Pergamon

Thrombosis Research 109 (2003) 207–215

THROMBOSIS RESEARCH

Regular Article

Characterization and functional analysis of TFPI-2 gene promoter in a human choriocarcinoma cell line

F. Hubé[a], P. Reverdiau[a,*], S. Iochmann[a], C. Cherpi-Antar[b], Y. Gruel[a]

[a] *Laboratoire d'Hémostase, EA 3249 "Cellules Hématopoïétiques, Hémostase et Greffe", IFR 120 Faculté de Médecine, 2 bis Bd Tonnellé, 37032 Tours Cedex, France*

[b] *Laboratoire de Biochimie et de Biologie Moléculaire, INSERM U316, IFR 120 Faculté de Médecine, 2 bis Bd Tonnellé, 37032 Tours Cedex, France*

Received 5 December 2002; received in revised form 30 January 2003; accepted 6 February 2003

Abstract

Tissue factor pathway Inhibitor-2 (TFPI-2) is associated with extracellular matrices and plays a major role in cell migration and tumor invasion. In this study, a 4.8-kb human TFPI-2 gene 5′-flanking region was isolated, cloned and sequenced. Promoter region analysis revealed a high GC-rich content without canonical TATA and CAAT boxes but three transcription initiation sites were identified. Moreover, several putative binding sites for transcription factors were identified (MyoD, LYF1, NF-Y, GATA, oct-1, AP-1, Sp1, NF1, NF-κB and egr-1). To characterize potential regulatory regions, TFPI-2/luciferase promoter constructs were then transfected in human choriocarcinoma JEG-3 cells. We first showed that the minimal TFPI-2 promoter is located between −166 and −111 from the translation start site. Luciferase activity consistently increased after stimulation of JEG-3 cells by phorbol 12-myristate 13-acetate indicating that NF1, NF-κB and egr-1/Sp1 binding sites are crucial in inducible TFPI-2 expression. Moreover, negative regulatory regions included AP-1 binding sites were identified. This study demonstrates that the TFPI-2 gene promoter exhibits typical features of a housekeeping gene.

Keywords: TFPI-2; Choriocarcinoma cells; GC-rich sequence; Transcription initiation site; Reporter gene assay

1. Introduction

Tissue factor pathway inhibitor-2 (TFPI-2), a 32-kDa member of the Kunitz-type serine proteinase inhibitor family, is associated with extracellular matrices and plays a major role in cell migration and tumor invasion. Despite similarities in amino acid sequence and structure to TFPI-1, the main inhibitor of TF/VIIa complex, TFPI-2 weakly inhibits factor Xa and thrombin generation, and thus poorly regulates TF-mediated coagulation [1]. In contrast, TFPI-2 efficiently decreases activation of metalloproteinases MMP-1, MMP-3, MMP-9, and MMP-13, by inhibiting plasmin and trypsin [2,3] and thus reduces tumor invasion and metastasis [4,5]. In addition, TFPI-2 could also regulate plasmin in atherosclerotic plaques modulating extracellular proteolytic mechanisms [6].

TFPI-2 is synthesized and secreted by endothelial, mesenchymal and epithelial cells, monocytes/macrophages and the syncytiotrophoblast [7–9]. On the other hand, the expression of TFPI-2 has been reported to be undetectable or at a very low level in a variety of invasive tumor cell lines [4,5,10–12] and downregulation of TFPI-2 gene could thus favor cancer invasion.

The complete nucleotide sequences of human and murine TFPI-2 genes consist of five exons and four introns [13,14]. The human TFPI-2 gene, located on chromosome 7q22 [15], spans approximately 7 kb and a recent analysis of the promoter region up to −3564 identified a single transcription initiation site without canonical TATA or CAAT box [14]. In contrast, the 5′-flanking region of the murine TFPI-2 gene contains a prototypic TATA box, a GC box and two CAAT boxes [13].

We recently reported that TFPI-2 mRNA synthesis by a human placenta cell line isolated from a choriocarcinoma

Abbreviations: TFPI-2, tissue factor pathway Inhibitor-2; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor; RT-PCR, reverse transcription-polymerase chain reaction; AMV, avian myeloblastosis virus; PCR, polymerase chain reaction; RACE, rapid amplification of the 5′-cDNA end; βGal, beta-galactosidase; TSS, translation start site; TrBMEC, transformed bone marrow microvascular endothelial cells; MIS, major transcription initiation site.

* Corresponding author. Tel.: +33-2-47-36-60-67; fax: +33-2-47-36-60-95.

E-mail address: reverdiau@med.univ-tours.fr (P. Reverdiau).

0049-3848/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0049-3848(03)00147-6

(JEG-3) was stimulated by phorbol 12-myristate 13-acetate (PMA), whereas tumor necrosis factor (TNF-α) had no effect [16]. This cell line was therefore considered of interest to identify regulatory elements essential for transcriptional regulation of TFPI-2 expression.

By using a method for walking upstream in genomic DNA, the present study further defined the sequence of the human TFPI-2 promoter up to −4767 in the 5′-flanking region. We thus identified two new minor transcription initiation sites, and also compared the human TFPI-2 promoter to the murine sequence already published [13]. In addition, we studied TFPI-2 promoter activity with various promoter/luciferase reporter gene constructs transfected into human placenta choriocarcinoma JEG-3 cells. Finally, this approach allowed us to define further the regions required in the promoter for basal and inducible TFPI-2 expression in this trophoblastic cell line.

2. Materials and methods

2.1. Cell culture and stimulation

Human trophoblast cell line JEG-3 was cultured in RPMI-1640 medium (Invitrogen Life Technologies, Cergy Pontoise, France) supplemented with 10% endotoxin-free heat-inactivated fetal calf serum (ATGC Biotechnologie, Noisy le Grand, France), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, 15 mM sodium bicarbonate and 2 mM glucose. Cells were grown at 37 °C in an atmosphere of 95% air and 5% CO_2.

Confluent cells were washed with Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution and detached by 0.05% trypsin–0.02% EDTA. Cell viability was determined by the Trypan blue dye exclusion test and ranged between 90% and 95%. Trophoblast cells were then seeded in 24-well plates (Falcon, Becton Dickinson, Le Pont de Claix, France) at 2×10^5 cells per well and cultured for 18 h in complete medium. JEG-3 cells were incubated with 20 ng/ml recombinant human TNF-α (specific activity: 2×10^7 IU/mg, Genzyme, Cambridge, MA, USA) for 60 min or with 100 ng/ml PMA (Sigma Aldrich Chimie, Saint Quentin Fallavier, France) for 4 h.

2.2. mRNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total mRNA was isolated from 2×10^5 JEG-3 cells either unstimulated or stimulated with TNF-α or PMA, using the Dynabeads mRNA Direct kit (Dynal France, Compiègne, France) according to the manufacturer's instructions. Total mRNA (40 ng for 2×10^5 cells) was then reverse-transcribed for 1 h at 42 °C in 1 × incubation buffer containing 250 μM of each deoxynucleotide triphosphate, 5 μM oligo$(dT)_{20}$, 25 U of RNase inhibitor and 20 U of avian myeloblastosis virus-reverse transcriptase (AMV, Roche Diagnostics, Meylan, France). Polymerase chain reaction (PCR) was then performed using first-strand cDNA obtained from 5×10^4 cells in a total reaction volume of 50 μl containing 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 50 μM of each deoxynucleotide triphosphate, 0.5 U of Super *Taq* DNA polymerase (ATGC Biotechnologies), and 1 μM of forward and reverse synthesized oligonucleotide primers (Table 1) (Genset, Paris, France). PCR was set up in a GeneAmp PCR system 2400 (Applied Biosystems, Courtaboeuf, France) programmed for an initial denaturation step of 3 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s, and the final extension step was performed at 72 °C for 7 min. PCR products were then analyzed by electrophoresis through 1.6% agarose gel in TBE buffer (90 mM Tris–HCl, 90 mM borate acid, 2.5 mM EDTA) containing 1 μg/ml ethidium bromide and visualized by UV transillumination (Gel Doc 1000 system, Bio-Rad). RT-PCR products were sequenced using the dideoxynucleotide chain termination method on a Perkin Elmer Abi Prism 377 DNA sequencer. Sequencing was performed on both strands with the reverse and forward primers specific for TFPI-2 and GAPDH used for RT-PCR.

2.3. Isolation and cloning of the TFPI-2 promoter fragment

In order to clone the human TFPI-2 promoter, we used the Human Genome Walker kit (Clontech, Palo Alto, CA, USA), containing five uncloned adaptator-ligated DNA fragments obtained after the digestion of a human genomic library with five different restriction enzymes. The first PCR was performed using 1 μM of TFPI-2 gene-specific primer (Ext-2) and 1 μM of the outer adaptor primer located in adaptor-ligated oligonucleotide (ALP-1) (Table 1). PCR amplifications were performed in the Gene Amp PCR system 2400 with a touchdown program to increase the specificity. The temperature profile was 5 min at 95 °C followed by amplification for 20 cycles with a 0.5 °C decrease in the annealing temperature after each cycle. The starting cycle consisted of 10 s at 94 °C, 30 s at 70 °C, and 4 min at 68 °C. Twenty additional cycles of the same program were conducted with a fixed annealing temperature of 60 °C, and a final extension for 10 min at 68 °C. A second PCR reaction was then undertaken using a 100-fold dilution of PCR1 products with nested TFPI-2-specific (Int-2) and adaptator (ALP-2) oligonucleotide primers. An Advantage-GC Genomic PCR kit (Clontech) including an optimized buffer (5% DMSO and 0.5 M GC-melt) was used to facilitate amplification of GC-rich sequences. Moreover, 0.1 U of a DNA polymerase mixture (Tth and $Vent_R$®) with 0.01 μg/μl anti-Tth DNA polymerase antibody were also used for efficient and specific amplification of long fragments. PCR products were then purified by the GFX PCR DNA and Gel Purification Kit (Amersham Pharmacia Biotech Europe, Paris, France) and were cloned into the pCR2.1 vector (Invitrogen Life Technologies). Both strands were then sequenced and the TFPI-2 promoter

Table 1
Oligonucleotide primers used in this study

Name	Sequence	Strand	Position[a]	Assay[b]
TFPI-2	5′-cagatgaagctacttgtatgggcttc-3′	+	+419/+444	RT
TFPI-2	5′-ggcaaagcgaagctttggcatc-3′	–	+669/+690	RT
GAPDH	5′-acagtccatgccatcactgcc-3′	+	+529/+549	RT
GAPDH	5′-gcctgcttcaccaccttcttg -3′	–	+774/+794	RT
ALP-1	5′-gtaatacgactcactatagggc-3′	+	[c]	GW
ALP-2	5′-actatagggcacgcgtggt-3′	+	[c]	GW
Ext-2	5′-agtgcagcctccgtcaggaaaagcagc-3′	–	+62/+36	GW, R
Int-2	5′-aaaagcagcagaatcgacagccccaggg-3′	–	+44/+17	GW, R
RACE 1	5′-cgactggagcacgaggacactga-3′	+	[c]	R
RACE 2	5′-ggacactgacatggactgaaggagta-3′	+	[c]	R
Tronq 2a	5′-c**aa**g**c**t**t**ggcaaggcgtccgagaaagc-3′	–	–20/–46	PC
P10	5′-acg**c**t**c**g**ag**ccgcataaagcgggca-3′	+	–117/–93	PC
P9	5′-acagtcc**t**c**ga**gcatgaatcagccac-3′	+	–176/–151	PC
P8	5′-gtccccgtctggactac**tc**gaggaaag-3′	+	–285/–260	PC
P7	5′-cattactgac**t**c**ga**gcgctccctcag-3′	+	–314/–289	PC
P6	5′-gag**c**t**c**g**ag**tccggcttctttacagcgcaa-3′	+	–382/–353	PC
P5	5′-gc**t**c**g**aggtccattgcaacgaatcccgc-3′	+	–481/–454	PC
P4	5′-ctgtccacc**c**t**c**g**a**gatattataagcctg-3′	+	–588/–560	PC
P3	5′-ctgaattc**t**c**ga**gatccacccgccttgg-3′	+	–714/–687	PC
P2	5′-ga**c**t**c**gagtgtttgcccctggcctaagg-3′	+	–1053/–1026	PC
P1	5′-ctggtcaaatc**t**c**g**agaactccccaaggc-3′	+	–1596/–1568	PC

The *Xho*I and *Hin*dIII restriction sites are underlined and bold letters indicate mutated nucleotides.
[a] Nucleotide position relative to the translation start site (+1).
[b] Primers used for amplification of RT-PCR products (RT), the 5′-flanking region (GW), for 5′-RACE (R) and for plasmid contructs (PC).
[c] Position is on the adaptator sequence.

sequence obtained has been deposited in the GeneBank database under accession number AY044097.

2.4. Rapid amplification of the 5′-cDNA end (RACE)

A modified 5′-rapid amplification of cDNA end (RACE) reaction based on a capfinder method for full-length mRNA (GenRacer kit, Invitrogen Life Technologies) was performed to localize the transcription initiation site in the 5′-flanking region of human TFPI-2. Total mRNA (300 ng) was isolated from 1.5×10^6 JEG-3 cells using the Dynabeads mRNA Direct kit. After dephosphorylating using 10 U calf intestinal phosphatase, the 5′-cap structure was removed from full-length mRNA using 10 U tobacco acid pyrophosphatase. mRNA was then ligated to "Genracer RNA oligo" using 5 U T4 RNA ligase and reverse-transcribed using 20 U AMV-RT and Ext-2 TFPI-2-specific primer. RACE products were then amplified by PCR using 2 μM RACE 1 and Ext-2 oligonucleotide primers (Table 1) and 1 U *Taq*/*Pfu* DNA polymerase mixture (80:1 unit ratio). Nested PCR was then performed using 2 μM RACE 2 and 2 μM Int-2 primers to identify products of interest. The amplified fragments were subcloned into pCR2.1 vector and sequenced on both strands.

2.5. Construction of TFPI-2/luciferase reporter plasmids

To characterize potential the regulatory regions in the TFPI-2 promoter, various deletion constructs of the 5′-flanking region were generated by PCR using modified primers that contained restriction sites (Table 1). The *Xho*I and *Hin*dIII restriction enzyme-digested fragments obtained were subsequently subcloned in the promoterless, enhancerless expression vector pGL3-Basic (Promega, France) upstream of the luciferase reporter gene. Sequences of the 10 promoter constructs were then confirmed by dideoxynucleotide chain-termination sequencing.

2.6. Transient DNA transfections and luciferase assays

The human TFPI-2/luciferase reporter constructs (1.1 nM) were transiently transfected into the choriocarcinoma cell line JEG-3 (2.5×10^5 cells cultured in a 24-well plate) using LipofectAMINE Plus™ Reagent (Invitrogen, The Netherlands) and following the manufacturer's instructions. The βGal plasmid (pSVbeta-galactosidase control vector; Promega) was always co-transfected to

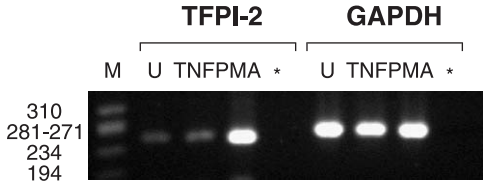

Fig. 1. Detection of TFPI-2 and GAPDH mRNA in JEG-3 trophoblast cells. Cells were unstimulated (U) or incubated with 20 ng/ml TNF-α for 1 h or 100 ng/ml PMA for 4 h. Lane M: ϕX174 RF DNA/*Hae*III DNA ladder.

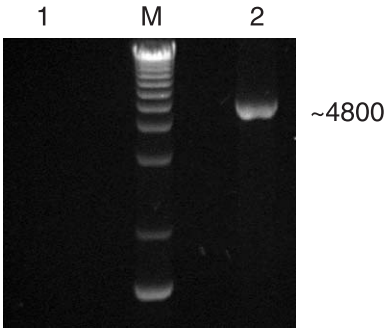

Fig. 2. Amplification of a 4.8-kb sequence from the 5′ -flanking region of the human TFPI-2 gene. First PCR (lane 1) and nested PCR (lane 2) were performed using TFPI-2 gene-specific and outer adaptor primers located in an adaptor-ligated oligonucleotide from a human genomic library digested with the *Pvu*II restriction enzyme. The PCR product was detected by electrophoresis through 1% agarose gel containing ethidium bromide. Lane M: 1-kb ladder of DNA size marker containing DNA fragments ranging from 75 to 12,216 bp.

normalize transfection efficiency. Transfections were performed on at least three independent experiments for each construct. Cells were then harvested 24 h after transfection and luciferase activities, measured using a Wallac Victor^2 multilabel reader luminometer (Perkin Elmer, France), were expressed in relative light units (RLU). Results were adjusted to the βGal activity (β-galactosidase Enzyme Assay System; Promega). A positive control containing CMV promoter sequence (pGL3 control, generous gift of Dr A. Touzé of EMI-U0010, Tours, France) and a negative control (pGL3-Basic) were used for each experiment.

3. Results

3.1. Basal and inducible TFPI-2 mRNA expression

TFPI-2 mRNA expression was studied in 5×10^4 JEG-3 trophoblast cells using a specific RT-PCR method. The optimal TNF-α and PMA concentrations and incubation times defined for use for cell stimulation were 60 min in the presence of 20 ng/ml TNF-α and 4 h with 100 ng/ml of PMA. The obtained RT-PCR product appeared as a single band of 253 bp, the expected size of TFPI-2 cDNA fragment (Fig. 1). The sequencing of this product was similar to the previously published sequence [17] and confirmed the specificity of the technique. GAPDH used as control (265 bp) was not affected in any experiment, regardless of cell stimulation. As shown by the relative intensities of cDNA bands, basal synthesis of TFPI-2 mRNA occurred in JEG-3 cells and appeared unmodified by TNF-α. In contrast, the intensity of the TFPI-2 cDNA band was increased after incubation of cells for 4 h with PMA.

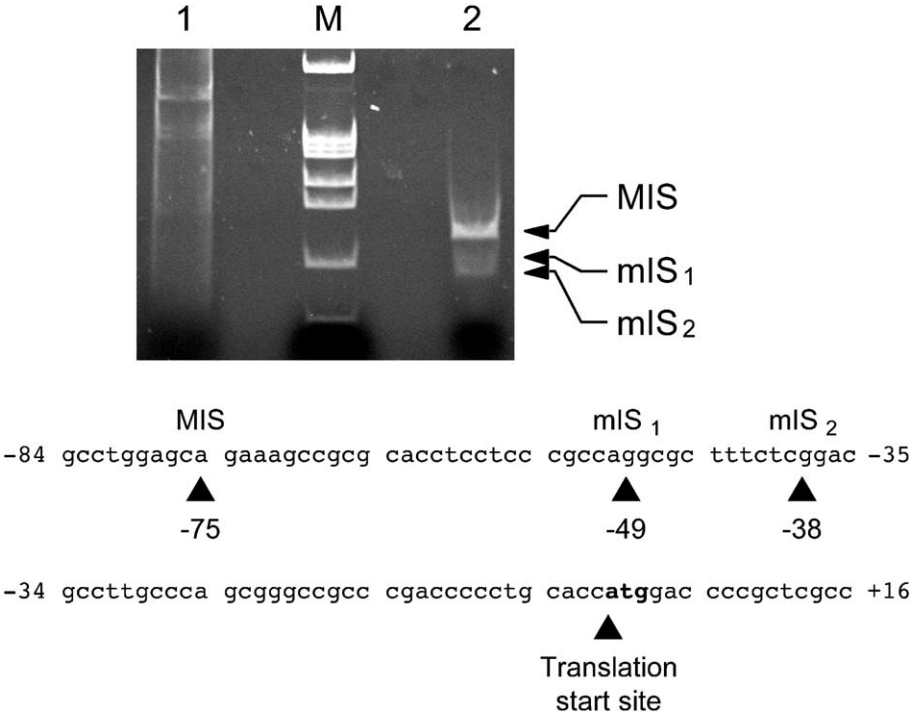

Fig. 3. Mapping of transcription initiation sites by RACE analysis. The RACE reaction was based on a capfinder method for full-length mRNA followed by a first PCR (lane 1) and a nested PCR (lane 2). The major initiation site (MIS) and the two minor initiation sites (mIS_1 and mIS_2) are indicated by arrows and located at positions −75, −49, and −38 upstream of the translation initiation site, respectively. The PCR products were detected by electrophoresis through 6% acrylamid gel. Lane M: ϕX174 RF DNA/*Hae*III fragments ranging from 72 to 1353 bp.

```
Human       TCCCTCCTCT AATAATATTT TGAAGGAACT GGGAGGGTCT AATCTGACTT TACTCTGAAT  -2328
Human       GTGTCATTCC AAAACTGGCT GTCTGTCTCA GTGGTTGGTG CATTAGAGCC AGCTCACACA  -2268
Human       GATTTTGGCC ATGGGGAGCT CTGGCTCTTG GAGATCTTTT CCCACCTCGG CATTCAGTGA  -2208
Human       TTCTGCAGTG GCAGCTTGAA ATTGACCTTA GTGAGCACAT TTACATCACA GAAATCAGTT  -2148
Human       AACTATACAA AGCATCCCCA TCCCATTTAT TTTTCCCTAC TGGAGAGGTG ATAATTAAAC  -2088
Human       ATTTATCTTT ATAGTTTCAA CTATCATTCT TAATCATAGA CATCTGCTAA TCAAAGCTGA  -2028
Human       CAGCATTTCC AGATCAGGCT GGTTTTCCTA GGACTGTGAC CTTGTAACAT GACATCAGAA  -1968
Human       TGACTGAAAA CTGGCACAAG GATATAATGT GCAAAATACC TCAGATTATT AACCCACTTT  -1908
Human       GAGCAAGTGT GTCCTATACT TTTCAGGGCT TCCTGAAAAC TTGAAAGAGA ACTTCTGTAA  -1848
Human       ACTACCCCCA GTATCCTCAG AAGTCAAGAA TAATTATTTA CTTGAGAATG TTGAATACAA  -1788
Human       CAAGAGAAAA GGGGAAGGGA AGACAAGTAT ATTAAAATGT CTTCAAAAAT ATCTTTAATA  -1728
Human       TTTTATGTAA AATTTCACTG TAGGTAATTG TTTGTATTAA AAAAAAAAGT GGATCACAAG  -1668
Human       CTAATATGTC AAAGGAACAG GGAACTTTAA TGGATGCACT GACATTAGGA AATGTGTGGG  -1608
Human       AATACACTGA TCTGGTCAAA TCTTCAGAAC TCCCCAAGGC TATCTCTCTG GGCTGCTATG  -1548
Human       GTTCTTCTCC AAAAACATAG ATACATACAA AAACATAGCT TCATACATGC TTGGTTGGGG  -1488
Human       TGTAAAATGG TACAATCATC TTGAAAAAAT CCTTTGGCAG TGTCTAACAT TTCTAAGTAT  -1428
Human       TTTATCCAAG AGTAATAAAA ATTGCAGGTC CACACAAAGC AGCTTTTTTC ATAAGAGCCC  -1368
Human       CCAAACAAAC TAGAAACAAC ACAAATTCCA GTAATAAGAC ATTTGTGATA TATTAGAATA  -1308
Human       TTACTCAATG ATTTAAAGGA ACAGACTATT GATACACACA GCAACATATT TATACATTGT  -1248
Human       GGGATATTCA TACGTTTTGG TAAATTCATA CCTTGGACTA CGCAGGAATT AAAAGAAACA  -1188
Human       GACTATTGAT GCAGTGACCT GGGCGTATCT CAAAAACAGG AAGTGTGAGA AAGAAATACT  -1128
Human       ACACAGTGAA TACTTTATAA TTCCGTTTAT ATGAAATTCA AGAATAGGCA AATCTAATCT  -1068
Human       ATGGTAATCG AAATGAGAAG AGTGTTTGCC CCTGGCCTAA GGATGGAAGG ATTGATTTAG  -1008
Human       GAAAAAGTCT GCAGGAGCTT TCTGGAGTGA TGGAAATGTT CTAACTCTTT TTTTTTCTT    -948
Human       CTTCTTCTTC TTTTTCTTCG TTTCGAGACG GAGTTCCACT CTGCCGCCCA GACTGGAGTG   -888
Human       CAGTGGCGCC ATCTGGGCTC ACTGCAGCCT CCGCCTCCTG GGTTCAAGCC ATTCTCCTGC   -828

                                        MyoD
Human       CTCAGCCTCC CGAGGGGCTG GGACTACAGG TGCCCGCCAC CACGCCCGGC TAATTTTTTG   -768
Human       TGTTTTTAAT AGAGACGGGG TTTCGCCATG TTGGCCAGGC TAGTCTCGAA CTCCTGATTT   -708

                                    LYF1
Human       CAGGTGATCC ACCCGCCTTG GCCTCCCAAA GTGCTGGGAT TACAGGCGCG AGCTACCGCG   -648

                                  NF-Y                          GATA
Human       CCCAGCCAAA ATGTTCTAAT TCTTTATTGG TGGCTACACA GTTGTCAAAA TTTATCAAAC   -588

                                       Oct-1
Human       TGTCCACCTA CGATATATTA TAAGCCTGTA TTAAATGTAA ATTAGAACTC GATTGAAATC   -528
Human       TGTGTGTATA AATATAAATA TACACTTATT TACATTTCTA ATCTAATCTG AAGGTCCATT   -468

                                 AP-1
Human       GCAACGAATC CCGCTCCGCT CTGACCCAAG AACTCCGCAC TTTCTCTCCA CCAGCCTTTC   -408
Human       AAATACCTGA ACTTCATGCC TTCAAGAGGT GGATTCCGGC TTCTT-TACA --GCGCAATC   -351
Mouse                                      GCAAATTGGC AACTGGTGCG TTGAGCAATC   -310

                AP-1                                        AP-1
Human       ACTAGCAGGT CATTTCCGTC TAGCTTGCAG ATTTCCCATT A---CTGACA CAAACGCTCC   -294
Mouse       CTCCACCG-T AATTTCCCTC TGGCTAGCCC ACTTCCCATC ATCACTGACA CACATGCACT   -251

              Sp1
Human       CTCAGGGCGT CCCCGTCTGG ACTACAGGAG AAAGTTTGGG AGGCAGGTTC AACTTTTCAA   -234
Mouse       CCACAGGCAT CACC-CCTTG GCTGCAAGCA AAACGTTGGG AATCAGGCTC AACTTCTCAA   -192

             NF1     NF-kB                                  egr-1   Sp1
Human       CTTGGCGGGG AATTCCTCTC CCTCTTACAC AGTTTGCAGC GCGGGGGCGG CGGGGTGA-C   -175
Mouse       -TTCCCAGGA AATTCCTCT- --TGGAACAC A--CTG-AAC GCGGGGGCG- --GGATGAGC   -142

                        AP-1                      egr-1   Sp1
Human       AGTCCCCGTG -CATGAATCA GCCACCCCTC AGGCTCCGCC CCGGCGGGGG TCGGCCGGAC   -116
Mouse       TCTGCCTAAG GCGTGAATCA GCCACGTTAT GCGCTCCGCC CCGTCGGGGG TCCAGGGGTG    -82

Human       GCTCGCCCCG CATAAAGCGG GCACCCGGGC CGCCTGGAGC AGAAAGCCGC GCAC-CTCCT    -57
Mouse       GCTTA----- ---AAGGCTG ---------- CTCTTCTAGC AGGCGACTA- GCACTCTCCT    -41

Human       CCCGCCAGGC GCTTTCTCGG ACGCCTTGCC CAGCGGGCCG CCCGACC-CC CTGCACCATG     +3
Mouse       CCCTCCAGGC GT-------- ---------C CAGAGAACCA CAGCACCACC CAACACTATG     +3
```

Fig. 4. Nucleotide sequence of the 5′-flanking regions of human and murine TFPI-2 genes. A 5′-flanking sequence (2.3 kb) of human TFPI-2 gene is aligned with 342 bp of corresponding murine sequence (Genbank AF180353). A total of 4.8 kb of human 5′-flanking sequence was sequenced and has been deposited in the Genbank datase under number AY044097. The three last nucleotides (ATG) indicate the translation initiation site and gray boxes indicate putative binding sites for transcription factors.

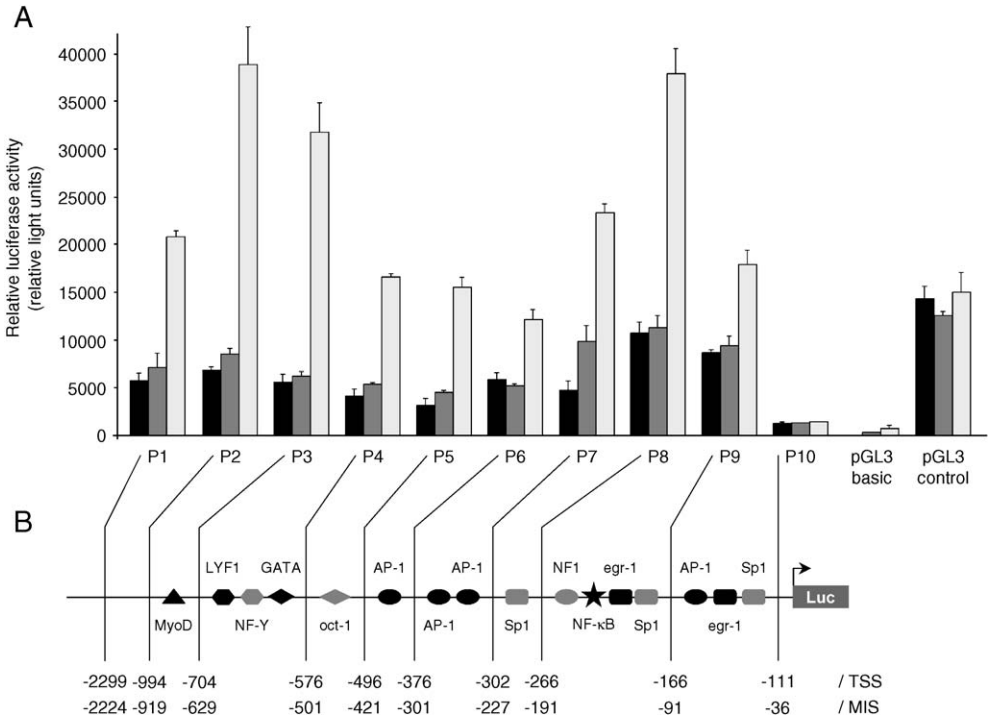

Fig. 5. Functional analysis of the human TFPI-2 promoter. (A) Relative luciferase activity of each promoter construct (P1 to P10) in unstimulated JEG-3 cells (■) and in cells incubated with 20 ng/ml TNF-α (▩) or with 100 ng/ml PMA (□). The luciferase activity of each reporter construct was normalized to the β-galactosidase activity. "pGL3 basic" and "pGL3 control" were used as negative and positive controls, respectively. The data are the mean ± S.E.M. of at least three independent transfections. (B) Schematic representation of TFPI-2 promoter and consensus sequences for binding transcription factors as determined by TESS 1.2.2 and MatInspector v2.2 programs, both based on the database Transfact 4.0. The numbers indicate nucleotide positions relative either to the translation start site (TSS) (higher numbers) or to the major transcription initiation site (MIS) (lower numbers) located 75 bp upstream of the TSS.

3.2. Analysis of the TFPI-2 promoter sequence

The nucleotide sequence of the 4.8-kb DNA region flanking the human TFPI-2 gene was cloned (Fig. 2) and sequenced using a method for walking upstream in genomic DNA. We defined an additional 1200-bp region compared to the previously reported TFPI-2 sequence [14] and the total sequence has been deposited in the Genbank Nucleotide Sequence Database under accession number AY044097. Six bases were different at positions −3087 and −2488 (T → C), −2380 and −2080 (C → T), −1271 (G → C) in our sequence, and there was no T between −2943 and −2942 upstream from the translation start site (TSS) (results obtained reproducibly). An 81-nucleotide region present as a single copy in our sequence between −1310 and −1230 was duplicated in the promoter described by Kamei et al. [14].

Sequence analysis revealed GC-rich content in the 1000-bp region upstream of the TSS, with 77% of GC in a region of 200 bp and a complete CpG island region spanning exon 1 with an observed/expected presence of CpG>0.85. We also showed that the TFPI-2 human promoter region contained neither a canonical TATA box nor a CAAT box.

To localize the transcription initiation sites in the 5′-flanking region of the human TFPI-2 gene precisely, we used a RACE reaction allowing amplification of only full-length mRNA with a CAP structure. Total mRNA (300 ng) was isolated from 1.5×10^6 JEG-3 cells. RACE products were obtained as one major band of 149 bp and two faint bands of 123 and 112 bp, respectively (Fig. 3).

Using TESS 1.2.2 and MatInspector v2.2 programs based on TRANSFAC database 4.0 [19], several putative binding sites for transcription factors were identified in the human TFPI-2 promoter, i.e. MyoD, LYF1, NF-Y, GATA, oct-1, AP-1, Sp1, NF1, NF-κB and egr-1 (Fig. 4). We also compared the human TFPI-2 promoter region to the murine sequence already published [13] and several potential binding sites for transcription factors, NF-κB (−226/−218), two overlapping egr-1/Sp1 (−193/−179 and −140/−126) and two AP-1 (−308/−302 and −162/−156) binding sites appeared in both sequences.

3.3. Analysis of basal and inducible TFPI-2 promoter activity in transfected JEG-3 cells

The human TFPI-2/luciferase promoter constructs were transiently transfected in unstimulated JEG-3 trophoblast cells, and luciferase activity of each promoter deletion construct normalized with βGal values was compared to those of pGL3 control (Fig. 5).

The basal activity of the largest promoter constructs (P1, P2, P3) was approximately one-third to one-half of those of

the pGL3 control. On the other hand, the highest luciferase activity measured without any stimulation was obtained in JEG-3 cells transfected with P8 and P9 constructs. In contrast, this basal promoter activity was completely abolished after transfection of the P10 construct.

Expression levels of all promoter constructs were not significantly modified when choriocarcinoma JEG-3 cells were incubated with TNF-α, except for cells with the P7 construct in which the luciferase activity increased by about 2-fold compared to the basal activity.

The luciferase activity of each promoter construct transfected into JEG-3 cells was always enhanced after PMA stimulation. The highest effects were recorded with P2 and P8. On the other hand, the luciferase activity of cells with P9 was strongly decreased compared to those obtained with the P8 construct. NF1, NF-κB and egr-1/Sp1 binding sites were identified in the sequence which was deleted in P9. The lowest promoter activities after stimulation by PMA were measured with JEG-3 cells transfected with P4, P5 and P6 constructs. One oct-1 and three AP-1 binding sites were present between positions −576 and −302 from the TSS.

4. Discussion

TFPI-2, a 32-kDa member of the Kunitz-type serine proteinase inhibitor family, is synthesised by many different cells, but is particularly abundant in the placenta [9,16,20]. It might therefore play a role in the regulation of hemostasis, and particularly of fibrinolysis within placenta, but it also could regulate trophoblast invasion and differentiation. Until recently, little was known about the regulation of the human TFPI-2 gene. In the present study, and in agreement with previous results [16], we showed that PMA strongly increased TFPI-2 mRNA synthesis in JEG-3 choriocarcinoma cells. This cell line was therefore considered of interest to study the transcriptional regulation of TFPI-2 and to define the minimal promoter sufficient to transcription. We cloned and sequenced the 4.8 kb DNA region flanking the human TFPI-2 gene.

We defined an additional 1200-bp region compared to the previously reported TFPI-2 sequence [14]. Morever, six bases were not identical and, more importantly, an 81-nucleotide region present as a single copy in our sequence between −1310 and −1230 was duplicated in the promoter described by Kamei et al. [14]. No canonical TATA box or CAAT box was found immediately upstream from the transcription start site as previously reported. Moreover, sequence analysis revealed GC-rich content in the 1000-bp region upstream from the TSS, with 77% of GC in a region of 200 bp and a complete CpG island region spanning exon 1 with an observed/expected presence of CpG>0.85. GC-rich sequences have often been reported in other promoters lacking TATA or CAAT boxes in the 5′-flanking region [18]. Interestingly, no GC-rich region was found in the murine TFPI-2 gene while the presence of TATA and CAAT boxes has been demonstrated [13]. The regulation of the TFPI-2 gene might therefore be different from one species from another. However, despite relatively low sequence similarity between murine and human TFPI-2 5′-flanking regions (51%), several potential binding sites for transcription factors, NF-κB (−226/−218), two overlapping egr-1/Sp1 (−193/−179 and −140/−126) and two AP-1 (−308/−302 and −162/−156) binding sites, were highly conserved. This indicates that these sequences probably play an important role in regulating TFPI-2 gene transcription both in the human and the mouse.

We then localized precisely the transcription initiation sites in the 5′-flanking region of the human TFPI-2 gene and, in agreement with Kamei et al. [14], sequencing of the major band of 149 bp confirmed that the adenosine located at position −75 bp upstream of the TSS is the major transcription initiation site (MIS). Moreover, the two additional faint bands allowed identification of two new minor transcription initiation sites located at −49 and −38. The site identified at −38 probably corresponds to the 5′-end of TFPI-2 cDNA previously cloned by Sprecher et al. [17]. Moreover, we reproduced the above results obtained with JEG-3 cells with another cell line, i.e. transformed bone marrow microvascular endothelial cells (TrBMEC).

The presence of several transcription initiation sites in this promoter, in which there is no consensus TATA box sequence, might be therefore critical for the regulation of the TFPI-2 gene that can be classified as a housekeeping gene [21]. This latter hypothesis is also supported by the high GC content (approximately 75%) in the TFPI-2 gene promoter, with the presence of typical GC boxes known as binding sites for the transcription factor Sp1 [22]. Nevertheless, a non-consensus TATA box located 30 bp upstream from the −75 bp major initiation site (CATAAA, −106/−101 from the TSS) could function in concert with binding sites for Sp1 to regulate TFPI-2 gene transcription.

Human TFPI-2/luciferase promoter constructs were then transiently transfected in unstimulated JEG-3 trophoblast cells in order to identify potential regulating regions. The basal activity of the largest promoter constructs (P1, P2, P3) was strong, and in agreement with the fact that TFPI-2 is normally expressed in JEG-3 cells [16]. On the other hand, we showed that the minimal TFPI-2 promoter in JEG-3 cells is located between −166 and −111 from the TSS. In a recent study conducted with glioma cells, Konduri et al. [23] showed a minimal promoter in a larger 5′-region, i.e. located between −312 and −81 from the TSS. Kamei et al. [14] had reviously identified a 85-bp region between −289 and −214 from the TSS (i.e., −224 to −139 from the MIS) sufficient for TFPI-2 gene transcription in TrBMEC. In comparison, the minimal promoter that we have defined is closer to the major transcription initiation site located at −75 bp. It includes one AP-1 (−162/−156) and one overlapping egr-1/Sp1 (−140/−126) binding site known

to play an activating role in promoters lacking TATA boxes [18]. The maximal basal luciferase activity recorded with the P8 construct could be attributed to the presence of binding sites for NF1 (−233/−228), known to facilitate assembly of the basal transcription complex [24], and for Sp1 (−185/−179), previously identified as mediating constitutive transcriptional activation in GC-rich regions of several other genes [18,25].

Levels of expression of promoter constructs were not significantly modified when JEG-3 cells were incubated with TNF-α. Nevertheless, we identified a potential NF-κB binding site, which is highly conserved in the murine sequence. TNF-α is a recognized NF-κB-dependent gene inducer, but its effect upon the TFPI-2 promoter in JEG-3 cells could be suppressed by negative regulatory elements present in the gene upstream from −302 since a slight increase in luciferase activity was observed with P7 to P9. Surprisingly, luciferase activity remains unchanged in P9 lacking NF-κB binding site suggesting the existence of at least one another positive regulatory element.

The luciferase activity of each promoter construct transfected into JEG-3 cells was always enhanced after PMA stimulation, the highest effects being recorded with P2 and P8. These results are in accordance with those of a previous study during which we showed a 7.8-fold increase in TFPI-2 mRNA synthesis in JEG-3 cells stimulated by PMA [16]. In addition, after deletion of the −266/−166 region, which contains NF1, NF-κB and egr-1/Sp1 binding sites, the luciferase activity of cells with P9 was strongly decreased compared to the P8 construct. Stimulation of several PMA-induced genes has been shown to be associated with a rapid increase in immediate-early egr-1 gene expression [26–28]. In addition, PMA-induced egr-1 has been reported to displace Sp1 from the overlapping recognition element, and thus to increase gene expression [26,29]. Interestingly, the two overlapping egr-1/Sp1 binding sites (−193/−179 and −140/−126) in the human TFPI-2 gene were also present in the murine promoter sequence, suggesting that they play an important role in the control of basal and inducible gene expression. On the other hand, the lowest promoter activities were measured with P4, P5 and P6, which probably contains negative regulatory elements between −576 and −302 from the TSS. Indeed, three AP-1 binding sites were identified and could thus be important in the negative control of TFPI-2 gene expression. In contrast, AP-1 elements have been shown to enhance the promoter expression of several other genes including MMP-9 [30,31]. This suggests that regulation of MMP-9 and TFPI-2 genes might result from opposite effects of AP-1. As TFPI-2 and MMP-9 exert inverse consequences in cell migration and tumor invasion [4,5], further studies allowing better definition of the mechanisms that regulate the transcription of these two genes are essential.

In conclusion, this study demonstrates that the TFPI-2 gene promoter exhibits typical features of a housekeeping gene. The TFPI-2 promoter contains NF1, NF-κB and egr-1/Sp1 binding sites which appear critical for TFPI-2 gene upregulation. Moreover, negative regulatory elements such as AP-1 binding sites are also present in the promoter and might in certain circumstances such as cancer contribute to downregulation of TFPI-2 synthesis.

Acknowledgements

The authors thank Doreen Raine for editing the English text. We particularly thank Antoine Touzé (EMI-U0010, Tours) for useful suggestions. This study was supported by the Institut pour la Recherche sur la Thrombose et l'Hémostase in Tours, France.

References

[1] Petersen LC, Sprecher CA, Foster DC, Blumberg H, Hamamoto T, Kisiel W. Biochemistry 1996;35:266–72.
[2] Rao CN, Mohanam S, Puppala A, Rao JS. Biochem Biophys Res Commun 1999;255:94–8.
[3] Herman MP, Sukhova GK, Kisiel W, Foster D, Kehry MR, Libby P, et al. J Clin Invest 2001;107:1117–26.
[4] Konduri SD, Rao CN, Chandrasekar N, Tasiou A, Mohanam S, Kin Y, et al. Oncogene 2001;20:6938–45.
[5] Rao CN, Lakka SS, Kin Y, Konduri SD, Fuller GN, Mohanam S, et al. Clin Cancer Res 2001;7:570–6.
[6] Crawley JT, Goulding DA, Ferreira V, Severs NJ, Lupu F. Arterioscler Thromb Vasc Biol 2002;22:218–24.
[7] Iino M, Foster DC, Kisiel W. Arterioscler Thromb Vasc Biol 1998; 18:40–6.
[8] Udagawa K, Miyagi Y, Hirahara F, Miyagi E, Nagashima Y, Minaguchi H, et al. Placenta 1998;19:217–23.
[9] Udagawa K, Yasumitsu H, Esaki M, Sawada H, Nagashima Y, Aoki I, et al. Placenta 2002;23:145–53.
[10] Rao CN, Cook B, Liu Y, Chilukuri K, Stack MS, Foster DC, et al. Int J Cancer 1998;76:749–56.
[11] Konduri SD, Tasiou A, Chandrasekar N, Nicolson GL, Rao JS. Clin Exp Metastasis 2000;18:303–8.
[12] Jin M, Udagawa K, Miyagi E, Nakazawa T, Hirahara F, Yasumitsu H, et al. Gynecol Oncol 2001;83:325–33.
[13] Kazama Y, Kamei S, Kuijper JL, Foster DC, Kisiel W. Thromb Haemost 2000;83:141–7.
[14] Kamei S, Kazama Y, Kuijper JL, Foster DC, Kisiel W. Biochim Biophys Acta 2001;1517:430–5.
[15] Miyagi Y, Yasumitsu H, Eki T, Miyata S, Kkawa N, Hirahara F, et al. Genomics 1996;35:267–8.
[16] Iochmann S, Reverdiau-Moalic P, Hubé F, Bardos P, Gruel Y. Thromb Res 2002;105:1–7.
[17] Sprecher CA, Kisiel W, Mathewes S, Foster DC. Proc Natl Acad Sci U S A 1994;91:3353–7.
[18] Smale ST. Biochim Biophys Acta 1997;1351:73–88.
[19] Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, et al. Nucleic Acids Res 2000;28:316–9.
[20] Hubé F, Reverdiau P, Iochmann S, Trassard S, Thibault G, Gruel Y. Biol Reprod 2003 (in press).
[21] Kang M, Matsudo Y, Sasagawa K, Tokuhisa T, Hatano M. Biochim Biophys Acta 2001;1519:167–74.
[22] Boyer TG, Maquat LE. J Biol Chem 1990;265:20524–32.
[23] Konduri SD, Osman FA, Rao CN, Srinivas H, Yanamandra N, Tasiou A, et al. Oncogene 2002;21:921–8.
[24] Dusserre Y, Mermod N. Mol Cell Biol 1992;12:5228–37.

[25] Petersohn D, Thiel G. Eur J Biochem 1996;239:827–34.
[26] Khachigian LM, Williams AJ, Collins T. J Biol Chem 1995;270: 27679–86.
[27] Minc E, de Coppet P, Masson P, Thiery L, Dutertre S, Amor-Gueret M, et al. J Biol Chem 1999;274:503–9.
[28] Akuzawa N, Kurabayashi M, Ohyama Y, Arai M, Nagai R. Arterioscler Thromb Vasc Biol 2000;20:377–84.
[29] Silverman ES, Khachigian LM, Lindner V, Williams AJ, Collins T. Am J Physiol 1997;273:H1415–26.
[30] Liu WH, Chen XM, Fu B. Acta Pharmacol Sin 2000;21:641–5.
[31] Simon C, Simon M, Vucelic G, Hicks MJ, Plinkert PK, Koitschev A, et al. Exp Cell Res 2001;271:344–55.