Characterization of the Gene Encoding Human TAFI (Thrombin-Activable Fibrinolysis Inhibitor; Plasma Procarboxypeptidase B)†

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ABSTRACT: Thrombin-activable fibrinolysis inhibitor (TAFI) is a recently described human plasma zymogen that is related to pancreatic carboxypeptidase B. The active form of TAFI (TAFIa), which is formed by thrombin cleavage of the zymogen, likely inhibits fibrinolysis by removal from partially degraded fibrin of the carboxyl-terminal lysine residues which act to stimulate plasminogen activation. We have isolated and characterized genomic clones which encompass the entire human TAFI gene from λ phage and bacterial artificial chromosome genomic libraries. The complete TAFI gene contains 11 exons and spans approximately 48 kb of genomic DNA. The positions of intron/exon boundaries are conserved between the TAFI gene and the rat pancreatic carboxypeptidase A1, A2, and B and the human mast cell carboxypeptidase A genes, indicating that these carboxypeptidases arose from a common ancestral gene. However, the intron lengths diverge significantly among all of these genes. The TAFI promoter lacks a consensus TATA sequence, and transcription is initiated from multiple sites. Transient transfection of reporter plasmids containing portions of the TAFI 5′-flanking region into mammalian cells allowed localization of the promoter and identified a ~70 bp region crucial for liver-specific transcription. Sequence analysis of cDNA clones obtained from human liver RNA indicated that the TAFI transcript is polyadenylated at three different sites. Our findings will facilitate the assessment of the regulation of TAFI expression by transcriptional and/or posttranscriptional mechanisms. Furthermore, knowledge of the genomic structure of the TAFI gene will aid in the identification of mutations that may be associated with the tendency to either bleed or thrombose.

The coagulation and fibrinolytic cascades are defined by series of zymogen to enzyme conversions which lead to the generation of the enzymes thrombin and plasmin, respectively. Thrombin catalyzes the conversion of fibrinogen to fibrin monomers, which then spontaneously polymerize to form the insoluble fibrin matrix that is the essential structural component of blood clots. Plasmin, on the other hand, is responsible for the degradation of fibrin that ultimately leads to clot dissolution.

Inherent to both cascades are internal feedback loops which serve to amplify or to dampen the activity of the cascades. The coagulation cascade is generally thought to be initiated through the extrinsic pathway by the activity of Factor VIIa bound to tissue factor; the relatively small amount of thrombin formed through activation of prothrombin by Factor Xa generated by this pathway is then capable of activating components of the intrinsic pathway resulting in an accelerated rate of thrombin formation. The endothelial cell transmembrane protein thrombomodulin, however, changes the specificity of thrombin from fibrinogen to protein C. Thrombin in complex with thrombomodulin generates activated protein C, an anticoagulant enzyme that specifically inactivates Factors VIIIa and Va, two essential cofactors in the intrinsic pathway, thereby attenuating further thrombin formation.

In the fibrinolytic cascade, fibrin is not only the terminal substrate of the cascade but also an essential cofactor for tissue-type plasminogen activator (tPA)-mediated[1] plasminogen activation. Plasminogen, tPA, and fibrin form a ternary complex necessary for efficient plasminogen activation (*1*); carboxyl-terminal lysines exposed in fibrin via limited proteolysis by plasmin convert fibrin into a more effective cofactor for plasminogen activation (*2*), protect plasmin from consumption by its major inhibitor α_2-antiplasmin (*3*), and promote the plasmin-mediated conversion of Glu-plasminogen to Lys-plasminogen (*2*), the latter of which is a better substrate for tPA (*1*).

Recent studies from several laboratories have delineated a novel pathway through which the thrombin−thrombomodulin complex acts and which represents an explicit molecular link between the activities of the coagulation and

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[1] Abbreviations: tPA, tissue-type plasminogen activator; TAFI, thrombin activable fibrinolysis inhibitor; TAFIa, activated TAFI; pfu, plaque-forming units; bp, base pairs; kb, kilobase pairs; AMV, avian myeloblastosis virus; CPB, carboxypeptidase B; CPU, carboxypeptidase U; CPA1/CPA2, carboxypeptidase A1/A2; MC-CPA, mast cell carboxypeptidase A; BAC, bacterial artificial chromosome; RT-PCR, reverse transcriptase-polymerase chain reaction.

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fibrinolytic cascades. Bajzar and co-workers (*4*) purified a zymogen from human plasma which was termed TAFI (thrombin-activable fibrinolysis inhibitor) on the basis of its ability to inhibit fibrinolysis in vitro when sustained thrombin generation occurs within the clot. Cleavage of the zymogen by high concentrations of thrombin leads to the formation of an enzyme (TAFIa) which displays basic carboxypeptidase activity and was therefore postulated to attenuate fibrinolysis by removal from partially degraded fibrin of the carboxyl-terminal lysine residues that are required for maximally efficient plasminogen activation. Further studies by this group (*5*) revealed that the thrombin−thrombomodulin complex activates TAFI with a 1250-fold higher catalytic efficiency than thrombin alone, suggesting that the former is the physiological activator of TAFI.

The entity that has been termed TAFI has been independently described by other groups. Partial sequence analysis of the purified TAFI protein showed that it was identical to a zymogen previously characterized by Eaton and co-workers (*6*), which was termed plasma procarboxypeptidase B. Sequence analysis of a cloned cDNA corresponding to this protein revealed that plasma procarboxypeptidase B is related (sharing approximately 40% amino acid identity) to procarboxypeptidase B, the zymogen form of a digestive enzyme secreted by the pancreas. Significantly, all of the residues in pancreatic carboxypeptidase B which have been shown to be required for substrate recognition and binding and catalysis are conserved in plasma procarboxypeptidase B (i.e., TAFI) (*6*).

Hendriks and co-workers (*7*) detected an unstable basic carboxypeptidase activity in human serum that was not present in plasma; these workers subsequently purified the enzyme responsible for this activity and termed it carboxypeptidase U (CPU) (*8*) since it is an "unstable" carboxypeptidase. It is likely that proCPU and TAFI are the same protein, since both proCPU and TAFI bind to plasminogen (*4*, *8*, *9*) and TAFIa activity is also intrinsically unstable with a half-life of about 10 min at 37 °C (*10*).

There is currently little data concerning the role of TAFI in hemostasis in vivo. A recent study in a canine model of thrombolysis revealed that an inducible carboxypeptidase activity (presumably TAFIa) is formed as a consequence of thrombosis (*11*). Inhibition of this activity by potato carboxypeptidase inhibitor (PCI) during thrombolytic therapy with tPA decreased the time required for reperfusion in this model. Additionally, a positive correlation was demonstrated between the amount of inducible carboxypeptidase activity formed and the time required for reperfusion. Studies in a rabbit jugular vein model of thrombolysis (*12*) revealed that inhibition of either Factor XI activation or TAFIa activity increased endogenous fibrinolysis; inhibition of Factor XI activation and TAFIa activity simultaneously did not further increase endogenous fibrinolysis. These results were taken as evidence for a novel role of the intrinsic pathway of coagulation in which fibrinolysis is attenuated in a manner dependent on activation of TAFI. This concept is supported by the findings of Broze and Higuchi (*13*) who found that the premature lysis of clots formed from hemophilic plasma in vitro could be wholly ascribed to the failure of the intrinsic pathway to generate sufficient thrombin to activate TAFI. Hemophilia, therefore, may be attributable not to the inability to form a clot per se, but rather to the inability to generate sufficient thrombin to stabilize the clot via activation of TAFI.

A recent study suggests that variation exists in the plasma TAFI antigen levels in the population (*14*). This variation was shown to affect fibrinolytic potential in the population examined, which is in accordance with the fact that the concentration of TAFI in plasma (~70 nM) (*15*) is far below the K_m for TAFI activation by thrombin or thrombin/thrombomodulin (*5*). The reasons for the variation in plasma levels of TAFI are unknown, however. Furthermore, no data exist pertaining to the potential ability of TAFI levels to be regulated by hormonal or other physiological or pathological stimuli. The gene encoding human TAFI has been mapped to 13q14.11 (*16*). No disorders of hemostasis have been mapped to this chromosomal region to date, however. At least two polymorphisms in the TAFI coding region which result in amino acid substitutions in the TAFI protein exist,[2] although it is not known whether these polymorphisms are associated with a tendency to bleed or thrombose or whether they affect the plasma levels or potential activity of TAFI. In the present study, we have cloned the gene encoding human TAFI and characterized it with respect to its genomic organization, sequences of the 5′- and 3′-flanking regions, location of the start sites for gene transcription, analysis of the 5′-flanking region for liver-specific promoter activity, and identification of the sites for polyadenylation of the transcript.

EXPERIMENTAL PROCEDURES

Screening of a Human Genomic DNA Library. A human genomic library in the λFIXII vector was purchased from Stratagene. Approximately 10^6 plaque-forming units (pfu) were plated on the *Escerichia coli* strain XL-1 Blue (Stratagene) (approximately 50 000 pfu/15 cm Petri dish) and duplicate plaque lifts were obtained using Hybond N+ nylon filters (Amersham). The filters were incubated for 24 h at 65 °C in prehybridization solution [6× SSC (1× SSC is 0.015 M sodium citrate pH 7.0, 0.15 M NaCl), 2× Denhardt's solution (1× Denhardt's is 0.5 mg/mL Ficoll, 0.5 mg/mL poly(vinylpyrrolidone), 5 mg/mL bovine serum albumin), 10 μg/mL sheared, denatured salmon sperm DNA]. The filters were then hybridized with a ^{32}P-labeled probe consisting of nucleotides 10−1355 of the TAFI cDNA (*6*) (encompasses the entire open reading frame) for 24 h at 65 °C in prehybridization solution containing 0.5% SDS. The probe was prepared using 20 ng of DNA and was labeled with [α-^{32}P]dATP (10 mCi/mL; Dupont/NEN) and the Prime-It II random primer labeling kit (Stratagene). Following hybridization, the filters were rinsed for 1 h at room temperature in 1X SSC, and then washed in 1× SSC, 0.5% SDS for 2 h (with three changes of solution) at 65 °C. The filters were then exposed to X-ray film (Kodak X-OMAT AR) for ~48 h at −70 °C with two intensifying screens. Eleven positive clones were obtained in duplicate and were plaque-purified by two further rounds of screening performed as described above.

Characterization of λ Phage Clones. Phage DNA was prepared from the plaque-pure clones using the Wizard kit (Promega). The starting material for these preparations was

[2] Boffa, M. B., Koschinsky, M. L., and Nesheim, M. E., unpublished observations.

plate lysates from two 15 cm Petri dishes each initially seeded with 25 000 pfu/plate of phage stock. Purified phage DNA was digested with *Sac*I and subjected to agarose gel electrophoresis in order to characterize the genomic DNA inserts. This analysis revealed that there were five unique clones represented in the 11 clones obtained; one representative of each unique clone was chosen for subsequent analysis. *Sac*I fragments from each phage clone were inserted into the *Sac*I site of the plasmid pBluescript II SK+ (Stratagene); these fragments were then subjected to Southern blot analysis and a progressive subcloning strategy in order to localize the exons and to construct a restriction map of the clones. DNA sequence analysis of subclones was accomplished using primers hybridizing either within the pBluescript II SK+ plasmid or within the TAFI cDNA sequence; sequencing reactions were performed using [α-^{35}S]dATP (12.5 mCi/mL, Dupont/NEN) and the Sequenase kit (Amersham). The sizes of individual fragments were determined by agarose gel electrophoresis using standard curves relating the distance of migration of fragments obtained from a *Hin*dIII digest of λ DNA to the logarithm of their length.

Isolation and Characterization of Bacterial Artificial Chromosome Clones. A human bacterial artificial chromosome (BAC) library in pBACe3.6 (the RPCI BAC library of Dr. Pieter de Jonge, Roswell Park, Buffalo, NY) was screened using ^{32}P-labeled probes consisting of either the cDNA fragment encompassing the TAFI open reading frame (see above) or an approximately 2.5 kilobase pair (kb) genomic fragment representing the 5′-most fragment obtained from the phage clones. Seven BAC clones were obtained (30F20, 55K4, 106H11, 139H14, 179M2, 213H14, and 234M21). DNA representing each clone was obtained and was digested either with *Eco*RI or *Sac*I and subjected to agarose gel electrophoresis followed by Southern blot analysis using ^{32}P-labeled probes representing either the 5′-most 100 base pairs (bp) of the TAFI cDNA or the 5′-most genomic fragment isolated from the phage clones. Hybridizing fragments were subsequently isolated and inserted into the pBluescript II SK+ plasmid and were analyzed using the methods and strategies described above. Two of the BAC fragments encompassed the 5′-flanking region of the TAFI gene. The DNA sequence of this region was determined by a progressive subcloning strategy; primers used either hybridized within the pBluescript II SK+ plasmid or were designed based on the existing genomic sequence.

Preparation of Human Liver RNA. A human liver sample was obtained (with institutional approval) from a patient undergoing liver resection and was snap-frozen in liquid nitrogen; the tissue was stored at −70 °C prior to use. Two grams of frozen liver was homogenized in the presence of guanidinium isothiocyanate (*17*) and total RNA was isolated from the homogenate by lithium chloride extraction (*18*). Poly(A)$^+$ RNA was subsequently isolated by affinity chromatography of the total RNA over oligo(T)-cellulose (Sigma) (*19*). This procedure yielded approximately 100 μg of poly(A)$^+$ RNA, which was stored at −70 °C.

Primer Extension Analysis. Antisense oligonucleotides corresponding to the 3′ end of exon 1 of the gene were designed based on the TAFI cDNA sequence (*6*). The oligonucleotide sequences are as follows: TAFI-PE1 (nucleotides 70−91), 5′-CTCTGAAACGCGAAGACATGC-3′; TAFI-PE2 (nucleotides 57−91), 5′-CTCTGAAACGCGAAGACATGCTGCTCACAGAAGAG-3′. The oligonucleotides were end-labeled using [γ-^{32}P]ATP (10 mCi/mL; Dupont/NEN) and T4 polynucleotide kinase (Promega). Reactions were performed essentially as described previously (*20*) using 20 μg of poly(A)$^+$ liver RNA and ∼2.5 × 10^6 cpm of either end-labeled oligonucleotide. Annealing of the respective oligonucleotides to the RNA was carried out at 25 °C (TAFI-PE1) or 30 °C (TAFI-PE2) overnight. Reactions were electrophoresed on a 6% polyacrylamide sequencing gel containing 7 M urea. As markers for the sizes of the primer-extended products, a dideoxy-sequencing reaction using TAFI-PE1 as the primer and a cloned genomic fragment encompassing the 5′-flanking region of the TAFI gene as the template was performed; the sequencing reaction was electrophoresed alongside the products of the primer extension reaction.

Reverse Transcription-Polymerase Chain Reaction of Human Liver RNA. Poly (A)$^+$ human liver RNA (2 μg) was heated to 90 °C for 5 min and then placed on ice in order to disrupt secondary structure in the RNA. To the RNA was added AMV reverse transcriptase buffer (to 1×), each dNTP (to 1 mM), 40 U RNAguard, $(dT)_{18}$ (20 pmol) as the primer for cDNA synthesis, and 10 units of AMV reverse transcriptase in a final volume of 20 μL. Reactions were incubated for 90 min at 42 °C, at which time the reactions were diluted to 150 mL with water. cDNA was stored at −20 °C prior to use. To isolate cDNA species encompassing the 3′-untranslated region of the TAFI mRNA transcript, the human liver cDNA was used as the template for the polymerase chain reaction (PCR) which was performed using *Pyrococcus furiosus* (*Pfu*) polymerase. The 5′ primer is a sense strand primer, designed based on the TAFI cDNA sequence, that spans nucleotides 1263−1280. The sequence of this primer is 5′-GCTTGGCATGTCATTAGG-3′. The sequence of the 3′ primer is 5′-$(T)_{17}(N)_4$-3′, where N represents any of the four deoxynucleotides. This primer would be expected to hybridize within the poly(A) tail of the template, but because of the presence of the four random nucleotides at the 3′ end, the primer would be expected to be "anchored" at the junction between the poly(A) tail and the 3′-untranslated region, thus improving the specificity of the amplification. The amplification reactions contained 1× *Pfu* polymerase buffer, 0.25 mM each dNTP, 50 pmol of the 5′ primer, 100 pmol of the 3′ primer, and 1/5 vol of cDNA. Reaction conditions were as follows: 5 min at 94 °C, at which time 2.5 units of *Pfu* polymerase was added, 40 cycles of 20 s at 94 °C, 20 s at 45 °C, 20 s at 75 °C, and a final 15 min at 75 °C. PCR products were inserted into the *Eco*RV site of pBluescript II SK+ for DNA sequence analysis.

Reporter Plasmids. Reporter plasmids containing genomic fragments encompassing the 5′-flanking region were constructed in the luciferase reporter vector pGL3 Basic (Promega). The 3′ boundary of all of the genomic fragments was the *Hin*dIII site in exon 1 that resides immediately 3′ to the initiator methionine codon; in all cases the initiator methionine codon was changed from ATG to TTG by PCR-mediated mutagenesis such that initiation of translation would only occur within the luciferase portion of the resultant fusion transcripts. Deletions of increasing portions of 5′-flanking sequence was accomplished using either convenient restriction sites or PCR.

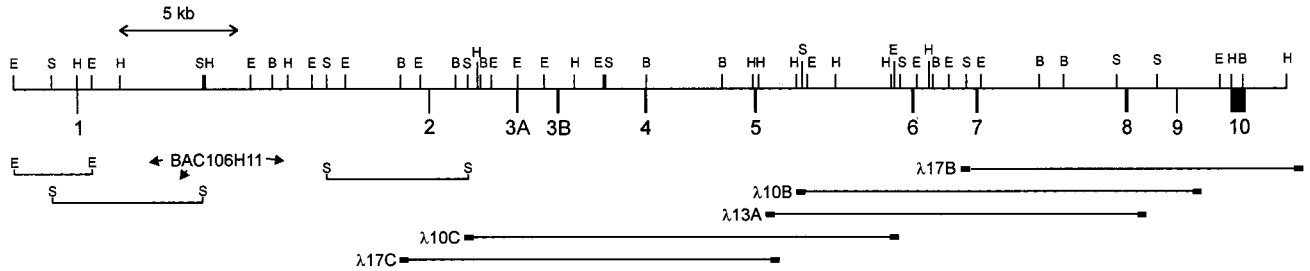

FIGURE 1: Restriction endonuclease map and genomic organization of the human TAFI gene. The map of the TAFI gene is presented to scale, with the positions of *Eco*RI (*E*), *Sac*I (*S*), *Hin*dIII (*H*), and *Bam*HI (*B*) restriction sites indicated and the lengths of the exons (numbered 1, 2, 3A, 3B, and 4−10) represented by the widths of the corresponding boxes. Shown below the map are the locations of the five overlapping λ phage genomic clones and the three genomic fragments isolated from the BAC clone 106H11. The rectangles on either end of each λ phage clone represent the phage arms.

For use in transient transfections (see below), large-scale preparations of all luciferase reporter plasmids were obtained using the Plasmid MAXI Kit (QIAGEN); the mass concentration of each plasmid was determined by measurement of absorbance at 260 nm (where an absorbance at 260 nm of 1.0 corresponds to a DNA concentration of 50 μg/mL). The approximate molecular mass of each plasmid was determined assuming an average molecular mass of 635 g/mol per base pair; the plasmid preparations were diluted such that equivalent molar concentrations of each plasmid were obtained.

Transient Transfection of Mammalian Cells. HepG2 (human hepatoma), 293 (human embryonic kidney), BHK (baby hamster kidney), and HeLa (human cervical carcinoma) cells were obtained from the American Type Culture Collection. Transient transfections were performed in 6-well plates (9.62 cm^2/well; Corning); cells were seeded in the wells approximately 18 h prior to transfection at a density of 1/7 (BHK, HeLa) or 1/5 (HepG2, 293) confluence. Transfections were performed by the method of calcium phosphate coprecipitation (*21*); each well received approximately 1 pmol of luciferase reporter plasmid. To control for transfection and harvesting efficiency, each well also received 0.5 μg of a plasmid containing the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) promoter (*22*). After a 6 h incubation with the precipitate (24 h for HeLa cells), the wells were washed three times with phosphate-buffered saline (PBS), fresh medium was added, and the cells were incubated for a further 42 h prior to harvesting of the cells for preparation of cytoplasmic extracts. The methods used for preparation of the extracts and for assays of luciferase and β-galactosidase activity have been reported previously (*23*). For each sample, the relative luciferase activity was calculated to be the luciferase activity per unit β-galactosidase activity per unit volume of cell extract.

Computer Methods. Sequences of the TAFI gene and protein were aligned with the sequences of other carboxypeptidase genes and proteins (obtained from GenBank) using ClustalX (version 1.64b) software. Dendritic trees were assembled using Treeview (version 1.5.2) (*24*) software based on the alignment data. Searches for consensus sequences for transcription factor binding sites in the TAFI 5′-flanking region were performed with Matinspector (version 2.2) (*25*) software using the Transfac (version 3.4) database of matrixes corresponding to known transcription factor binding sites.

RESULTS

Isolation and Characterization of the Human TAFI Gene. One million recombinants of a human genomic library in λFIXII were screened with a cDNA probe that encompassed the entire open reading frame of the TAFI transcript. This screen yielded five unique overlapping phage clones which were then subjected to Southern blot analysis and a progressive subcloning strategy in pBluescript II SK+ in order to localize the exons and characterize the intron/exon boundaries (Figure 1).

Since the phage clones did not encompass the first exon or the 5′-flanking region of the TAFI gene, the genomic library was rescreened using a cDNA probe spanning only exon 1. This screen, however, failed to identify any additional phage clones. Therefore, bacterial artificial chromosome (BAC) clones were isolated from a human genomic BAC library using as a probe either the cDNA fragment encompassing the entire open reading frame of TAFI or a genomic DNA fragment representing the 5′-most boundary of the phage clones. This screen yielded seven unique BAC clones which were then subjected to Southern blot analysis in order to identify restriction fragments that contained exon 1 and which overlapped with the phage clones. Hybridizing fragments were inserted into pBluescript II SK+ for analysis. Three unique BAC fragments were isolated from a single BAC clone (106H11) using this strategy: a ~6.3 kilobase pair (kb) *Sac*I fragment was found to overlap with the 5′-most phage clone (λ17C), and overlapping ~3.5 kb *Eco*RI and ~6.8 kb *Sac*I fragments were found to contain exon 1 and the 5′-flanking region (Figure 1).

The information contained in the BAC fragments and the phage clones allowed for the construction of a partial restriction map of the TAFI gene and for the localization of the exons within the genomic structure (Figure 1). The TAFI gene consists of 11 exons which are distributed within approximately 48 kb of genomic sequence. The locations of *Sac*I, *Eco*RI, *Bam*HI, and *Hin*dIII restriction sites within the TAFI gene were also determined. Since the two *Sac*I BAC fragments are not contiguous, the length and restriction map of the intervening genomic sequence was inferred by Southern blot analysis of the BAC clones using probes derived from each of these fragments (Figure 1).

The DNA sequence of each exon was determined and was found to correspond exactly to the cDNA sequence for plasma procarboxypeptidase B (i.e., TAFI) reported by Eaton et al. (*6*), except for an A→G substitution that converts a

Table 1: Intron/Exon Boundaries in the Human TAFI Gene[a]

Exon	3' splice acceptor	5' splice donor	Exon length (rat CPB)	Codon phase (rat CPB)
1		91 ...TCAGAG**gt**aacccaatagaatcttag	74 bp[b] (65 bp)[b]	2° (2°)
2	92 tcttttatttttttcccc**ag**TGGCCA...	167 ...TATGAG**gt**aatttctccctaatttat	76 bp (76 bp)	0° (0°)
3A	168 ttttgccccttaaactgt**ag**ATTGTT...	292 ...ATGCAG**gt**aggcacccgttcaatacg	125 bp (125 bp)	2° (2°)
3B	293 ctctctgtttcatcacct**ag**TGTCTT...	401 ...AATGAA**gt**aagccatcacacagctct	109 bp (100 bp)	0° (0°)
4	402 tctggtttccattttgct**ag**ATCTAT...	503 ...TTAAAG**gt**atgttgtggggaaagttg	102 bp (102 bp)	0° (0°)
5	504 tttggggtttctttgagc**ag**GTTTCT...	608 ...GGCCAT**gt**aagtattcacattctctt	105 bp (102 bp)	0° (0°)
6	609 atacttggtttaatttgc**ag**ATAACT...	719 ...AAAAAG**gt**aggagaaaaggcaaagaa	105 bp (111 bp)	0° (0°)
7	720 tttgtattttgtttatgt**ag**AATCGA...	813 ...GGTGTG**gt**aggttgttggctttattt	94 bp (91 bp)	1° (1°)
8	814 ccttaacaatttcgttac**ag**AGGAAG...	1016 ...GAACTG**gt**aagtgctacttaattatt	203 bp (203 bp)	0° (0°)
9	1017 ctatatctttttgcttcc**ag**TCTCTA...	1104 ...CCTTAT**gt**aagtatttcttcttatga	88 bp (85 bp)	1° (1°)
10	1105 tttctttcttcttcttttt**ag**ACCTAG...		182 bp[b] (185 bp)[b]	

[a] Intron/exon boundaries were characterized by DNA sequence analysis of human genomic clones. The exon sequences are presented in upper case letters while the intron sequences are in lower case letters. Nucleotide numbering is based on ref *6*. The consensus splice acceptor and donor sequences are in boldface type. The length of each exon and the codon phase is given with those of the rat pancreatic carboxypeptidase gene (*25*) presented in brackets. [b] Refers to the number of nucleotides in the open reading frame.

threonine codon (ACA) to an alanine codon (GCA) at the position specifying residue 147 in the TAFI protein. Analysis of cDNA clones derived from human liver RNA by RT-PCR (data not shown) or of PCR products amplified from human genomic DNA (*26*) suggests that this is indeed a common polymorphism in the human population, present in almost all individuals examined. The sequences of the intron/exon boundaries are presented in Table 1. Interestingly, we found that the location and phasing of these boundaries correspond exactly to those which have been reported for the rat pancreatic carboxypeptidase A1, A2, and B genes (*27*, *28*) and the human mast cell carboxypeptidase A gene (*29*) (Table 1 and Figure 2). The only exception to this rule is that in the rat carboxypeptidase A1 gene, exons 3A and 3B are fused into a single exon (*27*) (Figure 2). Despite the conservation of the locations of the exons in all of these carboxypeptidases, the lengths of the introns are widely divergent (Figure 2). Additionally, there are small differences in the lengths of certain exons between the TAFI gene and the rat carboxypeptidase B gene (Table 1).

Identification of the Transcription Start Site of the TAFI Gene. Primer extension analysis of human liver poly(A)$^+$ RNA was performed in order to identify the start site(s) of TAFI gene transcription. The primer used was an end-labeled 21mer antisense oligonucleotide specific for the 3′ end of exon 1 (TAFI-PE1; see the Experimental Procedures). A DNA sequencing ladder prepared using the same oligonucleotide as the primer and using a cloned fragment of the TAFI gene as the template was electrophoresed on the same gel to identify specific nucleotides corresponding to the transcription start site(s). The results (Figure 3) revealed multiple sites for the initiation of TAFI gene transcription. Nine of these sites can be considered to be major transcription start sites (indicated by the larger arrows and the boxed nucleotides in Figure 3) and appear to be similar in the frequency of their usage since the abundance of the respective primer-extended products is similar. Two other start sites (indicated by the smaller arrows in Figure 3) appear to be used with a lower frequency. Essentially identical results were found when a different end-labeled oligonucleotide (TAFI-PE2; see the Experimental Procedures) was used as the primer (data not shown). The boxed nucleotide designated +1 on Figure 3 is the first nucleotide of the cDNA sequence reported by Eaton and co-workers (*6*); we have arbitrarily designated this nucleotide as +1 since there does not appear to be a single major transcription start site. The location of the initiator methionine codon is also indicated in Figure 3. Thus, the 5′-untranslated region of the TAFI mRNA transcript varies from between 9 and 46 bp in length.

Characterization of the 5′-Flanking Region of the TAFI Gene. On the basis of the results of the primer extension analysis, we concluded that the genomic sequence immediately upstream of exon 1 indeed constitutes the 5′-flanking region of the TAFI gene. Therefore, we determined the complete nucleotide sequence of this region, up to 2699 bp upstream of the +1 nucleotide. The sequence of the 5′-flanking region is presented in Figure 4. Of note is the lack of a consensus TATA sequence located at an appropriate

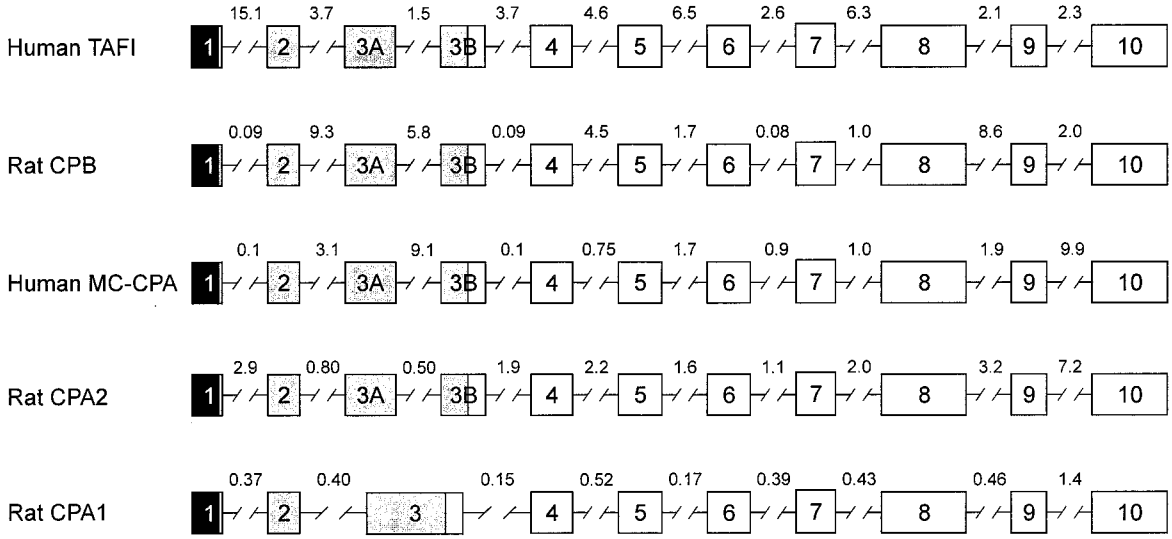

FIGURE 2: Schematic representation of the genomic organization of the human TAFI gene compared to related carboxypeptidase genes. The exons are represented by the boxes, numbered 1−10 and 3A, 3B. The black filled portions of the exons represent sequences encoding the signal peptides, the grey filled portions represent the sequences encoding the activation peptides and the white filled boxes represent the sequences encoding the catalytic domains. The broken lines connecting the exons indicate the positions of the introns; the length of each intron (in kilobases) is presented above each broken line.

distance (~30 bp) upstream of any of the transcription start sites. Thus, it can be concluded that the TAFI gene is analogous to the genes for several of the blood clotting factors including Factor VII (*30*, *31*), Factor IX (*32*), Factor X (*33*), Factor XII (*34*), and protein C (*35*) in that it is a liver-expressed gene whose transcription is initiated from multiple sites and whose promoter lacks a consensus TATA box.

Transcriptional Activity of the 5′-Flanking Region of the TAFI Gene in Cultured Mammalian Cells. To assess whether the 5′-flanking region of the TAFI gene can support transcription in mammalian cells, luciferase reporter plasmids containing increasing lengths of the TAFI 5′-flanking region were constructed (Figure 5). To ensure that all elements required for transcription were present, a unique *Hin*dIII site that spans the second and third codons in exon 1 (see Figure 4) was utilized such that the reporter constructs each contained the entire 5′-untranslated region of the TAFI gene; to prevent translation initiation of the fused TAFI/luciferase transcript at an inappropriate location, the initiator methionine codon derived from the TAFI gene was mutated from ATG to TTG.

TAFI reporter plasmids were transfected into HepG2 (human hepatoma), HeLa (human cervical carcinoma), BHK (baby hamster kidney), and 293 (human embryonic kidney) cells by the method of calcium phosphate coprecipitation. Each well received an equimolar amount of the respective reporter plasmids, as well as a fixed amount of a β-galactosidase-expressing internal control plasmid in order to correct for differences in transfection and harvesting efficiencies. Luciferase activity was expressed relative to that of a negative control promoterless luciferase reporter plasmid (pGL3 Basic). Transfection of a reporter plasmid containing 2699 bp of the TAFI 5′-flanking region upstream of the transcription start site into HepG2 cells resulted in a 10-fold higher luciferase activity than pGL3 Basic (Figure 5), indicating that the 5′-flanking region of the TAFI gene contains sequences capable of directing transcription in hepatic cells. Progressive 5′ deletions of the 5′-flanking region resulted in small but reproducible variations in luciferase activity while deletion of sequences upstream of −73 resulted in luciferase activity in HepG2 cells that was only marginally above background. These data indicate that sequences between −141 and −73 harbor promoter elements crucial for expression of the TAFI gene in hepatic cells. The TAFI reporter plasmids had little or no activity, relative to pGL3 Basic, in the nonhepatic cell lines HeLa and BHK. However, the reporter plasmids containing sequences beyond −73 but less than −417 resulted in luciferase activity higher than baseline levels in 293 cells (Figure 5).

Analysis of the 3′-Flanking Region of the TAFI Gene. The carboxyl-terminus of the TAFI protein and the 3′-untranslated region of the TAFI transcript are encoded by a single large exon (exon 10) in the TAFI gene (Figure 1 and Table 1). The sequence of the 3′ end of the gene is presented in Figure 6; the numbers in parentheses to the left of the sequence correspond to the numbering of the TAFI cDNA. Comparison of the genomic DNA sequence of this region to that of the TAFI cDNA sequence published by Eaton et al. (*6*) revealed that the 13 nucleotides immediately 5′ to the poly(A) tail in this cDNA (nucleotides 1695−1707) are not present in the gene. Therefore, we obtained clones corresponding to the 3′-untranslated region of the TAFI cDNA by RT-PCR using first-strand human liver cDNA as the template. Clones were isolated that ended with a poly(A) sequence at three different positions: after nucleotides 1677, 1710, and 1836 (indicated by the arrows in Figure 6). The placement of these polyadenylation sites affords the generation of TAFI transcripts containing 3′-untranslated regions of 390, 423, and 549 bp, respectively. Each of the three polyadenylation sites are preceded by a consensus polyadenylation signal (AUUAAA or AAUAAA 12−19 bp upstream; see Figure 6). These clones likely represent the full spectrum of possible polyadenylated products since no other consensus polyadenylation signals are present in this region of the gene (Figure 6). None of the clones contained the 13 nucleotides present between nucleotides 1695 and 1707 in the published cDNA sequence (*6*).

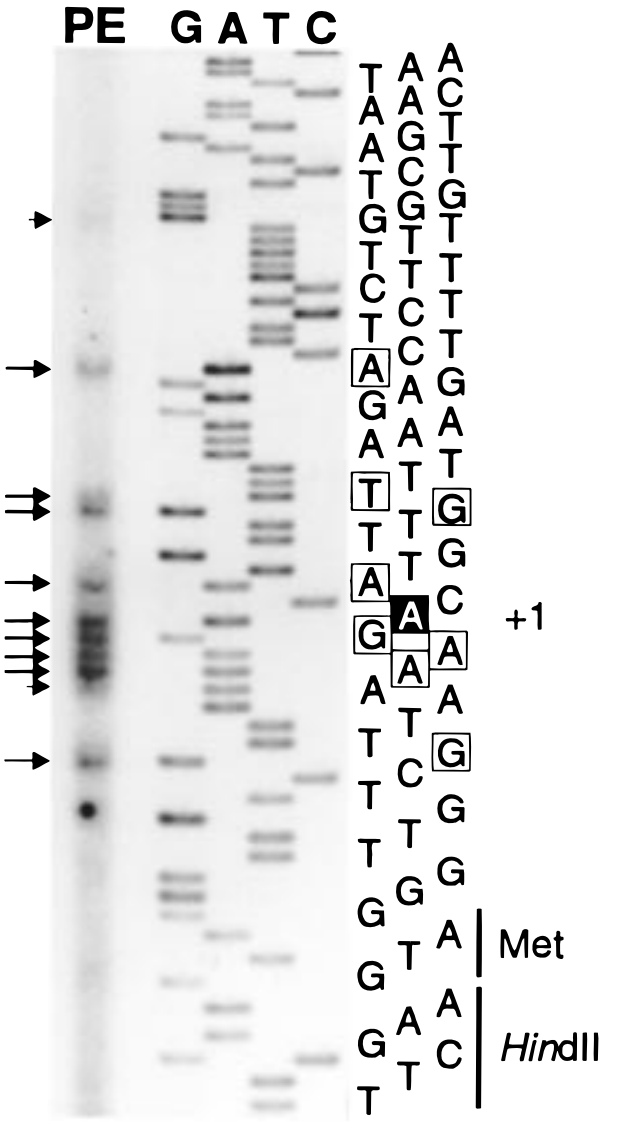

FIGURE 3: Identification of the start sites for transcription of the human TAFI gene by primer extension analysis. Primer extension analysis was performed on human liver RNA using reverse transcriptase and an antisense oligonucleotide specific for the 3′ end of exon 1 and end-labeled with ^{32}P. The products of the primer extension reaction (*PE*) were electrophoresed on a sequencing gel beside a dideoxy sequencing reaction (*G, A, T, C*) using a cloned DNA fragment encompassing the 5′-flanking region of the TAFI gene as the template and using the same antisense oligonucleotide as the primer. Indicated to the left of the resulting autoradiogram are the positions of bands corresponding to major (large arrows) and minor (small arrows) start sites. To the right of the autoradiogram is the sequence of the 5′-flanking region; the nucleotides representing the major start sites are boxed and the first nucleotide of the cDNA reported by Eaton and co-workers (6) is labeled *+1* and is shown in the black filled box. Also indicated are the positions of the initiator methionine codon (*Met*) and a *Hin*dIII restriction site.

DISCUSSION

Multigene families consist of genes encoding products which share common functional motifs but which have diverged in terms of primary sequence, physiological role and site of synthesis. The members of these gene families not only share substantial sequence homology in certain portions of their coding regions but also often share a highly conserved genomic organization with respect to the location of the intron/exon boundaries within the coding region. Thus, it has been concluded that multigene families have evolved by duplication of a common ancestral gene followed by divergent evolutionary processes leading to specialization of function of the gene products.

The genes encoding the carboxypeptidases are an example of such a multigene family. The pancreatic carboxypeptidases are digestive exopeptidases secreted by pancreatic acinar cells and specifically hydrolyze carboxyl-terminal residues containing aromatic or branched aliphatic side chains (CPA1, CPA2) or basic side chains (CPB) in protein substrates (*27*, *28* and references therein). Mast cell carboxypeptidase A is specifically expressed in the secretory granules of certain populations of mast cells (*29*) and possesses the same substrate specificity as CPA1 and CPA2 while sharing a higher degree of amino acid similarity with CPB (*36*). The gene encoding TAFI can now be added to this multigene family. TAFI shares substantial sequence similarity with the other carboxypeptidases (between 34 and 40% amino acid identity; data not shown) and exact conservation of amino acid residues thought to be involved in substrate binding and catalysis (*6*), as well as a strikingly similar genomic organization (see Figure 2 and Table 1). Other mammalian basic carboxypeptidases (reviewed in ref *37*), including carboxypeptidase N, a constitutive plasma carboxypeptidase, carboxypeptidase M, a membrane-bound carboxypeptidase, and carboxypeptidases H and D (*38*) which are components of secretory granules, are structurally distinct from the above carboxypeptidases and constitute a subfamily known as the "regulatory" basic carboxypeptidases since they are thought to be involved in regulating the activity of peptide hormones (*37*). One of the introns in the carboxypeptidase H gene, however, is in a position comparable to that in the pancreatic carboxypeptidase genes (*39*), indicating that the regulatory carboxypeptidases may have evolved from the same ancestral gene as the pancreatic carboxypeptidases, MC-CPA and TAFI.

Knowledge of the genomic structure of the TAFI gene allows conclusions to be made regarding the evolutionary relationship between TAFI and the other carboxypeptidases to which it is related. As depicted in Figure 2, all of these genes share an identical genomic organization except for exon 3, which is a single large exon in CPA1 but is split into two exons (3A and 3B) in the other carboxypeptidases (*27*−*29*). The intron dividing exons 3A and 3B was likely lost in the CPA1 gene following the gene duplication event which gave rise to separate CPA1 and CPA2 genes (*27*). Furthermore, exon 3A of CPA2 contains an additional seven codons at its 3′ end (these codons are included in exon 3 of CPA1) that are missing in CPB, MC-CPA and TAFI (*27*−*29*). Thus, it can be concluded that CPB, MC-CPA, and TAFI have arisen from a common ancestral gene that diverged from the lineage leading to the CPA1/CPA2 genes. Consistent with this idea, MC-CPA shows greater amino acid sequence similarity to CPB than to CPA1 or CPA2 despite the fact that MC-CPA has a similar substrate specificity to CPA1 and CPA2 (*29*). Alignment of the amino acid sequences of the human orthologues of all five carboxypeptidases reveals that CPB is more closely related to MC-CPA than it is to TAFI (data not shown). Thus, the ancestral gene leading to TAFI may have arisen from a gene duplication event that also gave rise to a common CPB/MC-CPA ancestor.

In contrast to the clear amino acid sequence similarity and conservation of genomic organization that characterizes the

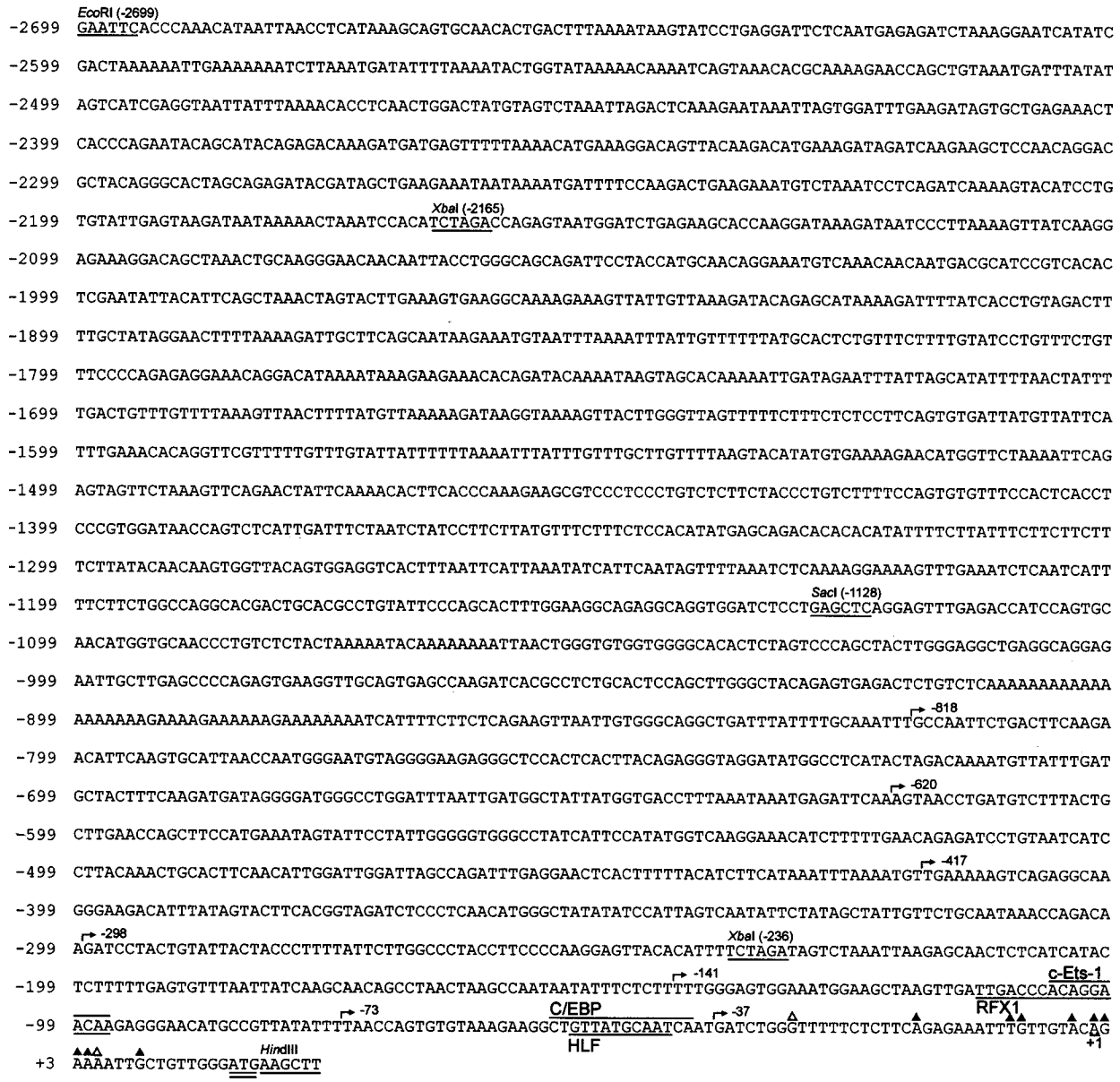

FIGURE 4: Nucleotide sequence of the 5′-flanking region of the human TAFI gene. The numbering of the nucleotides to the left of the sequence is given relative to the first nucleotide of the cDNA reported by Eaton and co-workers (6) which is labeled *+1*. The positions of *Eco*RI, *Xba*I, *Sac*I, and *Hin*dIII restriction sites are underlined. The positions of major (filled triangles) and minor (open triangles) transcription start sites are indicated. The initiator methionine codon is double underlined. The locations of selected potential transcription factor binding sites identified using Matinspector software (*25*) are indicated.

carboxypeptidase gene family, there is little nucleotide sequence similarity between the 5′-flanking regions of all of these genes (*27*−*29* and data not shown). Indeed, although CPB is expressed in the same tissue as CPA1 and CPA2, the 5′-flanking region of the CPB gene is only slightly more similar to that of the CPA1 and CPA2 genes than it is to the 5′-flanking region of the TAFI gene (data not shown). However, it should be noted that selective pressure in the 5′-flanking regions of genes is reflected to a greater degree in the sequences of binding sites for transcription factors than it is in the surrounding sequences. Indeed, the positions of TATA and CAAT sequences are conserved between all three rat pancreatic carboxypeptidase genes (*27*, *28*); in addition, the 5′-flanking region of all three rat genes contain (albeit in different positions) at least one copy of a sequence thought to confer specific expression in pancreatic acinar cells (*27*, *28*).

Analysis of the 5′-flanking region of the TAFI gene reveals that the promoter lacks a TATA box (Figure 4) and that transcription is initiated from multiple sites (Figure 3), similar to what has been observed for the genes encoding several of the vitamin K-dependent coagulation factors including Factors VII, IX, X, and XII as well as protein C (*30*−*35*). This contrasts with what has been observed for the genes encoding the pancreatic carboxypeptidases and MC-CPA which all apparently contain a TATA box and have unique transcriptional start sites (*27*−*29*). Thus, in the course of evolutionary events on the TAFI gene which led to the acquisition of its ability to be expressed in the liver, the promoter region of this gene acquired characteristics commonly associated with the promoters of the vitamin K-dependent coagulation factor promoters, which are also liver-specific in their expression.

The TATA sequence in the promoters of RNA polymerase II-transcribed genes binds a TATA-box-binding protein

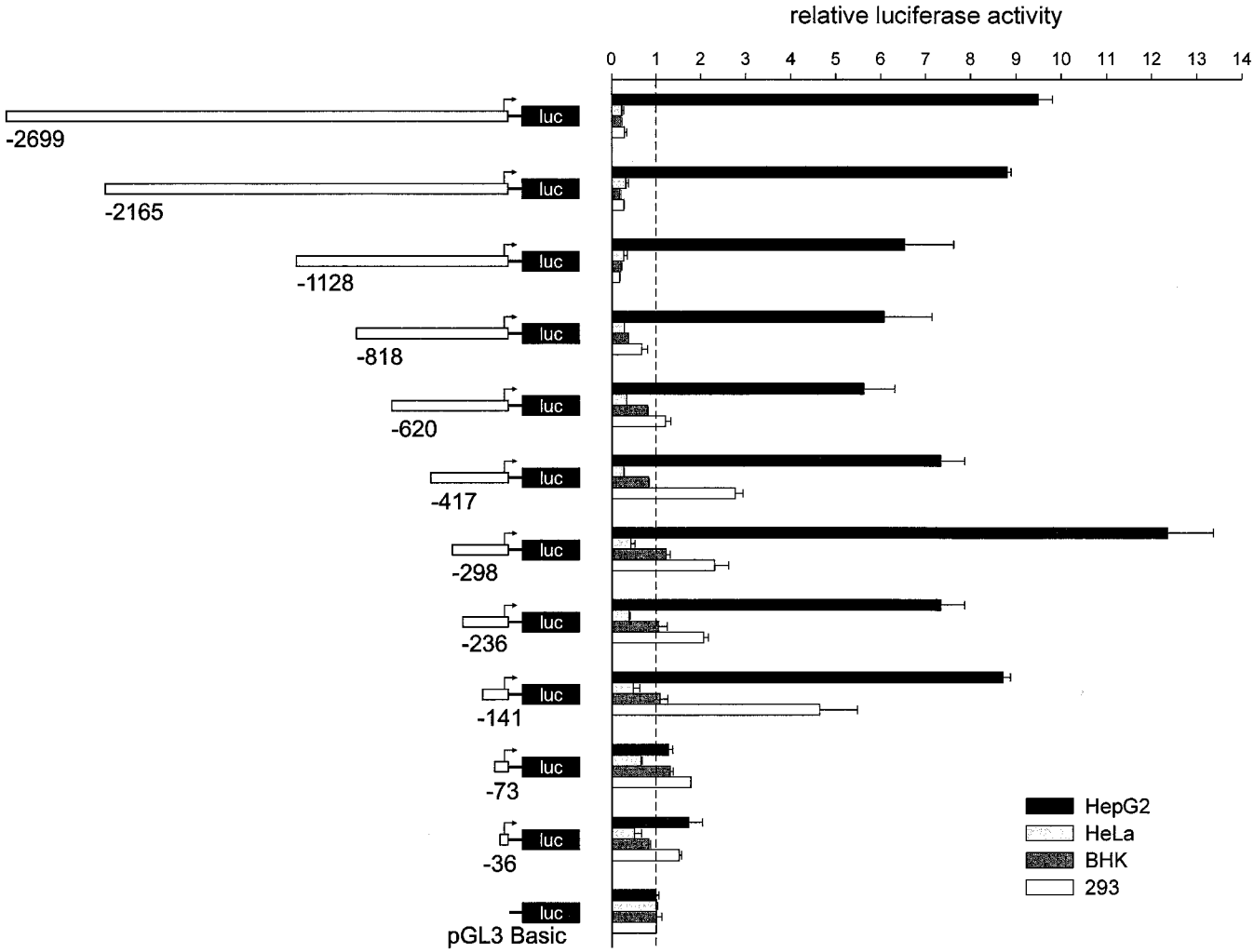

FIGURE 5: Analysis of the promoter activity of the TAFI 5′-flanking region by transient transfection of reporter plasmids into cultured mammalian cells. HepG2, HeLa, BHK or 293 cells were transiently transfected with equimolar concentrations of the reporter plasmids shown schematically on the left by the method of calcium phosphate coprecipitation. Included in each transfection was an equal amount of a β-galactosidase expression plasmid to control for differences in transfection and harvesting efficiency. Luciferase and β-galactosidase activity assays were performed on cytoplasmic extracts prepared from transfected cells; luciferase activities were normalized by dividing by the β-galactosidase activities. The relative luciferase activity is defined as that divided by the luciferase activity observed in cells transfected by a promoterless luciferase reporter plasmid (pGL3 Basic). The data shown are the mean of duplicate transfections; the error bars represent the standard errors of the mean. The data presented are representative of at least two independent experiments.

(TBP), which is a component of the preinitiation complex, formation of which is required for transcription to occur. The binding of TBP to the TATA sequence accounts for the existence of a unique transcriptional start site in most TATA-box-containing promoters. In the absence of a TATA box, however, the interaction of other transcription factors with sequences in the promoter is presumably required to localize the preinitiation complex to the appropriate location in the promoter. The weaker binding affinity and lower specificity of these interactions results in the appearance of multiple sites for transcription initiation, as we have observed for the TAFI gene. Promoters lacking TATA sequences often contain GC-rich regions in the neighborhood of the transcription start site(s) which bind the ubiquitous transcription factor Sp1 and have been postulated to account for the localization of the preinitiation complex in these promoters (*40*, *41*). For example, an Sp1 binding site is important for the transcription of the human Factor VII promoter in HepG2 cells (*31*). The TAFI promoter, however, contains no consensus Sp1 binding sites in the region of the proximal promoter (Figure 4).

The results of transient transfection experiments using luciferase reporter plasmids containing portions of the TAFI 5′-flanking region show that this region of the TAFI gene contains a functional promoter that is active in hepatic cells (Figure 5). Deletion of 5′-flanking nucleotides from −2699 to −142 had relatively minor effects on promoter activity, whereas deletion of 5′-flanking nucleotides beyond −141 resulted in a precipitous drop in promoter activity. These data indicate that nucleotides −141 to −73 of the TAFI gene are crucial for basal activity of the promoter. None of the TAFI reporter plasmids resulted in luciferase activity higher than background when transfected into the nonhepatic cell lines HeLa (human cervical carcinoma) and BHK (baby hamster kidney), indicating that the TAFI 5′-flanking region also contains sequences that restrict the transcription of the promoter to liver cells. Certain TAFI reporter plasmids did result in activity in 293 (human embryonic kidney) cells (Figure 5). However, RT-PCR (*6*) or Northern blot (data not shown) analysis of human kidney RNA failed to detect expression of TAFI in this organ, suggesting that the promoter activity observed in 293 cells is not physiologically

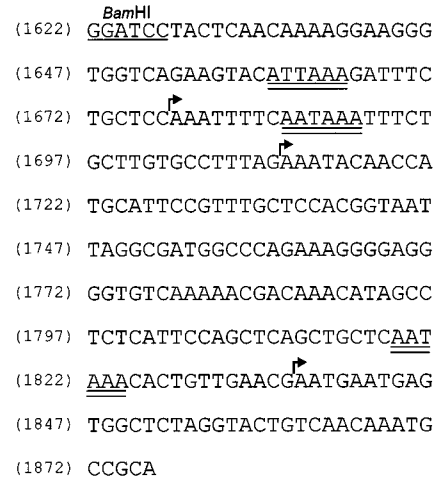

FIGURE 6: Nucleotide sequence of the 3′-flanking region of the human TAFI gene. The numbering of the nucleotides presented in brackets to the left of the sequence is based on the numbering of the published cDNA (6). The location of a *Bam*HI restriction site is underlined. The locations of polyadenylation sites of TAFI mRNA transcripts, as assessed by DNA sequence analysis of TAFI cDNA clones isolated from human liver RNA by RT-PCR, are indicated by the arrows. Each polyadenylation site is preceded by a consensus polyadenylation signal (double underlined).

relevant. It is important to note that the 293 cell line is transformed with a replication-defective form of adenovirus (*42*); thus, adenoviral proteins, including the potent transcription factor E1A (*43*), are present in 293 cells that are not present in human kidney. It should also be noted that although the activity of the reporters in 293 cells was dependent on the presence of the sequences within −141 that are required for promoter activity in liver cells, reporters containing 5′-flanking sequence beyond −417 lacked activity in 293 cells (Figure 5). It is possible that the activity of the TAFI reporter plasmids in 293 cells involves binding of transcription factors to sequences in the pGL3 Basic backbone. The plasmid-binding sites may need to be within a certain distance of the TAFI promoter in order to exert their effects and result in luciferase activity in 293 cells; the presence of large genomic inserts, for example in reporter plasmids containing sequences upstream of −417 (see Figure 5), would prevent the interaction of factors bound at these sites with those bound within the TAFI core promoter.

The mechanisms underlying the expression of genes specifically in the liver has been extensively studied and has been shown to be a function of the activity of certain liver-specific or liver-enriched transcription factors. The role of such factors in the liver-specific expression of coagulation factor genes has also been studied in detail. The human Factor VII (*30*, *31*) and Factor X (*33*, *44*) promoters contain functional binding sites for HNF-4, the human Factor IX promoter contains functional binding sites for HNF-4 as well as C/EBP (*45*), and the human protein C promoter contains functional binding sites for HNF-1, -3, and -6 (*35*, *46*−*49*). Examination of the TAFI 5′-flanking region sequence downstream of nucleotide −141 indicated several potential transcription factor binding sites that may mediate liver-specific transcription of the TAFI gene (see Figure 4). These include very good matches [i.e., core similarity = 1; matrix similarity > 0.95 (*25*)] to the consensus sequences for binding of the liver-enriched transcription factors C/EBP (*50*) (−53 to −40) and hepatic leukemia factor [HLF (*51*)] (−51 to −42). Although these binding sites are included in the −73 reporter plasmid that is not active in HepG2 cells (see Figures 4 and 5), the activity of these sites may depend on upstream sequences lacking in this plasmid. For example, HLF has been shown to cooperate with the ubiquitous Ets transcription factor GABPα/β (*52*) in transcription of the Factor IX promoter in HepG2 cells (*53*); a good match (core similarity = 0.926; matrix similarity = 0.873) to the c-Ets-1 binding site (*54*) is located between −105 and −96. A good match (core similarity = 0.882; matrix similarity = 0.874) to the ubiquitous transcription factor RFX1 (*55*) is present between −112 and −96 (Figure 4); the ability of this factor to participate in liver-specific transactivation is dependent on certain liver-specific factors (*56*) which may bind downstream of −73 in the TAFI promoter. Further studies will be required to definitively establish which transcription factors mediate expression of TAFI in liver cells.

Although Northern blot analysis revealed that TAFI transcripts are detectable in liver but not in kidney, brain, placenta, lung, or skeletal muscle (data not shown), we have not performed a definitive survey of TAFI expression in various tissues. Expression of primarily liver-expressed coagulation factors in extrahepatic tissues has been described. For example, protein C mRNA is detectable in human umbilical vein endothelial cells (*57*) and in cells of human male reproductive tissues (*58*). Recent reports have also indicated that the prothrombin gene is expressed in the kidney (*59*) and skeletal muscle (*60*).

By RT-PCR of human liver RNA, we isolated cDNA clones which represent TAFI transcripts in which three different polyadenylation sites were utilized (Figure 6). All three polyadenylation sites are preceded by a consensus polyadenylation signal and result in the formation of TAFI mRNA species containing 3′-untranslated regions of 390, 423, and 549 bp. The relative abundance of each form of the TAFI transcript could not be assessed by this methodology, however. None of the cDNA clones contained the sequence "CTTCTTCTCCTTT", which spans nucleotides 1695−1707 of the published TAFI cDNA sequence (*6*); this sequence is also absent from the corresponding genomic sequence (Figure 6). The origin of this sequence in the published cDNA is not clear at present.

The utilization of multiple polyadenylation sites during mRNA processing is frequently observed and may play a role in determining transcript abundance by specifying the inclusion or exclusion of sequences which modify the stability of the transcript. Such sequences can take the form of 50−150 bp long A/U-rich regions, often containing multiple copies of the AUUUA pentanucleotide (*61*). Although the TAFI 3′-untranslated region is relatively A/U-rich and contains one AUUUA sequence (beginning at nucleotide 1325), the region that would be differentially represented in TAFI transcripts based on alternative usage of polyadenylation sites is less A/U-rich and lacks this pentanucleotide sequence (Figure 6). While other sequences capable of modifying mRNA stability have been described (*61*), it remains to be determined whether TAFI mRNA abundance is regulated at this level and whether variation in the efficiency with which alternative polyadenylation sites are used during TAFI mRNA processing affects this process. Differential selection of polyadenylation sites has also been proposed as a mechanism by which tissue-specific expression

of particular transcripts is achieved (*62*). However, as discussed above, it is not yet clear if TAFI is expressed in tissues other than the liver.

There is currently no information concerning the ability of TAFI gene expression to be regulated by such factors as hormones or growth factors. It will be of particular interest to assess if TAFI, like the fibrinogen α, β, and γ chains, is an acute-phase reactant and is thus inducible by interleukin-6 (*63*). A recent study suggests that levels of TAFI antigen in plasma vary between 60 and 138% of the mean value (*14*). Polymorphisms in the 5′-flanking regions of the plasminogen activator inhibitor type 1 and Factor VII promoters have been demonstrated to alter plasma levels of these proteins by altering the rate at which transcription is initiated from the respective promoters (*31*, *64*); whether polymorphism in the 5′-flanking region of the TAFI gene is responsible for the variation in TAFI plasma levels remains to be determined. The characterization of the human TAFI gene which we have described here will be an invaluable tool for answering these important questions.

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