Cloning and characterization of lipopolysaccharide-induced tumor necrosis factor α factor promoter

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Abstract

We have recently identified lipopolysaccharide tumor-induced tumor necrosis factor α factor (LITAF) as a novel transcription factor controlling necrosis factor (*TNF*)-α expression in the human monocytic cell line, THP-1. To characterize the human (*h*) *LITAF* promoter, we isolated a 1.2-kb DNA fragment and followed this by a screening of human genomic DNA with a *hLITAF* cDNA probe. A 34-bp sequence domain located from nucleotides −74 to −43 in the *hLITAF* promoter exhibited the highest basal reporter gene activity; however, the activity was not elevated by lipopolysaccharide (LPS) stimulation. The sequence domain included a consensus sequence for hepatocyte nuclear factor (HNF)-3α, regulating the transcription of many kinds of genes. Interestingly, the DNA sequence position between −542 and −538 in the *hLITAF* promoter contained the CTCCC motif, which has been reported to act as a specific binding site for hLITAF protein. Electrophoretic mobility shift assays demonstrated that LPS induced the binding of THP-1 nuclear factors to a 22 bp probe containing the CTCCC motif. In addition, *hLITAF* mRNA and nuclear hLITAF protein increased significantly in the THP-1 cells immediately after LPS stimulation. These results suggest that the consensus sequence for HNF-3α, or a nuclear binding protein to the CTCCC motif, may play an important role in regulating LPS-dependent *LITAF* transcription.

Introduction

Lipopolysaccharide (LPS) is a potent stimulator of monocytes and macrophages, causing secretion of tumor necrosis factor-α (TNF-α) and other inflammatory mediators. Given the deleterious effects to the host of TNF-α, it is likely that *TNF*-α expression is tightly regulated. In studies pertaining to macrophage response to LPS, we identified a novel DNA-binding domain that contains transcriptional activity located from −550 to −487 in the human (*h*) *TNF*-α promoter, and sequence analysis of this fragment revealed the absence of any known binding sites for nuclear factor κB (NF-κB) (Takashiba *et al.*, 1995). We isolated, purified, and partially sequenced a 60-kD protein binding to this DNA fragment. For this binding protein, we identified a novel transcription factor, termed the LPS-induced TNF-α factor (LITAF), controlling *TNF*-α gene expression in the human monocytic leukemia cell line, THP-1 (Myokai *et al.*, 1999). Inhibition of human (*h*) *LITAF* mRNA expression in THP-1 cells resulted in a reduction of *hTNF*-α transcripts. Northern blot analysis detected a high level of *hLITAF* mRNA expression predominantly in the placenta, peripheral blood leukocytes, lymph nodes, and spleen. These findings suggest that hLITAF contributes to the activation of *hTNF*-α in response to LPS.

We have recently identified the signal transducer and activator of transcription (STAT)6(B) as a regulatory cofactor for hLITAF (Tang *et al.*, 2005). Cotransfection of STAT6(B) and hLITAF to THP-1 cells induced an interaction between the two proteins, which then formed a complex that subsequently translocated into the nucleus and upregulated the transcription of inflammatory cytokines including hTNF-α. Moreover, the level of secreted hTNF-α protein induced by either hLITAF alone or the hLITAF–STAT6(B) complex was significantly decreased by the silencing of hLITAF using short-hairpin RNA in cells. These findings suggest that the hLITAF expression and subsequent translocation of the hLITAF-STAT6(B) complex into the

nucleus play an important role in the regulation of inflammatory cytokines, including TNF-α, in response to LPS stimulation in mammalian cells.

In this study, we cloned and sequenced 1.2 kb of genomic DNA, which is 5′-upstream of *hLITAF* cDNA, and determined the transcription start site of *hLITAF*. The *hLITAF* promoter activity was examined by reporter gene assay in THP-1 cells and human T-cell leukemia (Jurkat) cells. Furthermore, we examined the specific binding of nuclear factors to a *hLITAF* promoter sequence in THP-1 cells using electrophoretic mobility shift assay (EMSA).

Materials and methods

Genomic cloning and sequencing

A human genomic DNA P1 library (P1-2535; Genome Systems Inc., St Louis, MO) was screened by the method described previously (Pierce *et al.*, 1992). A full-length *hLITAF* cDNA (GenBank accession no. U77396) was used as a probe for the screening. The primer (5′-TTTCTCTC CCTGCCCCCGCG-3′) was designed at the 5′-noncoding region of *hLITAF* cDNA, and it was used for sequencing. The clones obtained were sequenced using the dideoxy sequencing procedure (Sanger *et al.*, 1977) with the Automatic 377 sequencer (Perkin-Elmer, Foster City, CA). The positives were subcloned into pZErO-2 (Invitrogen, Carlsbad, CA), and their insert DNAs were analysed by Southern hybridization with a 300-bp probe prepared from the 5′ region of *hLITAF* cDNA. The insert DNAs were subcloned into pUC18 vector (Takara, Otsu, Japan), and the plasmid DNA was prepared using a Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). The DNAs were sequenced by the method described above.

Human genomic DNA was amplified by the method described previously (Barnes, 1994; Cheng *et al.*, 1994). The gene-specific primers used were designed at the 5′ noncoding region of *hLITAF* cDNA, and nested PCR was performed using a Genome Walker Kit (PT1116-1; Clontech Laboratory Inc., Palo Alto, CA). The PCR-based DNA fragment was cloned into pCR-Blunt Vector (Invitrogen), and the plasmid DNA was prepared, and sequenced in the same way as described above.

Similarity search, structural analysis, and construction of reporter plasmids

The nucleotide sequence determined was analysed for similarity to known human genes by the BLASTN program using GenBank human genome databases (final searches on 12 October 2005). The sequence was analysed for the presence of consensus transcription factor binding sites using the TFExplorer (http://mars.kribb.re.kr:8080/tfExplorer/), TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCHJ.html), and SIGNAL SCAN search program (http://bimas.dcrt.nih.gov/molbio/signal). Potential transcription start sites were analysed using the Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/), the Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html), and the Dragon Promoter Finder (http://sdmc.lit.org.sg/promoter/promoter1_4/DPF.htm).

Reporter plasmids were constructed from the genomic DNA isolated and the firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI). To obtain series of deletants, pGL3-Basic vectors containing the DNA were digested using the exonuclease III/Mung Bean Deletion Kit (Promega), and they were cloned again. The sequences of all constructs were confirmed by sequencing.

Cell cultures

THP-1 and Jurkat cells were purchased from the American Tissue Culture Collection (Bethesda, MD), and the cells were grown in complete medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 5% heat-inactivated fetal bovine serum). The LPS-free tissue culture reagents were purchased from GIBCO (Grand Island, NY), and used for all experiments.

Primer extension analysis

THP-1 cells were maturated by 200 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO) for 24 h, and stimulated with 100 ng mL^{-1} *Escherichia coli* LPS (055:B5; Sigma-Aldrich) for 2 h. Total RNA was recovered from the cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987), and poly(A)$^+$ RNA was collected using Dynabeads Oligo$(dT)_{25}$ (Dynal, Lake Success, NY). An oligo (5′-GGCCTCGGGTGTCCTTCCCGGATCTTGCGC-3′), whose sequence started 206 bases upstream from the translation–initiation codon, was labelled at the 5′ end with [γ-^{32}P]ATP and T4 kinase according to the method described previously (MaKnight & Kingsbury, 1982). Ten micrograms of poly(A)$^+$ RNA from the cells was annealed with the labelled *hLITAF*-specific primer. The extension reaction was carried out with Moloney murine leukemia virus reverse transcriptase (Promega) at 42 °C for 30 min followed by annealing of the primer to poly(A)$^+$ RNA. The reaction products were separated on an 8% polyacrylamide sequencing gel and the sequencing ladders of M13mp18 were used as size markers. The gel was dried under vacuum and visualized by autoradiography with BIOMAX MR film (Kodak, Rochester, NY).

Reporter gene assay

Reporter constructs were cotransfected with a control plasmid in which the *Renilla* luciferase gene was coupled to the herpes virus thymidine kinase promoter (pRL-TK; Promega). The pGL3-Control vector (Promega) and pGL3-Basic vector were used for a positive and negative control of promoter assay, respectively. THP-1 cells (1×10^6 well^{-1}) or Jurkat cells (5×10^5 well^{-1}) were plated on 6-well dishes, and they were incubated with the complete medium described above for 24 h until transfections.

Ten microliters of LipofectAMINE solution (Invitrogen) was diluted with 200 μL serum-free RPMI 1640 medium containing 2.5 μg DNA (2 μg reporter plasmid and 0.5 μg pRL-TK), and this solution was incubated at room temperature for 30 min to allow DNA-liposome complex to form. The cells were transfected for 12 h by incubating with the solution, followed by removal of the solution and washing with phosphate-buffered saline (PBS) (pH 7.2). The transfected cells were incubated in the complete medium for 48 h. In addition, THP-1 cells were induced to maturation by incubation in 200 nM PMA for 24 h prior to the LPS stimulation.

The cells were incubated for 12 h with or without 100 ng mL^{-1} LPS, and were lysed and analysed for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay Kit (Promega) and the luminometer (Wallac, Gaithersburg, MD). Three independent transfections were performed, and the *Renilla* luciferase value was normalized to its firefly luciferase value for each reporter construct. Statistical analysis was performed with Student's *t*-test.

EMSA

Double-stranded oligonucleotides (22 bp, AGCTCAGACCTCCCGGCTCGAC) were designed so as to cover the CTCCC motif within the *hLITAF* promoter. They were synthesized (Takara), and end-labelled with [γ-^{32}P]dATP (Amersham Bioscience, Tokyo, Japan) using a MEGALABEL Kit (Takara). The labelled DNA was separated from unincorporated [γ-^{32}P]dATP using the QIAquick Nucleotide Removal Kit (Qiagen), eluted in DNase-free water, and kept at 4 °C until used for EMSA.

Nuclear extracts were prepared from THP-1 cells by the method described previously (Mineshiba *et al.*, 2005), with slight modifications. Briefly, 5×10^6 cells were maturated with 200 nM PMA for 24 h and stimulated with 100 ng mL^{-1} LPS for 15 or 120 min. The cells were washed twice with PBS at 4 °C, and recovered. Nuclear protein was extracted from the cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to the manufacturer's instructions. The amount of protein was measured using Protein Assay Kits (Bio-Rad Laboratories, Hercules, CA). Four micrograms of the extract was incubated at room temperature for 30 min in a reaction buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 4% glycerol, 0.05 mg mL^{-1} poly(dI-dC), and 10 fmol labelled probe in a total volume of 11 μL. The DNA–protein complexes were mixed with 1 μL of 10× loading buffer (Gel Shift Assay Systems, Promega), and then they were analysed by electrophoresis on a 5% polyacrylamide gel using 0.5× Tris-borate-EDTA running buffer (45 mM Tris-HCl, 45 mM sodium borate, and 1mM EDTA). The signals were visualized using a Bio Imaging Analyzer (BAS 2000; Fuji, Tokyo, Japan). For competition experiments, the nuclear extracts were preincubated on ice for 30 min with a 500-fold molar excess of the unlabelled oligonucleotides.

Quantitative analysis of mRNA by real-time PCR

THP-1 cells (1×10^6 well^{-1}) were plated on 6-well dishes, and cultured in the complete medium described above. The cells were maturated for 24 h with 200 nM PMA, and incubated for various periods (1 to 12 h) with or without 100 ng mL^{-1} LPS. Total RNA was extracted from the cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987), and first stranded cDNA was synthesized from the RNA using Superscript II Reverse Transcriptase and oligo dT_{8-12} primer (both from Invitrogen). Two microliters of first stranded cDNA solution corresponding to approximately 40 ng total RNA was used for quantitative analysis of *hLITAF* and β-*actin* mRNA expression. The experiments were performed in triplicate for each data point. Statistical analysis was performed with Student's *t*-test.

The real-time PCR was performed by the method previously described (Maeda *et al.*, 2003). In brief, 50 μL of reaction mixture contained 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 20 pmol of gene-specific primers, 10 pmol of TaqMan probe, and the first stranded cDNA. The amplification conditions consisted of an initial 10 min denaturation step at 95 °C, followed by 40 cycles of denaturation at 94 °C for 15 s, and annealing at 60 °C for 1 min for *hLITAF* and β-*actin*. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical caps (Applied Biosystems). At each cycle, the accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye from dsDNA-binding SYBR Green. All data were analysed using the GensAmp 5700 SDS software. The PCR primers used were *hLITAF* sense, 5′-CCTCCTTCG TATTATACCCAGCCA-3′; anti-sense, 5′-GGAAGGACAA-CACATT TGGATAGG-3′; β-*actin* sense, 5′-TAGCGGAACC GCTCATTGCC-3′; and anti-sense, 5′-TTCACCCACACT GTGCCC-3′. The relative accumulation of *hLITAF* mRNA

was shown after normalization against the expression of β-*actin* mRNA.

Western blot analysis

THP-1 cells (1×10^6) were maturated for 24 h with 200 nM PMA, and stimulated with 100 ng mL^{-1} LPS for various periods (0, 0.5, 2, 12 or 24 h). The cells were washed twice with PBS and recovered, and the nuclear and cytoplasmic protein was extracted and prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). The amount of protein was measured in the same way as described above. THP-1 cells cultured without LPS were used for a negative control.

Western blot analysis was performed using the methods described previously (Towbin *et al.*, 1979), with slight modifications. In brief, nuclear protein (20 μg) was precipitated with 10% trichloroacetic acid, and the pellet was suspended in a sample buffer (50 mM Tris-HCl [pH 6.8], 2 mM EDTA, 10% glycerol, 2% β-mercaptoethanol, and

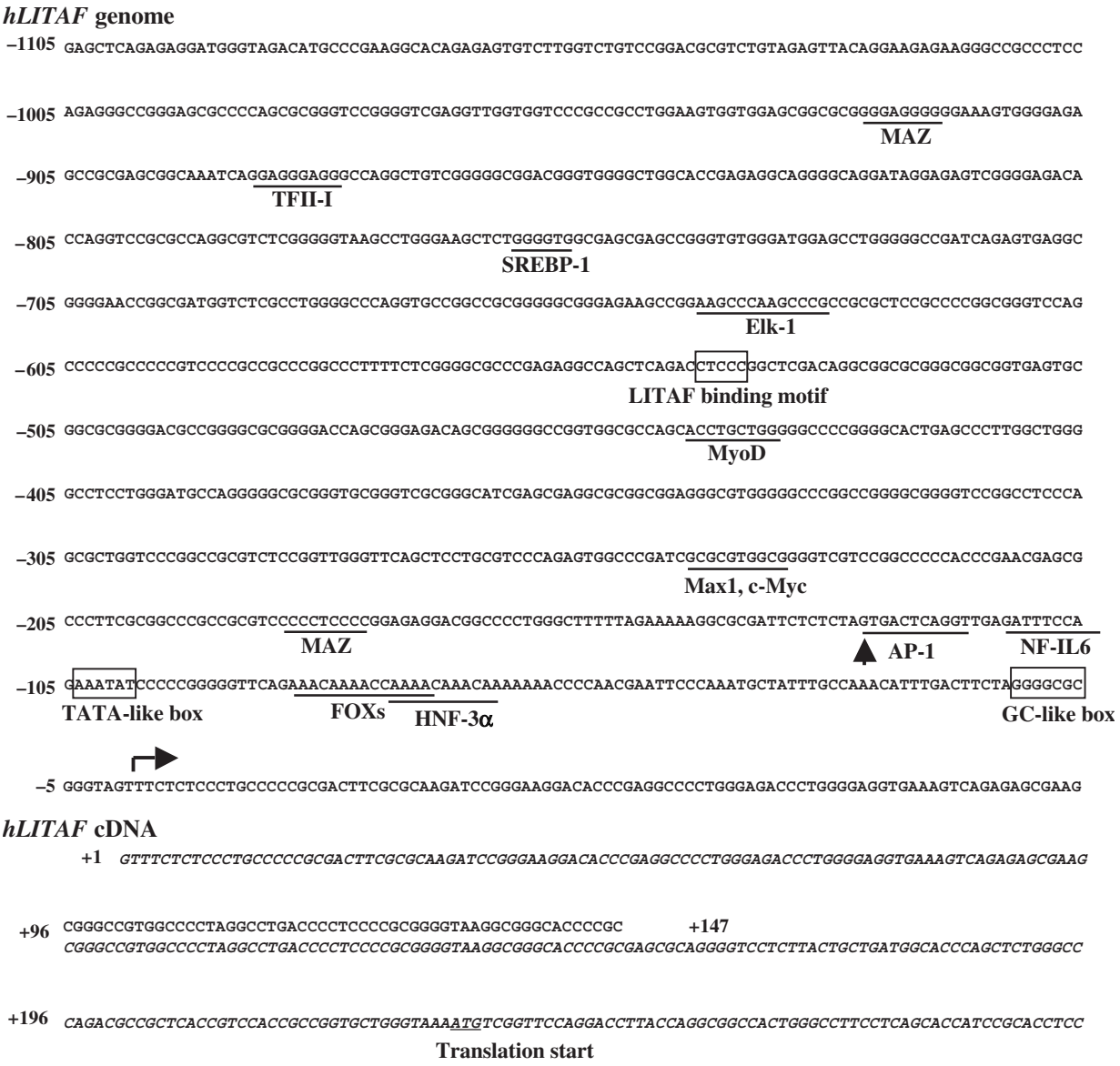

Fig 1. Nucleotide sequence of the 5′-flanking region of the *hLITAF* gene. The 5′-flanking sequence of the *hLITAF* genome and 5′ region of *hLITAF* cDNA (GenBank accession no. U77396) are aligned. Putative transcription factor binding sites (TFExplorer; score, >0.95) are indicated by underlining, and boxes indicated are the TATA-like box, GC-like box, and LITAF binding motif. FOXs include FOX-K1a, E1, C2, J1, F1, F2, M1a, M1b, O1a, O3a, O4, H2, D3, and J2. The transcription starting site is predicted by computer analysis (indicated by an arrow head), and determined by primer extension (indicated by a bent arrow).

0.025% bromophenol blue). Samples were heated at 100 °C for 5 min, and separated by a 10% SDS-PAGE. The cytoplasmic protein (20 μg) was mixed and heated in the same way, and separated by SDS-PAGE. The proteins were transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and blocked with 5% dry milk in TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20) for 1 h.

For immunological detection, the membranes were incubated in TBST containing 50 ng mL^{-1} anti-LITAF antibody (BD Biosciences, San Jose, CA) for 1 h, then incubated with 0.02% goat antimouse horseradish peroxidase-conjugated antibody, and finally they were developed using an ECL detection kit (Amersham BioScience, Piscataway, NJ). The signal for LITAF protein was normalized against that of actin protein. Statistical analysis was performed using Student's *t*-test.

Immunocytochemistry

THP-1 cells (5×10^3) plated on an 8-well culture slide (BD Biosciences) were maturated with 200 nM PMA for 24 h, and were stimulated with 100 ng mL^{-1} LPS for 2 h. The cells were fixed with 4% paraformaldehyde, washed twice with PBS, and incubated with 1 μg mL^{-1} anti-LITAF antibody for 30 min. For a negative control, the cells were incubated with mouse IgG (BD Biosciences). After washing with PBS, they were incubated with 1 : 1000 diluted anti-mouse IgG antibody conjugated with horseradish peroxidase (Amersham Bioscience), and developed using a DAB staining kit (Cat. 00-2088; Nichirei, Tokyo, Japan). The cells stained were observed through differential interference contrast microscopy (BX50; Olympus, Tokyo, Japan).

Results

Human LITAF promoter sequence and transcription initiation site

The 1252-bp DNA fragment was isolated by genomic library screening and subcloning, and then was sequenced. The DNA contained the 5′-upstream sequence of *hLITAF* cDNA (Fig. 1). The result suggests that the DNA sequence included the *hLITAF* promoter, and the sequence has been submitted to DDJB (accession no. AB074031). A human genomic BLAST search revealed that the DNA sequence was matched at 99% with the *hLITAF* genome (GenBank accession no. NT010 393). In addition, we succeeded in cloning the PCR-based DNA from the human genome, and its nucleotide sequence coincided with that of the *hLITAF* promoter (data not shown).

The typical TATA-like box was detected in the *hLITAF* promoter; however, the transcription start site predicted was located upstream of the TATA-like box (Fig. 1). Therefore, we examined the transcription start site of the *hLITAF* by primer extension. Two prominent products of 32 and 34 bases corresponded to transcription initiation sites 232 and 234 nucleotides upstream of the ATG translation start codon in the *hLITAF* cDNA, and the longer product was located at the first nucleotide of *hLITAF* cDNA (Figs 1 and 2). The results indicate that the transcription start site of the *hLITAF* gene is located 234 nucleotides upstream of the translation start codon.

Consensus transcription factor binding sites

Computational analysis of the *hLITAF* promoter sequence revealed potential DNA-binding sites specific to mammalian gene regulatory proteins (Fig. 1). From 130 to 105 bases upstream of the transcription initiation site, putative consensus sequence binding with activator protein-1 (AP-1) and nuclear factor IL-6 (NF-IL6), which are known to be LPS-induced transcription factors, was present just before the TATA-like box. However, there was no binding site for nuclear factor κB (NF-κB) in the promoter region.

Reporter gene assay

To elucidate the regulatory effects of the various areas within the *hLITAF* promoter on transcriptional activity, a series of reporter constructs was transfected into Jurkat or THP-1

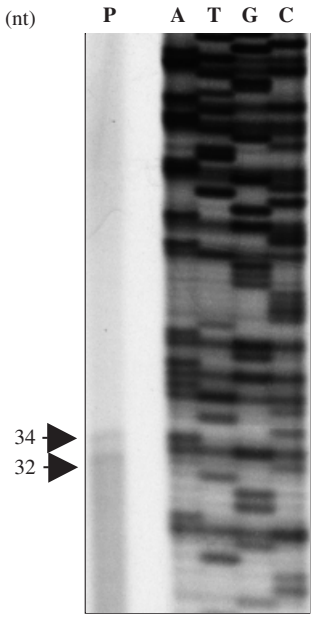

Fig 2. Determination of the transcription initiation site of the *hLITAF*. The poly(A)$^+$ RNA was annealed with an *hLITAF*-specific primer labelled with [γ-^{32}P]ATP. The extension reaction was carried out by reverse transcription, and the product (P) was separated on a polyacrylamide gel; the sequencing ladders of M13mp18 (A, T, G, C) were used as size markers.

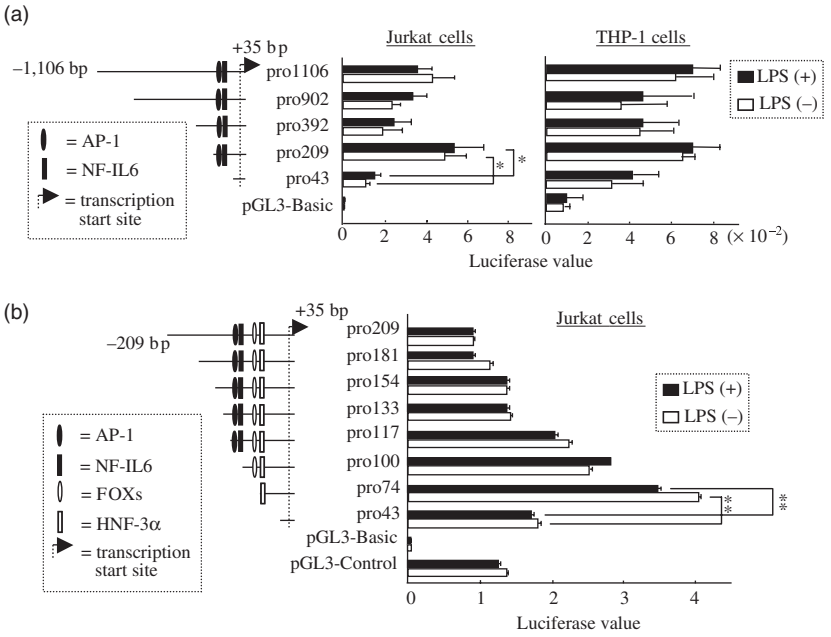

Fig 3. Analysis of the transcriptional activity of the *hLITAF* promoter. (a) Left: schematic description of the five *hLITAF* reporter constructs containing promoter fragments of various lengths cloned into the pGL3-Basic vector. The numbers in the names of the constructs indicate their respective lengths in nucleotides. Positions of the transcription start site, structural domains for AP-1 and NF-IL6 site are indicated. Middle and right: relative firefly luciferase activity of each construct in 5×10^5 Jurkat cells (middle) and 1×10^6 THP-1 cells (right). Both were transfected with the various reporter gene constructs together with the pRL-TK vector, which serves as an internal control. The figure shows the results carried out in triplicate for each construct. The firefly luciferase value of each sample has been normalized to its *Renilla* luciferase value, and this value has subsequently been compared with basal and LPS-induced values. Error bars indicate standard deviation. LPS (+): LPS-induced value; LPS (−): basal value. $^*P < 0.05$, compared between the constructs. (b) Left: schematic description of the eight *hLITAF* reporter constructs; position of the transcription start site, structural domains for AP-1, NF-IL6, FOXs and HNF-3α site are indicated. Right: relative luciferase activity of each construct in 1×10^5 Jurkat cells was counted by the methods described in (a). The relative promoter value in each construct is indicated as a fold-increase in the luciferase activity relative to that in the construct pro209. The figure shows the results carried out in triplicate for each construct. Error bars indicate standard deviation. LPS (+): LPS-induced value; LPS (-): basal value. $^{**}P < 0.01$, compared between the constructs.

cells, and reporter gene production was monitored (Fig. 3a). The construct pro209 exhibited the highest basal promoter value in both cell lines, and this value was significantly higher than that in the construct pro43. As shown in Fig. 3a, the construct pro209 includes the binding site for AP-1 and NF-IL6. Similar results were obtained in the case of the LPS-induced value. However, no significant elevation of the promoter activity by LPS stimulation was found, even in the cells transfected with the construct pro209.

We further examined precisely the reporter gene activity in Jurkat cells (Fig. 3b), because the efficiency of transfection was much higher in the cell line (data not shown). The construct pro74 containing a DNA sequence domain located from −74 to −43 in the promoter exhibited the highest value. However, no significant elevation of the promoter activity by LPS stimulation was found, even by transfection with the construct pro74. These results suggest that a transcription factor HNF-3α, or a nuclear protein binding to the CTCCC motif, can contribute to the *LITAF* gene expression constitutively.

Specific interaction of oligonucleotides with nuclear extract

EMSA showed that the DNA − protein complex was increased immediately by LPS stimulation (Fig. 4). In the competitive EMSA, the complex of radio-labelled probe and extract was competed by the excess of unlabelled probe (Fig. 4).

Human *LITAF* mRNA accumulation

The *hLITAF* mRNA expression level was analysed in the PMA-maturated THP-1 cells, followed by incubation with or without LPS (Fig. 5). The accumulation was up-regulated by LPS between 2 and 4 h, and recovered to the basal level thereafter.

Kinetics of LITAF protein

Western blot analysis revealed the kinetics of hLITAF protein in the nuclei and cytoplasm of the PMA-maturated THP-1

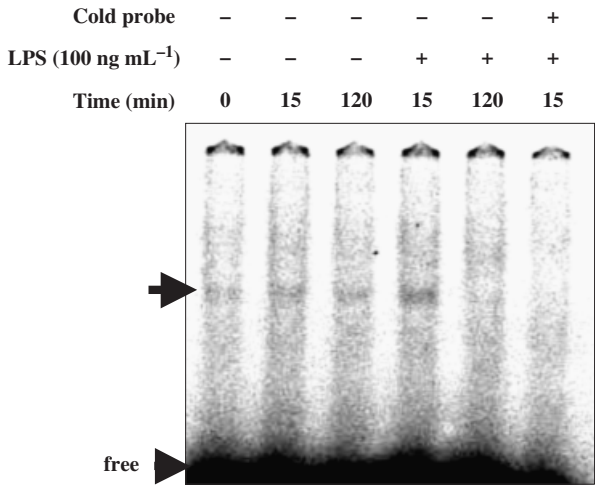

Fig 4. Interaction of nuclear factors with the oligonucleotides. EMSA was performed using a 22-bp oligonucleotide containing the CTCCC motif. The ^{32}P-labelled double-stranded oligonucleotides were incubated with nuclear extract prepared from THP-1 cells incubated with or without LPS. Two independent assays were performed, and a typical result is shown. 22-bp unlabelled oligonucleotides used as cold probe. Arrow, DNA – protein complex.

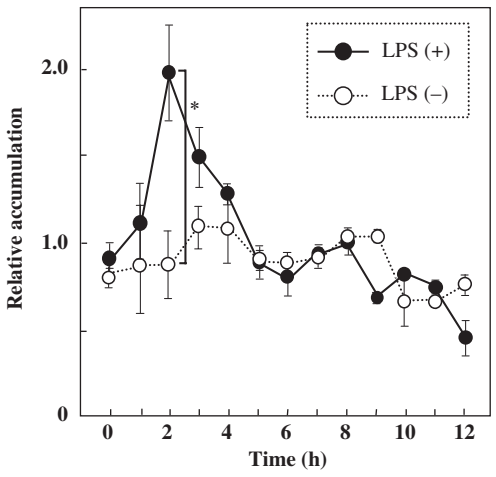

Fig 5. Human *LITAF* mRNA expression in THP-1 cells. Human *LITAF* mRNA was quantified by real-time PCR. Relative expression of *hLITAF* mRNA was shown after normalization against the expression of β-*actin* mRNA. The figure shows the result of experiments carried out in triplicate for each time. Error bars indicate standard deviation. LPS (+): cells cultured with LPS; LPS (–): cells cultured without LPS. $^*P < 0.05$, compared with the cells cultured without LPS.

cells (Fig. 6). A marked increase of the protein by LPS stimulation was detected in the nuclei at 2 h.

Localization of hLITAF protein in the PMA-maturated THP-1 cells was examined by immunocytochemistry (Fig. 7). In the nuclei of the cells, dense staining was observed at 2 h of LPS stimulation, whereas light staining was seen in the absence of LPS stimulation.

Discussion

In this study, we identified the *hLITAF* promoter, and performed a functional analysis of the promoter to gain insight into the mechanism for the regulation of *hLITAF* transcription. A transcription start site was predicted to be located 25 bases upstream of the TATA-like box in the promoter (Fig. 1). However, the transcription start site is almost always located 25 to 30 bases downstream of an active TATA element of higher eukaryotes (Sawadogo & Sentenac, 1990). Therefore, we performed primer extension analysis with a probe covering the proximal 5′-flanking region of *hLITAF* cDNA. Two products were detected, and the longer one was located at the first nucleotide of *hLITAF* cDNA (Figs 1 and 2). Theses results suggest that transcription is initiated at 234 bases from the translation start site This TATA-like box element is unlikely to be involved in transcription because of its distance of 100 bases from the transcription start site.

Computer analysis of the *hLITAF* promoter sequence indicated several putative binding sites for known transcription factors (Fig. 1). It is very important to know which region of the promoter regulates the *hLITAF* transcription. Using a series of sequentially deleted *hLITAF* promoters (-1106 to $+35$) ligated into pGL3-Basic plasmid for expression in Jurkat or THP-1 cells, the highest basal promoter activity was found to be located in the sequence from position -74 to -43 relative to the transcription start position (Fig. 3). This sequence contained the consensus sequence for HNF-3α, which contains a forkhead or winged helix domain as the DNA binding domain and regulates the transcription of many kinds of genes (Costa *et al.*, 1989; Lai *et al.*, 1990). Moreover, we could not find any other consensus sequence in this region. The findings suggest that the consensus sequence plays an important role in LPS-independent *hLITAF* transcription.

Predicted consensus sequences implied that the putative binding sites for AP-1 and NF-IL6 were responsible for activation of the *hLITAF* by LPS (Fig. 1). However, in the cells transfected with any construct containing these binding sites, the promoter activity was not increased by LPS (Fig. 3b). Interestingly, the DNA sequence position between -542 and -538 in the *hLITAF* promoter contained the CTCCC motif (Fig. 1), which is likely to act as a specific binding site for hLITAF protein (Tang *et al.*, 2003). EMSA indicated that LPS induced binding of the oligonucleotides encompassing the CTCCC sequence with nuclear factors prepared from THP-1 cells (Fig. 4). Competitive EMSA using unlabelled oligonucleotides showed specific binding of the motif with a nuclear protein (Fig. 4). These results suggest that the CTCCC motif within the *hLITAF* promoter is important for the binding to the nuclear factors in LPS-stimulated THP-1 cells. In addition, the motif is found in

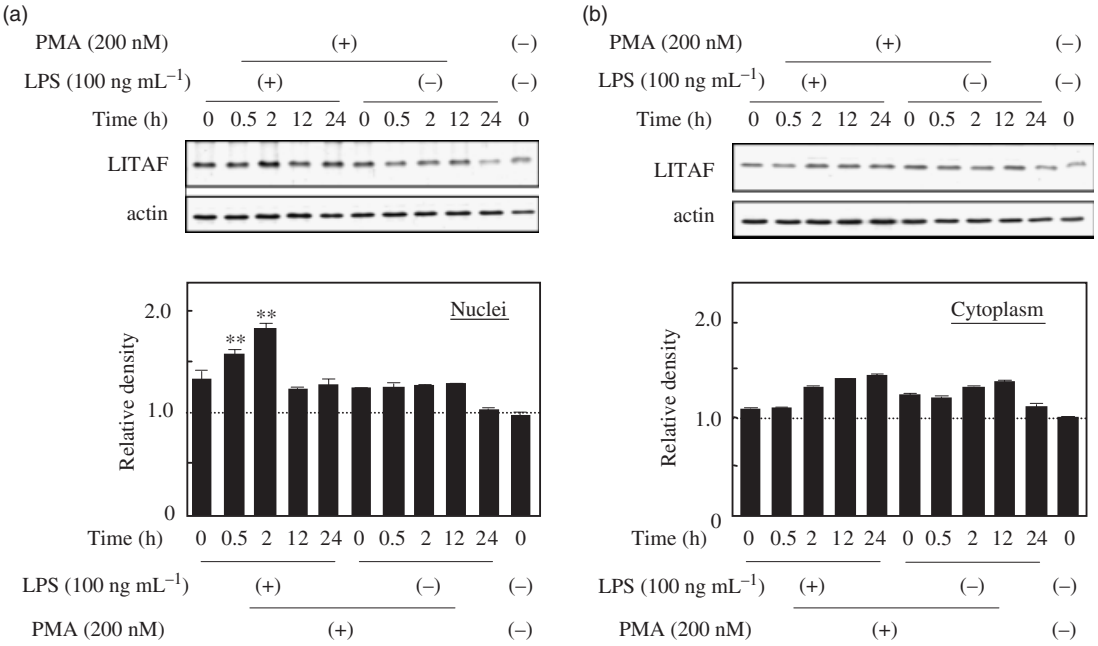

Fig 6. Kinetics of hLITAF protein in THP-1 cells. hLITAF protein in (a) nuclei and (b) cytoplasm was detected by Western blotting. Relative density of the LITAF protein was shown after normalization against the density of actin. The figure shows the result of experiments carried out in triplicate for each time. Error bars indicate standard deviation. $^{**}P < 0.01$, compared with the cells cultured without LPS.

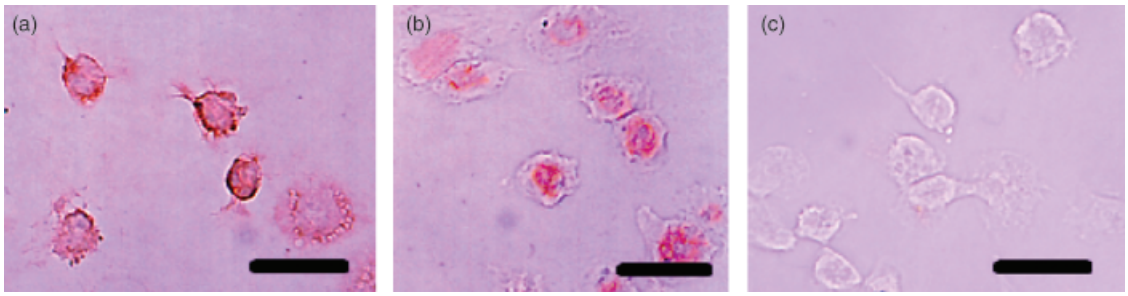

Fig 7. Localization of hLITAF protein in THP-1 cells. THP-1 cells were stained by immunocytochemistry using anti-LITAF antibody. In the nuclei of the cells, dense staining was observed at 2 h of LPS stimulation (b), whereas light staining was seen in the absence of LPS stimulation (a). No signal was observed in the LPS-stimulated cells followed by staining using mouse IgG (c). Bar equals 50 μm.

human *interleukin* (*IL*)*-6* and *IL-8* promoters (data not shown); therefore, a reporter gene assay using mutations in the binding site is currently under way.

A significant increase of *hLITAF* mRNA and nuclear hLITAF protein occurred in the THP-1 cells immediately after LPS stimulation (Figs 5–7). Analysis of protein levels over time after transfection of hLITAF and STAT6(B) into THP-1 cells indicated that the hLITAF–STAT6(B) complex is formed and begins translocating into the nucleus between 4 and 8 h after transfection (Tang *et al.*, 2005). In contrast to our expectations, cytoplasmic LITAF protein was not reduced at 0.5 and 2 h after LPS stimulation (Fig. 6). It is possible that phosphorylated hLITAF protein may be detected by the antibody used here, and that nuclear hLITAF protein is activated immediately by LPS stimulation. In our preliminary examination, LPS-dependent increase of nuclear hLITAF protein was inhibited in the THP-1 cells immediately after a treatment with a mitogen-activated protein kinase inhibitor (data not shown). The activation of hLITAF protein may reflect a discrepancy in changes of nuclear and cytoplasmic hLITAF protein in the LPS-stimulated cells.

In conclusion, we have cloned and characterized the *hLITAF* promoter sequence by reporter gene assay and EMSA. The consensus sequence for HNF-3α contributes the LPS-independent *hLITAF* transcription. A nuclear protein binding to the CTCCC motif, probably to act as a

specific binding site for hLITAF protein, may play an important role in regulating LPS-dependent *hLITAF* transcription.

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