Lipopolysaccharide-Inducible and Salicylate-Sensitive Nuclear Factor(s) on Human Tumor Necrosis Factor Alpha Promoter

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Lipopolysaccharide (LPS) is one of the most potent trigger substances for monocytes and macrophages causing secretion of tumor necrosis factor alpha (TNF- α) and other inflammatory mediators. The nature of the nuclear factors involved in human TNF- α gene regulation is still unknown. Nuclear factor kappa B (NF- κ B) proteins have been suggested to play an important role in gene transcription of inflammatory mediators when monocytes are stimulated with LPS. However, it remains unclear whether these nuclear factors are the only ones involved in human TNF- α gene regulation. In this report, to further the identification of nuclear factor(s) involved in TNF- α gene regulation, human monocytic THP-1 cells were transfected with a series of truncated versions of human TNF- α promoter. A 98-bp region located from nucleotides –584 to –487 demonstrated strong promoter activity. Electrophoretic mobility shift assays demonstrated that a 64-bp fragment located within the 98-bp region and lacking any potential NF- κ B-binding sites avidly bound LPS-challenged THP-1 nuclear protein. Although this binding was inhibited in salicylate-treated cells, as was binding of NF- κ B, the pattern of binding was found to differ from that noted for NF- κ B. Analysis of this 64-bp fragment disclosed the absence of an NF- κ B consensus sequence, suggesting a novel nuclear DNA-binding protein necessary for the initiation of human TNF- α transcription other than, or in addition to, NF- κ B.

Lipopolysaccharide (LPS) is a major component of the gram-negative bacterial outer membrane (30) and one of the most potent stimulators of monocytes and macrophages for the synthesis of a variety of inflammatory mediators, including tumor necrosis factor alpha (TNF- α) (24). TNF- α exhibits pleiotropic effects on different tissues and cells in vivo as well as in vitro (3, 4). A dose-dependent pattern of TNF- α biological activity has been widely demonstrated. Yet, overproduction of TNF- α has been shown to be deleterious for the host, with associated pathology including cachexia, irreversible organ failure, and death (40). Therefore, TNF- α gene expression must be tightly regulated. In inflammatory and immunological processes, TNF- α induces the expression of cell adhesion molecules (6, 27, 28), secretion of cytokines such as interleukin-8 (37), interleukin-6 (29), and macrophage-specific chemotaxin (12). These molecules may promote the local recruitment and tissue infiltration of neutrophils and immunocompetent cells such as T and B lymphocytes, monocytes, and macrophages and may modulate bone turnover. TNF- α is known to be produced in abundance by activated monocytes and tissue macrophages; however, the signaling pathways by which LPS stimulates monocyte/macrophage activation are still unknown.

TNF- α biosynthesis is controlled at multiple levels. In response to LPS, TNF- α gene transcription is increased approximately threefold. In addition, TNF- α mRNA levels rise by a factor of 100, indicating increased stability of the message. Furthermore, TNF- α secretion, which is undetectable in quiescent cells, rises by a factor of about 10,000 after LPS challenge (15). However, under specific circumstances, TNF- α

mRNA may be detected within cells in the absence of TNF- α protein production or release (5, 10, 16). The regulation of TNF- α gene expression has been studied under various conditions. The TNF- α gene is transcriptionally active in thioglycolate-elicited mouse macrophages, as demonstrated by Northern (RNA) blot analysis (5, 10). However, the TNF- α mRNA is not translated into protein (5), indicating a regulation, at least in part, at the translational level. Furthermore, it has been shown that TNF- α gene expression is controlled at the transcriptional level in resting human monocytes (20).

Gene transcription has been shown to be controlled by DNA-binding proteins (31). Nuclear factor kappa B (NF- κ B) was suggested to be an important factor for TNF- α gene transcription in LPS-challenged monocytes and macrophages because of the existence of NF- κ B-binding motifs in the human TNF- α promoter region (25, 26) and its translocation to the nuclei of LPS-stimulated monocytes (11, 23, 42). However, the evidence for the involvement of NF- κ B in TNF- α gene transcription is still controversial. It has been demonstrated that mutation(s) or deletion(s) of NF- κ B-binding motifs on the TNF- α promoter failed to show reporter gene activation in transfected cells (9, 13, 33). However, independent studies pertaining to the regulation of the human TNF- α promoter in transfected cell lines found no evidence for LPS-inducible enhancer activity of the NF- κ B-binding motifs (17). Drouet et al. (13) discussed possible explanations for this discrepancy between the regulation of the mouse TNF- α gene by NF- κ B and that of the human gene. They suggested that enough NF- κ B is constitutively expressed to sustain a high-level baseline expression of the human TNF- α gene, as in the study by Goldfeld et al. (17), and that significant differences between the regulation mechanisms of the mouse and human TNF- α promoters exist. Therefore, the nature of the nuclear factor(s) involved in human TNF- α gene regulation needs better clarification.

Anti-inflammatory drugs such as salicylate have been recently suggested to play an important role in gene transcription (19). Particularly, sodium salicylate and aspirin were shown to be able to inhibit NF- κ B activation by preventing the degra-

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dation of NF- κ B inhibitor I κ B, blocking thereby the translocation of NF- κ B in the nuclear compartment. Because salicylates are anti-inflammatory drugs that also appear to affect transcription, we examined the effect of sodium salicylate on the activity of a purported nuclear factor in TNF- α gene regulation. The first segment of our work consisted of sequencing the human TNF- α promoter, in which we have reported the existence of several potential binding sites specific to human gene regulatory proteins other than NF- κ B that are inducible by LPS (38).

The purpose of our study was to further the identification of the DNA-binding protein(s) involved in LPS-induced human TNF- α gene transcription, with the human monocytic cell line THP-1 (41) as a model. THP-1 cells were transfected with a series of truncated versions of the human TNF- α promoters fused to a reporter gene. A 98-bp region located from nucleotides (nt) -584 to -487 demonstrated a strong promoter activity. Furthermore, a 64-bp domain within the 98-bp region, lacking any potential NF- κ B-binding sites, was found to bind avidly to LPS-challenged THP-1 nuclear protein extracts. Analysis of this domain demonstrated a lack of any NF- κ B consensus binding sequences. Finally, salicylate was found to modulate the 64-bp region-binding LPS-induced nuclear protein activity as for NF- κ B (19). Taken together, our data suggest that other transcription factors probably acting in synergism with NF- κ B are involved in human TNF- α gene transcription in THP-1 cells.

MATERIALS AND METHODS

Cells. The human monocytic cell line THP-1 was purchased from American Type Culture Collection (Rockville, Md.). Cells were maintained in complete RPMI (C-RPMI) (RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], penicillin [100 U/ml], and streptomycin [100 μ g/ml]) and 5% fetal bovine serum (all from GIBCO, Gaithersburg, Md.). LPS-free tissue culture reagents were used throughout of the experiments.

Plasmids, transfection, and CAT assay. Serial 5' deletions of human TNF- α promoter (nt -1052 to +126) (38) were subcloned into pCAT-Enhancer plasmid (Promega, Madison, Wis.) which has a simian virus 40 enhancer and chloramphenicol acetyltransferase (CAT) gene. A complex of promoter sequences of cytomegalovirus (CMV) and human T-cell leukemia virus 1 long terminal repeat (HTLV-1 LTR) obtained from CMV-LTR-CAT (7) was inserted into pCAT-Enhancer, and was used as a positive control. The β -galactosidase gene was inserted into pRC/CMV (Invitrogen, San Diego, Calif.), which has a promoter sequence for CMV and the polyadenylation signal and transcription termination sequences from the bovine growth hormone gene, and was used as an internal control. All of the plasmid DNAs were purified by using a plasmid purification kit (Qiagen, Düsseldorf, Germany), sterilized with ethanol, dissolved in sterilized TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) at a 1- μ g/ μ l concentration, and kept at -20°C.

Cells were collected by centrifugation, washed once with RPMI 1640 medium, resuspended in ice-cold RPMI 1640 medium supplemented with 10% fetal calf serum (5×10^6 cells per 0.25 ml per transfection), and then put on ice until use. The suspended cells were transferred to a cuvette (0.4-cm gap), 20 μ g of test plasmid and 5 μ g of internal control plasmid were added, and the mixture was kept on ice for 10 min. The cells were electroporated with a Bio-Rad Laboratories (Hercules, Calif.) Gene Pulser set at 250 V and 960 μ F and then were kept on ice for 10 min. The electroporated cells were then transferred to a six-well plate with 4.75 ml of C-RPMI supplemented with 10% fetal calf serum per well. LPS (34) was added at 100 ng/ml, and the cells were incubated for 15 to 17 h.

The plate was put on ice for 5 min, and cells were harvested by scraping gently with a cell lifter (Costar, Cambridge, Mass.) before transfer to a 15-ml centrifuge tube for centrifugation. After the medium was aspirated, the cells were washed with 10 ml of phosphate-buffered saline (PBS; 2.7 mM KCl, 1.2 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , 138 mM NaCl [pH 7.4]), resuspended in 1 ml of PBS, and transferred to a 1.5-ml microcentrifuge tube. After brief centrifugation and aspiration of PBS, cells were resuspended in 100 μ l of Tris-HCl buffer (0.25 M, pH 8.0) and were lysed by three freeze-thaw cycles. The cell lysates were assayed for β -galactosidase activity with a kit (Promega) and for CAT activity by a simple phase-extraction method following the protocol of Kingston and Sheen (18). The normalized TNF- α promoter activity was expressed in arbitrary units of CAT activity divided by β -galactosidase activity. Induction fold was obtained by dividing the normalized CAT activity of the LPS-challenged transfected cells by the normalized CAT activity of the LPS-free transfected cells. All transfection and

FIG. 1. Identification of regions in the human TNF- α promoter including nt +126, downstream of the transcription starting site important for induction by LPS. The map shows putative NF- κ B-binding site homology of >80% (shaded boxes, with darker shading indicating higher homology). THP-1 cells were transfected with 25 μ g of DNA and were treated immediately after transfection with 100 ng of LPS per ml. Cell lysates were analyzed for CAT activity 15 to 17 h later. Induction fold was obtained by dividing the normalized CAT activity of the LPS-challenged transfected cells by the normalized CAT activity of the LPS-free transfected cells. The mean values and standard errors (T bars) were obtained from two individual experiments using same DNA preparation. SV40, simian virus 40.

CAT assays were performed in two independent experiments with the same DNA preparations.

Electrophoretic mobility shift assay (EMSA). Cells (5×10^6 cells per 5 ml per well) were cultured with various concentrations of sodium salicylate (Sigma, St. Louis, Mo.) to inhibit NF- κ B activation (19) in C-RPMI supplemented with 5% human serum for 15 min and then were directly stimulated with 100 ng of LPS per ml (34) for 2 h. The concentrations of sodium salicylate used in this study did not alter the amount of total cellular protein in a well.

Nuclear and cytoplasmic extracts were prepared by the combination of the methods described previously (1, 32), with modifications. After being washed with cold PBS, cells were suspended in 400 μ l of hypotonic buffer (10 mM HEPES, pH 7.9, at 4°C; 1.5 mM MgCl_2 ; 10 mM KCl; 5 $\times$ proteinase inhibitor mix [4-amidinophenyl-methanesulfonyl fluoride (20 μ g/ml), antipain (25 μ g/ml), aprotinin (20 μ g/ml), nitrobestatin (5 μ g/ml), chymostatin (20 μ g/ml), 3,4-dichloroisocoumarin (25 μ g/ml), E-64 (50 μ g/ml), leupeptin (10 μ g/ml), and pepstatin A (10 μ g/ml), phosphoramidon (10 μ g/ml), 50 μ M benzamide, 50 μ M sodium metabisulfite]; 1 mM phenylmethylsulfonyl fluoride; 1 mM dithiothreitol) on ice for 15 min. Cytoplasmic extracts were obtained in 50 μ l of replaced hypotonic buffer with 0.6% Nonidet P-40. Nuclear extracts were obtained in 50 μ l of extraction buffer (0.3 M KCl, 20 mM HEPES [pH 7.9 at 4°C], 25% glycerol, 1.5 mM MgCl_2 , 0.2 mM EDTA, 1 $\times$ proteinase inhibitor mix, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and were shaken vigorously for 30 min on ice. Cell extracts were stored at -70°C.

The EMSA was performed by the method of Chodosh (8). NF- κ B consensus double-stranded oligonucleotide (Promega) and human TNF- α promoter were end labeled with [γ - 32 P]ATP (3,000 Ci/mmol; NEN Research Products, Boston, Mass.). Extracts were preincubated in binding buffer [4% glycerol, 1 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 50 ng of poly(dI:dC)] at room temperature for 5 min and then were incubated with labeled oligonucleotide probe for 20 min. Binding proteins were separated by electrophoresis with high-ionic-strength 4% polyacrylamide gel (acrylamide-bisacrylamide [80:1], 50 mM Tris-HCl, 380 mM glycine, 2 mM EDTA, 2.5% glycerol) at 100 V. After drying of the gel, autoradiography was performed at -70°C.

RT-PCR. In parallel with the cell culture for EMSA, another set of cell cultures was performed under the same conditions. At the end of the culture period, total cellular RNA was recovered and the amount of TNF- α mRNA was measured by quantitative reverse transcription (RT)-PCR with 6.6 nM [α - 32 P]dCTP (3,000 Ci/mmol; NEN) (34, 39). Successful isolation of RNA was monitored by detection of β -actin mRNA by using RT-PCR performed in parallel. One-tenth of the PCR product was analyzed on 3% NuSieve GTG (FMC BioProducts, Rockland, Maine)-1% agarose (GIBCO) gel and was stained with ethidium bromide (0.5 μ g/ml). After photographs were taken, the signal bands were cut from the gel and the radioactivity was determined by liquid scintillation spectroscopy. Counts per minute were converted to molecules of mRNA by using a standard curve with double-stranded cDNA (data not shown). In all experiments, the presence of possible contaminants and background radioactivity in the gel were evaluated by control reactions in which RT and amplification were carried out on RNA-free samples.

RESULTS

LPS-sensitive human TNF- α promoter region. THP-1 cells transfected with a series of 5' deletants revealed an LPS-sensitive region (Fig. 1). As shown in Fig. 1, deletant Δ #3 (from

FIG. 2. Identification of regions in the human TNF- α promoter from nt -694 to -356 important for induction by LPS. The deletant numbers used in Fig. 1 are shown on the map. The putative binding sites for transcription factors (>80% homology) (arrows) are shown under the map. CAT activity was determined as described in the legend for Fig. 1. Broken lines, missing region.

nt -641 to +126) and to a certain extent deletant $\Delta\#4$ were shown to have the highest LPS inducibility of CAT activity. The subsequent deletant $\Delta\#5$ (from nt -477 to +126) had a substantially reduced LPS inducibility of CAT activity. An important regulatory region for LPS-induced human TNF- α gene activation was identified in the vicinity of nt -645 upstream of the transcription starting site, which prompted us to engineer smaller deletants (designated by an S) in the region between $\Delta\#3$ and $\Delta\#6$. To further narrow the nucleotide sequence of the regulatory domain in human TNF- α gene transcription, the DNA sequence from nt -694 to -356 (the region between $\Delta\#3$ and $\Delta\#6$) was subjected to another series of deletants, as shown in Fig. 2 (S#0 from nt -694, S#1 from nt -619, S#2 from nt -593, S#3 from nt -583, S#4 from nt -488, S#5 whole promoter lacking nt -549 to -487, and S#6 whole promoter lacking nt -612 to -487). Deletant S#3 showed the strongest induction of CAT activity by LPS, approximately 2.5-fold more than the baseline level (Fig. 2).

Further sequence analysis of the fragment between nt -584 and -484 (adjacent to the area of S#3 and S#4 starting points) disclosed the presence of both palindromic sequences (CCTGAGG [nt -552 to -546 {forward} and nt -491 to -485 {reverse}] and TGAGGCCTCA [nt -547 to -540 {forward}] and repeated sequences (AGGCCTCA [nt -547 to -540 and nt -493 to -486 {forward} and nt -550 to -493 {reverse}]) (Fig. 3). Furthermore, 11-bp regions (TCCTGAG GCCTCA) in this fragment are found to be complementary (nt -583 to -573 [forward] and nt -494 to -484 [reverse]),

suggesting the possibility of the formation of DNA secondary structure (i.e., loops) involved in TNF- α gene regulation.

Nuclear proteins binding to LPS-sensitive region. In order to identify and characterize the purported binding protein(s) involved in TNF- α gene regulation, two fragments of the TNF- α promoter active region exhibiting the strongest CAT activity were used as probes in EMSA: S#3 minus S#4, called S#3/4, a 98-bp probe from nt -584 to -487; and S#3/4 lacking the potential NF- κ B-binding site, called S#3/4^(NF- κ B), a 64-bp probe from nt -550 to -487 (Fig. 2). The pattern of binding was found to differ from that noted with probes containing NF- κ B-like binding domains.

When either of the probes was used, three highly shifted bands (bands 1, 2, and 3 in lanes 2 of Fig. 4) were found to increase in density after LPS challenge. To further determine the nature of the nuclear factor(s) involved in TNF- α gene regulation, cells were cultured in the presence of salicylate, a known inhibitor of NF- κ B activation. The amount of LPS-induced nuclear NF- κ B (lane 2 in Fig. 5A) was found to be reduced in a dose-dependent manner by the treatment of sodium salicylate (lanes 4 to 6 in Fig. 5A). Under this condition, TNF- α mRNA accumulation was completely inhibited by treatment with sodium salicylate at a concentration greater than 20 mM (Fig. 5B). The three highly shifted bands (bands 1, 2, and 3 in lanes 2 of Fig. 4) were found sensitive to salicylate treatment (lanes 4 to 6 in Fig. 4).

With an approximately 50 $\times$ excess amount of cold competitors (the cold probe itself, NF- κ B, CREB, AP1, and AP2), highly shifted bands 1 and 2 did not completely disappear except in the presence of the cold probe itself (Fig. 6). None of the nuclear factor consensus sequences used were able to obliterate the binding pattern observed with the 98-bp (S#3/4) and 64-bp [S#3/4^(NF- κ B)] probes. However, with the 98-bp probe which contains a potential NF- κ B motif, cold CREB and AP2 were able to slightly decrease the density of bands 1 and 2 (lanes 5 and 7 in Fig. 6A).

DISCUSSION

In this study, the transfection of truncated versions of the human TNF- α promoter region (Fig. 1 and 2) along with EMSA data (Fig. 5 and 6) strongly suggests the involvement of a salicylate-sensitive nuclear protein(s) probably acting in synergism with NF- κ B in LPS-induced human TNF- α gene regulation. The DNA sequence of that binding site(s) has been identified and was found to be unique (Fig. 3).

FIG. 3. Sequence analysis of the fragment between nt -584 and -484. Palindromic sequences (--- and ---), repeated sequences (==), and complementary 11-bp regions (boldface type) were found. A putative NF- κ B-binding domain (nt -583 to -574 [*], discrepancy) and an AP2-binding domain (nt -567 to -560) were also found. The 98-bp fragment (nt -584 to -487 [in uppercase letters]) and the 64-bp fragment (-550 to -487 [in uppercase letters]) were used as EMSA probes for further studies.

FIG. 4. EMSA gel probed with the 98-bp (S#3/4) and 64-bp [S#3/4(NF- κ B)⁰] fragments from TNF- α promoter. Cells were either unstimulated (–) or stimulated (+) with LPS at 100 ng/ml for 2 h in the presence or absence of the indicated concentration of sodium salicylate. Nuclear extract (7.5 μ l) was incubated with 320 fmol of a ³²P-labeled 98-bp (A) or 64-bp (B) probe. Three highly shifted bands (bands 1 to 3 shown by the arrows) were detected with both probes. Both bands 1 and 2 were increased by stimulation with LPS and were decreased by treatment with sodium salicylate at 20 and 40 mM. Data were representative of two independent experiments.

In LPS-induced mouse TNF- α gene transcription, NF- κ B has been shown to be essential for transcription (9, 13, 33). NF- κ B is induced by a variety of stimuli and controls the expression of a large number of genes (2). However, it has been difficult to establish a cause-and-effect relationship between NF- κ B and TNF- α gene transcription after LPS stimulation (13, 17). In addition, synergism between NF- κ B and other transcription factors has been reported in cytokine gene transcription (21, 22, 36). These data would therefore tend to support the fact that transcription factors in addition to NF- κ B could be involved in regulation of cytokine genes whose promoters include NF- κ B-binding consensus sequences. Thus, it appeared reasonable to search for transcription factors other than NF- κ B in human TNF- α gene regulation.

Recently, we reported a wide variety of potential DNA-binding sites in the TNF- α promoter region (38), which suggested that several DNA-binding proteins could participate in the activation of human TNF- α gene transcription. TNF- α

FIG. 5. Effects of sodium salicylate. Cells were either unstimulated (–) or stimulated (+) with LPS at 100 ng/ml for 2 h in the presence or absence of the indicated concentration of sodium salicylate. Nuclear extracts were prepared and were subjected to EMSA using the NF- κ B probe (A). Inducible NF- κ B is shown by the arrow on the left. Nonspecific binding is shown by the arrowheads on the right. Five microliters of nuclear extract was incubated with 7 fmol of the ³²P-labeled NF- κ B probe. Total cellular RNA was isolated and was subjected to RT-PCR (B). The ratio of TNF- α to β -actin mRNA molecules is shown. β -Actin mRNA accumulation was stable throughout this experiment. Sodium salicylate inhibited both active NF- κ B in the nucleus and TNF- α mRNA accumulation. Data are the means and are representative of two independent experiments.

promoter region exhibits several nuclear factor-binding sites, including Egr-1, NF-IL6, and Oct-2, in addition to the NF- κ B-binding site (38), and these factors have been advocated to be LPS inducible (14). Our results show that deletant Δ #3, which includes some NF- κ B consensus sequences, had a strong induction activity, suggesting the existence of an important regulatory region in the vicinity of nt –641 upstream of the transcription starting site. When the region between nt –694 and –356 was further analyzed by using small deletants in a reporter gene system, deletant S#3 was the only one to demonstrate the strongest induction. The region from nt –583 to –486 (S#3/4) appeared to exhibit nuclear protein-binding domains for DNA-binding proteins crucial for human TNF- α gene transcription. In addition, when either a 61-bp region (nt –549 to –489) or a 124-bp region (nt –612 to –489) was deleted (deletant S#5 or S#6, respectively), the transcriptional activity returned to the baseline level. None of the deletants S#0, S#1, and S#2 exhibited any LPS-induced CAT activity above the baseline level, which could suggest the existence of potential down-regulatory domains located between nt –694

FIG. 6. Competitive EMSA gel probed with the 98-bp (S#3/4) and 64-bp [S#3/4^(NF- κ B)] fragments from TNF- α promoter. A mixture of nuclear extracts (4 μ l) obtained from LPS-challenged cells as described for Fig. 4 was incubated with 160 fmol of a ³²P-labeled 98-bp (A) or 64-bp (B) probe. An approximately 50 $\times$ excess amount of cold competitor was preincubated before the probe was added. As a control, NF- κ B from 2 μ l of mixture of nuclear extracts was detected with the ³²P-labeled NF- κ B probe (1.75 fmol). The densities of band 1 and band 2 (arrows on the left) detected with the 98-bp probe seemed to decrease when cold CREB and AP2 competitors were added.

and -584. EMSA using both 98-bp (S#3/4) and 64-bp [S#3/4^(NF- κ B)] probes demonstrated an intense nuclear protein binding whose pattern differed from that obtained with the NF- κ B probe and which was not displaced by competing cold NF- κ B consensus double-stranded oligonucleotide. This finding along with the fact that none of the NF- κ B consensus binding sequence was found on the 64-bp probe substantiates the hypothesis that nuclear factors other than NF- κ B may be also involved in LPS-induced human TNF- α gene transcription. Our findings, questioning the involvement of only NF- κ B in LPS-induced human TNF- α gene transcription, have been supported by those of other laboratories (17). Differences in nucleotide sequences of mouse and human TNF- α promoters and variability within the two species of the nature of nuclear factors involved in TNF- α gene transcription could account for the discrepancy.

Nucleotide sequences in the region of the 64-bp fragment [S#3/4^(NF- κ B)] did not exhibit any homologies with any nucleotide sequences which serve as binding sites for known transcriptional factors (searched against data from reference 14), thereby providing a strong argument for the uniqueness of these sequences and their potential binding proteins. Nevertheless, the existence of DNA secondary structures that could be involved in TNF- α gene regulation could not be ruled out, especially in light of the palindromic and repetitive sequences observed in the adjacent region of the 64-bp fragment [S#3/4^(NF- κ B)]. The identification of these proteins should definitely shed some light on TNF- α gene regulation.

Sodium salicylate has been used as an anti-inflammatory drug to relieve pain and fever. Recently, its function has been suggested to be based on the inhibition of NF- κ B activation by blocking the disruption of I κ B from NF- κ B in cytoplasm (19). We attempted to use this drug to further characterize the isolated DNA-binding domain involved in human TNF- α gene transcription other than that by NF- κ B by eliminating NF- κ B activation. Salicylate treatment on LPS-challenged THP-1 cells inhibited both NF- κ B activation and TNF- α mRNA accumulation in cells. However, it inhibited not only NF- κ B but also the LPS-induced nuclear protein binding to the 98-bp (S#3/4) and 64-bp [S#3/4^(NF- κ B)] probes. Thus, this LPS-induced nuclear protein is also sensitive to sodium salicylate.

Several cytokines have been suggested to be regulated by DNA-binding proteins that stimulate gene transcription upon activation of the cyclic AMP (cAMP) signaling pathways (14, 36). Cold probes for cAMP-inducible transcription factors such as AP2 and CREB were found to compete for binding with the 98-bp (S#3/4) probe (Fig. 6A). However, no putative CREB-binding domains were found either in the 98-bp (S#3/4) region or in the 64-bp [S#3/4^(NF- κ B)] region. Because CREB constitutes a gene family of at least 10 different transcription factors (14), an unknown CREB-related factor(s) in human TNF- α gene regulation could be envisioned. With respect to AP2, one AP2-binding domain was found in the 98-bp (S#3/4) region (Fig. 3), explaining the binding inhibition (Fig. 6A). However, no AP2-binding sites were found in the 64-bp [S#3/4^(NF- κ B)] region, thereby ruling out the probable involvement of AP2. Previous data from our laboratory (35) which substantiate this last observation demonstrated that H-89, a specific cyclic nucleotide-dependent protein kinase inhibitor, did not affect TNF- α and interleukin-1 β secretion by LPS-stimulated human monocytes. Thus, cAMP-inducible transcription factors may not be essential for LPS-induced TNF- α gene transcription, and this uncertainty underlines the necessity for further studies to determine the nature of the nuclear protein involved in LPS-induced human TNF- α gene regulation. This nuclear protein appears to be affected by salicylate treatment.

The data presented in this paper suggest the involvement of transcription factors other than NF- κ B in LPS-induced human TNF- α gene regulation and highlight the role of the salicylate-sensitive nuclear factor described in this paper in addition to that of NF- κ B in the initiation of human TNF- α gene transcription. The 64-bp [S#3/4^(NF- κ B)] fragment of human TNF- α promoter, which was found devoid of NF- κ B consensus sequence, provides evidence for the involvement of nuclear proteins other than NF- κ B in LPS-induced human TNF- α gene transcription. This nuclear factor(s) appears to act in synergism with NF- κ B in TNF- α gene regulation, given that the control of gene expression of deleterious cytokines must be tightly regulated.

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