Lipopolysaccharide-mediated Transcriptional Activation of the Human Tissue Factor Gene in THP-1 Monocytic Cells Requires Both Activator Protein 1 and Nuclear Factor κB Binding Sites

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Summary

Lipopolysaccharide (LPS) activation of cells of monocytic lineage leads to rapid and transient expression of a set of inflammatory gene products, including tissue factor (TF). This transmembrane receptor is the major cellular initiator of the blood coagulation cascades, and induced expression of TF is postulated to play a role in inflammation. Functional studies using transfected THP-1 monocytic cells revealed the presence of a 56-bp LPS response element (LRE) within the TF promoter that conferred LPS responsiveness to a heterologous promoter. LPS stimulation of these cells activated proteins that bound to nucleotide sequences within the LRE resembling consensus binding sites for activator protein 1 (AP-1) and nuclear factor κB (NF-κB). Induction of the TF gene may represent a prototypic example of gene activation in monocytic cells by assembly of transcription factor complexes, and may clarify the role of AP-1 and NF-κB in the regulation of other LPS-responsive genes.

Bacterial endotoxin (LPS) is responsible for many of the cellular responses to Gram-negative bacterial infections in higher primates. These responses may be induced after the association of LPS with the plasma protein LBP (LPS binding protein) and the binding of this complex to the cell surface receptor CD14 (1, 2). LPS stimulates cells of monocytic lineage to rapidly and transiently express a defined set of gene products, including TNF (3), IL-6 (4), IL-1 (5), and the transmembrane protein tissue factor (TF)[1] (6). It is suspected that common pathways may exist in these cells to coordinate transcriptional activation during inflammation. Induced expression of both the TF receptor and TNF have been shown to be mediators of fatal bacteremic shock (7–9), indicating that antibodies against these molecules may offer a potential therapy to life-threatening infection.

TF is the receptor and cofactor for plasma factors VII/VIIa, and mediates the cellular initiation of the coagulation serine protease cascades (10, 11). Thus, TF is also responsible for thrombin generation and the associated inflammatory consequences via the thrombin receptor (12). In addition, expression of TF by monocytic cells has been postulated to play a role in inflammation (13) and the cellular immune response (14, 15). Antigen-specific CD4 T cells induce TF expression in monocytes and macrophages by cell-cell interaction as well as by lymphokine collaboration (16–18). Pathologic activation of the coagulation cascades by TF within the vasculature has been implicated in several disease states, including septic shock, disseminated intravascular coagulation, and various thromboembolic disorders (9, 13, 19).

In this study, we attempted to identify DNA sequence elements and their corresponding transcription factors that are required for LPS-mediated induction of the TF promoter in THP-1 monocytic cells. We found that a 56-bp regulatory region was required, which we have designated an LPS response element (LRE). Moreover, exposure of these cells to LPS activated two distinct proteins that bound to nucleotide sequences within the LRE that resemble consensus binding sites for both activator protein 1 (AP-1) (20) and nuclear factor κB (NF-κB) (21). We propose that engagement of these binding sites is required to mediate the pattern of transcriptional induction of the TF promoter observed for cells of monocytic lineage.

Materials and Methods

Plasmids. The promoterless luciferase reporter vectors p19LUC (22), pRSVLUC (23), and pRSVCAT (24) were used as controls in the transfections. The construction of pTF(−2106)LUC, pTF(−948)LUC, pTF(−383)LUC, pTF(−278)LUC, pTF(−111)LUC, pTF(−21)LUC, pTFM2(−278)LUC, pTFM3(−278)LUC, and

[1] *Abbreviations used in this paper:* AP-1, activator protein 1; CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; LRE, LPS response element; NF-κB, nuclear factor κB; TF, tissue factor.

pTFM4(−278)LUC has been described previously (25). 5′ deletion of the TF promoter was achieved by Exonuclease III (Promega Biotec, Madison, WI) digestion of a 504-bp fragment (−383 to +121) in pGEM3Zf(−) and subsequent insertion of DNA fragments into p19LUC (Fig. 1). pPAI-LUC (Fig. 2) was made by inserting a 106-bp DNA fragment, containing the minimal promoter from the human plasminogen activator inhibitor type 1 (PAI-1) gene (−31 to +75 [22]), upstream of the luciferase reporter gene in p19LUC. This fragment was kindly provided by Drs. M. Keaton and D. Loskutoff. Various DNA fragments or double-stranded oligonucleotides from the TF promoter (Table 2) were inserted upstream of this minimal promoter in pPAI-LUC (Fig. 2). pTFM5(−278)LUC and pTFM6-(−278)LUC were made by site-directed mutagenesis of a template, pTF(−278)LUC (Fig. 1), using oligonucleotides M17 and M18, respectively (Table 2). pTFM7(−278)LUC and pTFM8(−278)LUC were made in a similar way but used a template, pTFM4(−278)LUC, which contains mutations in both AP-1 sites (Fig. 3).

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared as described previously (26) either from control THP-1 cells or from cells stimulated with 10 μg/ml of LPS *Escherichia coli* 0111:B4 (Calbiochem Behring Corp., San Diego, CA) for 45 min. EMSAs were performed using a modification of the protocol described previously (27). Extracts were incubated on ice for 30 min with 6 μg of poly (dI·dC) (Pharmacia Fine Chemicals, Uppsala, Sweden) in 18 μl of buffer (25 mM Hepes, pH 7.9; 40 mM KCl; 5 mM $MgCl_2$; 0.125 mM EDTA; 0.75 mM DTT; 7.5% glycerol). Next, 0.1–0.5 ng (10,000–50,000 cpm) of oligonucleotide probe was added and incubation continued for a further 15 min on ice and 15 min at 25°C. Oligonucleotides were labeled with [^{32}P]ATP (Amersham Corp., Arlington Heights, IL) using either the Klenow fragment of *E. coli* DNA polymerase I or T_4 polynucleotide kinase (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Samples were subjected to electrophoresis on a 6% polyacrylamide gel containing 45 mM Tris-borate, 45 mM boric acid, and 1 mM EDTA.

Cell Culture. The human monocytic leukemia cell line, THP-1, was obtained from the American Type Culture Collection (Rockville, MD) (No. TIB 202). For this study we used a subline of THP-1, kindly provided by Dr. D. Dialynas, which exhibited low levels of TF activity under standard culture conditions and could be induced to express high levels of TF activity in response to LPS. These cells were cultivated at a density of 2–4 × 10^5 cells/ml in RPMI 1640 supplemented with 20 mM Hepes (adjusted to pH 7.4 with NaOH), 50 μM 2-ME, 2 mM L-glutamine, and 7% FCS.

DNA Transfection and LPS Induction of the Cloned TF Promoter. A DEAE-Dextran transfection procedure was used (28). Briefly, 2 × 10^7 cells were harvested and incubated for 15 min at 37°C in 1 ml of STBS (25 mM Tris/Cl, pH 7.4; 5 mM KCl; 0.7 mM $CaCl_2$; 137 mM $NaCl_2$; 0.6 mM Na_2HPO_4; 0.5 mM $MgCl_2$) containing 4 μg of plasmid DNA and 150 μg/ml of DEAE-Dextran (Pharmacia Fine Chemicals). 2 μg of pRSVCAT was used as an internal control in the transfections. Cells were washed and resuspended in complete media and cultivated for 46 h. At this time, each transfection was divided into two equal portions which were incubated at 37°C for a further 5 h either in the presence or absence of 10 μg/ml LPS. Cells were harvested and luciferase activity was determined.

Activity Assays. One-half (50 μl) of the cell lysate (100 μl) from transfected THP-1 cells was assayed for luciferase activity using a Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA) as described previously (23). Chloramphenicol acetyl transferase (CAT) activity was determined from 25 μl of the extract using a diffusion-based assay as described previously (29).

Results

LPS Induction of the Human TF Promoter. A series of TF promoter plasmids was constructed by inserting DNA fragments, containing sequential 5′ truncations of the TF pro-

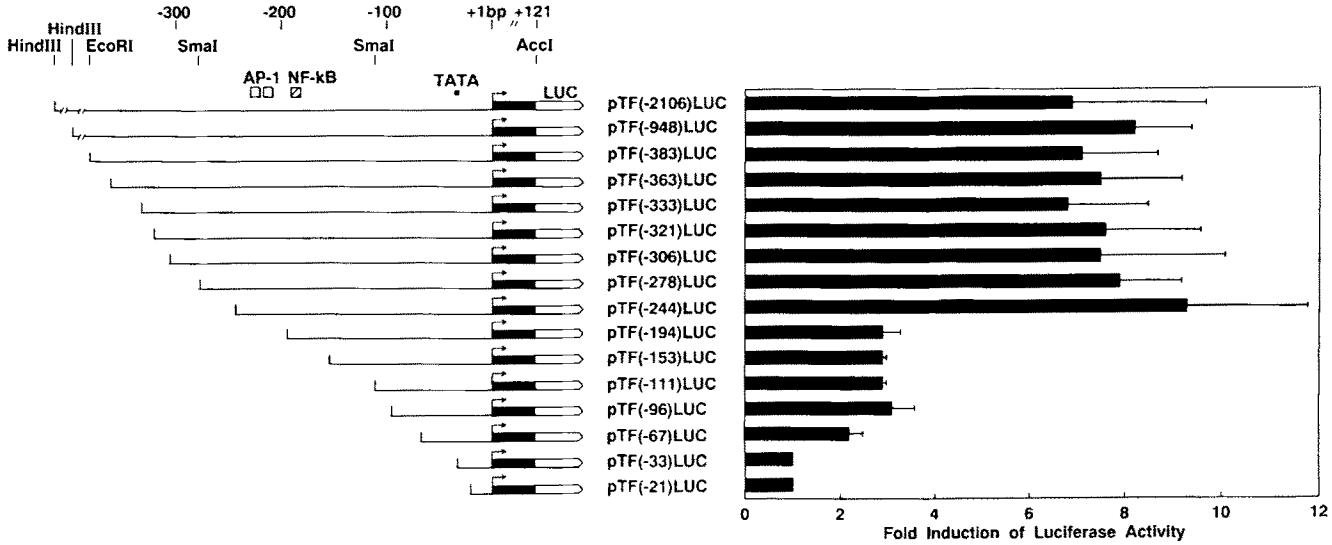

Figure 1. LPS induction of TF promoter plasmids from the 5′ deletion series. The 5′ boundaries of 16 plasmids containing various truncations of the TF promoter are shown. Some key restriction endonuclease sites are indicated. The positions of the TATA consensus promoter element, AP-1 sites, and NF-κB site are marked. The bent arrow represents the start site of transcription upstream of the luciferase reporter gene (LUC). Total luciferase activity (light units) from transfected THP-1 cells was measured and normalized for the amount of DNA uptake by using pRSVCAT as an internal control. The average fold induction of luciferase activity expressed by each plasmid in response to LPS in three independent experiments is shown with the standard deviation.

moter between −2106 and −21, upstream of the firefly luciferase reporter gene in p19LUC (Fig. 1). Transcriptional activity of the TF promoter was quantified by measuring luciferase activity expressed by transiently transfected human THP-1 monocytic cells. TF promoter constructs exhibited low promoter activity in unstimulated cells (Table 1), consistent with a low rate of transcription of the endogenous TF gene (30).

To determine the approximate location of LREs within the 5′ flank of the TF gene, we analyzed luciferase activity expressed by these plasmids in THP-1 cells stimulated with LPS. Plasmids could be divided into two classes, "high" or "low," depending on the magnitude of the response to LPS (Fig. 1). The first class included nine plasmids, pTF(−2106)LUC to pTF(−244)LUC, which all exhibited high levels of induction averaging 7.6-fold. The second class averaged only 2.8-fold induction and included five plasmids, pTF(−194)LUC to pTF(−67)LUC. Removal of DNA between −194 and −68 did not significantly influence the level of LPS induction, although the basal promoter activities associated with pTF(−96)LUC and pTF(−67)LUC were significantly reduced (Table 1). Finally, pTF(−33)LUC and pTF(−21)LUC were not transcriptionally active.

Table 1. *Luciferase Activity Expressed by TF Promoter Plasmids in Unstimulated THP-1 Cells*

Plasmid	Luciferase activity (-LPS)	pRSVLUC
		%
pTF(-2106)LUC	2,884	11
pTF(-948)LUC	4,868	18
pTF(-383)LUC	4,735	18
pTF(-363)LUC	3,884	14
pTF(-333)LUC	5,452	20
pTF(-321)LUC	4,186	16
pTF(-306)LUC	5,509	20
pTF(-278)LUC	4,507	17
pTF(-244)LUC	5,413	20
pTF(-194)LUC	4,379	16
pTF(-153)LUC	3,249	12
pTF(-111)LUC	2,884	11
pTF(-96)LUC	1,176	4
pTF(-67)LUC	258	1
pTF(-33)LUC	216	<1
pTF(-21)LUC	215	<1
pRSVLUC	26,951	-
p19LUC	210	-

Total luciferase activity for a typical experiment is shown as light units and also as a percentage of the level expressed by pRSVLUC. pRSVLUC and p19LUC were used as positive and negative controls, respectively. Transfection efficiencies between various TF promoter plasmids were normalized using expression by pRSVCAT.

These studies indicated the presence of at least one LRE within the TF promoter with a 5′ boundary at approximately −244. In addition, DNA sequences between −2106 and −245 did not appear to be required for LPS induction in these monocytic cells. Removal of DNA (−244 to −195) containing two AP-1 sites decreased the level of LPS induction of the TF promoter from high to low, suggesting that AP-1, or a related transcription factor, may be required to mediate full LPS induction of the TF promoter.

Localization of the LRE from the TF Promoter. To more accurately define the 5′ and 3′ boundaries of this putative LRE, we analyzed the ability of various DNA fragments from the TF promoter to confer LPS responsiveness to a heterologous promoter. A 106-bp DNA fragment from the human PAI-1 gene (−31 to +75), which includes the minimal transcriptional initiation information, was inserted upstream of the luciferase reporter gene to create pPAI-LUC (Fig. 2). Based on the data described above, we inserted a 167-bp fragment (−278 to −112) from the TF promoter upstream of the human PAI-1 promoter to create pTF(278/112)PAI-LUC (Fig. 2). This plasmid contained two AP-1 sites and an NF-κB site and directed a 6.3-fold induction of luciferase activity in THP-1 cells exposed to LPS, indicating that the 167-bp fragment included at least one LRE. Smaller DNA fragments were subsequently analyzed to more precisely define the boundaries of the LRE, and to elucidate the role of the AP-1 and NF-κB sites. The 133-bp fragment of pTF(244/112)PAI-LUC and the 100-bp fragment of pTF(227/128)PAI-LUC each contained all three sites and both exhibited an equivalent 8.5-fold induction. Deletion of a DNA segment containing the two AP-1 sites created pTF(207/112)PAI-LUC and drastically reduced the level of induction to 3.2-fold. These data more accurately defined the 5′ boundary of this LRE to −227. Furthermore, removal of DNA between −179 and −112 disrupted the NF-κB site in pTF(227/180)PAI-LUC and abolished LPS induction of luciferase activity. These data indicate that one or both of the AP-1 sites together with the NF-κB site are required for full functional activity of the LRE.

As a next step in the localization and identification of functional binding sites within this LRE, a 56-bp oligonucleotide (−227 to −172) was designed to include both AP-1 sites and the NF-κB site. This DNA was inserted upstream of the minimal PAI-1 promoter in pTF(227/172)PAI-LUC and directed an 11-fold induction of luciferase activity in transiently transfected THP-1 cells stimulated with LPS (Fig. 2). Therefore, the 56-bp region contains most, if not all, of the information necessary for binding transcription factors that mediate LPS induction of this LRE. Furthermore, a similar level of induction was observed when this 56-bp region of the TF promoter was inserted in the opposite orientation (data not shown).

By dividing the 56-bp region into two subregions, we wished to determine if either the 5′ subregion, designated region I (−227 to −189), or the 3′ subregion, designated

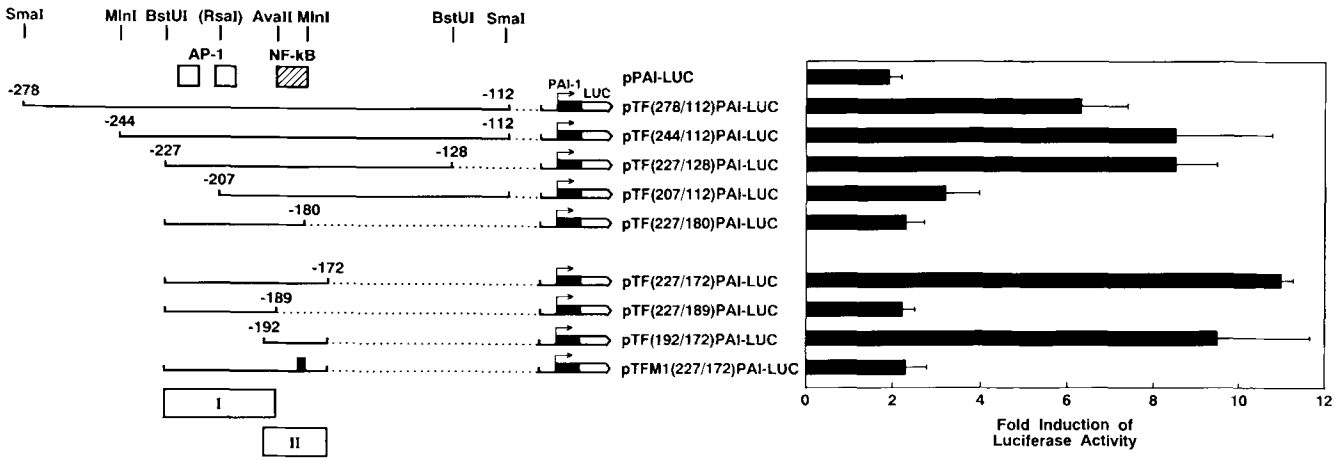

Figure 2. LPS induction of TF promoter plasmids from the heterologous promoter series. All plasmids in this series, including the control pPAI-LUC, contain the minimal PAI-1 promoter (PAI-1) upstream of the luciferase reporter gene (LUC). Restriction endonuclease sites used to generate some of these plasmids are shown. pPAI-LUC expressed ~1,800 light units of luciferase activity in a typical experiment. Addition of DNA fragments from the TF promoter increased basal expression from the heterologous promoter by an average of 3.6-fold, which is comparable with levels associated with the wild-type TF promoter. Regions I and II of the LRE are shown together with AP-1 and and NF-κB binding sites. The 3-bp substitution in the NF-κB site is indicated by the filled box. Total luciferase activity (light units) from transfected THP-1 cells were measured as described in Fig. 1 in three independent experiments. The RsaI site was introduced by site-directed mutagenesis (25).

region II (−192 to −172), could function independently. Cells transfected with pTF(227/189)PAI-LUC, which contained region I and included the two AP-1 sites, did not exhibit a significant response to stimulation with LPS (Fig. 2). In contrast, cells transfected with pTF(192/172)PAI-LUC, which contained region II and included the NF-κB site, exhibited a 9.5-fold induction of luciferase activity in response to LPS. However, it must be noted that the NF-κB site within the 21-bp insert of this plasmid is removed from its normal context, and the observed high level induction compared with that directed by the larger NF-κB-containing plasmid, pTF(207/112)PAI-LUC (see above), may reflect loss of a postulated downstream binding site(s) for negative regulatory factors. Nevertheless, we examined the role of the NF-κB site within the 56-bp region. A 3-bp substitution within the NF-κB site between −181 and −179 in pTFM1 (227/172)PAI-LUC abolished the ability of the 56-bp region to direct LPS induction of luciferase activity. These data strongly demonstrate the requirement for the NF-κB site in the LPS induction of the TF promoter.

Mutagenesis of the LRE in the Wild-type TF Promoter. To examine whether the LRE identified using the heterologous promoter is indeed valid for LPS induction of cells bearing the wild-type TF promoter, we mutated the two AP-1 sites and the NF-κB site within the LRE (−227 to −172) either separately or in combination (Fig. 3). pTF(−278)LUC was chosen

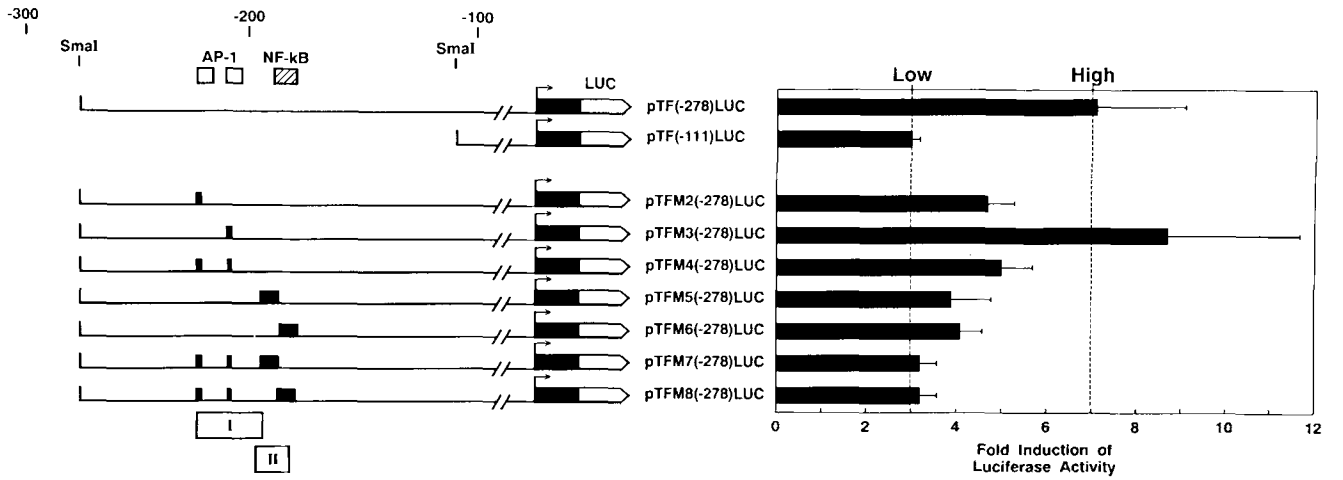

Figure 3. LPS induction of TF promoter plasmids from the mutant series. The position of base pair substitutions in these various plasmids is indicated by filled boxes. Mutation of the AP-1 sites and the NF-κB site did not significantly reduce the basal levels of luciferase activity associated with the seven mutant plasmids, pTFM2(−278)LUC to pTFM8(−278)LUC. Regions I and II of the LRE are shown together with AP-1 and NF-κB binding sites. Total luciferase activity (light units) from transfected THP-1 cells were measured as described in Fig. 1 in four independent experiments. The level of induction from the "low" and "high" classes is indicated.

as the template for site-directed mutagenesis because it contained a fully functional LRE and directed a high level of induction (7.1-fold) in response to LPS. It should be noted that complete disruption of this LRE would still be expected to yield a threefold induction in response to LPS.

When compared to the induction levels associated with the wild-type promoter in pTF(−278)LUC, mutation of the distal AP-1 site (−223 to −217) in pTFM2(−278)LUC decreased the level of the induction to 4.7-fold in THP-1 cells stimulated with LPS, whereas mutation of the proximal AP-1 site (−210 to −204) in pTFM3(−278)LUC failed to decrease the level of induction (Fig. 3). Mutation of both AP-1 sites in pTFM24(−278)LUC decreased the induction level to fivefold. Furthermore, mutation of two overlapping 10-bp regions in pTFM5(−278)LUC and pTFM6(−278)LUC, both of which disrupted the NF-κB site (−188 to −179) in region II, reduced induction levels to fourfold. Finally, when both AP-1 sites and the NF-κB site were mutated simultaneously, the levels of LPS induction associated with either pTFM7(−278)LUC or pTFM8(−278)LUC were further reduced to only 3.2-fold. This low level of induction is comparable with that exhibited by pTF(−111)LUC, in which the complete upstream LRE had been deleted. These data indicate that both the NF-κB site and the distal AP-1 site are required to mediate full LPS induction of the wild-type TF promoter.

Analysis of Transcription Factors That Bind to the LRE from the TF Promoter. To identify transcription factors that bind to the LRE from the TF promoter, we performed EMSAs. First, we analyzed the ability of NF-κB, or a related protein, to bind to region II (−192 to −172) of the LRE. Nuclear extracts, prepared from either THP-1 cells exposed to LPS for 45 min or control cells, were incubated with M19/20 (Table 2), a double-stranded 21-bp oligonucleotide spanning the NF-κB site within the TF promoter. A protein-DNA complex was observed using LPS-induced nuclear extracts but not using control extracts (Fig. 4). This protein-DNA complex was specifically competed by a consensus NF-κB site (Table 2), but not by a 100× molar excess of either M23/24 (Table 2), an oligonucleotide containing a 3-bp substitution in the NF-κB site from the TF promoter, or an irrelevant oligonucleotide. A similar complex was observed when an oligonucleotide containing a consensus NF-κB (Table 2) was incubated with an LPS-induced nuclear extract (Fig. 4). Therefore, it appears that NF-κB, or a closely related protein, binds to region II of the LRE.

Second, we investigated whether a member of the AP-1 family could bind to region I (−227 to −189) of the LRE. Nuclear extracts were incubated with M14/15 (Table 2), a double-stranded 35-bp oligonucleotide from the TF promoter that included both AP-1 sites. A specific protein-DNA com-

Table 2. *Oligonucleotides Used in This Study*

Oligonucleotides	
	-227 -193
M 14	5′-CGGTTGAATCACTGGGGTGAGTCATCCCTTGCAGG-3′
M 15	3′-GCCAACTTAGTGACCCCACTCAGTAGGGAACGTCCCAGG-5′
	-192 -172
M 19	5′-GTCCCGGAGTTTCCTACCGGG-3′
M 20	3′-GCCTCAAAGGATGGCCC-5′
	-192 -172
M 23	5′-GTCCCGGAGTTAGATACCGGG-3′
M 24	3′-GCCTCAATCTATGGCCC-5′
	-204 -175
M 17	5′-ATCCCTTGCACCTCGAGGAAGTTTCCTACC-3′
	-195 -166
M 18	5′-AGGGTCCCGGCCTCGAGGAACGGGAGGAGG-3
AP-1	Consensus 5′-GAGCCGCAAGTGACTCAGCGCGGGGCGTGTGCAGG-3′
NF-κB	Consensus 5′-CAGAGGGGACTTTCCGAGA-3′

Binding sites for AP-1 and NF-κB are underlined. Numbering of the 5′ → 3′ DNA strand is relative to the transcription start site of the TF promoter at +1. M14/15 represents region I and was used to examine AP-1 binding in EMSA; plasmids containing this region were used to assess the functional activity of the AP-1 sites in THP-1 cells. M19/20 represents region II and was used to study NF-κB binding and functional activity of this region. M23/24 contains a 3-bp substitution in the NF-κB binding site. The oligonucleotides M17 and 18 were used for site-directed mutagenesis. Oligonucleotides containing consensus binding sites for either NF-κB or AP-1 were used as positive controls in the EMSAs. The oligonucleotide containing a consensus NF-κB binding site was derived from the mouse Ig κ enhancer. The AP-1 consensus oligonucleotide was kindly provided by Dr. K. Kaushansky and was derived from the human metallothionein-II_A enhancer.

* Base pair substitutions differing from that of the wild-type TF promoter.

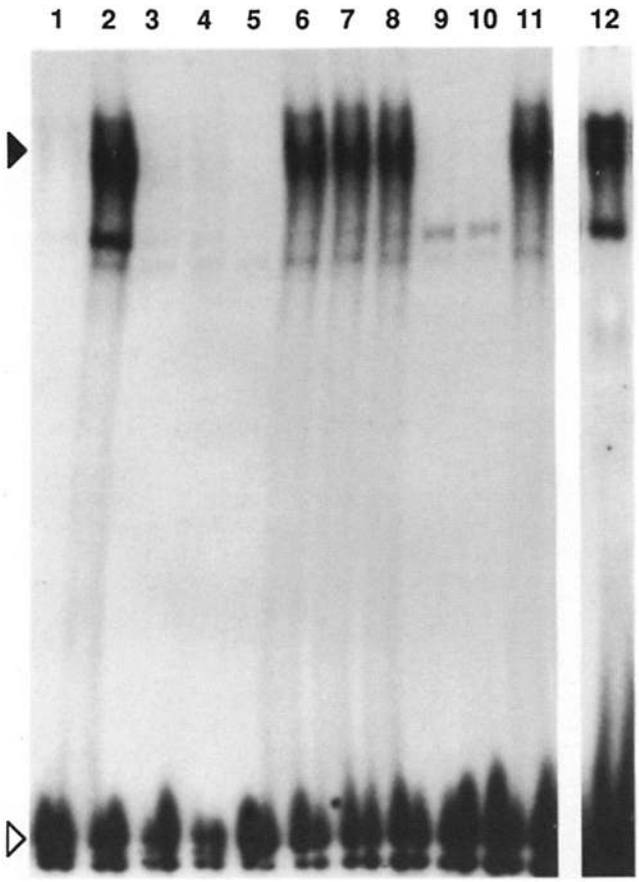

Figure 4. Electrophoretic mobility shift assay of NF-κB binding. Nuclear extracts were prepared from either control THP-1 cells or LPS-stimulated cells and incubated with a 21-bp oligonucleotide, M19/20 (Table 2). 50,000 counts of M19/20 were incubated with 10 μg of nuclear extract in the presence of 6 μg poly(dI·dC). Lane *1*, M19/20 with control extract; lane *2*, M19/20 with LPS-induced extract. Lanes *3–11* contain M19/20 with LPS-induced extract and various amounts of unlabeled competitors: lanes *3*, *4*, and *5* contain 25×, 50×, and 100× molar excess, respectively, of M19/20; lanes *6*, 7, and *8* contain 25×, 50×, and 100× molar excess, respectively, of M23/24, a mutated NF-κB site (Table 2); lanes *9* and *10* contain 25× and 50× molar excess, respectively, of the NF-κB consensus oligonucleotide. Lane *11* contains a 100× molar excess of an irrelevant oligonucleotide, M14/15. Lane *12* contains 10,000 counts of the NF-κB consensus oligonucleotide incubated with 7.8 μg of LPS-stimulated extract. The position of the protein-DNA complex corresponding to NF-κB binding is indicated with a filled triangle, whereas the free oligonucleotide is marked with an open triangle. The band below the NF-κB complex appears to represent a nonspecific complex. Lanes *1–11* were exposed for 48 h, whereas lane *12* was exposed for 16 h at −80°C with an intensifier screen.

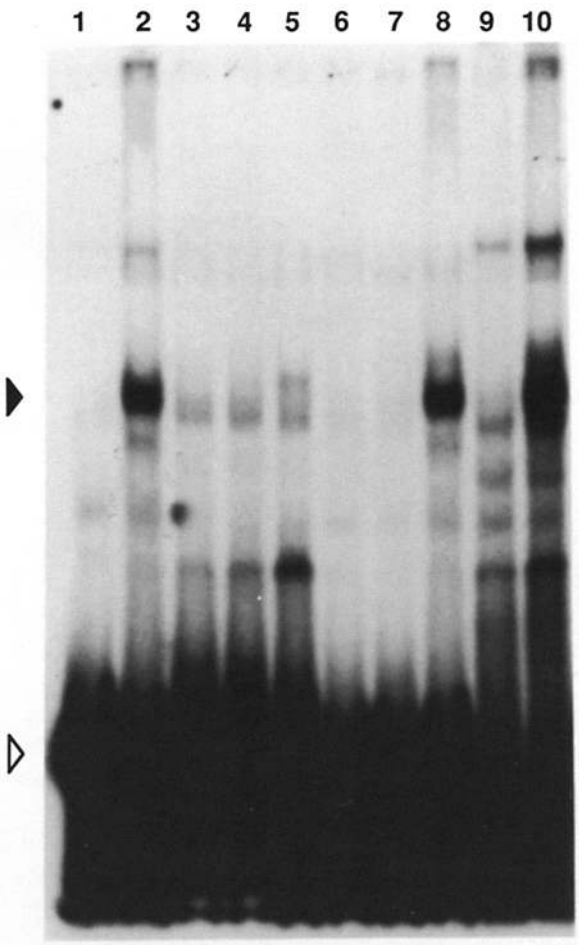

Figure 5. Electrophoretic mobility shift assay of AP-1 binding. Nuclear extracts were prepared from either control THP-1 cells or LPS-stimulated cells and incubated with a 35-bp double-stranded oligonucleotide, M14/15 (Table 2). 50,000 counts of M14/15 were incubated with 1.68 μg of nuclear extract in the presence of 6 μg poly (dI·dC). Lane *1*, M14/15 with control extract; lane *2*, M14/15 with LPS-induced extract. Lanes *3–7* contain M14/15 with LPS-induced extract and various amounts of unlabeled competitors: lanes *3*, *4*, and *5* contain 25×, 50×, and 100× molar excess, respectively, of M14/15; lanes *6* and 7 contain 25× and 50× molar excess, respectively, of the AP-1 consensus oligonucleotide (Table 2). Lane *8* contains 100× molar excess of an irrelevant oligonucleotide, M19/20 (Table 2). Lanes *9* and *10* contain 50,000 counts of the labeled AP-1 consensus oligonucleotide incubated with control and LPS-induced extract, respectively. The position of the protein-DNA complex proposed to contain AP-1 is indicated with a filled triangle, whereas the free oligonucleotide is marked with an open triangle. The autoradiogram was exposed for 16 h at −80°C with an intensifier screen.

plex was observed only when nuclear extract from LPS-stimulated cells was used (Fig. 5). This complex was specifically competed by an AP-1 consensus oligonucleotide (Table 2) but not by a 100× molar excess of an irrelevant oligonucleotide. Incubation of the AP-1 consensus oligonucleotide with the LPS-induced nuclear extract produced a similar complex (Fig. 5). These results suggest that LPS stimulation of THP-1 cells activates AP-1, or an AP-1-like protein, and allows binding to the LRE of the TF promoter.

Discussion

LPS is a potent pathogenic molecule in higher primates that is implicated in several disease states including septic shock (31). Inhibition of TF activity in vivo by a mAb has provided therapeutic benefit in a baboon model of *E. coli* bacteremia (9). We have previously shown that induced expression of TF in monocytes in response to LPS is controlled primarily at the level of transcription (6). Therefore, in the present study we have investigated the regulation of TF gene transcription in cells of monocytic lineage. We have identified a 56-bp LRE

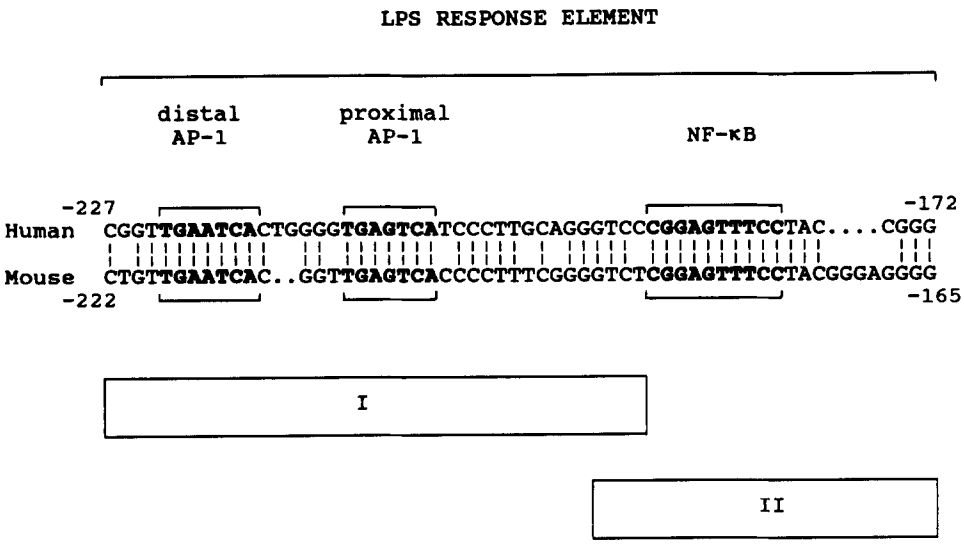

Figure 6. Conservation of the LPS response element from the human and mouse TF promoters. Binding sites for distal and proximal AP-1 sites and the NF-κB site are shown in regions I and II of the LRE. Numbering is from the start site of transcription at +1 (52).

within the human TF promoter that conferred LPS responsiveness to a heterologous promoter and functioned in a position and orientation-independent manner. The LRE was composed of two subregions, region I and region II (Fig. 6), which bind AP-1- and NF-κB-like proteins.

Deletion of region I or mutation of the distal AP-1 site within this region both decreased the level of LPS induction and indicated that this distal AP-1 site was required for full functional activity of the LRE in situ in the TF promoter. The proximal AP-1 site within region I appeared to have no major role in LPS induction, although the close tandem arrangement of these two AP-1 sites may suggest functional cooperativity. An AP-1-like transcription factor was present in LPS-induced nuclear extracts of THP-1 cells that specifically bound to region 1. The AP-1 family is involved in the transcriptional regulation of several inducible genes such as collagenase (32) and IL-2 (33).

Region II contains an NF-κB site that is essential for full functional activity of the LRE. Mutation of this NF-κB site abolished the ability of the 56-bp LRE to direct LPS induction of luciferase activity. Moreover, a 21-bp DNA fragment containing this site conferred LPS responsiveness to a heterologous promoter. A specific protein-DNA complex was observed in EMSA when this 21-bp oligonucleotide was incubated with nuclear extract from LPS-induced THP-1 cells, which was competed by a consensus NF-κB site. A recent study (34) is consistent with our data revealing that LPS stimulation of THP-1 cells activates NF-κB. Thus, engagement of this binding site in region II by NF-κB appears to be necessary to mediate full transcriptional induction of the TF promoter in response to LPS. NF-κB has been established as a member of the rel family of proteins, all of which display cytosolic-nuclear translocation (35, 36).

In the wild-type TF promoter the functional activity of the NF-κB site appears to be modulated by the binding of regulatory proteins to flanking or overlapping DNA sequences. We speculate that the NF-κB site may be negatively regulated by the binding of proteins to 3′ or overlapping sites, similar to the binding of a family of constitutively expressed C/EBP-like proteins to the NF-κB site in the angiotensinogen gene (37). In contrast, the presence of a protein bound to an upstream AP-1 site may stabilize the binding of NF-κB via protein-protein interactions. We hypothesize that the LRE is a combinatorial unit that requires the presence of both the NF-κB site, which may act as the "core," and the distal AP-1 site. We propose that assembly of a transcriptional enhancing complex involves cooperative interactions between proteins of the NF-κB and AP-1 families. It is of note that DNA binding of both of these proteins is regulated by phosphorylation (38, 39). The exact identity of the transcription factors that bind to this LRE will require further characterization using purified proteins and specific antibodies.

Isolation of the complete murine TF gene, including 5′ flanking sequences, has allowed identification of conserved regions of these two promoters that may serve regulatory roles. The overall sequence identity of regions between −300 and +1 was 64% (data not shown). In contrast, the 56-bp LRE exhibited 90% sequence identity between murine and human sequences with 100% conservation of the two AP-1 sites and the NF-κB site (Fig. 6). This supports our conclusion that the 56-bp region is a major regulatory element in the TF promoter.

In this study, we have shown that NF-κB is activated in THP-1 monocytic cells. This transcription factor has been implicated in the regulation of genes involved in inflammation, including IL-6, TNF, and IL-8. For instance, the NF-κB site within the human IL-6 promoter is required to mediate both LPS inducibility of the IL-6 gene in U937 monocytic cells (40) and the response to the cytokines IL-1 and TNF (41). LPS induction of the murine TNF gene involves several regions containing four NF-κB sites (42) that were shown to bind NF-κB (43). NF-κB- and NF-IL6-like proteins bound to two regions of the IL-8 promoter that conferred responsiveness to IL-1 and TNF (44). This 24-bp region of the IL-8 promoter contains an NF-IL6 site (45) immediately upstream of an NF-κB site and shows a similar organization to that

of the distal portion of the 56-bp LRE of the TF promoter. In contrast, other reports concluded that the NF-κB site is not a major contributory element for induction of the IL-6 gene (46). In fact, IL-1 responsiveness of the IL-6 promoter was found to be mediated by a separate NF-IL6 binding element (47). A recent study on the human TNF promoter concluded that three NF-κB sites were not involved in LPS induction, despite NF-κB binding to these sites (48). Moreover, activation of NF-κB alone in LPS-desensitized Mono-Mac-6 monocytic cells was not sufficient to induce transcription of the TNF gene (49). These discrepancies may reflect difficulties in defining combinatorial response elements. In addition, NF-κB-binding activity may not correspond to a single polypeptide but rather to a family of DNA-binding proteins (50), some of which may be modulated by other proteins to bind to lower affinity κB-related sequences (51).

This study using THP-1 monocytic cells has identified a 56-bp LRE that is composed of two distinct regions that are required to mediate LPS induction of the TF gene. This combinatorial element includes both AP-1 and NF-κB sites and may serve as a prototypic example for regulated assembly of transcription factor complexes. NF-κB sites could represent common *cis*-acting elements that are required for the activation of genes encoding inflammatory mediators. As postulated from the present studies, the context of NF-κB sites within these various promoters appears to dictate their regulation. We propose that NF-κB sites act synergistically with other binding sites to assemble two or more transcription factors in a supramolecular complex to mediate full transcriptional activation.

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