Lipopolysaccharide Induction of Tissue Factor Expression in THP-1 Monocytic Cells

PROTEIN-DNA INTERACTIONS WITH THE PROMOTER*

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Tissue factor, the cellular receptor for factor VII/VIIa, activates both the intrinsic and extrinsic pathways of blood coagulation. In this analysis we have used DNase I footprinting to map the sites of protein-DNA interaction along the promoter (-383 to +8) using nuclear extracts prepared from uninduced and lipopolysaccharide-induced THP-1 cells. We have identified six regions that interact with nuclear factors in both uninduced and induced extracts. Four footprints are contained within a region reported to confer base-line high level expression and lipopolysaccharide and serum induction. Two additional footprints map to a region reported to reduce basal transcription by 50%. The only qualitative change in the footprint pattern with uninduced and induced extracts is the appearance of two hypersensitive sites with uninduced extracts. In addition, changes in the level of protein-DNA binding are detected with only one probe by DNA mobility shift analysis. A combination of well characterized transcription factors (AP1), primarily lymphoid cell specific regulatory proteins (NF-xB- and/or Ets-1-related proteins), as well as additional, uncharacterized proteins appear to interact with these sequences. Our data suggest that post-translational modification of existing transcription factors, and not induction of new DNA-binding activity, mediates the lipopolysaccharide induction of tissue factor synthesis in THP-1 cells.

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The abbreviations used are: TF, tissue factor; LPS, lipopolysaccharide; DMSA, DNA mobility shift assay; DMSP, DNA mobility shift probe; PCR, polymerase chain reaction; PEA3, Polyomavirus enhancer activator 3; bp, base pair.

er (6, 7). Inappropriate induction of TF synthesis is associated with a number of diseases, including acute myelogenous leukemia (8), fibrosarcoma (9), macrophages associated with ulcerated atherosclerotic plaques (10), and herpes simplex virus-infected endothelial cells (11). Induction of TF expression by monocytes and endothelial cells is associated with disseminated intravascular coagulation, a rapidly fatal disease in humans. The causative role of TF expression in human disseminated intravascular coagulation is strengthened by experiments, using a baboon model, that block the lethal effects of bacterial infection by administration of either anticoagulants (12) or blocking antibodies to TF (13).

Analysis of TF gene promoter-luciferase reporter gene constructs following transfection into Cos 7 cells defined the minimal promoter required for high level expression. Results from both transcriptional activation and an increase in mRNA stability (4, 5). Transfection of a deletion series of TF promoter-luciferase reporter gene constructs into THP-1 cells identified a 20-base pair (bp) region between -192 and -172 relative to the cap site to be sufficient to mediate LPS-induced transcriptional activation (16).

To begin to define the molecular events that modulate TF gene transcription, these studies have characterized the interaction of nuclear factors with the TF promoter. We have prepared nuclear extracts from both uninduced and LPS-induced THP-1 cells in order to compare binding activity with the promoter under conditions where the gene is either expressed at low level (uninduced) or actively transcribed (induced) in these cells. An induction period of 1.5 h was used in these studies since our data, as well as data from other groups, have determined that TF mRNA synthesis is rapidly induced with maximal expression between 1 and 2 h in THP-1 cells (5). We have used a combination of DNase I footprinting to position areas of DNA-protein interaction and DNA mobility shift assays (DMSA) to both quantify and more precisely characterize the nature of the proteins that interact with these sites.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Plasmids—For DNase I footprinting experiments an EcoRI to SacI tissue factor promoter fragment of 381 bp was cloned into the pBluescript II polylinker (KS, Strategene) to generate pTFP. pTFFP was digested with SacI and the 3' overhang was removed by the 3'-5' exonuclease activity of T4 DNA polymerase. XbaI linkers (New England Biolabs) were ligated into the blunt-ended SacI site to generate pTFFXSma. For DNase I footprinting the following constructs were made. pTFFXASma contains a 167-bp deletion of an internal SmaI fragment that fuses sequences from -383 to -280 to -113 to +8. pTF- F5Spma contains the 167-bp SmaI fragment with promoter sequences...
between -280 and -113. Fragments and orientation relative to plasmid polylinker sites are described in Fig. 2. Promoter DNA fragments were digested with EcoRI and XhoI (New England Biolabs), separated by polyacrylamide gel electrophoresis (5% acrylamide, 29:1), and eluted into sterile water. Fragments were 3'-end-labeled on one strand with the large fragment of DNA polymerase I (Klenow) at either the EcoRI site with dATP or the XhoI site with dCTP.

Oligonucleotides were synthesized on an Applied Biosystems model 394 DNA synthesizer. Several small fragments used in DNA mobility shift experiments were either purified from TF subclones (Sty 84) or synthesized by polymerase chain reaction (PCR) using promoter fragments as template and standard PCR conditions (PCR1). Oligonucleotides were synthesized to encompass binding sites identified by DNase I footprinting. DNA fragments were annealed in 0.1 M NaCl and were 5'-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (Boehringer).

Cell Lines—THP-1 cells were grown in spinner flasks (BeloC Glass) in RPMI supplemented with 5% heat-inactivated fetal calf serum (low endotoxin, HyClone) and 10 μg/mL b-mercaptoethanol as described elsewhere (18). For induction experiments, cells were centrifuged, washed in Ca2+/Mg2+-free phosphate-buffered saline (Dulbecco); 137 mM NaCl, 2.7 mM KCl, 16 mM KH2PO4; pH 7.2, and grown in serum-free RPMI containing E. coli LPS (Sigma) at a final concentration of 1 μg/mL for 1.5 h. To ensure induction of TF mRNA under these conditions, mRNA was prepared from both the uninduced and LPS-induced cells (19), and expression of TF mRNA was examined by Northern blotting and hybridization with a radiolabeled TF cDNA probe (20).

Footprint Analysis of Nuclear Extracts—Nuclear and SI00 (cytoplasmic) extracts were prepared from THP1, WI38, and HEPG2 cells according to the method of Dignam et al. (21). All extracts were dialyzed into sterile Buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) for 5 h at 4 °C. Extracts were aliquoted, frozen in dry ice-ethanol, and stored at -80 °C. Protein concentration was determined by the Bradford method (Bio-Rad) (22). As a control for extract quality, all nuclear extracts were incubated with labeled oligonucleotides containing an AP1 consensus binding site, and protein-DNA interactions were determined. All extracts used in these studies exhibited binding to this control sequence (data not shown).

DNase I Footprinting—DNase I footprinting was performed essentially as previously described (23), with modifications (24). 0.5 ng (3000 cpm) of probe labeled at one end was added for 30 min on ice in 25 μl of buffer containing 22.4 mM HEPES, pH 7.9, 61.6 mM KCl, 6.0 mM MgCl2, 1.25 mM dithiothreitol, 0.05 mM mg/mL bovine serum albumin; 0.1 mM EDTA; 0.26 mM phenylmethylsulfonyl fluoride; and 1-4 μg poly(dIdC) (Pharmacia LKB Biotechnology Inc.). Control reactions did not contain protein, and experimental lanes contained 20 μg of nuclear protein. Immediately prior to use, DNase I (Worthington) was diluted into buffer containing 25 mM HEPES, pH 7.5, 25 mM CaCl2, 1 mM MgCl2, 0.5 mM dithiothreitol, and 0.1 mg/mL bovine serum albumin. Control reactions were incubated with 12 ng of DNase I, and experimental lanes were incubated with 266 ng of DNase I for 5 min on ice. 75 μl of stop buffer containing 20 mM Tris, pH 7.5, 20 mM EDTA, 0.5% SDS, 270 mM NaCl, 0.133 mg/mL sonicated herring testes DNA, and 0.1 mg/mL bovine serum albumin were added to each reaction and incubated at 40 °C for 5 min. The mixture was phenol-chloroform (50:50, v:v)-extracted, and the aqueous phase was ethanol-precipitated. DNA pellets were resuspended in 95% formamide, 0.1% bromphenol blue, and 0.1% xylene cyanol, denatured at 95 °C for 3 min, cooled on ice for 5 min, and electrophoresed in 1 x TBE (0.089 M Tris borate, 1 mM EDTA) on 6% acrylamide gel, 0.7% agarose denaturing polyacrylamide gels. Gels were transferred to Whatman No. 3MM paper, dried in vacuum, and autoradiographed at -80 °C.

Analysis of TF mRNA Expression—The increase in steady state levels of TF mRNA occurs rapidly following addition of LPS to the growth medium. Fig. 1 compares TF mRNA expression in monocyte and control cell lines. In this analysis, THP-1 and U937 cells were induced for 60 min with 1 μg/mL LPS. WI38 cell mRNA was included as a positive control for TF expression. Within 60 min following incubation of THP-1 cells with LPS the steady state levels of the TF-specific 2.3-kilobase transcript increases.

DNase I Footprint Analysis—DNase I footprinting was used to determine the sites of protein-DNA interaction between -383 and the mRNA cap site. To detect all sites which may interact with protein we included areas which showed perturbation in intensity of banding pattern when compared with the control lane even when no classical footprint was detected. To resolve protected regions more easily, the 383-bp TF promoter fragment was subdivided into two fragments. Diagrams of the subclones which were used in this analysis are shown in Fig. 2. Footprint analysis of pTFPXASma is shown in Fig. 3. The footprint pattern on the noncoding strand (Panel A) and coding strand (Panel B) shows three protected regions (footprints 1 (22 to +8), 6 (-332 to -313), and 7 (-363 to -343). Footprint analysis of pTFPASma is shown in Fig. 4. The footprint pattern of the noncoding strand (Panel A) and the coding strand (Panel B) shows three additional protected regions (footprints 2 (-191 to -151), 3/4 (-231 to -198), and 5 (-277 to -258).

Although nuclear extracts from uninduced and LPS-induced monocytes were incubated with the probes, there is no obvious change in the pattern of protected fragments with induction. However, two hypersensitive sites are identified only with uninduced extract. In Fig. 3 (Panel B), a weak but reproducible
In order to determine the level of NF-κB binding in THP-1 cell extracts, an oligonucleotide containing the consensus binding site was synthesized, end-labeled, and subjected to DMSA (Fig. 6, Panel B). Uninduced THP-1 cells contain proteins that interact with the NF-κB consensus sequence and, following induction, the level of this binding activity increases.

DMSP 3 and 4 (footprint 3/4) bind with different affinities to proteins whose binding is competed by oligonucleotides containing an AP1 binding site. Although AP1 binding to DMSP 3 is unchanged in uninduced and induced extracts (Fig. 7, Panel A, lanes 2 and 3) there is a substantial increase in AP1 binding to DMSP 4 with induction (Fig. 7, Panel B, lanes 2 and 3). In fact, there is very little protein binding to this site in uninduced extracts (Fig. 7, Panel B, lane 2). Our data confirm that the promoter sequence between -231 and -198 contains two adjacent AP1 sites. In addition, these results indicate that the affinity of the two sites for AP1 binding activity differs, and the binding kinetics for the distal AP1 binding site change with induction.

DMSP 5 (footprint 5) binds to a nuclear protein that is not competed by AP1, AP2, or SP1 (Fig. 8, Panel A, and Table I). In addition, there is no change in the level of binding for this probe with induction (data not shown). DMSP STY84 (an 84-bp restriction fragment containing footprints 6 and 7) generates several specific mobility shift bands (Fig. 8, Panel B). These factors are localized to the nucleus, and none of these binding activities is competed with consensus oligonucleotides containing AP1, AP2, or NF-κB binding sites. In addition, there is no binding activity for these factors in extracts prepared from fetal lung fibroblasts (WI38 cells) or hepatocellular carcinoma (HepG2) cells (Fig. 8, Panel B, lanes 4 and 5). DMSP PCR 1 (footprints 5, 6, and 7) binds the same factors as the Sty 84 fragment (Fig. 8, Panel C, open arrows). In addition, this probe binds the DMSP 5-specific factor (dark arrow) which is specifically competed by unlabeled DMSP 5 oligonucleotide.

The sequences protected by footprints 2 and 7 contain PEA3 core motifs; therefore, we determined the availability of nuclear factors in THP-1 cells to interact with consensus oligonucleotides containing a PEA3 consensus sequence (Fig. 9). THP-1 extracts contain protein(s) that interact with oligonucleotides containing the PEA3 motif, indicated by the two specific mobility shift complexes that are detected. Neither complex is competed by NF-κB or AP1 oligonucleotides (lanes 9–12). By contrast, both of the complexes are competed with excess unlabeled PEA3 oligonucleotide (lanes 3 and 4) and with 200-fold excess DMSP 7 (lanes 7 and 8), an oligonucleotide spanning footprint 7 that also contains a PEA3 core motif.

Since DMSP 2 contains a PEA3 core motif, this sequence was also used as a competitive inhibitor of Ets-1 binding to its consensus. Although DMSP 2 is not as effective as DMSP 7, there is approximately a 30% reduction in Ets-1-specific complex formation when a 200-fold excess of DMSP 2 is included in the reaction. The fold molar excess of DMSP 2 in this reaction is deceptive in this case because DMSP 2 appears to contain both a weak PEA3 motif and a weak NF-κB motif, and the binding sites overlap (Fig. 5). The presence of overlapping binding sites suggests that occupancy by either factor might preclude binding by the other. Furthermore, it suggests that there may be a competition between these factors for binding and regulation at this site.

**DISCUSSION**

The TF gene promoter contains several regions that are resistant to DNase I cleavage when labeled DNA fragments are preincubated with nuclear protein prepared from uninduced and induced THP-1 cells. The results are summarized in Table I and Fig. 10, which also identifies functional regions of the TF
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**FIG. 3. DNase I footprint analysis of pTFPXΔSma.** The 224-bp fragment was labeled at the EcoRI site (noncoding strand, Panel A) or the XhoI site (coding strand, Panel B). Nucleotides protected and footprints are indicated by the brackets and corresponding footprint number. Hypersensitive sites are denoted by the open circles. The cluster of hypersensitive sites in Panel B identifies the TTATA box. P, probe; G/A, Maxam Gilbert G+A reaction sequencing ladder; -, no extract; U, 20 pg of nuclear extract prepared from uninduced THP-1 cells; I, 20 pg of nuclear extract prepared from LPS-induced THP-1 cells.

The promoter described in the literature (4, 14, 16). With the exception of footprint 1 (−22 to +8) the additional five footprints are contained within a 200-bp region between −374 and −172 relative to the cap site. Four of the footprints are contained in a region of the promoter reported to be involved in transcriptional activation and induction (footprints 1–5) and two additional footprints map to a region that may be involved in silencing gene expression (footprints 6 and 7).

Since footprint 1 (−22 to +7) was very strong we considered that this region, between the TTATA box and the cap site, might bind a negative factor capable of preventing occupancy by RNA polymerase II and its accessory proteins in uninduced THP-1 cell extracts. To explore this possibility DMSP 1, a 29-bp double-stranded oligonucleotide, was synthesized, labeled, and incubated with nuclear extracts. As Table I indicates no specific mobility shift bands were detected even though a range of conditions were employed. A computer search for consensus binding sites in this sequence was also nonproductive. One explanation is that this strongly protected region interacts with RNA polymerase II and its accessory factors in the context of the native promoter, preventing digestion by DNase I. However, in the DNA mobility shift assay this complex may be unable to form on the 29-bp fragment due to physical constraints that preclude the generation of protein-DNA complexes.

Sequences between −191 and −172 appear to be important for LPS induction of TF expression, since these sequences have been reported to be sufficient to mediate LPS induction of reporter gene constructs (16). Using DMSA, we detect weak binding activity to DMSP 2 (footprint 2, −191 to −151) in both uninduced and induced nuclear extracts. This binding is completely abrogated when an unlabeled oligonucleotide containing the NF-κB consensus site is included in the reaction. Other groups have reported a strong induction of NF-κB binding ac-
activitiy to both its consensus binding sequence and to oligonucleotides spanning -193 to -172 in nuclear extracts prepared from LPS-induced THP-1 cells (16). Our data confirm that the level of NF-κB binding activity in THP-1 cells increases with induction; however, we do not see comparable levels of binding to oligonucleotides containing the NF-κB-related sequence found in the TF promoter (-191 to -172). In contrast to the strong signal observed when a probe containing the consensus NF-κB binding site is incubated with induced THP-1 cell extracts, DMSP 2 oligonucleotides bind very weakly to factors in both uninduced and induced extracts. One explanation for the reduced binding activity may be differences between the nucleotide sequence contained in DMSP 2 (CGGAGTTTCC) in comparison with the nucleotide sequence of the NF-κB consensus binding site (GGGACTTTCC). In addition, we do not see a dramatic difference in binding to DMSP 2 with uninduced and induced extracts. The two nucleotide sequence differences present in the TF sequence (indicated by bold letters) are not choices that conform to the NF-κB consensus alternatives GGG(G/A)(A/T/C/T/T/C/T/C)(T/C)CC). Since the binding to DMSP 2 is very low level, these two nucleotide differences must be critical for high affinity binding by NF-κB.

Computer homology analysis of the TF gene promoter indicates that, on the noncoding strand, there are two regions with homology to the PEA3 consensus core sequence (AGGA(A/C)) (26). One is contained in footprint 2 (-191 to -172), and an additional site is contained within footprint 7 (-363 to -343). Ets-1 as well as related family members have been reported to bind to the PEA3 motif (27, 28). We are currently evaluating Ets-related protein interactions with these sequences. Preliminary data indicate that THP-1 nuclear extracts contain a binding activity that interacts with a consensus oligonucleotide containing the PEA3 enhancer sequence (Fig. 9). Ets-1 binding is reported to be relatively low affinity; however, binding to the consensus oligonucleotide is partially competed by 200-fold excess of DMSP 2 and more effectively competed by 200-fold excess DMSP 7 and Ets-1 consensus sequence (Fig. 9). Although DMSP 2 is a less effective competitor than DMSP 7, the
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The inability of DMSP 2 to completely abrogate Ets-1 binding suggests that the protein interactions with this sequence might be more complex. Our data suggest that there might be a competition for binding to this site by both NF-κB- and Ets-1-related factors. In this case, binding of NF-κB to the DMSP 2 competitor probe would reduce the concentration of probe available to compete for Ets-1 binding to its consensus binding site. This may be important in the function of the promoter. Although TF expression is induced by serum and the sequences between -383 to the cap contains enough information to mediate this expression is induced by serum and there are two reiterations within the TF proximal promoter, factor binding to this region may also be responsible for serum induction.

The phosphoprotein Ets-1, or another member of this family of transcriptional activators, interacting with the PEA3 motif, provides an attractive candidate for a key regulator of TF expression in THP-1 cells for several reasons. The PEA3 motif can mediate serum induction as well as phorbol ester induction of gene expression (29). Other groups have described a type II proto-enhancer that combines several transactivation domains, each of which is weak in isolation, in sequence to form an enhancer element with stronger activation potential (30).

Footprint 3/4 contains two closely linked binding sites for AP1 (DMSP 3 and 4). DMSP 3 binds with high affinity to AP1 in uninduced and induced nuclear extracts. By contrast, DMSP 4 binds to AP1 with barely detectable affinity with uninduced extracts and this binding increases with LPS induction. The authenticity of AP1 binding to DMSP 4 was confirmed since the extracts and this binding increases with LPS induction. The Polyomavirus enhancer, as well as the collagenase promoter, are included in this subclass (31). In addition, they point out that several genes, including stromelysin, interleukin-2, and Fos, contain linked PEA3 and AP1 motifs. Our data suggest that the TF gene may also be included in each of these groups, since AP1 and PEA3 motifs are also linked, and this association may be important in the function of the promoter. Although TF expression is induced by serum and the sequences between -383 to the cap contains enough information to mediate this induction, there is no consensus serum response element located in this region (14, 17). Since the PEA3 element is serum responsive and there are two reiterations within the TF proximal promoter, factor binding to this region may also be responsible for serum induction.

Footprint 3/4 contains two closely linked binding sites for AP1 (DMSP 3 and 4). DMSP 3 binds with high affinity to AP1 in uninduced and induced nuclear extracts. By contrast, DMSP 4 binds to AP1 with barely detectable affinity with uninduced extracts and this binding increases with LPS induction. The authenticity of AP1 binding to DMSP 4 was confirmed since the binding activity is competed by unlabeled oligonucleotides containing either the AP1 consensus site or by unlabeled DMSP 3 which also binds AP1. Comparison of the DNA sequence in

![DNA sequence and regions of protein-DNA interaction on the TF promoter](image)

**Figure 5.** DNA sequence and regions of protein-DNA interaction on the TF promoter. The dark lines delineate footprinted regions. Footprint numbers are indicated above each line. Confirmed proteins and their consensus DNA binding sites are indicated by the boxed regions and regular typeface print. Putative DNA binding sites are boxed and indicated in boldface type. CAP site indicates the mRNA transcription start site. Negative numbers to the left of the figure indicate nucleotide distances relative to the CAP.

### Table 1
Summary of DNA mobility shift probes and results

| Footprint no. | DMSP no. | Location     | Sequence                                      | No. of mobility shift | Binding competed by |
|---------------|-----------|--------------|-----------------------------------------------|-----------------------|---------------------|
| 1             | 1         | -22 to +7    | GCGCGGGGACCCGGCGCCCGTCCTCCAAGACTG            | ?                     |                    |
| 2             | 2         | -191 to -172 | GTCGGGGGAGTTTCTACCCGG                       | 2                     | Self, NF-κB         |
| 3/4           | 3         | -215 to -198 | TCGGGTGCTAGTCTCCCTT                        | 1                     | Self, AP1           |
| 3/4           | 4         | -231 to -212 | GCAGGTTTGAATCTACCGG                        | 1                     | Self, AP1, DMSP 3   |
| 5             | 5         | -277 to -262 | GTGCGGGGCTACGGACCC                        | 1                     | Self               |
| 7             | 7         | -363 to -343 | GATCCCTTTCCCTGCAATGACCT                     | 4                     | Self, one DMSP 5    |
| 5, 6, 7      | PCR1      | -363 to -262 | GATCGAGGGGCTTCTCTCCTAG                     | 3                     | Self               |
| 6, 7         | Sty 84    | -374 to -290 | TACGCCCCCTGAAGAGACAGTCC                    | 2                     | Self               |
| 6, 7         | NF-κB     |              | GATCGAGGGGCTTCTCTCCTAG                     | 2                     | Self, DMSP 2, DMSP 7|

[Further details and explanations regarding the table entries are provided in the text body.]
DMSP 3 and DMSP 4 reveals that DMSP 3 contains a canonical AP1 binding site (TGAGTCA) which differs at one nucleotide in DMSP 4 (TGAATCA). This single change may account for the difference in affinity of these two probes for AP1 binding. Since there are a number of heterodimers that recognize the AP1 binding site, one explanation for the dramatic increase in binding to the DMSP 4 probe in induced nuclear extract might be that a novel heterodimer that forms post-induction might recognize this oligonucleotide. This may be the same heterodimer or a different heterodimer from the complex that recognizes DMSP 3. In addition, the absence of binding activity to DMSP 4 in uninduced extract suggests that a subset of AP1 binding activity is not able to recognize the single nucleotide change from the consensus present in this oligonucleotide. By DNase I footprinting these two binding sites are contiguous along the promoter. In addition, the degree of protection by protein in uninduced and induced extracts is equivalent (Fig. 4). This suggests that AP1 interactions with the two contiguous binding sites may be cooperative in the context of a larger DNA fragment. Thus, in contrast to the difference in affinity between DMSP 3 and 4, determined by DMSA, there may not be a substantial difference in the footprint pattern on the TF promoter since constitutive binding to the proximal site in uninduced extract may protect the distal site also.

Finally, we have also identified two regions of the TF promoter (footprints 5, -277 to -262; and 6, -332 to -313) that bind nuclear factors both by DNase I footprint analysis and DNA mobility shift analysis. Unlike most of the other protected regions, footprint 6 is characterized by an uneven intensity of DNase I fragments compared with the control lane, which does not contain added extract, suggesting that protein interactions with this sequence may not be very strong. Although the foot-

**Fig. 6. DMSA with probes corresponding to footprint 2.** Panel A, DMSP 2; Panel B, NF-κB, PROTEIN: no extract (Panels A and B, lane 1); 10 μg of uninduced THP-1 cell nuclear extract (Panel A, lane 2; Panel B, lanes 2, 6-10); 10 μg of induced THP-1 cell nuclear extract (Panel A, lanes 3-7; Panel B, lane 3); 10 μg of uninduced THP-1 cell cytoplasmic extract (Panel B, lane 4); 10 μg of induced THP-1 cell cytoplasmic extract (Panel B, lane 5). Arrows at the right of the panels indicate the specific mobility shift bands. Specific competitors: -, no competitor; S, unlabeled AP2 consensus oligonucleotide containing AP2 binding site (GATCGAACTGACCGCCCGGCCCGT); NF-κB consensus oligonucleotide containing NF-κB binding site (GATCGAGGG-)

**Fig. 7. DMSA with probes corresponding to footprint 3/4.** Panel A, DMSP 3; Panel B, DMSP 4. Protein: no extract (Panels A and B, lane 1); 10 μg of uninduced THP-1 cell nuclear extract (Panels A and B, lane 2); 10 μg of induced THP-1 cell nuclear extract (Panels A and B, lanes 3-7). Arrow at the right of the panels indicates the specific mobility shift band. Specific competitors: -, no competitor; S, unlabeled probe; AP1, consensus oligonucleotide containing AP1 binding site; AP2, consensus oligonucleotide containing AP2 binding site; DMSP 3, DMSP 3 oligonucleotide. Unlabeled competitors are indicated and the fold molar excess is given in parentheses.
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**Fig. 8.** DMSA with probes corresponding to footprints 5, 6, and 7. Panel A, DMSP 5 (footprint 5); Panel B, DMSP Sty 84 (footprints 6 and 7); Panel C, DMSP PCR1 (footprints 5, 6, and 7). Protein: no extract ( Panels A, B, and C, lane 1) or 10 µg of extract were included in each reaction. THP-1 cell uninduced nuclear extract ( Panel B, lane 2); THP-1 cell-induced nuclear extract ( Panel A, lanes 2–6; Panel B, lanes 3 and 8–11; Panel C, lanes 2 and 3); THP-1 cell-uninduced S100 extract ( Panel B, lane 6); THP-1 cell-induced S100 extract ( Panel B, lane 7); fetal lung fibroblast nuclear extract ( W138, Panel B, lane 4); hepatocellular carcinoma cell nuclear extract ( HepG2, Panel B, lane 5). Arrows and open arrowheads at the right of the panels indicate the specific mobility shift bands. Specific competitors: –, no competitor; S, unlabeled probe; AP1, consensus oligonucleotide containing AP1 binding site; AP2, consensus oligonucleotide containing AP2 binding site; SP1, consensus oligonucleotide containing SP1 binding site ( GATCGATCGGGGCGGGGCGATC); NF-KB, consensus oligonucleotide containing NF-KB binding site; DMSP 5, oligonucleotide spanning footprint 5. Unlabeled competitors are indicated and the fold molar excess is indicated in parentheses.

**Fig. 9.** DMSA with probes containing PEA3 consensus binding sites and competition with TF promoter DMSP. Protein: no extract, lane 1. All other lanes contain 10 µg of induced THP-1 nuclear extract. Arrows at the right indicate the specific mobility shift bands. Specific competitors: –, no competitor; S, unlabeled probe; DMSP 2, oligonucleotide from footprint 2; DMSP 5, oligonucleotide from footprint 5; NF-xB, consensus oligonucleotide containing NF-xB binding site; AP1, consensus oligonucleotide containing AP1 binding site. Fold molar excess is indicated in parentheses.

print pattern is unusual, we have determined that proteins do interact with labeled probes containing this sequence (Fig. 8, **PCR1** and **Sty 84**). We have not been able to compete the binding to either the DMSP 5 oligonucleotide or the Sty 84 fragment with unlabeled oligonucleotides containing several consensus binding sites (Table 1). Although footprint 5 contains an SP1 binding motif, the binding to this oligonucleotide is not competed by unlabeled SP1 consensus oligonucleotides. Computer homology search for reported transactivator binding sites did not reveal any additional consensus sites in these two regions.

At least for the Sty 84 fragment, our data indicate that the factors binding to this probe are not present in W138 cells (fetal lung fibroblasts), which express high levels of TF mRNA, and HEPG2 cells (hepatocellular carcinoma cells), which do not express TF mRNA (data not shown). Since footprints 6 and 7 reside in the "silencer" region of the promoter (14), factors binding to these sites could facilitate the repression of TF synthesis in uninduced monocytes. Subsequent modification of these factors following an induction stimulus could reactivate transcription suggesting that these proteins might be "modulatory" in function. Since W138 cells constitutively express TF mRNA, and HEPG2 cells do not express TF mRNA, modulatory proteins would not be needed. In fact, our analysis suggests that they are not present in extracts prepared from these cells.

At least two of our footprints (5 and 6) appear to interact with nuclear binding proteins although the sequences contained in these regions do not share homology with consensus binding sites in the database. We have prepared nuclear extracts from a number of different cell lines in order to determine if the proteins that bind to these sequences are monocyte specific. By Southwestern bloting we have identified a 150-kDa monocyte-
specific protein that binds to probes containing footprints 5 and 6 (data not shown). We are beginning to purify these binding activities in order to characterize them further.

In summary, we have identified six regions between -383 and -277 that are resistant to DNase I cleavage when DNA is preincubated with nuclear extract. Four of these footprints map to regions reported to be involved in transcriptional activation (footprints 2–5, -277 to -172). Two additional footprints localize to a region that has been reported to reduce base-line expression of the TF promoter by 50% in transfected COS 7 cells (footprints 6 and 7, -383 to -278). With the exception of two single, hypersensitive sites that are detected with probes incubated with uninduced extracts (Figs. 3B and 4B) and an increase in binding of AP1 to DMSP 4 oligonucleotides, there are no additional differences in protein-DNA interactions when uninduced or induced extracts are compared. This analysis does not preclude that post-translational modification of pre-existing proteins shifts the promoter from an inactive state to a transcriptionally active state following LPS induction. Alternatively, additional proteins not detected in crude nuclear extracts may contribute to this process.

Although several of the regions seem to bind well characterized factors such as AP1, two additional sites contain a PEA3 core enhancer motif that may interact with a member of the Ets-1 family of transcriptional activators. In addition, the role of Sp1 binding to the regulation of TP synthesis remains to be evaluated. Finally, we have also identified binding to sequences that appear to be important for constitutive expression (footprint 5) and repression of expression (footprints 6 and 7). Two of these sites (footprints 5 and 6) may bind novel factors since they do not contain homology to reported consensus binding sites by computer sequence analysis. Experiments to further characterize the proteins that interact with these sequences are in progress and will contribute to a better understanding of the molecular events that modulate TP synthesis in monocyte cell lines.

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