

A Transcriptional Repressor of the Tissue Factor Gene in Endothelial Cells

Harry Holzmüller, Thomas Moll, Renate Hofer-Warbinek, Diana Mechtcheriakova,
Bernd R. Binder, Erhard Hofer

Abstract—Tissue factor, the high-affinity receptor and cofactor for the plasma serine protease VII/VIIa, is the primary cellular initiator of the blood coagulation cascade. Inside the vasculature, expression of the tissue factor gene must be tightly controlled. Whereas the endothelium normally does not express tissue factor, on stimulation with inflammatory cytokines or endotoxin the gene is transcriptionally upregulated leading to a procoagulant state. We have now detected a repressive cis-acting element in the tissue factor promoter that downmodulates tissue factor transcription in endothelial cells. In reporter gene assays, deletion of this element leads to an increase of tissue factor transcription and insertion of a trimerized site reduces transcription. Specific protein/DNA complexes are formed on the element with nuclear extracts in electrophoretic mobility shift assays and cross-linking of the proteins followed by SDS-PAGE detects the presence of at least 2 subunits of ≈ 40 and 60 kDa, respectively. After transfection of different cell types with the reporter genes, the suppressive effect of the element can only be revealed in endothelial cells. These data suggest that this element represents a novel transcription factor target sequence that functions to suppress expression of the tissue factor gene, preferentially in endothelial cells thereby supporting a noncoagulant state. (*Arterioscler Thromb Vasc Biol.* 1999;19:1804-1811.)

Key Words: tissue factor ■ endothelium ■ transcriptional repressor

Tissue factor (TF) is a transmembrane glycoprotein with distant structural similarity to the cytokine receptor family and functions as the cellular activator of the extrinsic and intrinsic coagulation pathways.¹⁻³ It forms a complex with the plasma serine protease factor VII/VIIa, which binds and activates factors IX and X. Whereas TF is constitutively produced on cells of the adventitia surrounding blood vessels, it is not normally expressed on cells exposed to circulating blood, such as endothelial cells (ECs) or immune cells.⁴⁻⁶ However, after exposure to endotoxin or inflammatory cytokines, ECs rapidly upregulate TF expression. Inappropriate expression of TF on ECs is associated with several diseases and can cause various forms of intravascular coagulation.⁷⁻⁹

The mechanisms that lead to TF gene upregulation in ECs or monocytes after exposure to inflammatory stimuli have been investigated in some detail.¹⁰ DNase I footprinting studies on both the human and the porcine TF promoters have revealed the occupancy of several sites, including nuclear factor- κ B (NF κ B), AP-1, and Sp1 elements, in the respective promoters.^{11,12} To investigate the roles of these transcription factor binding sites in TF gene expression, reporter genes containing various parts of the TF promoter have been analyzed.¹²⁻¹⁴ Whereas primarily the NF κ B, and to some degree also the 2 AP-1 elements, which are clustered between -186 and -142 of the porcine TF promoter, are involved in

the activation of the gene in monocytic and ECs after stimulation with endotoxin, the Sp1 elements are likely required for basal transcription from the TF promoter. The region containing the Sp1 elements has been further implicated in TF expression induced by phorbol 12-myristate 13-acetate in HeLa cells.¹⁵ The TF κ B element, which deviates in 1 position from the NF κ B consensus site, binds p65 and c-Rel subunits.^{12,16,17} These subunits quickly accumulate in the nucleus after stimulation but are virtually absent from nuclear extracts of untreated ECs.

However, it has remained elusive by which mechanisms TF expression is prevented in quiescent ECs compared with the subendothelium and which mechanisms contribute to the fine-tuning of TF expression. Simply the absence of the respective NF κ B subunits in the nucleus might explain the lack of expression in quiescent ECs and transcription could be upregulated in relation to the amount of κ B accumulating in the nuclei. In contrast, repressive elements could be present on the TF promoter and prevent inappropriate expression in the absence of any stimuli. We have therefore investigated additional regions of the TF promoter previously shown by us to be occupied by proteins in footprint analysis.¹² In these studies, we noticed that deletion of a region at -300 bp of the porcine TF promoter leads to a severalfold higher transcription rate in reporter gene assays. We define here the bound-

Received October 13, 1998; revision accepted December 18, 1998.

From the Department of Vascular Biology and Thrombosis Research, Vienna International Research Cooperation Center, University of Vienna, Austria. Present address of T.M., Cardiogene AG, Max-Planck-Strasse 15a, D-40699 Erkrath, Germany.

Correspondence to Erhard Hofer, Department of Vascular Biology and Thrombosis Research, Vienna International Research Cooperation Center, University of Vienna, Brunnerstrasse 59, A-1230 Vienna, Austria. E-mail erhard.hofer@univie.ac.at

© 1999 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

aries and sequence of the element responsible for this effect and demonstrate that specific protein/DNA complexes are formed on the porcine and the homologous human version of the element. In addition, we present data indicating that the repressor functions selectively in ECs.

Methods

Cell Culture and Transient Transfection

ECs were isolated from porcine and bovine aortas and cultured as described.^{12,18} Human umbilical vein ECs (HUVECs), primary human vascular smooth muscle cells, and primary human fibroblasts were obtained as described^{19–21} and cultured in M199 medium supplemented with 20% newborn calf serum (HyClone), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. HUVEC cultures contained, in addition, 50 µg/mL EC growth supplement (Technoclone) and 5 U/mL heparin. Cells were used up to passage number 12. Lipopolysaccharide (LPS) and recombinant human interleukin-1α and tumor necrosis factor-α (TNF-α) used for inductions were purchased from Sigma and from Genzyme Inc, respectively.

Transfections of porcine aortic ECs were performed by using psoralen- and UV-inactivated biotinylated adenovirus and streptavidin/poly-L-lysine as a vector for DNA delivery according to the protocol of Cotten et al,²² with minor modifications as described by Moll et al.¹² Bovine aortic ECs, HUVECs, human fibroblasts, or human smooth muscle cells were transfected by using LipofectAMINE (GIBCO) according to the protocol provided by the manufacturer.

Reporter Gene Constructs and Assays

The basal TF promoter/luciferase reporter gene construct containing the region from -330 to +118 of the porcine TF promoter cloned into a modified pUBT-luc vector²³ has been described previously.¹² The -295/+118 TF promoter fragment was synthesized by PCR from the -330/+118 construct, using an oligonucleotide providing a *NotI* cleavage site fused to the sequence starting at position -295. The PCR fragment was cleaved with *NotI* (at the 5'-end) and *HindIII* (at the 3'-end, position +118) and cloned into the respective sites of the pUBT-luc vector. The construct with a deletion of the pyrimidine-rich repressor element (Py-box) was obtained from (1) a synthetic oligonucleotide covering the sequences from -330 to -307 with a *NotI* site at the 5'-end and an *XbaI* site at the 3'-end, and (2) a PCR fragment synthesized similarly as described above, using an oligonucleotide providing an *XbaI* cleavage site fused to the 5'-end of the sequence starting at position -287 of the TF promoter, the PCR product extending to the *HindIII* site at +118. The resulting construct has the sequences from position -306 to -288 substituted by an *XbaI* cleavage site (GCTCTAGAGC). The TFκB deletion construct has been described,¹² the double deletion of the Py-box and κB sites was obtained by using a fragment generated by PCR from the κB deletion construct as described above.

A trimerized Py-box (GCTCTAGACCCCTTCTTCCCCACAG-ACCTGC ATCGAT CCCCTTCTTCCCCACAGACCTGC ATCG-ATCCCCTTCTTCCCCACAGACCTGCTCTAGAGC) was synthesized with an Applied Biosystems DNA Synthesizer and the oligonucleotide inserted into the *XbaI* site, substituting the Py-box sequence in the corresponding deletion construct. All constructs were partially sequenced to show fidelity of the PCR and subcloning procedures. Luciferase assays were performed with cellular lysates of transfected cells as described.²⁴ Between 10³ and 10⁵ luciferase units were obtained with the basal TF promoter constructs, using ≈10⁵ cells per well for the transfections, respectively.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay

Nuclear extracts from confluent EC cultures, human vascular smooth muscle cells, human fibroblasts, THP-1, and K562 cells were prepared essentially as described by Moll et al.¹² For the electrophoretic mobility shift assays, 2.5 to 5 µg of nuclear protein were incubated with 0.3 to 1.0 ng of radioactively labeled oligonucleotide

hTF	-363	CCCTTTCCTGCC . ATAGACCTGC	-342
		*** ** * ** * *****	
pTF	-308	CCCCTTCTTCCCCACAGACCTGC	-286
		***** ** * ** * **	
hVWF	-381	CCCCTTCTTCCAAACACAGCAGC	-359

Figure 1. Sequence alignment of the porcine and human Py-box elements. The sequence of the -300 region of the porcine promoter was used to search the human tissue factor (TF) promoter, using the Bestfit program of the Genetics Computer Group Sequence Analysis Software Package at default values with the gap weight reduced to 3.0. The 23 bp shown to be important for the formation of the nuclear complexes on the porcine TF Py-box are given and the corresponding region in the human TF promoter is displayed. Further, a similar sequence detected in the human von Willebrand factor promoter is shown. hTF indicates human TF Py-box; pTF, porcine TF Py-box; and hVWF, human von Willebrand factor Py-box-like sequence.

(10⁵ cpm/µL) in binding buffer (20 mmol/L HEPES-KOH, pH 7.9, 1 mmol/L EDTA, 5 mmol/L MgCl₂, 50 mmol/L KCl, 1 mmol/L DTT, and 10% glycerol) for 20 to 30 minutes at room temperature. Protein/DNA complexes were resolved on 5% PAGE in 0.5× Tris-borate/EDTA electrophoresis buffer.²⁵

The double-stranded synthetic oligonucleotides were radioactively labeled by filling in the overhangs with Klenow enzyme in the presence of [α-³²P]dATP and subsequently purified over a 7% polyacrylamide gel. The sequences of the probes used were as follows:

porcine TF Py-box: 5'-ctaCCCCTTCTTCCCCACAGACCTGCgaatt-3';

5'-deletions: 2 bp, 5'-ctaCCTTCTTCCCCACAGACCTGCgaatt-3'; 4 bp, 5'-ctaTTCTTCCCCACAGACCTGCgaatt-3';

3'-deletions: 2 bp, 5'-ctaCCCCTTCTTCCCCACAGACCTgaatt-3'; 4 bp, 5'-ctaCCCCTTCTTCCCCACAGACgaatt-3'; 6 bp, 5'-ctaCCCCTTCTTCCCCACAGgaatt-3';

human TF Py-box: 5'-ctaTCCCTTCTTCCCATAGACCTGCgaatt-3';

Ets-1: 5'-ctaCAGAGCGGAAGTGACgaatt-3';

vWF Py-box: 5'-aattCCCCTTCTTCCAAACACAGCAGCgaatt-3';

Sp1: 5'-aattCGGGGGCGGGACCAGGGCGGGGCTCgaatt-3';

UV Cross-Linking and Southwestern Blotting

UV cross-linking experiments were performed according to Chodosh et al,²⁶ as previously described.¹² In brief, an elongated form of Py-box oligonucleotide (5'-CCCTTGCGATATCCCCCTTCTTCCCCACAGACCTGC-3') was body-labeled on the top strand with [α-³²P]dATP, using Klenow enzyme and a short oligonucleotide as primer (5'-CCCTTGCGATATC-3'). The reaction was performed in the presence of bromodeoxyuridine triphosphate (BrdUTP). Nuclear extract (10 µg) was then incubated with 1.5 to 2×10⁶ cpm of gel-purified radioactive probe. After native gel electrophoresis, the protein/DNA complexes were covalently cross-linked by UV irradiation (302 nm, 12 minutes) in the gel. The bands containing the complexes of interest were excised and the gel slice boiled for 5 minutes in ≈1 volume 2× SDS sample buffer. Subsequently, both gel slice and sample buffer were loaded onto a 7.5% polyacrylamide gel and analyzed by SDS-PAGE and autoradiography.

Results

A Novel Conserved Sequence Element in the TF Promoter

We have previously characterized protein binding regions on the porcine TF promoter by footprint analysis and detected binding of Sp1, NFκB, and AP-1 factors.¹² In these studies, an additional region at -300 bp of the TF promoter has been found to be protected from DNase I cleavage. A closer sequence comparison of the DNase I-protected region of the porcine promoter¹² with the human TF gene²⁶ identified a region of similar sequence ≈50 bp further upstream in the human promoter (Figure 1). In agreement with our findings in

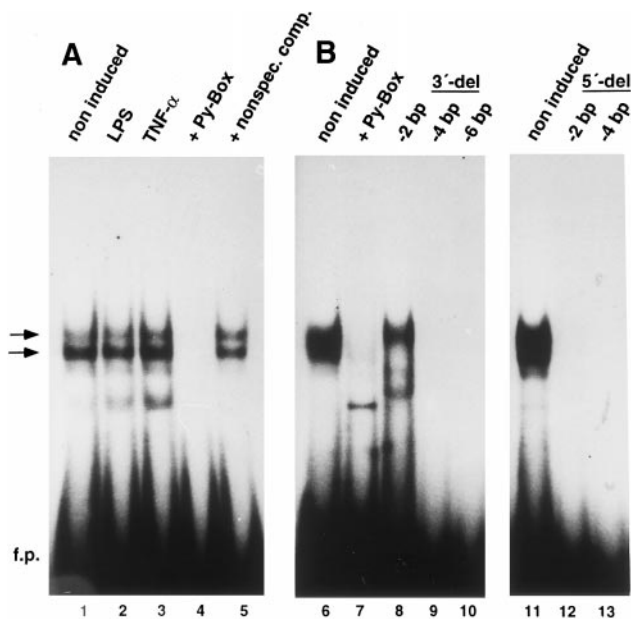


Figure 2. Binding of nuclear proteins to the Py-box element. A, Specific complexes are formed on the Py-box element. An oligonucleotide containing the conserved sequence of the tissue factor (TF) promoter was used for electrophoretic mobility shift assays with extracts from untreated porcine aortic endothelial cells (lane 1) or cells induced for 2 hours with 200 ng/mL lipopolysaccharide (LPS) (lane 2) or 100 U/mL tumor necrosis factor- α (TNF- α) (lane 3). Specificity of binding is demonstrated by complete competition with 100-fold excess of unlabeled Py-box oligonucleotide (lane 4) and absence of competition with a nonspecific oligonucleotide (lane 5). B, Twenty-three bp of the Py-box element are important for complete binding. A series of consecutive 2-bp deletions from the 3'-end (lanes 8 through 10) or 5'-end (lanes 12 and 13) of the Py-box sequence was generated and tested for binding of nuclear complexes. Lanes 6 and 11 show the mobility shift obtained with the wild-type sequence and lane 7, the competition with an excess of unlabeled oligonucleotide. The arrows indicate the position of the 2 major specific DNA complexes formed. f.p., free probe.

the porcine system, this region in the human promoter has previously been described to be protected from DNase I digestion in footprint experiments.¹¹ We have termed the CT-rich region closely conserved between the human and porcine genes pyrimidine box (Py-box) element.

Specific Protein/DNA Complexes Are Formed With the Py-Box Element

To define the boundaries of the element and to characterize potential proteins interacting with this region, gel retardation assays were performed by using oligonucleotides covering the sequences of the region. With oligonucleotides containing the sequences from position -308 to -286 of the promoter, we observed the formation of 2 specific protein/DNA complexes of slightly differing mobilities with nuclear extracts from both quiescent as well as LPS- or TNF- α -treated cells (Figure 2A). To define the minimal DNA sequence required for complex formation, a series of oligonucleotides with sequential 2-bp deletions of bases from either the 5'- or the 3'-ends were created. Binding assays with these oligos are shown in Figure 2B. Removing 2 nucleotides from the 3'-end of the conserved region was tolerated in part, although the quantity of bound factors was reduced and the lower of the 2 complexes largely disappeared. When 2 nucleotides were

removed from the 5'-end, or 4 nucleotides from the 3'-end, binding activity was completely destroyed. This demonstrates that a minimum of 21 bp from -308 to -288 is essential for complex formation.

Deletion of the Py-Box Element Increases Transcription Rates

When we tested a deletion of the sequences from -330 to -295 of the basal TF promoter, which removes the 5'-half of the Py-box element, increased expression of a luciferase reporter gene was observed (Figure 3A). Further proof that the Py-box element mediates the repressive effect observed is provided by a substitution of the 19 central nucleotides of the element (positions -306 to -288) by an *Xba*I cleavage site. This substitution leads to a 3-fold increase in the transcriptional activity of the promoter, comparable with the effect seen with the -295 deletion construct (Figure 3B). The relative increase is similar for LPS-treated or nontreated cells.

As discussed previously,¹² we generally observe a partial activation of the TF gene by the transfection procedures, leading to partially upregulated levels of TF transcription in cells without LPS treatment. To determine whether the Py-box element would function to ensure suppression of the TF gene in the absence of NF κ B activity, we have tested whether removal of the Py-box element from the κ B-deleted version of the basal -330 -bp TF promoter would result in increased transcription. Removal of the κ B site from the promoter has been previously shown to lead to a strong reduction in promoter activity and virtual unresponsiveness to LPS activation.¹² Indeed, additional removal of the Py-box element from the κ B-deficient TF promoter results in increased transcription levels, suggesting a role of the Py-box element in suppressing the activity of constitutive transcription factors such as AP-1 and Sp1 in EC (Figure 3C).

To exclude an effect of the *Xba*I linker sequence inserted on the position of the Py-box, we have further tested whether a trimerized Py-box element would function when reinserted into the *Xba*I site in the corresponding Py-box deletion construct. As expected, a 3-fold reduction in comparison with the Py-box deletion construct is observed (Figure 3C).

CT-Rich Sequences Within the Py-Box Are Essential for Binding Activity

To identify the parts of the Py-box sequence required for complex formation, we tested a series of 3-bp substitutions throughout the 21-bp region determined to be the minimal size of the element in mobility shift assays. Consecutive substitutions of 3 nucleotides of the Py-box sequence by the triplet T-C-A results in a complete loss of binding activity when nucleotides 1 to 12 and 19 to 21 are replaced. In contrast, substitutions of oligonucleotides 13 to 18 still allowed the formation of somewhat reduced levels of the complex. These data indicate that the pyrimidine parts of the sequence are essential for complex formation and the sequence of the stretch from oligonucleotides 13 to 18, which contains 4 purines, is partially dispensable (Figure 4).

Proteins in the Range of 40 and 60 kDa Bind to the Py-Box Element

To investigate the subunit composition of the protein complex interacting with the Py-box, we have analyzed which proteins

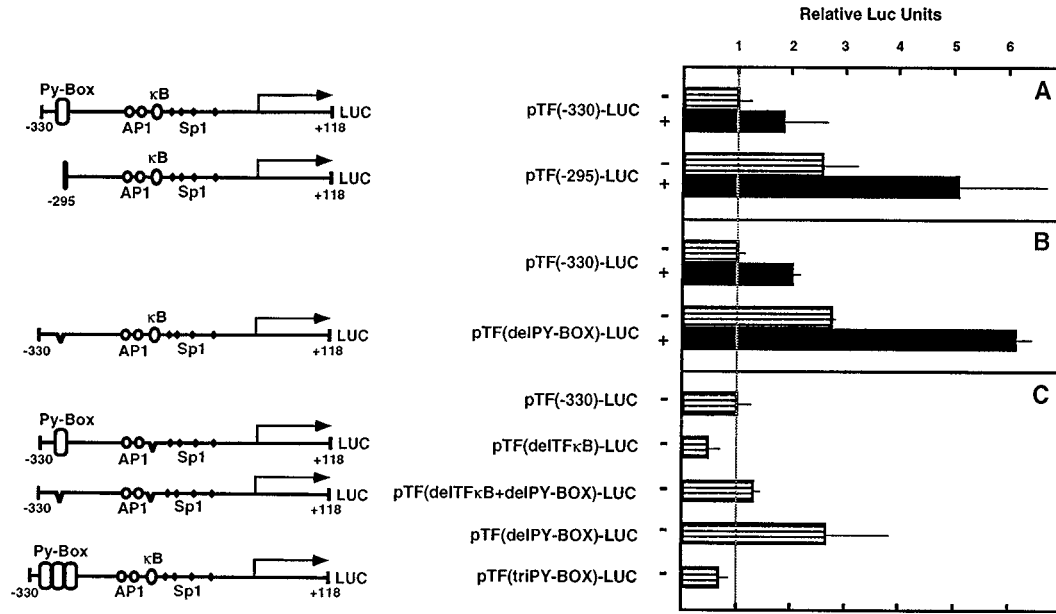


Figure 3. Transcriptional activities of Py-box deletion constructs. A, 5'-Deletion of the tissue factor (TF) promoter. The region from -330 to -295 of the basal transcription factor promoter containing part of a DNase I protected region was deleted and the transcriptional activity of the construct compared with the basal TF promoter (-330 to +118 bp) fused to a luciferase reporter gene. Forty-eight hours after transfection, primary porcine aortic endothelial cells were induced with 1 μg/mL lipopolysaccharide (LPS) (+) or left untreated (-). Luciferase activity obtained was normalized to the expression rates from a cotransfected cytomegalovirus promoter/β-gal expression vector. The relative luciferase activity obtained with the basal promoter in uninduced cells was arbitrarily set to 1. B, Deletion of the Py-box element. The region from -306 to -288 in the basal TF promoter containing the center of the Py-box sequence was substituted with an XbaI linker sequence. The construct was transfected and analyzed in comparison with the basal promoter in the presence (+) or absence (-) of LPS induction as described in A. C, Deletions of the Py-box and κB elements and reinsertion of a trimerized Py-box. Constructs with single deletions of the Py-box and the κB site and a double deletion of both sites from the TF promoter were tested. In addition, a construct containing a trimerized Py-box reinserted into the XbaI site, substituting the Py-box element in the TF promoter, was obtained and tested. The different elements of the TF promoter present on the various constructs and shown to be occupied by nuclear proteins in footprint analysis¹² are depicted in the left part of the figure. The deletions are indicated by triangular insertion.

can be covalently cross-linked to 5-bromodeoxyuridine (BrdU)-substituted DNA after UV irradiation of gel-purified protein/DNA complexes.²⁷ Proteins cross-linked to the Py-box were separated by SDS-PAGE, revealing a single strong band at 48 kDa and 3 weaker bands of ≈65 to 70 kDa (Figure 5). Assuming that the DNA in the complex would comprise ≈4 to 8 kDa, this

suggests the presence of 2 to 4 different DNA binding polypeptides of ≈40 and 60 to 65 kDa, respectively.

Competition Experiments With Potential Related Elements

Having defined the minimal size of the Py-box binding site, we have searched the nucleotide database for transcription factor binding sites with homologies to the Py-box. We have noticed the presence of a run of 5 nucleotides CTTCC, which could represent the core sequence of an ets binding site.²⁸ However, when a standard ets-1 binding site is used in competition experiments, the formation of specific complexes on the Py-box element is not affected (Figure 6A). Further, Ets-1 antibodies do not give a supershift when added to the electrophoretic mobility shift assay (data not shown).

Further, we have aligned the Py-box sequence to promoters of several genes expressed in ECs. In this analysis, we detect the best homology to a stretch of the human von Willebrand factor promoter (-381 to -359 bp; see Figure 1). It is noteworthy that this sequence is present in the von Willebrand promoter in a 174-bp fragment recently described to contain a repressive element.^{29,30} We have also tested the sequence from the von Willebrand promoter and found that this element partially competes with the complex formation on the TF Py-box (Figure 6A) and gives a similar sized, although somewhat weaker, shift in electrophoretic mobility

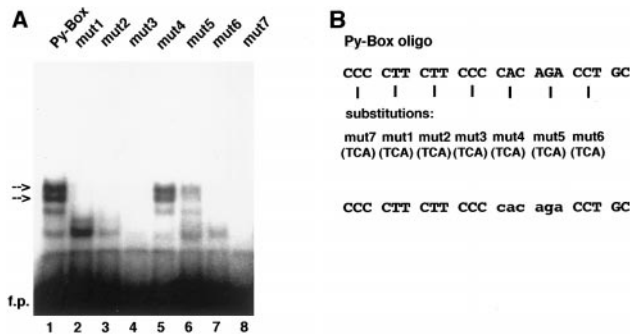


Figure 4. The CT-rich sequences of the Py-box are essential for binding activity. A, A series of oligonucleotides containing consecutive 3-bp substitutions in the Py-box sequence of the tissue factor (TF) promoter (lanes 2 through 8) were tested in parallel with the authentic sequence (lane 1) for complex formation in gel-retardation assays. B, The consecutive substitutions by TCA are shown for mutants 1 through 7. The sequence in the lower part indicates the regions essential for binding in uppercase letters, the regions partially dispensable for binding in lowercase letters. The arrows indicate the position of the 2 major specific DNA complexes formed; f.p., free probe.

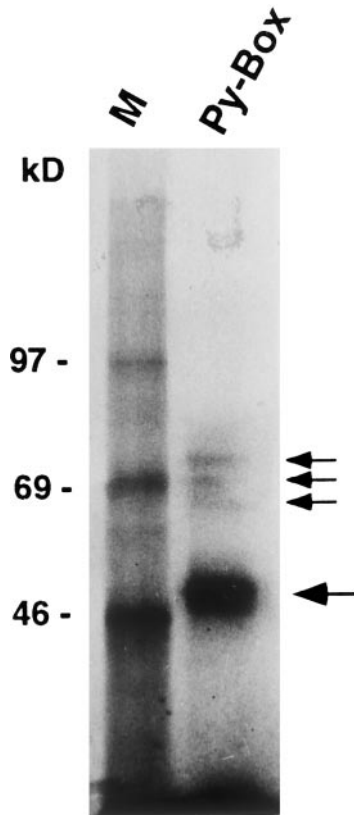


Figure 5. Definition of the subunit composition of the nuclear complexes binding to the Py-box element. UV cross-linking of nuclear proteins to the Py-box. Photoreactive bromodeoxyuridine-substituted Py-box oligonucleotides were synthesized by primer extension with Klenow polymerase and used for a preparative electrophoretic mobility shift assay. After separation of the complex bound to the Py-box oligonucleotide on a native polyacrylamide gel, the DNA was cross-linked to the proteins by UV irradiation. The cross-linked proteins in the retarded band were then eluted and separated on a 7.5% SDS-polyacrylamide gel. Arrows indicate the positions of the cross-linked proteins.

shift assays (data not shown). It is therefore possible that a Py-box-like element is present also in the von Willebrand factor gene.

The Py-Box Element Gives Mobility Shifts With Extracts From Human Umbilical Vein ECs and Other Human Cell Types

To test whether extracts from human ECs and the human version of the Py-box are capable of forming similar complexes, the porcine Py-box oligonucleotide was tested with extracts of porcine aortic ECs and HUVECs. Identical bands are obtained with extracts from the porcine and human cells (Figure 6B). In a similar manner, the human Py-box sequence gives the same shift (Figure 7). To further evaluate whether the complex-forming proteins are present in cell types of the subendothelium, extracts from human primary vascular smooth muscle cells and fibroblasts were tested. Again, similar complexes were detected with nuclear extracts from smooth muscle cells and fibroblasts (Figure 7).

Repressive Effect of the Py-Box Is Only Revealed in Endothelial Cells

A potential cell-type specific function of the Py-box element was tested by reporter gene assays by using the -330-bp

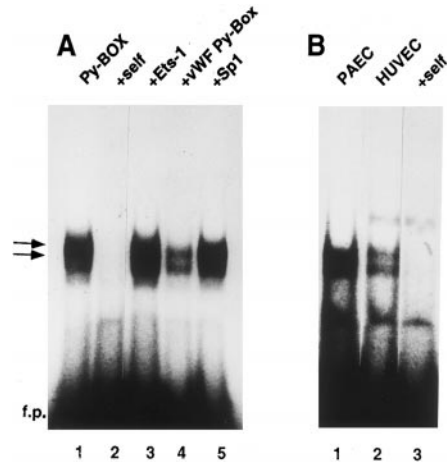


Figure 6. Competition of binding with potential related sequences and comparison of binding of nuclear extracts of porcine and human endothelial cells (ECs). A, Competition of the binding of nuclear proteins to the porcine Py-box element was tested by using the authentic Py-box sequence (lane 2), an ets-1 binding site from the Maloney murine sarcoma virus long-terminal repeat (lane 3), a Py-box-related sequence from the von Willebrand factor (vWF) promoter (lane 4), and an Sp1-binding site (lane 5). B, The binding of nuclear proteins of porcine aortic endothelial cells (PAEC) (lane 1) and human umbilical vein endothelial cells (HUVEC) (lane 2) to the porcine Py-box element is shown. The arrows indicate the position of the 2 major specific DNA complexes formed; f.p., free probe.

basal TF promoter and its Py-box deletion form. After transfection of HUVECs and 2 primary human cells constitutively producing TF, vascular smooth muscle cells and fibroblasts, increased expression of the Py-box deletion construct, compared with the control construct, could only be shown in the ECs (Figure 8).

Discussion

Whereas several cell types around blood vessels, such as adventitial fibroblasts and smooth muscle cells, keratinocytes

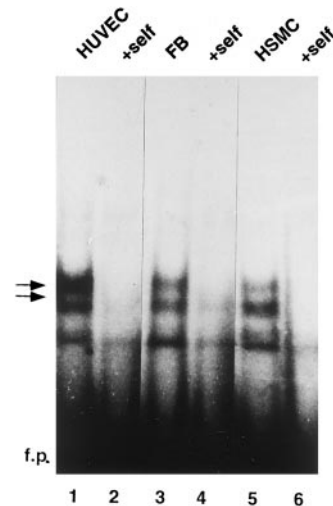


Figure 7. Complex formation of the human tissue factor (TF) Py-box with nuclear extracts from several cell types. Gel-retardation assays were performed by using nuclear extracts from human umbilical vein endothelial cells (HUVEC) (lane 1), primary human fibroblasts (FB) (lane 3), and primary human vascular smooth muscle cells (HSMC) (lane 5). The corresponding competitions with the unlabeled Py-box oligonucleotide are shown in lanes 2, 4, and 6. The arrows indicate the position of the 2 major specific DNA complexes formed; f.p., free probe.

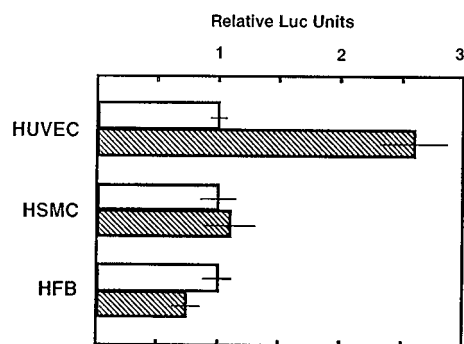


Figure 8. The repressive effect of the Py-box element is only revealed in endothelial cells. Reporter gene assays, using the -330 -bp tissue factor (TF) promoter construct and the corresponding Py-box deletion were performed after transfection of human umbilical vein endothelial cells (HUVEC), vascular smooth muscle cells (HSMC), and fibroblasts (HFB) as described in Methods and the legend to Figure 3. Open columns depict the basal TF promoter construct, hatched columns the corresponding Py-box-deleted construct.

of the skin and astroglia of the brain, constitutively express TF, vascular ECs and blood cells normally do not express the protein.^{4–6} This cell type-specific constitutive expression of TF suggests that the protein forms a hemostatic barrier around blood vessels that activates coagulation when the integrity of the vascular system is disrupted. In contrast, cells inside the vasculature such as ECs are only capable of upregulating TF in response to specific stimuli such as endotoxin or inflammatory cytokines. Inappropriate TF expression on these cells can lead to pathological conditions connected with inflammation, atherosclerosis, and intravascular thrombosis.^{7–9} Therefore, the TF gene must be tightly controlled in ECs. As recently shown by Mackman,¹⁰ Moll et al,¹² and Parry and Mackman,¹⁷ the TF promoter is upregulated after activation of ECs by an NF κ B-like site binding c-Rel and p65, the binding factors being absent from nuclei of unstimulated cells. However, it is still unclear how the absence of expression is ensured in the presence of additional transcription factors binding to the TF promoter in quiescent ECs such as AP-1 and Sp1.^{12,14} Further, the proper quantitative adjustment of only moderate transcription levels of TF in cytokine-activated ECs may need control mechanisms in addition to NF κ B activation.

Because we have previously observed a strong DNase I protected site around -300 bp of the porcine TF promoter,¹² we have explored the possibility that this region would contain a transcription factor binding site. Although our previous sequence comparison of the porcine and human TF promoters¹² has not revealed a conserved sequence in this region, a closer inspection of the neighboring sequences identified a closely related element in both genes occurring ≈ 50 bp further upstream of the transcription start site in the human promoter. Because the porcine promoter is lacking the sequences between -37 bp and -69 bp of the human promoter, the distance between the Py-box and the AP-1 and NF κ B sites is very similar in the human and porcine promoters. Remarkably, the region containing the human equivalent has also been described to contain a strong DNase I-protected site and has been suggested to bind regulatory proteins.¹¹ In addition, the region upstream of the AP-1 elements between

-383 and -279 bp in the human TF promoter has been shown to contain a negative regulatory element by reporter gene studies in COS cells, but no further characterization has been described.^{10,31}

When the binding of nuclear proteins to oligonucleotides containing the conserved sequences was tested, a specific binding pattern showing 2 complexes of slightly differing mobilities was revealed (Figure 2). The observed binding was similar with extracts from untreated, or LPS- or TNF- α -induced ECs, suggesting occupancy of the site independent of the activation status of the cells. This observation suggests that the binding factors work to suppress transcription in quiescent ECs and to fine-tune the rates in activated cells to moderate levels. The limits of the binding element were clearly defined to a stretch of 23 bp, largely conserved between the human and porcine genes. A further shortening of the sequence was not at all tolerated on the 5'-end and only partially on the 3'-end. This indicates a relatively large binding area.

To prove that the 23-bp element caused the repressive effect and not any other undetected site between -330 and -295 of the TF promoter, a deletion construct substituting the 19 bp from the center of the Py-box element was prepared and found to give an increased transcription rate comparable with the -295 deletion construct. Increased transcription rates of the deletion construct were observed for untreated and LPS-treated ECs (Figure 3B) and similar results were obtained for TNF- α -induced cells (data not shown). To further test the possibility that the Py-box element could function to ensure absence of expression in quiescent ECs in the presence of AP-1 and Sp1 factors, we have tested the effect of deleting the Py-box sequence from a promoter fragment without the κ B element. This resulted again in increased transcription levels, suggesting a role in the suppression of AP-1 and Sp1 transcription factors constitutively present in ECs. Because also κ B-mediated induced levels of transcription were reduced by the Py-box element, a second role could be the fine-tuning of TF transcription to levels appropriate to cytokine-stimulated ECs. In accordance with its function as a suppressive element, a trimerized site reintroduced into the *Xba*I site, substituting the Py-box in the deletion construct, showed 3-fold reduced transcription levels compared with the Py-box deletion construct (Figure 3C).

The core of the repressor binding site is extremely pyrimidine-rich on the plus strand, containing close to 80% pyrimidines. For this reason, we refer to this sequence as Py-box element. A series of 3-bp substitutions throughout the element supports the conclusion that the parts of the sequence that are pyrimidine-rich on the plus strand and purine-rich on the opposite strand are essential for the observed binding. A closer inspection of the sequence and comparison with a collection of transcription factor binding sites revealed some distant similarities to the binding sites of the Ets family members, which are GA-rich on one strand and CT-rich on the other strand.²⁸ The GGAA Ets-1 core site is present on the opposite strand of the porcine and human equivalents of the Py-box. However, we believe it unlikely that an Ets family member is actually binding to the Py-box. Our data show that a standard Ets-1 binding site could not compete for Py-box binding (Figure 6) and an Ets-1 antibody did not supershift the complex (data not shown). These

data challenge the presence of Ets-1 in the Py-box binding complex, although a distant relative of the large family cannot be excluded.

The proteins binding to the Py-box oligonucleotide have been further analyzed by using UV cross-linking (Figure 5). These data define a polypeptide in the size range of 40 kDa and 3 further polypeptides of similar size in the range of 60 to 65 kDa. It is possible that these 3 polypeptides are differentially modified forms of 1 subunit or 3 independent proteins.

Searching the promoters of other genes expressed in ECs, a striking similarity of the Py-box to a sequence in the von Willebrand factor promoter was detected (see Figure 1). This region from the von Willebrand factor promoter competed reasonably well for the binding to the Py-box element (Figure 6A) and gave a comparable, although weaker, complex with nuclear extracts from ECs (data not shown). The sequence is located in the von Willebrand promoter within a larger segment recently shown to exert strong repressive effects mediated by an NF1 binding site.^{29,30} It is possible that the pyrimidine-rich sequence in the same fragment of the von Willebrand factor promoter contributes to the repression of the gene.

Many transcription factors have been shown to be modulated in their activity by posttranslational modifications as phosphorylation.³² This in turn could either affect the binding properties of the factors to their respective binding site on DNA or could modulate the interaction with other transcription factors without influencing the binding to their cognate DNA element in the promoter. The latter mechanism could explain our observation that the inhibitive properties of the Py-box element can be only detected in ECs and not in primary fibroblasts or smooth muscle cells despite the presence of binding factors in these cells. It is possible that the inhibitive properties of the Py-box binding factors are only revealed after a certain modification of the factors in ECs. In contrast, in fibroblasts or smooth muscle cells, the inhibitive properties observed in ECs could be overcome in the context of different factors with strong activating properties.

Repressive transcription factors can exert their action through several mechanisms.³³ They could bind and then either directly interact with other enhancer binding factors or general transcription factors and thereby inhibit the transactivation of the transcriptional machinery. In contrast, they could compete for the binding of activatory transcription factors to the same region of the promoter. There is ample evidence that many transcription factors can act either as activating or as inhibitory factors, depending on the specific array of the additional transcription factors binding to an individual promoter. In the case of the Py-box binding factors, we would favor currently the direct interaction with other activating transcription factors constitutively binding to the TF promoter in ECs. This inhibition is likely to be overcome in ECs by the activity of NF κ B factors, which are strongly induced after inflammatory activation of the endothelium. It remains to be shown whether the described repressive element and its interacting proteins, for which we propose a role in maintaining a nonthrombotic environment inside the vasculature, define a mechanism active also on other genes expressed in ECs.

Acknowledgment

This work was supported by a grant from the Austrian Science Foundation to E.H. (SFB F005-10). We thank Mrs. Erni Scharzinger for excellent technical assistance.

References

- Edgington TS, Mackman N, Brand K, Ruf W. The structural biology of expression and function of tissue factor. *Thromb Haemost.* 1991;66:67-79.
- Ruf W, Edgington TS. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. *FASEB J.* 1994;8:385-390.
- Martin DM, Boys CW, Ruf W. Tissue factor: molecular recognition and cofactor function. *FASEB J.* 1995;9:852-859.
- Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues: implications for disorders of hemostasis and thrombosis. *Am J Pathol.* 1989;134:1087-1097.
- Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci U S A.* 1989;86:2839-2843.
- Fleck RA, Rao LV, Rapaport SI, Varki N. Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. *Thromb Res.* 1990;59:421-437.
- Morrissey JH, Drake TA. Procoagulant response of the endothelium and monocytes. In: Schlag G, Riedl H, eds. *Pathophysiology of Shock, Sepsis and Organ Failure.* Berlin, Germany: Springer-Verlag; 1993:564-574.
- Kirchofer D, Tschopp TB, Hadvary P, Baumgartner HR. Endothelial cells stimulated with tumor necrosis factor- α express varying amounts of tissue factor resulting in inhomogenous fibrin deposition in a native blood flow system: effects of thrombin inhibitors. *J Clin Invest.* 1994;93:2073-2083.
- Levi M, ten Cate H, Bauer KA, van der Poll T, Edgington TS, Buller HR, van Deventer SJ, Hack CE, ten Cate J, Rosenberg RD. Inhibition of endotoxin-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. *J Clin Invest.* 1994;93:114-120.
- Mackman N. Regulation of the tissue factor gene. *Faseb J.* 1995;9:883-889.
- Donovan-Peluso M, George LD, Hassett AC. Lipopolysaccharide induction of tissue factor expression in THP-1 monocytic cells: protein-DNA interactions with the promoter. *J Biol Chem.* 1994;269:1361-1369.
- Moll T, Czyz M, Holzmüller H, Hofer-Warbinek R, Wagner E, Winkler H, Bach FH, Hofer E. Regulation of the tissue factor promoter in endothelial cells: binding of NF κ B-, AP-1-, and Sp1-like transcription factors. *J Biol Chem.* 1995;270:3849-3857.
- Mackman N, Brand K, Edgington TS. Lipopolysaccharide-mediated transcriptional activation of the human tissue factor gene in THP-1 monocytic cells requires both activator protein 1 and nuclear factor κ B binding sites. *J Exp Med.* 1991;174:1517-1526.
- Parry GC, Mackman N. Transcriptional regulation of tissue factor expression in human endothelial cells. *Arterioscler Thromb Vasc Biol.* 1995;15:612-621.
- Cui MZ, Parry GC, Oeth P, Larson H, Smith M, Huang RP, Adamson ED, Mackman N. Transcriptional regulation of the tissue factor gene in human epithelial cells is mediated by Sp1 and EGR-1. *J Biol Chem.* 1996;271:2731-2739.
- Oeth PA, Parry GC, Kunsch C, Nantermet P, Rosen CA, Mackman N. Lipopolysaccharide induction of tissue factor gene expression in monocytic cells is mediated by binding of c-Rel/p65 heterodimers to a κ B-like site. *Mol Cell Biol.* 1994;14:3772-3781.
- Parry GC, Mackman N. A set of inducible genes expressed by activated human monocytic and endothelial cells contain κ B-like sites that specifically bind c-Rel-p65 heterodimers. *J Biol Chem.* 1994;269:20823-20825.
- Warren JB. Large vessel endothelial isolation. In: Warren JB, ed. *The Endothelium: An Introduction to Current Research.* New York, NY: Wiley-Liss; 1990:263-272.
- Wojta J, Hoover RL, Daniel TO. Vascular origin determines plasminogen activator expression in human endothelial cells. *J Biol Chem.* 1989;264:2846-2852.
- Wojta J, Gallicchio M, Zoellner H, Hufnagl P, Last K, Filonzi EL, Binder BR, Hamilton JA, McGrath K. Thrombin stimulates expression of tissue-type plasminogen activator and plasminogen activator inhibitor type 1 in cultured human vascular smooth muscle cells. *Thromb Haemost.* 1993;70:469-474.

21. Zhang JC, Fabry A, Paucz L, Wojta J, Binder BR. Human fibroblasts downregulate plasminogen activator inhibitor type-1 in cultured human macrovascular and microvascular endothelial cells. *Blood*. 1996;88:3880–3886.
22. Cotten M, Wagner E, Birnstiel ML. Receptor-mediated transport of DNA into eukaryotic cells. *Methods Enzymol*. 1993;217:618–644.
23. de Martin R, Vanhove B, Cheng Q, Hofer E, Cszimadia V, Winkler H, Bach FH. Cytokine-inducible expression in endothelial cells of an I kappa B alpha-like gene is regulated by NF kappa B. *EMBO J*. 1993;12:2773–2779.
24. de Wet J, Wood KV, DeLuca M, Helinski DR, Subramani S. Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol*. 1987;7:725–737.
25. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
26. Mackman N, Morrissey JH, Fowler B, Edgington TS. Complete sequence of the human tissue factor gene, a highly regulated cellular receptor that initiates the coagulation protease cascade. *Biochemistry*. 1989;28:1755–1762.
27. Chodosh LA, Carthew RW, Sharp PA. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. *Mol Cell Biol*. 1986;6:4723–4733.
28. Wasylyk B, Hahn SL, Giovane A. The Ets family of transcription factors. *Eur J Biochem*. 1993;211:7–18.
29. Jahroudi N, Lynch DC. Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol Cell Biol*. 1994;14:999–1008.
30. Jahroudi N, Ardekani AM, Greenberger JS. An NF1-like protein functions as a repressor of the von Willebrand factor promoter. *J Biol Chem*. 1996;271:21413–21421.
31. Mackman N, Fowler BJ, Edgington TS, Morrissey JH. Functional analysis of the human tissue factor promoter and induction by serum. *Proc Natl Acad Sci U S A*. 1990;87:2254–2258.
32. Ernst P, Smale ST. Combinatorial regulation of transcription I: general aspects of transcriptional control. *Immunity*. 1995;2:311–319.
33. Johnson AD. The price of repression. *Cell*. 1995;81:655–658.