Functional analysis of the human tissue factor promoter and induction by serum

(blood coagulation/transcriptional regulation/serum response element/AP-1 binding sites)

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ABSTRACT Tissue factor (TF) is the primary initiator of the coagulation protease cascades. This cell surface glycoprotein is the receptor and essential cofactor for the serine protease factor VIIa. TF is constitutively expressed in some extravascular cell types and is transiently induced in monocytes, endothelial cells, and fibroblasts. Inducible expression is implicated in cellular immune responses, inflammation, and intravascular coagulation. Transcriptional regulation of the TF promoter was analyzed in COS-7 cells under conditions of (i) high-level expression and (ii) serum induction. The region comprising nucleotides –209 to +121 (relative to the transcription start site) supports high-level transcriptional activity and can be divided into two distinct regions: a region (–111 to +121) that exhibited low promoter activity and a region (–209 to –112) that enhanced transcriptional activity to a high level. The role of further upstream sequences is still to be established, although two consensus binding sites for the transcriptional activator protein AP-1 did enhance low-level promoter activity. In serum-starved COS-7 cells TF expression was transiently increased 20-fold by serum. All transcriptionally active constructs were responsive to serum, indicating the presence of at least one serum response element, whose function was retained in the immediate 5' aspect of the gene, at –111 to +14. Based on this functional map, we propose that the elaborate pattern of TF expression by cells results from a relatively complex promoter.

Tissue factor (TF) is the cell surface glycoprotein that enables cells to initiate the coagulation protease cascades. It functions as the high-affinity receptor for the serine proteases factors VII and VIIa (reviewed in ref. 1). The resulting TF–VIIa complex provides a first catalytic event that is responsible for initiation of the coagulation protease cascades by specific limited proteolysis. Unlike the other cofactors of these protease cascades, which circulate as nonfunctional precursors, TF is a potent initiator that is fully functional when expressed on cell surfaces. Therefore, regulation of *de novo* TF expression in transcriptionally silent cells would appear to be a critical step in the control of this protease cascade and its inflammatory consequences. Indeed, TF is a transiently inducible effector molecule of endothelial cells and monocytes in inflammation (2, 3) and is implicated in the pathogenesis of several thrombohemorrhagic disorders (4).

Expression of the TF gene is markedly induced in response to serum in mouse 3T3 cells and, since this induction is rapid and does not require new protein synthesis, the TF gene has been classified as an immediate early gene (5). The mechanism by which TF gene expression is induced by serum and cytokines is not well defined. However, the observation that TF is coinduced with such genes as *c-fos* and *c-myc* has led to the intriguing suggestion that TF expression may play a

role in cell growth (5). The activation of *c-fos* by serum has been shown to be mediated in part by a well-defined cis element called the serum response element (SRE) (6).

Induction of TF expression is under transcriptional control in both monocytes and fibroblasts (3, 5). Therefore, understanding TF expression requires characterization of elements within the TF gene and its promoter that are responsible for regulation of transcription within the context of differentiation, transformation, and induction in normal and pathologic settings (7). We previously determined the organization of the human TF gene together with its complete DNA sequence (8). In the present study we have identified the promoter of the TF gene. DNA upstream of the initiation codon was used to drive expression of the luciferase gene in a reporter vector transfected into COS-7 cells. These cells express TF at a high level and, under conditions of serum starvation, show substantial induction of the TF gene in response to serum.

MATERIALS AND METHODS

Plasmids. DNA fragments from the TF promoter (8) were inserted into the multicloning site of p19luc (9) by using the restriction sites shown in Fig. 3. Plasmids pL8, pL9, and pL21 were made by site-directed mutagenesis using the oligonucleotides Mack 1 and Mack 2 (Fig. 1). The following oligonucleotides (Fig. 1) together with their complementary sequences were cloned into the unique *Sma* I site of pL6 to make the enhancer series: Mack 3 in pL22 and pL23, Mack 5 in pL25, Mack 7 in pL26, Mack 9 in pL27, and Mack 11 in pL12. The promoterless p19luc reporter vector (9) and the pRSVluc and pRSVcat vectors (11) were used as controls in the transfections.

Cell Culture. COS-7 cells (simian virus 40-transformed monkey kidney cells) were obtained from the American Type Culture Collection and cultivated as instructed. Cells were serum-starved by replacing the growth medium [Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum] with DMEM plus 0.25% serum for 24 hr before inducing with serum by shifting the cells to DMEM plus 20% serum.

DNA Transfections. Supercoiled plasmid DNA (4 µg) was used to transfect each subconfluent cell culture (10-cm plate; Costar) by the DEAE-dextran method (11) with addition of chloroquine. In standard transfections, cells were cultured in DMEM plus 10% calf serum for 48 hr before harvest. Alternatively, transfected cells were serum-induced.

Activity Assays. Cell lysates were prepared and assayed for luciferase activity as described (11). Chloramphenicol acetyltransferase (CAT) activity was determined by a diffusion-based assay (12). Cell lysates (frozen and thawed three times) were assayed for TF activity in a one-stage clotting assay (13).

Abbreviations: TF, tissue factor; SRE, serum response element.

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TF	-235	<u>AP-1</u>	<u>AP-1</u>	-196
	5'	CGGTGGCGCGGTGAATCACTGGGGTGAATCATCCCTTGC	3'	
Mack 1	5'	CGGTGGCGCGGTAAACATCACTGGGG-3'		
Mack 2		5'-ATCACTGGGGTACGTCATCCCTTGC-3'		
Mack 3	5'	GGGGGTGAATCACTGGGGTGAATCATCCCG-3'		
Mack 5	5'	GGGGGTACGATCACTGGGGTGAATCATCCCG-3'		
Mack 7	5'	GGGGGTGAATCACTGGGGTACGTCATCCCG-3'		
Mack 9	5'	GGGGGTACGATCACTGGGGTACGTCATCCCG-3'		
Mack 11	5'	GGGTGAATCACTGGGGTGAATCATCC-3'		

FIG. 1. Oligonucleotides used in the study. The TF genomic sequence in this region (−235 to −196) and the position of the two putative AP-1 sites are shown. Mutated bases are indicated by asterisks above the sequence and were chosen according to Lee *et al.* (10). The distal AP-1 site (TTGAATCA) was mutated to either TAACATCA or TACGATCA and the proximal AP-1 site (GTGAGTCA) was mutated to GTACGTC. The bases of the flanking *Sma* I half-sites are underlined.

RNA Hybridization. Total cellular RNA purified by the guanidine thiocyanate/CsCl method was subjected to denaturing electrophoresis in formaldehyde/agarose gels and blotted onto GeneScreen (New England Nuclear) (3). DNA probes were labeled by the random-primer method (Boehringer Mannheim), and hybridization and wash conditions were as described (3).

Primer Extension. Primer extension was performed (8) using a 29-mer oligonucleotide primer, 5'-GCATTCCGGTACTGTGGTAAATGGAAG-3', which is complementary to nucleotides 30–58 of the luciferase cDNA sequence (11).

RESULTS

Serum Induction of TF Activity in COS-7 Cells. COS-7 cells were adopted as a model system for the TF promoter studies because they produce a high level of TF activity (200 units per 10^6 cells) when continuously cultured in medium containing serum and can be transfected with high efficiency. By comparison, human monocytes stimulated with endotoxin produce only 10–25 units of TF activity per 10^6 cells. In addition, serum-starved COS-7 cells exhibited lower levels of TF activity but responded to serum by transiently increasing TF activity. All procoagulant activity derived from COS-7 cells was identified as due to TF since it was inhibitable by monoclonal antibodies to human TF (13).

COS-7 cells induced with 20% serum showed a 20-fold increase in TF activity, which peaked at 8 hr before decreasing over the next 14 hr to levels observed when cells were continuously cultivated in the presence of serum (Fig. 2A). TF mRNA levels increased 32-fold from low levels to a peak at 2 hr before slowly decreasing (Fig. 2B).

High-Level TF Promoter Activity in COS-7 Cells Transfected with the 5' Deletion Series. To study constitutive expression, TF promoter plasmids (Fig. 3) were transfected into COS-7 cells and the cells were cultivated in the presence of serum for 48 hr. The longest construct, pL1, exhibited promoter activity that was $\approx 3\%$ the level observed for pRSVluc, the vector driven by the strong Rous sarcoma virus promoter (Fig. 4A). Primer extension showed that the RNA transcripts derived from pL1 in COS-7 cells initiated at the same position as the TF mRNA transcripts in human fibroblasts (data not shown).

Plasmids with sequential 5' truncations of the TF promoter region (pL1, pL2, and pL3) down to nucleotide position −383 all expressed similar levels of total luciferase activity (Fig. 4A). Further deletion of the region from −383 to −279 (pL4) reproducibly led to an almost 2-fold increase in luciferase

FIG. 2. Time course for serum induction. COS-7 cells were serum-starved for 24 hr, then fed with medium containing 20% calf serum (0 hr). At the times indicated, disrupted cells were assayed for total TF activity (A) and the levels of TF mRNA were determined by hybridization (B) using a TF cDNA probe (8). In B, 10 μ g of total RNA was used in each lane and was normalized for the amount of the single 1.1-kilobase (kb) CHO-B housekeeping mRNA (14). Positions of 28S and 18S rRNA are indicated. Band intensity was quantified using an LKB Ultrosan XL and the adjusted values gave the following: 0 hr, 1 $\times$; 1 hr, 18 $\times$; 2 hr, 32 $\times$; 3 hr, 28 $\times$; 4 hr, 28 $\times$; and 5 hr, 16 $\times$.

expression, suggesting that elements in this region might bind regulatory factors that have a negative effect on transcription. Removal of the DNA from −278 to −210 (pL5), which contains two consensus AP-1 binding sites, resulted in a 2-fold decrease of transcription from the TF promoter. Deletion to create a plasmid (pL6) containing only 111 nucleotides upstream of the cap site reduced promoter activity to about 26% of the wild-type level. Finally, promoter activity from pL7, in which the TATA box and all upstream sequences had been deleted, did not differ from that of the promoterless control p19luc. This indicated that positively acting DNA sequence elements necessary for high-level TF promoter activity in COS-7 cells were located between −278 and +121. Notably, this region is rich in consensus sequences for binding regulatory factors and includes a predicted TATA box, two AP-1 binding sites (10) and two CACCC boxes (15), and four sequences that comply with the G+C-rich recognition site for transcription factor Sp1 (16) (see Fig. 6).

Serum Induction of Luciferase Activity Expressed by TF Promoter Plasmids. To establish whether the serum response of the endogenous TF gene in COS-7 cells could be attributed to DNA elements within the promoter region described above, COS-7 cells transfected with pL3 were serum-induced. Luciferase expression transiently increased 20-fold to a peak between 3 and 5 hr postinduction (Fig. 5). Therefore, pL3 appeared to contain at least one SRE.

To further delineate the DNA sequence element(s) required for this serum response, COS-7 cells transfected with TF

FIG. 3. TF promoter plasmids. Panel A, the 5' deletion series; panel B, deletion of either the TATA box or the 5' untranslated region; panel C, the mutant series; panel D, the enhancer series. pL12 contains two copies of a shorter, 26-base-pair (bp) oligonucleotide (Mack 11). The AP-1 sites are represented as boxes where mutated sites are filled; in the enhancer series the arrows indicate the orientation. The *Rsa* I site was introduced by site-directed mutagenesis.

promoter plasmids from the 5' deletion series (pL1–pL7) and pL14 (Fig. 3) were induced with serum. All transcriptionally active TF promoter constructs supported a 6- and 12-fold induction of luciferase activity (Table 1), which suggests that at least one SRE is located between –111 and +14. Previously described SRE nucleotide sequences (6) are not present in this region, indicating the presence of a novel SRE.

Removal of the TATA Box. Two plasmids were created from pL3 to examine the role of the TATA box and 5' untranslated region of exon 1 (Fig. 3, panel B). Excision of 21 nucleotides (–42 to –22), so as to eliminate the TATA box from the complete –383 to +121 DNA fragment, led to a modest, 27% reduction in total luciferase activity from pL15 as compared to wild-type activity (Fig. 4B). In addition, removal of the majority of the 5' untranslated region of exon 1, from +15 to +121 (pL14), reduced luciferase expression by 34%.

Role of the AP-1 Binding Sites. AP-1 binding sites are critical for basal activity of certain promoters and have key roles in the induction of other genes (10). Results above show that deletion of a region (–278 to –210) containing two AP-1 consensus binding sites led to a 2-fold decrease of TF promoter activity.

To test whether this decrease was due to deletion of the two AP-1 sites, we mutated each individual consensus site (Fig. 1) *in situ* and determined its effect on the ability of the TF promoter to regulate the high levels of luciferase expression. The maximum reduction expected was 50% because complete removal of this region (–278 to –210) gave levels of luciferase similar to those of pL3 (Fig. 4A). Mutation of either site alone (pL8 and pL9, Fig. 3, panel C) led to only a small reduction in luciferase expression, and mutation of both sites together (pL21) led to a 28% reduction in TF promoter

activity relative to the activity of the parental plasmid pL4 (Fig. 4C). These results show that the AP-1 sites do not play a major role in TF promoter activity, although mutation of both sites does reduce transcription. There are two hypotheses that may explain these data: (i) that the contribution of the AP-1 sites is difficult to detect because of the high levels of expression in COS-7 cells and (ii) that the activity of the AP-1 sites is modified by the binding of proteins to other DNA elements within this region.

To test the first hypothesis, we examined whether these AP-1 sites could act as enhancers in a system with low promoter activity. Previously, synthetic AP-1 binding sites have been shown to possess enhancer activity in HeLa cells when inserted upstream or downstream of the early promoter of simian virus 40 (10). A 32-bp oligonucleotide (Fig. 1) containing the TF upstream DNA sequence –226 to –201 and spanning the two putative AP-1 sites was analyzed for enhancer activity by adding it to pL6, since this plasmid exhibited low promoter activity. The results from pL22 and pL23 (Fig. 3, panel D) illustrate that this does increase luciferase expression relative to pL6 by more than 6-fold whether the oligonucleotide is in forward or reverse orientation (Fig. 4D). A shorter, 26-bp oligonucleotide (Fig. 1) gave similar results (data not shown). When two copies of the 26-bp oligonucleotide were added to create pL12, overall activity was increased 15-fold. To determine whether it was the putative AP-1 sites themselves that were influencing transcriptional activity, we mutated each AP-1 site alone (pL25 and pL26) and also made a double mutant (pL27; Fig. 3, panel D). The level of enhancement was reduced to only 2- and 3-fold for the single mutants pL25 and pL26, respectively, and was completely abolished for the double mutant.

FIG. 4. Functional analysis of TF promoter activity. Total luciferase activity (arbitrary units) from transfected COS-7 cells was measured and normalized for the amount of DNA uptake by using pRSVcat (2 μ g) as an internal control. Each transfection was performed in duplicate at least three times and the results of a typical experiment plus the standard deviation are shown. (A and B) The 5' deletion series and deletion of the TATA box, respectively. (C and D) The mutant and enhancer series, respectively.

These results indicated that each putative AP-1 site alone could act as an enhancer in our experimental system and that these activities combined to enhance TF promoter function. Furthermore, the mutations chosen were effective in abolishing this activity.

DISCUSSION

Transfection of COS-7 cells, which express the endogenous TF gene *in vitro*, revealed that a region within the TF promoter between -383 and +121 contained DNA sequence elements involved in regulating high levels of transcription, while additional 5' flanking sequence up to -2106 gave no

increase in promoter activity. In contrast, low-level transcription initiation was ascribed to the sequence -111 to +121. Deletion of the TATA box together with all upstream nucleotides completely abolished TF promoter activity. However, excision of a small DNA fragment (-42 to -22) spanning the TATA box had only a moderate effect on luciferase expression. Previous studies of the simian virus 40 early promoter established that the TATA-box region was involved in fixing transcription initiation precisely but was dispensable for gene expression (17). The TF promoter contains four predicted recognition sites for the transcription

FIG. 5. Time course for serum induction of luciferase activity. COS-7 cells transfected with pL3 (10 μ g per 10-cm plate) were serum-starved for 24 hr and induced with medium containing 20% calf serum. At the times indicated cells were harvested and luciferase activity per 10⁶ cells was determined.

Table 1. Serum induction of the TF promoter plasmids

Plasmid	Luciferase activity, arbitrary units per 10 ⁶ cells		Fold induction
	0 hr	3.5 hr	
pL1	920	11,023	12.0
pL2	1119	14,334	12.8
pL3	1318	14,535	11.0
pL4	2930	28,845	9.8
pL5	1778	12,728	7.2
pL6	333	3,307	9.9
pL7	66	111	1.7
pL14	2109	11,997	5.7

COS-7 cells were transfected (6 μ g per 10-cm plate) with various TF promoter plasmids (pL1-pL7 and pL14) and serum-starved for 24 hr before being induced with 20% calf serum. Samples were taken at the time of induction (0 hr) and 3.5 hr later. The combined results from three independent experiments are shown together with fold induction.

FIG. 6. DNA sequence of the TF promoter. The TATA sequence is underlined and the start of transcription (+1) is marked with a bent arrow. Restriction sites for *Eco*RI and *Acc*I are indicated. Sequences showing homology to the consensus sequences previously described (8) for binding sites of a variety of transcription factors are boxed. The position of the 32-bp oligonucleotide used in the enhancer series is indicated by bold underlining. The bases that were changed by site-directed mutagenesis or in the mutant 32-bp oligonucleotides are marked with dots above the sequence. The region proposed to contain a SRE is indicated by dashed underlining.

factor Sp1 (Fig. 6), and their close proximity to the cap site (−140 to −57) is consistent with the location of functional Sp1 binding sites (16). These data suggest that the TF promoter can be functionally divided into two regions. First, a region (−111 to +121) that exhibits low promoter activity and binds the basic transcriptional machinery; it includes a TATA box and three Sp1 binding sites. Second, a region (−209 to −112) that enhances TF promoter activity to high levels.

Serum-starved COS-7 cells were observed to transiently increase both TF procoagulant activity and TF mRNA in response to the addition of serum as a source of growth factors. The induction of the murine TF gene by serum shows similar kinetics to that of *c-myc*, with mRNA levels peaking at 2 hr, whereas other immediate early genes such as *c-fos* show mRNA levels that are less sustained, peaking at 20 min (5). A potent SRE has been defined as part of the *c-fos* promoter (6), but this consensus sequence is lacking in the *c-myc* gene. In this study a region within the TF promoter between -111 and +14 (Fig. 6) was sufficient for serum responsiveness. However, as for the *c-myc* gene, the 5' aspect of the TF gene including this region (-111 to +14) did not show consensus nucleotide sequences present in known SREs (6). We speculate that the TF promoter may contain a novel SRE that could be related to the element in the *c-myc* gene. It will be important to map more precisely the SRE within the TF promoter. TF expression in response to growth factors suggests a new role for this protein in cell growth (5). In support of this notion is the well-known capacity of thrombin, generated in the coagulation cascade, to act as a cytokine-like mitogen and activator of a wide variety of cell types (reviewed in ref. 18).

The pattern of TF expression in cells is complex and is influenced by differentiation (7) as well as various agonists such as endotoxin and the cytokines interleukin 1 and tumor necrosis factor α (1–3). Although the two AP-1 sites within the TF promoter do not play a major role in constitutive expression in COS-7 cells, they may represent an important agonist-responsive element in cell types that can be induced to express TF. For instance, an interleukin 1-responsive element within the human interleukin 2 gene was recently found to be similar to an AP-1 binding site (19). Analysis of the TF promoter indicates a degree of complexity that promises to explain the regulation of expression of this

important cell surface receptor and the initiation of the coagulation protease cascades in disease.

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