**KB-type Enhancers Are Involved in Lipopolysaccharide-Mediated Transcriptional Activation of the Tumor Necrosis Factor α Gene in Primary Macrophages**

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The activation of differentiated macrophages during an inflammatory reaction is accompanied by an increase in the transcription of many genes whose products are necessary for these cells’ effector functions, and by an accumulation of the corresponding mRNAs (1–3). Tumor necrosis factor (TNF-α, cachectin) is a major cytokine produced by macrophages. Among its pleitropic effects, TNF-α is a potent inducer of a variety of inflammatory reactions, and plays a major role in the development of endotoxic shock (4–6). Although TNF can also be produced by lymphocytes (7–10), macrophages seem to be its most abundant source.

The mouse TNF locus, which is located at the boundary of the class III and class I regions of the MHC, comprises two very closely linked genes, coding for TNF-α and TNF-β (lymphotoxin) (11–13). We have previously shown that the TNF-β promoter is silent in macrophages, while the TNF-α promoter is inducible by LPS and IFN-γ (2, 3, 10). In the present study, we attempted to map the DNA sequence elements that are required for LPS inducibility of the TNF-α promoter in cultured primary macrophages, and to identify LPS-induced factors that may bind to them. We found evidence suggesting major roles in LPS-mediated transcriptional activation for one or more enhancers of the "KB" family, and for a Y box similar to that found in the promoter of MHC class II genes.

Materials and Methods

Construction of DNA Clones. The plasmids containing the bacterial chloramphenicol acetyl transferase (CAT) gene linked to the SV40 early promoter (pSV2CAT), the promoterless pSV0CAT, and the pBLCAT-3 and pBLCAT-2 vectors, have been previously described (14, 15). The pSV2GH plasmid, with the human growth hormone (HGH) gene under the control

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Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; HGH, human growth hormone.
of the SV40 early promoter, was generated by inserting a 557-bp Hind III to Nde I fragment from pSV2CAT into pOGH (16) cut with Hind III and Nde I.

All of the TNF promoter constructs used in this study were derived from a λEML3 genomic clone containing most of the mouse (strain C57B1/6) TNF locus (11, 17) (Fig. 1). Bam HI to Nar I or Sac I to Nar I genomic fragments were cloned into the pBLCAT3 vector to generate TNF(-1059)CAT and TNF(-695)CAT, respectively. An Eco RI to Nar I genomic fragment (nt -655 to +138 relative to the TNFα cap site) was cloned in pGEM3 (Promega Biotec, Madison, WI), and deletions were generated by successive digestions with Eco RI, exonuclease III (New England Biolabs, Beverly, MA), Mung bean nuclease (Pharmacia, Uppsala, Sweden), and Hind III, and recloning of size-selected DNA fragments into pGEM3 cut with Sma I and Hind III. The deleted promoter fragments were then introduced (as Hind III to Eco R I fragments) into the Hind III site of pSVOCAT. The endpoints of the deletions were established by sequencing the TNF promoter/CAT hybrids (Fig. 5). Further sequencing detected the presence of a short (170 nt) piece of extraneous vector DNA 3' of the TNF sequence in the deletion mutants, which was found not to influence promoter activity (data not shown).

The αB/TK constructs were made by inserting a double-stranded oligonucleotide containing a mouse TNFα αB enhancer (Fig. 5; site 3 in Figs. 1 and 7) and Hind III cohesive ends into the Hind III site of the pBLCAT2 vector. The sequence of the oligonucleotidewas as follows:

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5'AGCTCAAAACAGGGGGCTTTCCCTCCTC
3'
3' GTTTGTCGCCCCGCCAGGGGAGGAGTCGA5'
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The copy number and orientation of the inserted oligonucleotides were established by sequencing.

S1 Nuclease Mapping. Single strand-specific nuclease protection mapping was done by standard protocols, using 500 U of S1 or Mung bean nucleases (Pharmacia) and 5 μg of total cytoplasmic RNA per hybridization.

Transfection of Bone Marrow-derived Macrophages. Macrophages were generated from bone marrow cells of C57B1/6 mice (Iffa Credo, L'Arbresle, France) by cultivating them for 10 d in Dulbecco's minimal essential medium (DMEM) supplemented with 20% horse serum and 30% L cell-conditioned medium (a source of macrophage [M]-CSF). These cell preparations contain >99% macrophages, as judged by standard histochemical techniques (18). For transfections, cells were washed twice in serum-free DMEM and resuspended at 10^7 cells/ml in DMEM containing 250 μg/ml DEAE-dextran (mol wt ~500,000, Pharmacia) and 50 mM

![Figure 1](https://example.com/figure1.png)

Figure 1. Structure of the mouse TNF locus. Numbering of the sequence is according to Semon et al. (17). The coding parts of the exons are shaded. The region of the locus used for studies of the TNF-α promoter and its salient features are marked above the map.
Tris/HCl (pH 7.5). After addition of DNA (5 μg/10⁷ cells), the cells were incubated for 90 min at 37°C with occasional shaking to avoid clumping. DMSO was added to a final concentration of 10%, the cells were kept for 2-3 min at room temperature, and diluted 10-fold with DMEM. After two washes in DMEM, the cells were returned to culture in complete medium. LPS (form W from Escherichia coli 055:B5; Difco Laboratories, Detroit, Michigan) was added between 24 and 36 h after transfection. CAT assays were performed on crude cell extracts 48 h after transfection, using ³²P-labeled chloramphenicol (Amersham plc, Amersham, UK) as a substrate, and thin-layer chromatography to separate the native and acetylated forms (14). The culture medium was assayed for human growth hormone using a sandwich RIA (Nichols Institute, San Juan Capistrano, CA).

Preparation of Nuclear Extracts from Peritoneal Macrophages. Peritoneal exudate cells were prepared from 3-mo-old CBA/CA mice 4 d after a single 1.5-ml i.p. injection of aged thioglycolate broth (Difco Laboratories). To obtain activated macrophages, 0.5 ml of LPS (20 μg/ml in PBS) was injected intraperitoneally 45 min before harvesting the total exudate population. Nuclei were obtained by breaking cells swollen in hypotonic buffer (10 mM Hepes, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM DTT, 5 mg/ml powdered nonfat milk, 0.5 mM PMSF, 1% (wt/vol) aprotinin, and 50 μg/ml each pepstatin A, leupeptin, chymostatin, and antipain) with three strokes of a B pestle in a Dounce homogenizer. The same cocktail of protease inhibitors was added to all subsequent buffers. Preparation of nuclear extracts essentially followed the procedure of Gorski et al. (19).

Gel Retardation Assays. For binding reactions, 0.1-1 ng of radiolabeled fragment (10,000-20,000 cpm) was mixed with 12 μg (protein) of nuclear extract in a total volume of 20 μl of 25 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 12% (vol/vol) glycerol, 40 mM KCl, 2 μg of poly(dI:dC) and 0.5 μg of denatured E. coli DNA were added to all reactions as nonspecific competitors. After 30 min of incubation at room temperature, the reaction mixtures were loaded on 4% polyacrylamide gels in low ionic strength buffer (0.25 x TBE). The gels were pre-run for at least 1 h at 20 V/cm and run for 3 h at the same voltage. After electrophoresis, the gels were dried and exposed for autoradiography for a few hours.

Results

Mapping of the 5' End of TNF-α mRNA. To have a reference point for the location of the TNF-α promoter elements, we precisely mapped the mRNA 5' end in single strand-specific nuclease protection assays. We 5' end-labeled a Xma III (nt 4339) to Nar I (nt 4498) fragment at the Nar I site and hybridized it to total cytoplasmic RNA extracted from resting or LPS-stimulated peritoneal macrophages. The sizes of the fragments protected from Mung bean or S1 nuclease digestion were compared with an A+G ladder of the probe (Fig. 2; the heavy band near the top of the gel corresponds to undegraded probe). The result shows some heterogeneity in the 5' end of the mRNA, with one major and three minor initiation sites. Three of the four sites contain the canonical CA dinucleotide found at the cap sites of most eukaryotic mRNAs. We used the major start site (nt 4360) as position +1 in a zero-less numbering scheme.

Transient Expression of Transfected DNA in Bone Marrow-derived Macrophages. Primary macrophages accumulate large amounts of TNF-α mRNA after exposure to LPS, and this accumulation is due at least in part to increased transcription of the TNF-α gene (2, 3, 10, 20). Bone marrow-derived macrophages could be successfully transfected (as judged by CAT expression from the pSV2CAT plasmid) up to 12 d after initial culturing. Transfection with the pSV2GH plasmid resulted in the secretion of significant amounts (up to 60 ng/ml) of HGH into the culture medium. In subsequent experiments, we co-transfected test plasmids containing the CAT reporter gene.
with pSV2GH, and controlled transfection efficiencies by measuring the HGH concentration in the culture medium. Co-transfection with pSV2GH affected neither the level of expression nor the inducibility of the TNF/CAT constructs (data not shown).

LPS-inducible Promoter Activity of 5' Deletions of the TNF-α Upstream Region. We constructed a series of nested deletions of the TNF-α 5' flanking region, starting at nt -1059, and with a common 3' end at nt +138, before the TNF-α initiation codon. These promoter fragments, coupled to the CAT coding region (see Materials and Methods), were co-transfected with the pSV2GH plasmid into bone marrow-derived macrophages, and cell extracts were assayed for CAT activity 2 d later. The results of one such experiment are shown in Fig. 3. Plasmids TNF(-1059)CAT, TNF(-695)CAT and TNF(-655)CAT had a strongly LPS-inducible promoter activity, which was greatly reduced in TNF(-451)CAT, and in all subsequent deletions. A second drop in inducibility by LPS occurred between nt -301 and nt -241 (Fig. 3). Constructs containing less than 241 nt of the TNF-α promoter were no longer able to respond to LPS. Although the level of inducibility by LPS varied between experiments, the reductions in LPS-induced expression between nt -655 and -451 and between nt -301 and -241 were observed in four independent experiments. Within the range (nt -1059 to -109) that we covered with our set of deletion mutants, there was no evidence indicating the presence of negative regulatory elements.

Sequence-specific DNA-binding Activities Induced by LPS. Using the gel retardation assay, we tested whether proteins capable of binding to the TNF-α upstream sequences defined above were induced by LPS in nuclear extracts of peritoneal macrophages. End-labeled restriction fragments spanning nt -695 to -432 (Sac I to Nco I) and
Figure 3. Inducibility of TNF-α promoter/CAT constructs by LPS. Macrophages were transfected with TNF/CAT constructs and pSV2GH as described in Materials and Methods, and split into control and LPS-induced (100 ng/ml) samples 24 h after transfection. CAT assays were performed on crude cell extracts 16 h later, and transfection efficiencies were monitored by measuring the concentration of HGH in the culture medium. The $\kappa B/TK$ plasmids were constructed by inserting a $\kappa B$-containing oligonucleotide upstream of the HSV TK gene of the pBLCAT2 vector, as described in Materials and Methods. Their structure is as follows, with (+ or −) indicating the orientation of the oligonucleotide relative to that found in the TNF-α gene: $\kappa B/TK$ 1 (−X+X−); $\kappa B/TK$ 3 (−); $\kappa B/TK$ 5 (X+X+X+X+X+X+); $\kappa B/TK$ 10 (−X+). The relative amounts of acetylated chloramphenicol generated by LPS-stimulated transfectants, determined by densitometry and corrected for protein in the extracts, were as follows: TNF(-1059)CAT: 91.2; TNF(-695)CAT: 85.0; TNF(-655)CAT: 77.4; TNF(-451)CAT: 14.7; TNF(-403)CAT: 11.6; TNF(-361)CAT: 15.9; TNF(-301)CAT: 28.6; TNF(-241)CAT: 15.9; TNF(-197)CAT: 2.1; TNF(-144)CAT: 2.5; TNF(-109)CAT: 1.8; $\kappa B/TK$ 1: 212.9; $\kappa B/TK$ 3: 120.4; $\kappa B/TK$ 5: 52.7; pBLCAT2: 3.9; pSV2CAT: 104.7.

−316 to −161 (Ava II to FnuD II), were used to assess the presence of DNA-binding proteins in nuclear extracts of resting versus LPS-activated cells. As a control for the DNA-binding activity of the nuclear extracts, we used a fragment of the c-fos proto-oncogene (nt −350 to −270; Pst I to Xma III) to which no new proteins bind after LPS induction (Collart, M. A., unpublished result). Fig. 4 shows the gel retardation patterns obtained with these three fragments, using nuclear extracts from untreated (R) and LPS-activated (L) peritoneal macrophages. Extracts from bone marrow-derived macrophages produced essentially identical patterns (data not shown). The −695 to −432 fragment formed two complexes (II and IV) with extracts from resting macrophages, and three additional major complexes (Ia, Ib, and III) with extracts from LPS-induced macrophages. The same results were obtained when using
a fragment spanning nt -655 (Eco RI) to -432 (Nco I). With the -316 to -161 fragment, no new complexes were seen in extracts from LPS-activated cells; however, we could reproducibly see a two- to threefold increase in the amount of the slowest-migrating complex formed with this fragment (Fig. 4). The c-fos SRE-containing fragment yielded identical patterns with extracts from resting or activated macrophages. All these complexes could be competed out by cold DNA from the same fragment used for labeling, but not by irrelevant DNA (data not shown).

The nucleotide sequence of the region between nt -655 and -427 (Fig. 5) contains two sequence elements with strong similarity to the κB enhancer originally found in the immunoglobulin κ chain gene. Additionally, the sequence around nt -250 is very similar to the MHC class II “Y box”. In order to test whether DNA-protein contacts within the complexes involved these consensus sequences, we added competing double-stranded oligonucleotides containing these sequences to the gel retardation assays. In the experiment depicted in Fig. 6 A, we analyzed the effects of added oligonucleotides on DNA-protein complexes generated with the -695 to -432 fragment. An oligonucleotide spanning nt -521 to -499 (κB; site 3 in Figs. 1 and 7) effectively competed for the formation of complexes Ia, Ib, and III, less well for complex II, and not at all for complex IV. A second oligonucleotide spanning nt -669 to -636 (CK-I; site 2 in Figs. 1 and 7) completely inhibited the formation of complex IV, and partially inhibited complexes Ia and Ib. An oligonucleotide spanning nt -263 to -223 (Y box) had no effect on protein binding to this fragment. The single protein-DNA complex mapping to the -316 to -161 fragment was effectively competed by the Y box oligonucleotide, but not by the κB oligonucleotide (Fig. 6 B). We conclude from these experiments that all of the detectable DNA-protein complexes mapping between -695 and -161 involve sequences from three sites: two κB-like enhancers, and a MHC class II Y box. A detailed analysis of these DNA-protein interactions will be published elsewhere (Collart, M. A., and P. Vassalli, manuscript submitted for publication).

The TNF-κ κB-type Enhancer Confers LPS Responsiveness to a Heterologous Promoter. To test whether the κB-like element centered around nt -510 (site 3) has an LPS-inducible

**Figure 4.** Gel retardation assays using TNF-κ promoter fragments and nuclear extracts from peritoneal macrophages. Restriction fragments from the TNF-κ or c-fos promoters were endlabeled and incubated with nuclear extracts from resting (R) or LPS-stimulated (L) peritoneal macrophages. Unmarked lanes contain fragments incubated without extracts. Gel retardation assays were performed as described in Materials and Methods.
Figure 5. Sequence of the TNF-α upstream region. The rabbit, human, and mouse upstream regions were aligned using the LineUp editor (UWGCG software package). The sequence of the rabbit upstream region was partly determined by us (Shakhov, A. N., et al., manuscript in preparation), and partly taken from published sequences (34). The human sequence is from reference 35, and the mouse sequence from references 13, 17, and 36, but corrected to reflect more recent sequencing data (EMBL/GenBank data libraries accession number Y00467). Regions of high sequence conservation are shaded, and the end points of the 5' deletions and restriction fragments used in this study are marked.
enhancer activity, we constructed a series of plasmids containing this element coupled to an enhancerless herpes simplex virus thymidine kinase (TK) promoter, and tested them for inducibility by LPS after transfection into bone marrow macrophages. The pBLCAT2 parental plasmid and the hybrid κB/TK 3 (one copy of the κB enhancer) were unresponsive to LPS (Fig. 3). The κB/TK 10 hybrid (two copies) was weakly inducible by LPS, while plasmids κB/TK 1 (three copies) and 5 (six copies) were inducible to a level higher than TNF(-1059)CAT, which contains 1,059 bp of TNF-α 5' flanking sequence.

Discussion

The data presented above demonstrate that primary macrophages derived from bone marrow cells could be transfected with exogenous DNA, that this DNA was expressed, and that LPS inducibility was maintained for the transiently expressed TNF-α promoter. There was a good agreement between the data obtained from the transient expression of deletion mutants, and those demonstrating LPS-induced
sequence-specific DNA binding activities. The region between nt -655 and nt -451 was required for maximal LPS induction, and fragments spanning nt -655 to -427 or -695 to -427 formed distinct new complexes with LPS-induced proteins. These complexes involved protein binding to sequences centered around nt -650 and -510. Additional deletion of the region between nt -301 and nt -241 abrogated inducibility; similarly, using a fragment spanning nt -316 to -161, we could see a two- to threefold LPS-mediated increase in the concentration of a specific protein-DNA complex that maps to a MHC class II Y box.

Fig. 5 shows the sequence of the TNF-α promoter region, with the potential regulatory elements identified in this study. The region between nt -521 and nt -499 contains an element with close similarity to the Ig κ light chain enhancer, the binding site for the NF-κB protein (21, 22), and to the related enhancers found in the MHC class I, β2-microglobulin, β-interferon, IL-2 receptor α chain, and IL-2 genes, in the long terminal repeats (LTRs) of HIV-1 and HTLV-1, and in the early enhancers of SV40 and human CMV (21, 23–25). There is ample evidence for the functional importance of the κB-related enhancers in all of these genes (21–26). We have found that two or more copies of the TNF-α κB site conferred LPS inducibility upon a heterologous promoter.

In addition, three other potential κB enhancers are found within 1 kb of the mRNA initiation site. The first (nt 862 to -852; site 1 in Figs. 1 and 7) conforms closely to the κB consensus. The two others (nt -659 to -649, site 2, and -208 to -198, site 4) are related to the “cytokine-1” motif found in the IL-2, IL-3, G-CSF, and GM-CSF promoters (27), and are located in regions of high conservation between the rabbit, human, and mouse sequences (Fig. 5). At least one nuclear protein bound to site 2 in the TNF-α promoter (Fig. 6). It has recently been shown that the “cytokine-1” site found in the IL-2 promoter actually binds the same proteins as the κB-type enhancer of the IL-2 receptor α chain promoter (25). Therefore, it is likely that the “cytokine-1” motif is in fact a variant of the κB enhancer. Fig. 7 shows a compilation of enhancer motifs originally assigned to either the κB or the “cytokine-1” classes, including those from the mouse TNF genes. The most striking features emerging from this compilation are: (a) the clear separation of purines (boldface) and pyrimidines (italics) in the enhancer motif; (b) the presence of three essentially invariant nucleotides.

The presence of one copy of the κB enhancer is probably not sufficient for LPS-inducible expression in macrophages, since the TNF-β gene, which contains a single copy of the motif, is not expressed in these cells, either naturally or in our transfection assay, and since the κB/TK construct with a single copy of the enhancer was not inducible either. Therefore, it is likely that more than one κB-type enhancer participates in activation of the TNF-α promoter. This interpretation is also consistent with the results of our deletion analysis, where loss of the second and third potential κB sites correlated with loss of LPS inducibility, and with preliminary data indicating that the deletion of a single κB site was not sufficient to abolish LPS inducibility.

A recent study (28) suggested that κB-type enhancers may be active in all mature macrophage populations; this is consistent with our earlier observation of a basal activity of the TNF-α promoter in resting macrophages (2, 10). However, our κB/TK constructs were expressed at very low levels in bone marrow-derived macrophages,
but could be fully activated by as little as 10 ng/ml LPS (data not shown). Therefore, we would argue that exposure to LPS or similar macrophage-activating agents is necessary for maximal expression of genes controlled by κB-type enhancers.

The region between nt −301 and −241, where residual LPS inducibility was lost in the 5′-deletion series, contains only a Y box, similar to those found in the promoters of MHC class II genes (29–31), and a (CA)₉ microsatellite. In addition, LPS induced an increase in the concentration of a protein binding to the Y box (nt −263 to −223). This indicates that the Y box may be important for the induction of TNF-α transcription, and that there may be common elements involved in the regulation of TNF-α and MHC class II genes. Experiments exploring the expression of MHC class II promoter mutants in transgenic mice suggest that the Y box has a particular importance for macrophage-specific gene expression, and for inducibility by IFN-γ (29, 30). It should be stressed, however, that LPS alone not only fails to induce MHC class II gene expression, but actually inhibits it (32). Therefore, it is very unlikely that the Y box alone could confer LPS inducibility. The presence of a Y box in the TNF-α, but not the TNF-β promoter argues for a role of this element in macrophage-specific expression.

A recent article (33) reported that 95 nt of the human TNF-α promoter region are sufficient for full induction by PMA in the U937 promonocytic cell line. These 95 nt do not contain either of the κB-like elements found in the human promoter (Fig. 5). On the other hand, a NF-κB-like protein is induced by PMA in U937 cells (28). It is conceivable that PMA, but not LPS, can trigger a κB-independent activation pathway; alternatively, the human gene may contain promoter proximal enhancer elements not found in the mouse. The latter explanation seems less likely, as the nucleotide sequence between −200 and +1 is highly conserved between species (Fig. 5).

The data presented in this article provide additional evidence for the ubiquitous role of NF-κB and related proteins as transducers of extracellular signals affecting the physiological state of a variety of cell types (26). In macrophages, NF-κB seems to have a central role in the initial activation event triggered by LPS, and in the transcriptional activation of the TNF-α gene. Other signals, carried by proteins interacting with the Y box, are probably also involved in modulating the activity of the TNF-α promoter.

Summary

We have explored the cis-acting elements necessary for the LPS-mediated activation of the mouse TNF-α promoter by transfecting a set of 5′ deletion mutants linked to the CAT reporter gene into primary bone marrow-derived macrophages. A major drop in inducibility by LPS was seen upon deletion of a region mapping between nt −655 and nt −451. Gel retardation assays revealed that LPS induced the appearance in this region of several specific DNA-protein complexes mapping to sequence motifs with strong homology to the κB enhancer. Constructs containing two or more copies of one of the κB enhancer motifs linked to a heterologous promoter were inducible by LPS. Additional deletion of a region between nt −301 and nt −241, which contains a MHC class II-like "Y box" and formed a Y box-specific complex with
a protein whose concentration was increased by LPS, caused a nearly complete loss of inducibility by LPS. We speculate that NF-κB and/or related proteins are involved in the LPS-induced transcriptional activation of the TNF-α gene, and that factors interacting with the Y box can additionally modulate the activity of the gene in macrophages.

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