J. Biochem. 125, 1039-1047 (1999)

Monkey Hepatocytes Efficiently Express Tissue Factor Pathway Inhibitor (TFPI), in Contrast with Human and Rat Hepatocytes[1]

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Received December 4, 1998; accepted March 9, 1999

It has been reported that tissue factor pathway inhibitor (TFPI), a Kunitz-type protease inhibitor that regulates the extrinsic blood coagulation pathway, is not expressed in human, bovine, rabbit, or rat liver. Here, we found that TFPI is efficiently expressed in Macaque monkey liver. Monkey hepatocytes were identified as the expression cells by Northern blot analysis. The hepatocytes were stained with anti-human TFPI antibody, as were endothelial cells of the small vessels. We isolated and sequenced the 5′-flanking 1.4 kb regions of monkey and human TFPI genes, and found them to show 92.6% identity in their nucleotide sequences. We measured their transcriptional activities using a luciferase reporter gene and showed that the activity of the monkey TFPI gene is higher than that of the human gene in monkey primary hepatocytes. Although the binding motif of hepatocyte nuclear factor-1 is present only in the monkey gene, the site does not seem to be involved in the transcriptional activity. Mutagenetic analyses revealed that the region from −138 to +28 in the monkey gene is important for the expression of TFPI in hepatocytes. The present study indicates that the expression of the monkey TFPI gene is regulated by different mechanisms from the human TFPI gene.

Key words: gene expression, hepatocytes, HUVEC, TFPI, thrombosis.

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor against both factor Xa and factor VIIa/tissue factor complex, and thus regulates the initial reactions of the extrinsic blood coagulation pathway. Although TFPI circulates in the blood stream in free and lipoprotein-associated forms, the majority of TFPI is thought to be associated with proteoglycans on the surface of vascular endothelial cells (see reviews, Refs. *1-5*). TFPI consists of three tandem Kunitz domains flanked by a cluster of acidic amino acid residues at the N terminus and basic residues at the C terminus. The human TFPI gene spanning 85 kb consists of nine exons and resides on the long arm of chromosome 2 (2q31-32.1), which is located 36 cM proximal to D2S43 (pYNZ15) and 13 cM distal to the crystalline γ-polypeptide locus CRYGP1 (p5G1) (*6-8*). It has been reported that human and rat TFPI mRNAs are expressed mainly in endothelial cells, especially in lung and kidney (*9-11*). It is also known that the expression of TFPI in cultured human umbilical vein endothelial cells (HUVEC) is not significantly affected by phorbol myristate acetate, endotoxin, interleukin-1, or tumor necrosis factor α, indicating that TFPI is constitutively synthesized in these cells. However, a recent report indicated that TFPI is also expressed in cultured human vascular smooth muscle cells, and that its expression is regulated by epidermal growth factor and platelet-derived growth factor-B (*12*). On the other hand, no evidence has been presented that TFPI is efficiently expressed in the liver. Ameri *et al.* observed that the level of TFPI mRNA is almost negligible in bovine and rabbit liver (*9*) and in cultured hepatocytes from normal human liver (*10*). The absence of TFPI in human hepatocytes was confirmed by an immunohistochemical technique (*13*). We also found no detectable TFPI mRNA in rat liver (*11*). Although the organization of the human TFPI gene and the nucleotide sequence upstream of the 5′-flanking region have been determined (*6-8*), the reason TFPI is expressed in vascular endothelial cells but not in hepatocytes remains to be explained.

We previously determined the primary structure of monkey TFPI by cloning its cDNA and prepared the recombinant protein (*14*). We then developed an enzyme-

[1] This work was supported in part by Special Coordination Funds for Promoting Science and Technology (Encouragement System of COE) from the Science and Technology Agency of Japan, by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by a Grant-in-Aid for Research Promoting Comprehensive Longevity Science and a Research Grant for Cardiovascular Diseases (8A-1) from the Ministry of Health and Welfare. This work was a cooperative research program of the Primate Research Institute, Kyoto University.

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Abbreviations: HBSS, Hanks' balanced salt solution; HNF, hepatocyte nuclear factor; HUVEC, human umbilical vein endothelial cells; LDL, low density lipoprotein; RACE, rapid amplification of cDNA ends; SSC, standard saline citrate; TFPI, tissue factor pathway inhibitor.

linked immunosorbent assay system using the recombinant monkey TFPI to evaluate the protein levels in monkey plasma, and found that the levels of low density lipoprotein (LDL)-associated TFPI increase in monkeys fed a high-cholesterol diet (*15*). During our studies on monkey TFPI, we found that TFPI mRNA is efficiently expressed in the monkey liver as well as in the lung and kidney, in contrast to the case in human, bovine, rabbit, and rat liver. We also detected the TFPI antigen in monkey liver by an immunohistochemical technique. In the present study, we examined the transcriptional activities of the 5′-flanking regions of the human and monkey TFPI genes in monkey primary hepatocytes to clarify the mechanism by which TFPI is efficiently synthesized in monkey liver.

EXPERIMENTAL PROCEDURES

Materials—Macaque monkeys maintained at the Primate Research Institute, Kyoto University, were used. Collagenase was purchased from Yakult (Tokyo). Insulin, dexamethasone and glucagon were from Sigma (St. Louis, MO, USA). Hybond N^+ nylon membranes were from Amersham (Buckinghamshire, England). Synthetic oligonucleotides were purchased from Japan Bio Service (Saitama).

Northern Blot Analysis—Monkeys were sacrificed under deep anesthesia with Ketaral and Nembutal according to the technique adapted from the "Guide for the Care and Use of Laboratory Primates" at the Primate Research Institute, Kyoto University. Tissues from the monkeys were immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA was prepared from the frozen tissues by the acid guanidium thiocyanate-phenol-chloroform method (*16*) using ISOGEN (Wako Chemicals, Osaka) according to the manufacturer's protocol. Total RNA was also isolated from cultured monkey primary hepatocytes. Human total RNA was purchased from OriGene Technologies (Rockville, MD, USA). The samples were electrophoresed in a 0.8% agarose gel containing 2.2 M formaldehyde. RNA in the gel was transferred onto a Hybond-N^+ charged membrane and hybridized with radiolabeled monkey or human TFPI cDNA containing the full length of the coding region as a probe. Autoradiography was carried out using an imaging plate on a bioimaging analyzer (model BAS-2000, Fuji Photo Film, Tokyo).

Immunohistochemical Analysis—Rabbit anti-human TFPI antibody was prepared as reported previously (*17*) and purified on a recombinant human TFPI-immobilized column. Tissues from monkey liver were dissected, rinsed with phosphate-buffered saline, and fixed in 3% paraformaldehyde at 4°C. After dehydration in serially-diluted ethanol, the tissues were embedded in paraffin and sectioned. The sections were rehydrated, treated with 5% skim milk to block the non-specific binding of IgG, and then incubated with the rabbit anti-human TFPI antibody overnight at 4°C. After washing, the TFPI antigen was detected using the DAKO ABC system (DAKO Japan, Kyoto). As negative controls, the sections were incubated with non-immunized rabbit IgG or rabbit anti-TFPI antibody pre-absorbed with excess amounts of recombinant human TFPI.

Cell Culture—A portion (approximately 20 g) of a small leaf of liver was aseptically removed from a monkey and placed on a clean bench. The liver was cannulated and perfused with 200 ml of warmed Hanks' balanced salt solution (HBSS) containing 0.5 mM EGTA, and then perfused with 200 ml of warmed collagenase solution (HBSS containing 0.05% collagenase, 0.01% soybean trypsin inhibitor, and 5 mM $CaCl_2$) at 20 ml/min. The liver was minced in chilled HBSS and filtered through a sterile gauze. The filtrate was then centrifuged at $85 \times g$ for 2 min and the pellet, consisting mainly of hepatocytes, was resuspended in 200 ml of chilled HBSS. Hepatocytes in the pellet were refined by repeating the centrifugation at $85 \times g$ for 2 min three times. The hepatocytes were resuspended in William's E medium containing 10% heat-inactivated fetal calf serum, 10 nM insulin, 10 nM dexamethasone, and 10 nM glucagon. The cells were inoculated onto 24-well plates coated with type I collagen at 1×10^6 cells/well for DNA transfection. For Northern blot analysis, the cells were inoculated onto 100-mm type I collagen-coated dishes. Six hours later, the medium was changed and the unattached cells were removed. The hepatocytes were subjected to the various assays 2 days after inoculation. Crude non-parenchymal cells were collected by centrifuging ($1{,}500 \times g$, 5 min) the supernatant from the first centrifugation.

5′-Rapid Amplification of cDNA Ends (RACE) of Monkey TFPI—Monkey liver poly A(+) RNA was prepared from total RNA using Oligotex-dT30 (Roche, Basel, Switzerland). 5′-RACE was carried out using a 5′-RACE kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Briefly, the first strand cDNA was synthesized with the TFPI-specific primer 5′-TTTCAGTGGTGGCAACTCAG-3′ (base Nos. 144–125), the numbers based on the monkey TFPI cDNA numbering from A of the first ATG in the coding region (*14*, GenBank accession No. S73337). A linker containing an *Eco*RI site, supplied in the kit, was ligated to the 3′-end of the first-strand cDNA using T4 RNA ligase, and then 30 cycles of PCR were performed using the anchor primer and a unique primer 5′-ATAGCATTAAGAGGAGCAG-3′ (base Nos. 87–68). The second PCR was performed with the anchor primer and the primer 5′-GCATCAGGCATATGGAAACC-3′ (base Nos. 55–36) linked to the *Hin*dIII site at the 5′-end. The product was analyzed by 2% agarose gel electrophoresis, cloned into pBluescript SKII(+) at the *Eco*RI and *Hin*dIII sites, and sequenced using a DNA sequencer ABI model 373A (Perkin Elmer, Norwalk, CT, USA).

Construction and Screening of Monkey and Human Genomic DNA Libraries—The genomic DNA library was constructed with monkey or human leukocyte genomic DNA and lambda DASH II vector (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol except that human genomic DNA was completely digested with *Hin*dIII and fragments approximately 25 kb in length were used. The probes for screening the genomic DNA libraries were prepared by PCR with the primers designed to amplify exon I of the monkey and human TFPI genes as follows:

Monkey:
MX1P: 5′-AGCAGAGACTTCAGGCTGG-3′
MX1M: 5′-CTGATGAAAGTTCTTGGACTG-3′
Human:
HX1P: 5′-CTCTGAGGCTCCCTCTTTGC-3′
HX1M: 5′-TCTTATGGGCTAGACAGTATATTG-3′

These probes were labeled with [^{32}P]dCTP using a Mega-

prime labeling kit (Amersham, Buckinghamshire, England). Hybridization was performed in 10% polyethylene glycol, 7% SDS containing 100 μg/ml of denatured salmon sperm DNA at 60 C overnight. The filters were washed twice with 2 × standard saline citrate (SSC) for 5 min, once with 1 × SSC for 30 min at room temperature, and twice with 2 × SSC at 60 C for 30 min. Overnight autoradiographic exposure of the filters was performed with X-OMAT film (Kodak) using an intensifying screen Quantum III at −70 C. The restriction enzyme map was determined by calculating the sizes of fragments obtained by digestions using a single enzyme or double enzymes combined with *Not*I, or by hybridization of partially digested inserts according to the procedures of Evans and Wahl (*18*). The precise location of the restriction enzyme sites was determined by PCR with exon-specific primers and the T3 or T7 primer, which locates in the vector, after subcloning the fragments into pBluescript SKII(+). The fragments containing Exon I were sequenced.

Construction of the Reporter Gene Plasmids—The 5′-flanking regions upstream of the transcription initiation site, including 28 base pairs of exon I of the monkey or human TFPI genes, were obtained by PCR using specific primers as follows: Fw1 (5′-GGCGAGCTCTCAAGAAAGAAAATCTGATTGGCT-3′) and Mrv1 (5′-CCGCTCGAGTTATCCAGCCTGAAGTCTCTGC-3′) for amplification of 1438 bp of the monkey TFPI gene, Fw1 and Hrv1 (5′-CCGCTCGAGTTATCCAGCCTAAAGTCGCTGC-3′) for 1427 bp of the human gene. Mfw2 (5′-TCGGTACCGAATTCAAATAACTTGGC-3′) and Mrv2 (5′-GCGAGCTCTTATCCAGCCTGAAGTCTCTGC-3′) for amplification of 566 bp of the monkey gene, Hfw2 (5′-TCGGTACCGAATTCAAATAACTGGGC-3′) and Hrv2 (5′-GCGAGCTCTTATCCAGCCTAAAGTCGCTGC-3′) for 548 bp of the human gene. The underlined sequences are restriction enzyme recognition sites: *Sac*I sites in Fw1, Mrv2, and Hrv2, *Xho*I sites in Mrv1 and Hrv1; and *Kpn*I sites in Mfw2 and Hfw2. The PCR products were inserted upstream of the firefly luciferase gene in PICA basic vector 2 (Toyo Ink, Tokyo) to make pM − 1410/ + 28, pH − 1399/ + 28, pM − 538/ + 28, and pH − 520/ + 28.

Site-Directed Mutagenesis—pM − 538/ + 28(Δ ATTT) containing a 4-bp deletion in the hepatocyte nuclear factor-1 (HNF-1) binding motif of the monkey gene was made by an overlap extension PCR method. Briefly, two separate PCR products were obtained using antisense- (5′-AAGTTTATTAACAATGTAA-3′) or sense- (5′-TTACATTGTTAATAAACTT-3′) mutated oligonucleotides lacking 4 bases (ATTT) in the region −329 to −326 of the monkey gene, and one end primer, Mfw2 or Mrv2. The two products were mixed, and a second PCR was performed using the two end primers, Mfw2 and Mrv2. The product was subcloned into PICA basic vector 2.

Chimeras, pMH, pHM, pHMH, and pMHM, were also constructed by the same method using oligonucleotides corresponding to the common region between the monkey and human genes as primers and the monkey or human TFPI gene as a template. The oligonucleotide regions are as follows: Com1, −386 to −367; Com2, −262 to −241; Com3, −167 to −148, based on the numbering of the monkey gene.

Six site-directed mutants, pM − 538/ + 28mut1, pM − 538/ + 28mut2, pM − 538/ + 28mut3, pM − 538/ + 28mut4, pM − 538/ + 28mut5, and pM − 538/ + 28mut6, were made using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) with either antisense- or sense-mutated oligonucleotides. The nucleotide sequences of the sense-oligonucleotides for mutagenesis are as follows: m1s (5′-TCCTTCATCTGTTTCCTCCACTAAAAAAAAGAAAGAAAGAAAGGAAAAAGAGGTTTAGACTA-3′), m2s (5′-AGAGAGAGAGAAAGAAAGAAAAAAGAGGTTTAGACTA-3′), m3s (5′-AAAGAGGTTTAGACTAAATAGAGTCAGAGTTGCAGTG-3′), m4s (5′-AGAGTTGCAGTGACCTAAACAGGAAGTTGGGCTATTC-3′), m5s (5′-AGTGATCTCTGAAGCCGACTCTGAGGCTCCCTCTTTGC-3′) and m6s (5′-CTCTAACAGACAGCAGCGACTTTAGGCTGGATAAGAGCT-3′). The sequences of the antisense-oligonucleotides are complementary to each sense-oligonucleotides.

Transient Transfection and Luciferase Assay—Monkey hepatocytes on 24-well plates were transfected with 0.5 μg per well of firefly luciferase expression vectors using Lipofectin (Life Technologies, Rockville, MD) according to the manufacturer's protocol. As an internal standard, 0.01 μg per well of pRL-SV40 vector (Toyo Ink) containing the SV40 early promoter and sea pansy luciferase gene was co-transfected. Five hours after transfection, the culture medium was changed, and after 48 h, the cells were lysed. The activities of firefly and sea pansy luciferases were measured by a PICA gene dual luciferase assay system (Toyo Ink) using LUMINOUS CT-9000D (DIA-IATRON, Tokyo).

Prediction of Transcription Factor Binding Sites—The

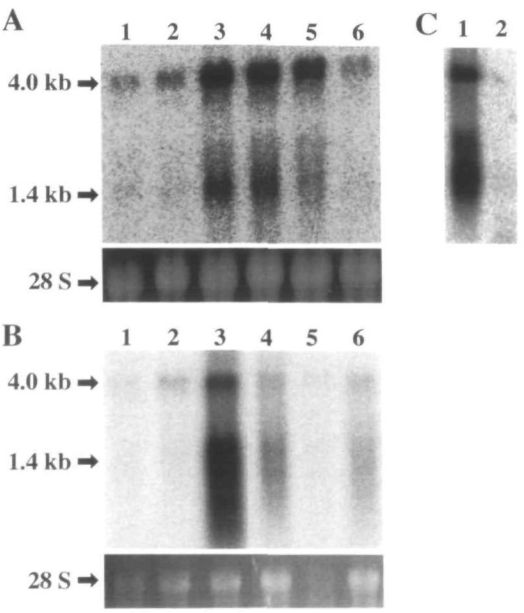

Fig. 1. **Northern blot analysis of monkey and human TFPI mRNA.** Samples of 10 μg of total RNA [(A) and (B)] or 2.5 μg of poly A(+) RNA (C) were subjected to 0.8% agarose gel electrophoresis, and probed with monkey TFPI cDNA [(A) and (C)] or human TFPI cDNA (B) as described in "EXPERIMENTAL PROCEDURES." (A) and (B): lane 1, brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, kidney; lane 6, spleen. (C): lane 1, cultured primary monkey hepatocytes; lane 2, non-parenchymal cells from monkey liver. The tops of (A) and (B) show autoradiography. The bottoms of (A) and (B) show ethidium bromide staining of 28 S ribosomal RNA.

transcription factor binding sites upstream of the human and monkey TFPI genes were searched using TFSEARCH ver.1.3 (*19*) with the threshold set at 85.0 points.

RESULTS

Northern Blot Analysis of Monkey and Human TFPI mRNA In order to identify the major tissues responsible for the expression of TFPI in monkeys, we evaluated the expression of TFPI mRNA by Northern blot analysis. Although it has been reported that TFPI is not expressed in human, bovine, rabbit (*9, 10*), or rat liver (*11*) by Northern blot analysis, the expression patterns of TFPI in various tissues have not been examined except in rat. Therefore we examined the expression of TFPI in humans as well as in monkeys. As shown in Fig. 1A, monkey TFPI mRNA with molecular sizes of 4.0 and 1.4 kb was detected, as observed for human (*20*) and rat (*11*) TFPI mRNA. It has also been suggested that two polyadenylation signals are present in monkey TFPI as reported in humans (*20*). TFPI mRNA was markedly expressed in monkey lung (lane 3) and kidney (lane 5) as well as in the human tissues (Fig. 1B). On the other hand, the level of TFPI expression in monkey liver (lane 4) was comparable to that in the lung and kidney. This observation differs from the case in humans. In humans, although the expression of TFPI was observed in the liver (Fig. 1B, lane 4), the level is considerably lower than that in the lung (lane 3).

To identify the cells responsible for the expression of TFPI mRNA in monkey liver, we isolated hepatocytes and non-parenchymal cells and examined the expression of TFPI mRNA by Northern blot analysis. As shown in Fig. 1C, hepatocytes expressed two sizes of TFPI mRNA in monkey liver, whereas the non-parenchymal cell fraction

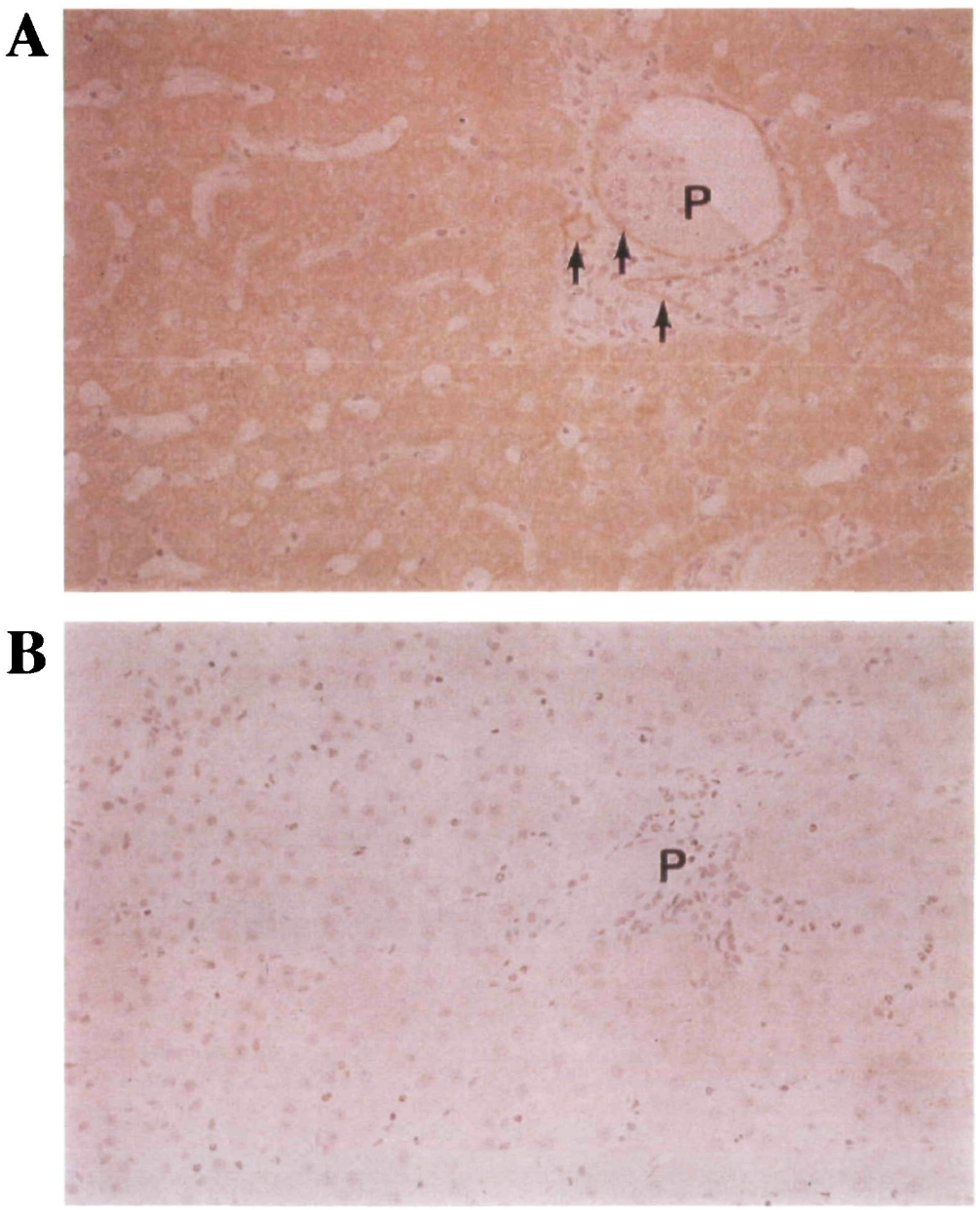

Fig. 2. **Immunohistochemical analysis of TFPI antigen in monkey liver.** Fixed sections were stained with rabbit anti-human TFPI polyclonal antibody as described in "EXPERIMENTAL PROCEDURES." (A): anti-human TFPI polyclonal antibody. (B): non-immunized rabbit IgG. P: Portal triad. Arrows indicate small vessels lined with endothelial cells positive for anti-TFPI antibody. (Original magnification, ×800)

comprising sinusoid endothelial cells and Kupffer cells, showed no TFPI mRNA.

Immunohistochemical Analysis of Monkey TFPI—Since the TFPI mRNA was detected in monkey hepatocytes, we performed immunohistochemical analysis of TFPI in monkey liver with anti-human TFPI antibody. As shown in Fig. 2A, positive signals were detected in hepatocytes, in addition to endothelial cells of small vessels, especially the venules in portal triads and central veins. None of the control sections treated with non-immunized IgG showed any significant staining (Fig. 2B). The immunoreactivity of the positive cells shown in Fig. 2A was completely abolished by preincubating the rabbit anti-TFPI antibody with recombinant human TFPI (data not shown).

Isolation of the 5′-Flanking Regions of Monkey and Human TFPI Genes—We tried to determine the transcription initiation site of the monkey TFPI gene prior to analyzing the transcriptional activities of the 5′-flanking region. 5′-RACE predicted that the probable transcription initiation site of the monkey gene was identical to that of the human gene.

To isolate the 5′-flanking region of the monkey TFPI gene, 4.8×10^5 plaques of a monkey genomic DNA library were screened with a probe containing exon I. We obtained three overlapping clones of λMTFPI5FL (Nos. 5, 7, and 15) covering a length of 60 kb including 30 kb upstream of the initiation site as shown in Fig. 3A. The restriction enzyme site map for clone No. 7 was determined with *Eco*RI, *Hin*dIII, and *Pst*I, since this clone had the longest 5′-flanking region. On the other hand, 11 clones containing the 5′-flanking region of the human TFPI gene were obtained from 4.8×10^5 plaques of a human genomic library, and the *Eco*RI site map was determined for one of the clones, λHTFPI5FL-3, since all clones showed the same fragments after digestion with *Eco*RI as shown in Fig. 3B. The nucleotide sequence of this region of the human TFPI gene was completely identical to that reported by Girard *et al.* (7). Figure 4 shows the nucleotide sequences of the 5′-flanking regions of the monkey and human TFPI genes, including the transcription initiation sites. Their identity is 92.6%. We searched the transcription factor binding sites in the regions using a database and found a sequence homologous with the HNF-1 binding motif (*36*) approximately 300 bp upstream of the transcription initiation site only in the monkey TFPI gene as shown in Fig. 4. Several previously reported binding sites such as AP-1, NF-1, C/EBPa, and c-Ets were found in both the human and monkey TFPI genes.

Luciferase Assay of Monkey and Human TFPI Genes—The luciferase activities were examined in monkey primary hepatocytes transfected with various constructs. As shown in Fig. 5A, the luciferase activity obtained from the construct containing up to 1,410 bp from the transcription initiation site of the monkey TFPI gene, pM−1410/+28, was twice that of the construct containing up to 1,399 bp of the human gene, pH−1399/+28. The ratio of the activities between monkey and human constructs containing shorter genes, pM−538/+28 and pH−520/+28, was almost the same as for pM−1410/+28 and pH−1399/+28. This indicates that the element for monkey liver-specific expression is present up to 538 bp upstream of the transcription initiation site of the monkey TFPI gene. On the other hand, the activities of pM−538/+28 and pH−520/+28 were obviously higher than those of pM−1410/+28 and pH−1399/+28, respectively. This suggests that the regions from −1410 to −538 in monkey and from −1399 to −520 in human negatively regulate the expression of the TFPI gene.

In order to examine whether the HNF-1 binding motif in the monkey gene is responsible for the efficient expression of monkey TFPI in hepatocytes, pM−538/+28(ΔATTT) was constructed. pM−538/+28(ΔATTT) is a mutant in which the HNF-1 binding motif in the monkey gene is replaced by the sequence for the corresponding region in the human gene by deletion of 4 nucleotides (−329 to −326 in monkey gene). No loss of luciferase activiy was observed in the construct pM−538/+28(ΔATTT). This indicates that the difference in the expression pattern between monkey and human TFPI is due to the presence of an element other than the HNF-1 binding motif in the 5′-flanking region of the monkey TFPI gene. We checked the level of HNF-1 expression in cultured monkey primary hepatocytes, because TFPI expression may be affected by the decrease of HNF-1 in cultured cells. However, no

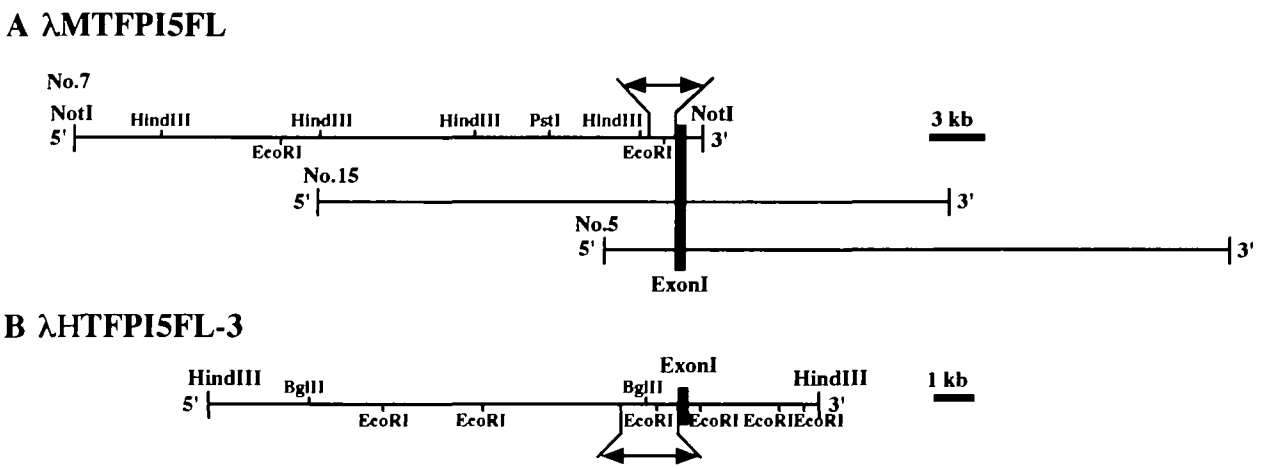

Fig. 3. **Restriction enzyme site map of the 5′-flanking regions of the monkey and human TFPI genes.** The thin horizontal bars represent the inserted DNA in each clone. Filled vertical bars indicate exons. The fragments indicated by arrows (↔) were cloned into PICA basic vector 2. (A): λMTFPI5FL for the monkey TFPI gene. (B): λHTFPI5FL-3 for the human TFPI gene.

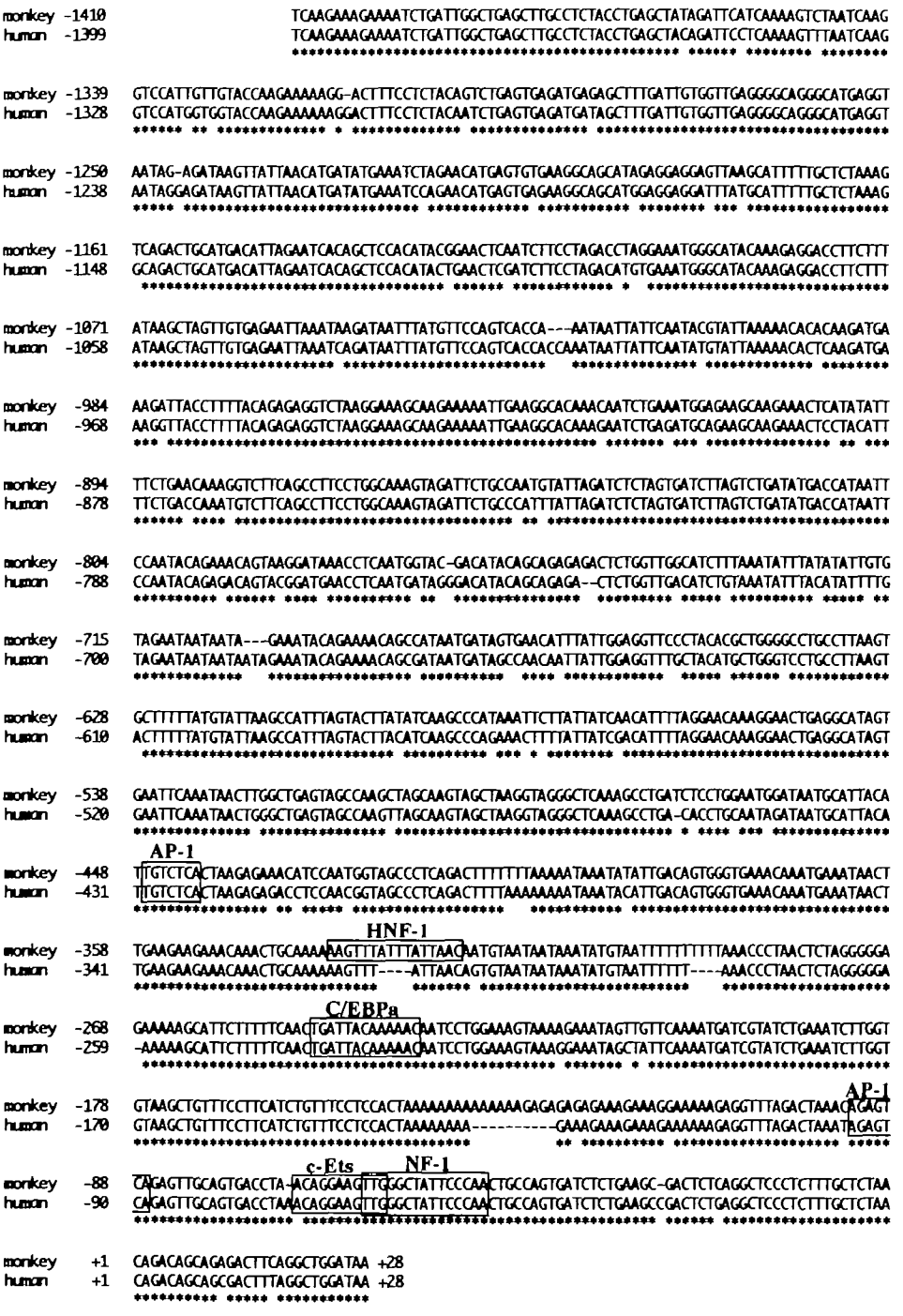

Fig. 4. **Nucleotide sequences of the 5′-flanking regions of the monkey and human TFPI genes including the transcription initiation sites.** The nucleotide sequence of the human TFPI gene is cited from Ref. 7. Sequences are shown with numbering from the major transcription initiation sites. Nucleotides that are identical in both the monkey and human sequences are shown by asterisks. Transcription factor binding motifs are boxed.

decrease in the level of the HNF-1 protein was observed until at least 4 days after inoculation (data not shown).

We then looked for the site in the 5′-flanking region of the monkey TFPI gene responsible for expression in monkey hepatocytes using four kinds of chimera constructs. The structures of the chimeras are shown on the left in Fig. 5B. The constructs pHM and pMHM showed activities equivalent to the monkey gene whereas pMH and pHMH showed lower activities comparable to pH-520/+28. Both pHM and pMHM, which have high expression activities like the monkey gene, contain the region from −138 to +28 of the monkey TFPI gene. This indicates that this region in the monkey gene is important for the expression of monkey TFPI in hepatocytes.

We extended our study of the site responsible for the efficient expression of the monkey TFPI gene in the region from −138 to +28 by site-directed mutagenesis. We made six kinds of mutants in which the nucleotide sequences of the monkey gene were replaced by the corresponding human sequences at the sites indicated in Fig. 6A. As shown in Fig. 6B, luciferase activities were lower in pM−538/+28mut1, pM−538/+28mut3, pM−538/+28mut5, and pM−538/+28mut6. On the other hand, the activities of pM−538/+28mut2 and pM−538/+28mut4 were not

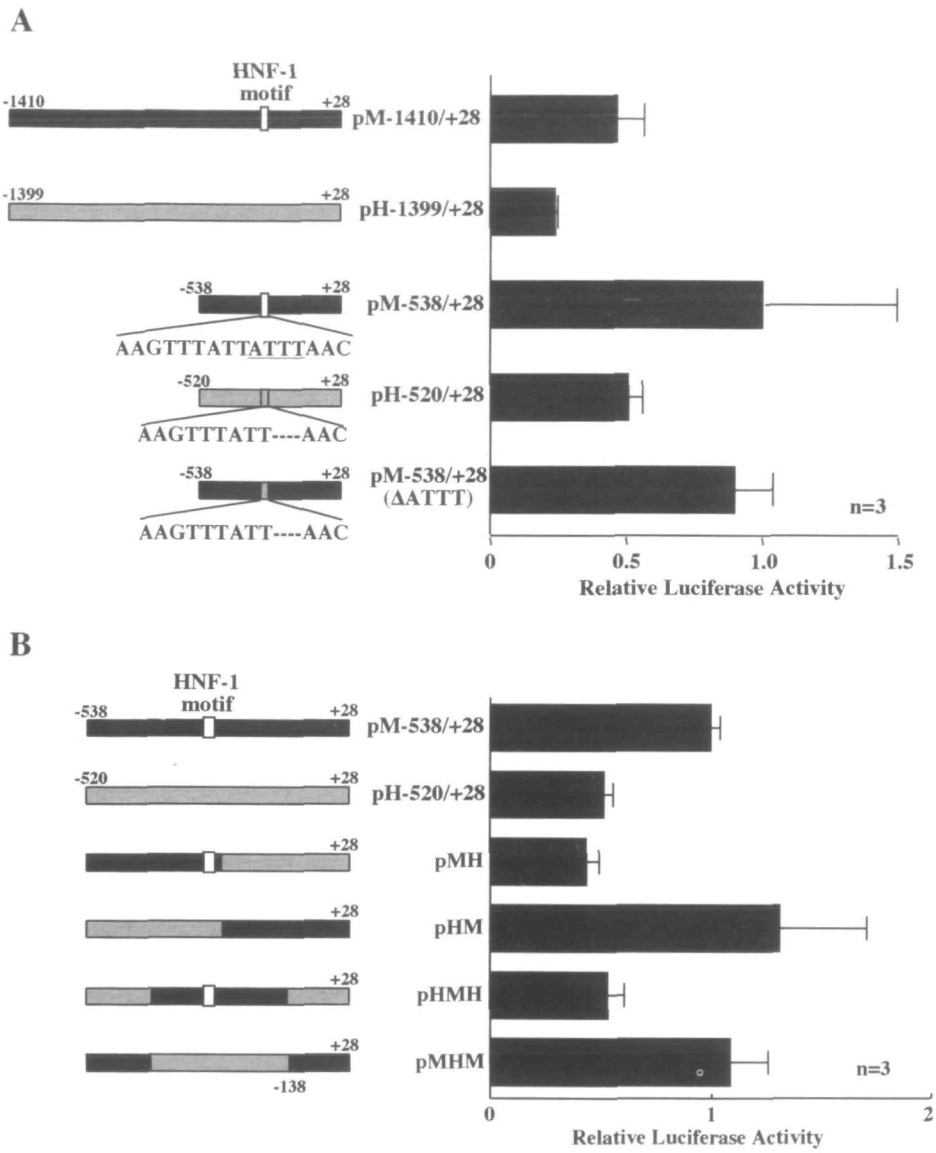

Fig. 5. **Transcriptional activities of the proximal region of the monkey and human TFPI genes.** (A) Constructs of approximately 1.4 or 0.5 kb from the 5′-flanking region of the monkey and human genes or site-directed mutants were transiently expressed in monkey primary hepatocytes, and the luciferase activities were measured as described under "EXPERIMENTAL PROCEDURES." The schemes of the TFPI promoter-luciferase fusion constructs are shown on the left. The solid and shaded bars represent the monkey and human sequences, respectively. (B) Chimera constructs of the monkey and human TFPI genes were expressed and the luciferase activities were measured as in (A). Results are the means and standard errors of three independent transfections, expressed relative to the activity of pM − 538/ + 28.

decreased. These data suggest that the difference in the expression activity between the monkey and human TFPI genes results from composite effects of several regions within − 138 to + 28.

DISCUSSION

Liver is an important tissue for the synthesis and clearance of many proteins including plasma proteins. Many of the genes for these proteins are transcriptionally regulated by liver-specific transcription factors such as HNF-1 (*21-23*). Most proteins in the plasma are cleared through hepatocytes by interacting with specific receptors. It has been shown that mouse and rat hepatocytes play an important role in the clearance of TFPI. Recombinant TFPI intravenously administered to rats and mice disappears very rapidly from the blood and accumulates in the liver (*24-27*). TFPI has been speculated to associate first with the surface of sinusoidal endothelial cells and then to be internalized in hepatocytes through interaction with proteoglycan and LDL-receptor-related protein on the surface (*25, 28, 29*). Human and rat TFPI mRNAs are markedly expressed in kidney and lung, but not in liver (*9-11, 13, 30*). The expression of TFPI mRNA in kidney and lung is thought to be mediated by vascular endothelial cells in these tissues. Cultured monocytes, smooth muscle cells, synovial cells, and chondrocytes, and mesangial cells from fetal kidneys have been demonstrated to synthesize TFPI (*31-35*). However, it has been shown that cultured human hepatocytes do not express TFPI mRNA (*9, 10*).

In the present work, the expression of TFPI in monkey hepatocytes was demonstrated by (i) the detection of TFPI mRNA by Northern blot analysis of monkey hepatocytes (Fig. 1C), (ii) immunostaining of monkey hepatocytes with anti-TFPI polyclonal antibody (Fig. 2A), and (iii) the binding that the transcriptional activity of the monkey TFPI gene is higher than that of the human TFPI gene in monkey primary hepatocytes (Fig. 5A). These data appear to be supported by the observation that the TFPI concentration of monkey plasma is higher than that of human

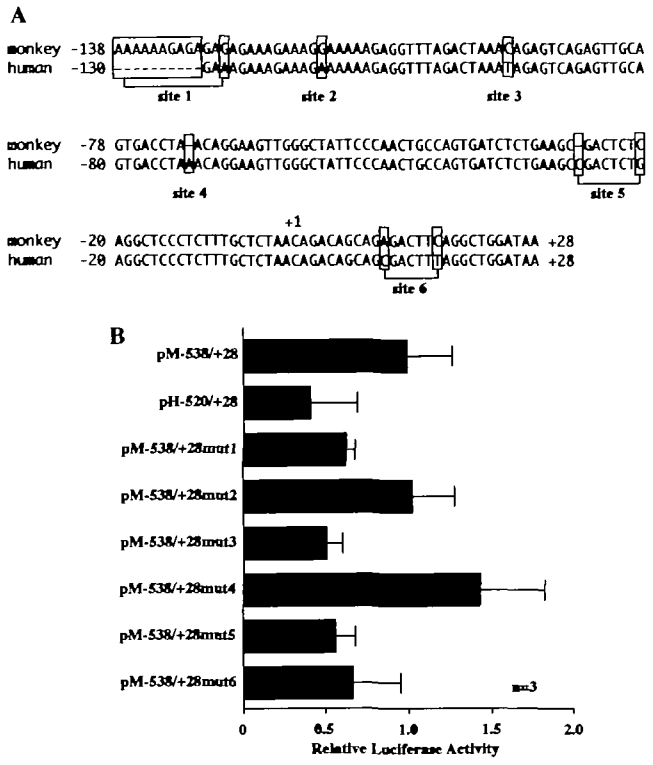

Fig. 6. **Transcriptional activities of site-directed mutants.** (A) Nucleotide sequence of the site in the 5′-flanking region responsible for the efficient expression of the monkey TFPI gene in comparison with the human gene. Open boxes indicate nucleotide sequence differences between the monkey and human genes. pM−538/+28mut1-6 are mutant in which the nucleotide sequences at sites 1-6 of the monkey gene are replaced with the corresponding human sequences by site-directed mutagenesis. (B) Site-directed mutants of the monkey TFPI gene were expressed and the luciferase activities were measured as described in Fig. 5. Results are the means and standard errors of three independent transfections, expressed relative to the activity of pM−538/+28.

plasma (Nakamura, S., Kumeda, K., and Kato, H., personal communication). In order to reveal the mechanism by which only monkey hepatocytes express TFPI efficiently, unlike human, bovine, rabbit, and rat liver, we determined the sequence of the 5′-flanking region of the monkey TFPI gene and surveyed the motif for the liver-specific expression in monkey. A nucleotide sequence of approximately 1.4 kb in the 5′-flanking region showed 92.6% identity to human TFPI. Because it was demonstrated that the element for monkey liver-specific expression is present up to 538 bp upstream of the transcription initiation site of the monkey TFPI gene, we searched transcription factor binding sites in these regions using a database. As shown in Fig. 4, we found a sequence homologous for the HNF-1 binding motif (*36*) only in the monkey TFPI gene, while several previously reported binding sites, such as AP-1, NF-1, C/EBPa, and c-Ets, are found in both the human and monkey TFPI genes (*18, 37*). HNF-1 is a well-known transcription factor important for the exclusive expression of various genes in the liver (*38*). However, it has been suggested that the HNF-1 binding site is not essential for the efficient expression of TFPI in monkey liver because (i) the transcriptional activity of the monkey TFPI gene is not changed by a mutation at the HNF-1 site (Fig. 5A) and (ii) the transcriptional activities of both the monkey and human TFPI genes are not significantly affected by the over-expression of HNF-1 (data not shown). On the other hand, analysis using chimeras and site-directed mutants suggests that several elements in the proximal region of the monkey gene are involved in the monkey-specific expression of TFPI in liver. However, we could not find any reported *cis*-acting sequences at the position of site-directed mutation indicated in Fig. 6 by a search using TFSEARCH ver 1.3 database. AP-1 might be related to the monkey-specific expression, because its binding site is close to mutation site 3. Accordingly, although we have not yet identified either the precise *cis*-acting elements or their binding factors, there is a possibility that the elements could be novel. More details concerning the sequence of this region of the rabbit, bovine, and rat TFPI genes will help us to elucidate the significance of the specific motif. A TFPI homologue TFPI-2, shows inhibitory activity different from that of TFPI (*39*) and is located on chromosome 7q22 (*40*). In contrast to TFPI, TFPI-2 is expressed efficiently in human liver and placenta. It will be interesting to compare the corresponding 5′-flanking region of the TFPI-2 gene with that of the TFPI gene. The accumulation of information about the upstream genes of TFPI and TFPI homologues from various mammals will clarify the tissue-specific and species-specific expression of TFPI.

TFPI mRNA is also expressed in the livers of a pregnant monkey and fetus (data not shown), whereas the human TFPI concentrations in cord blood and in plasma from neonates have been reported to be 50-70% those of adults (*24*). The expression patterns of TFPI mRNA similar to those of Macaque monkey in terms of size and tissue distribution were also observed in crab-eating monkey, rhesus monkey, and cotton-top tamarin (data not shown); all species of monkey tested express significant amounts of TFPI mRNA in their livers. Among non-human primates, New World monkeys (cotton-top tamarin) and Old World monkeys (Macaque monkeys) express TFPI in their hepatocytes, suggesting that TFPI expression in hepatocytes is a specific event that developed during the evolution of non-human primates. In this sense, the non-expression of TFPI in human hepatocytes is thought to be retrospective, that is, backward evolution of the newly-evolved property of non-human primates, which may lower anti-thrombotic potential in humans.

We thank Drs. Toshiyuki Miyata, Yuji Arai, and Kyoko Hidaka for helpful suggestions and discussion during the preparation of this manuscript. We also thank Ms. Kiyomi Tasaka and Eiko Takeda for their technical assistance.

REFERENCES

1. Rapaport, S.I. and Rao, L.V.M. (1992) Initiation and regulation of tissue factor-dependent blood coagulation. *Arterioscler. Thromb.* **12**, 1111-1121
2. Lindahl, A.K. (1995) Tissue factor pathway inhibitor in health and disease. *Trends. Cardiovas. Med.* **5**, 167-171
3. Broze, G.J., Jr. (1995) Tissue factor pathway inhibitor and the revised theory of coagulation. *Annu. Rev. Med.* **46**, 103-112
4. Wun, T.C. (1995) Tissue factor pathway inhibitor in *Molecular Biology of Thrombosis and Hemostasis* (Roberts, H.R. and High, K.A., eds.) pp. 331-353, Marcel Dekker, New York
5. Petersen, L.C., Valentine, S., and Hedner, U. (1995) Regulation

of the extrinsic pathway system in health and disease: The role of factor VIIa and tissue factor pathway inhibitor. *Thromb. Res.* **79**, 1-47
6. van der Logt, C.P., Reitsma, P.H., and Bertina, R.M. (1991) Intron-exon organization of the human gene coding for the lipoprotein-associated coagulation inhibitor: The factor Xa dependent inhibitor of the extrinsic pathway of coagulation. *Biochemistry* **30**, 1571-1577
7. Girard, T.J., Eddy, R., Wesselschmidt, R.L., MacPhail, L.A., Likert, K.M., Byers, M.G., Shows, T.B., and Broze, G.J., Jr. (1991) Structure of the human lipoprotein-associated coagulation inhibitor gene. *J. Biol. Chem.* **266**, 5036-5041
8. Enjyoji, K., Emi, M., Mukai, T., Imada, M., Leppert, M.L., Lalouel, J.M., and Kato, H. (1993) Human tissue factor pathway inhibitor (TFPI) gene: complete genomic structure and localization on the genetic map of chromosome 2q. *Genomics* **17**, 423-428
9. Ameri, A., Kuppuswamy, M.N., Basu, S., and Bajaj, S.P. (1992) Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators. *Blood* **79**, 3219-3226
10. Bajaj, M.S., Kuppuswamy, M.N., Saito, H., Spitzer, S.G., and Bajaj, S.P. (1990) Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor:evidence that endothelium is the principal site of its synthesis. *Proc. Natl. Acad. Sci. USA* **87**, 8869-8873
11. Enjyoji, K., Emi, M., Mukai, T., and Kato, H. (1992) cDNA cloning and expression of rat tissue factor pathway inhibitor (TFPI). *J. Biochem.* **111**, 681-687
12. Caplice, N.M., Mueske, C.S., Kleppe, L.S., Peterson, T., Broze G.J., Jr., and Simari, R.D. (1998) Expression of tissue factor pathway inhibitor in vascular smooth muscle cells and its regulation by growth factors. *Circ. Res.* **83**, 1264-1270
13. Werling, R.W., Zacharski, L.R., Kisiel, W., Bajaj, S.P., Memoli, V.A., and Rousseau, S.M. (1993) Distribution of tissue factor pathway inhibitor in normal and malignant human tissues. *Thromb. Haemost.* **69**, 366-369
14. Kamei, S., Kamikubo, Y., Hamuro, T., Fujimoto, H., Ishihara, M., Yonemura, H., Miyamoto, S., Funatsu, A., Enjyoji, K., Abumiya, T., Miyata, T., and Kato, H. (1994) Amino acid sequence and inhibitory activity of Rhesus monkey tissue factor pathway inhibitor (TFPI): Comparison with human TFPI. *J. Biochem.* **115**, 708-714
15. Abumiya, T., Nakamura, S., Takenaka, A., Takenaka, O., Yoshikuni, Y., Miyamoto, S., Kimura, T., Enjyoji, K., and Kato, H. (1994) Response of plasma tissue factor pathway inhibitor to diet-induced hypecholesterolemia in Crab-eating monkeys. *Arterioscler. Thromb.* **14**, 483-488
16. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
17. Abumiya, T., Enjyoji, K., Kokawa, T., Kamikubo, Y., and Kato, H. (1995) An anti-tissue factor pathway inhibitor (TFPI) monoclonal antibody recognized the third Kunitz domain (K3) of free-form TFPI but not lipoprotein-associated forms in plasma. *J. Biochem.* **118**, 178-182
18. Evans, G.A. and Wahl, G.M. (1987) Cosmid vectors for genomic walking and rapid restriction mapping. *Methods Enzymol.* **152**, 604-610
19. Akiyama, Y., Onizuka, K., Noguchi, T., and Ando, M. (1998) Parallel protein information analysis (PAPIA) system running on a 64-node PC Cluster in *Proc. the 9th Genome Informatics Workshop (GIW'98)*, pp. 131-140, Universal Academy Press
20. Girard, T.J., Warren, L.A., Novotny, W.F., Bejcek, B.E., Miletich, J.P., and Broze, G.J., Jr. (1989) Identification of the 1.4 kb and 4.0 kb messages for the lipoprotein associated coagulation inhibitor and expression of the encoded protein. *Thromb. Res.* **55**, 37-50
21. Hayashi, Y., Chan, J., Nakabayashi, H., Hashimoto, T., and Tamaoki, T. (1992) Identification and characterization of two enhancers of the human albumin gene. *J. Biol. Chem.* **267**, 14580-14585
22. Rouet, P., Raguenez, G., Tronche, F., Yaniv, M., N'Guyan, C., and Salier, J.P. (1992) A potent enhancer made of clustered liver-specific elements in the transcription control sequences of human alpha 1-microglobulin/bikunin gene. *J. Biol. Chem.* **267**, 20765-20773
23. Bancroft, J.D., McDowell, S.A., and Degen, S.J. (1992) The human prothrombin gene: transcriptional regulation in HepG2 cells. *Biochemistry* **31**, 12469-12476
24. Palmier, M.O., Hall, L.J., Reisch, C.M., Baldwin, M.K., Wilson, A.G., and Wun, T.C. (1992) Clearance of recombinant tissue factor pathway inhibitor (TFPI) in rabbits. *Thromb. Hamostas.* **68**, 33-36
25. Warshawsky, I., Broze, G.J., Jr., and Schwartz, A.L. (1994) The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. *Proc. Natl. Acad. Sci. USA* **91**, 6664-6668
26. Elsayed, Y.A., Nakagawa, K., Kamikubo, Y., Enjyoji, K., Kato, H., and Sueishi, K. (1996) Effects of recombinant human tissue factor pathway inhibitor on thrombus formation and its *in vivo* distribution in a rat DIC model. *Am. J. Clin. Pathol.* **106**, 574-583
27. Kamikubo, Y., Hamuro, T., Matsuda, J., Shinya, N., Miyamoto, S., Funatsu, A., and Kato, H. (1996) Antithrombotic effect of human recombinant tissue factor pathway inhibitor on endotoxin-induced intravascular coagulation in rats: concerted effect with antithrombin. *Thromb. Haemostas.* **76**, 621-626
28. Narita, M., Bu, G., Olins, G.M., Higuchi, D.A., Herz, J., Broze, G.J., Jr., and Schwartz, A.L. (1995) Receptor systems are involved in the plasma clearance of tissue factor pathway inhibitor. *J. Biol. Chem.* **270**, 24800-24804
29. Warshawsky, I., Herz, J., Broze, G.J., Jr., and Schwartz, A.L. (1996) The low density lipoprotein receptor-related protein can function independently from heparan sulfate proteoglycans in tissue factor pathway inhibitor endocytosis. *J. Biol. Chem.* **271**, 25873-25879
30. Erlich, J.H., Apostolopoulos, J., Wun, T.C., Kretzmer, K.K., Holdsworth, S.R., and Tipping, P.G. (1996) Renal expression of tissue factor pathway inhibitor and evidence for a role in crescentic glomerulonephritis in rabbits. *J. Clin. Invest.* **98**, 325-335
31. Weissbach, G., Harenberg, J., Wendisch, J., Pargac, N., and Thomas, K. (1994) Tissue factor pathway inhibitor in patients and children. *Thromb. Res.* **73**, 441-446
32. van der Logt, C.P., Dirven, R.J., Reitsma, P.H., and Bertina, R.M. (1994) Expression of tissue factor and tissue factor pathway inhibitor in monocytes in response to bacterial lipopolysaccharide and phorbolester. *Blood Coagul. Fibrinolysis* **5**, 211-220
33. McGee, M.P., Foster, S., and Wang, X. (1994) Simultaneous expression of tissue factor and tissue factor pathway inhibitor by human monocytes. A potential mechanism for localized control of blood coagulation. *J. Exp. Med.* **179**, 1847-1854
34. Brinkman, T., Kahnert, H., Prohaska, W., Nordfang, O., and Kleesiek, K. (1994) Synthesis of tissue factor pathway inhibitor in human synovial cells and chondrocytes makes joints the predicted site of bleeding in Haemophiliacs. *Eur. J. Clin. Chem. Biochem.* **32**, 313-317
35. Yamabe, H., Osawa, H., Inuma, H., Kaizuka, M., Tamura, N., Tsunoda, S., Fujita, Y., Shirato, K., and Onodera, K. (1996) Tissue factor pathway inhibitor production by human mesangial cells in culture. Thromb. *Haemostas.* **76**, 215-219
36. Courtois, G., Aumhueter, S., and Crabtree, G.R. (1988) Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. *Proc. Natl. Acad. Sci. USA* **85**, 7937-7941
37. Tyson, D.R., Kuppuswamy, M.N., Broze, G.J., Jr., and Bajaj, S.P. (1993) Revised DNA sequence of exon 1 and 5′-flanking region of the human tissue factor pathway inhibitor gene. *Thromb. Res.* **70**, 269-273
38. Bach, I., Galcheva-Gargova, Z., Mattei, M.G., Simon-Chazottes, D., Guenet, J.L., Crereghini, S., and Yaniv, M. (1990) Cloning of human hepatic nuclear factor 1 (HNF1) and chromosomal localization of its gene in man and mouse. *Genomics* **8**, 155-164
39. Petersen, L.C., Sprecher, C.A., Foster, D.C., Blumberg, H., Hamamoto, T., and Kisiel, W. (1996) Inhibitory properties of a novel human Kunitz-type protease inhibitor homologous to tissue factor pathway inhibitor. *Biochemistry* **35**, 226-272
40. Miyagi, Y., Yasumitsu, H., Eki, T., Miyata, S., Kishikawa, N., Hirahara, F., Aoki, I., Misugi, K., and Miyazaki, K. (1996) Assignment of the human PP5/TFPI-2 gene to 7q22 by FISH and PCR-based human/rodent cell hybrid mapping panel analysis. *Genomics* **35**, 267-268