) the overexpression of p65 and p50 subunits of NF-kB to activate α1-AT gene expression further attest to the role of NF-kB as a regulator of α1-AT gene induction. NF-kB is a family of pleiotropic inducible transcription factors originally identified as nuclear factors that bind to the kB enhancer motif of immunoglobulin κ light chain (Sen and Baltimore, 1986). The transcription factor is activated by a number of extracellular signals and cytokines including IL-1 and tumor necrosis factor. Recent numerous studies have shown that NF-kB regulates a wide variety of genes including those of IL-1β, IL-6, IL-8, IL-2, and serum amyloid A (Bensi et al., 1990; Libermann and Baltimore, 1990; Bensi et al., 1989; Ray et al., 1995). The involvement of NF-kB in the transcriptional induction of α1-AT broadens the range for this factor to yet another gene that is induced in response to inflammatory stress.

In this report, we have laid the foundation for more detailed analyses of the regulation of the inducible expression of the α1-AT gene in liver and monocyte/macrophages during the inflammatory response. Increased expression of α1-AT also poses a serious health problem in individuals carrying the PiZZ α1-AT mutant allele. During inflammation, synthesis of α1-AT in these patients is increased, causing greater intracellular accumulation of the defective protein (Barbay-Morrel et al., 1987). Understanding the molecular mechanisms of inducible expression of α1-AT will allow for development of therapies to intervene the increased synthesis and accumulation of the defective protein in the liver of a homozygous PiZZ individual. Further work along this line is currently in progress.

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Role of a Distal Enhancer Containing a Functional NF-κ B-binding Site in Lipopolysaccharide-induced Expression of a Novel α₁-Antitrypsin Gene
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