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Characterization of the 5′ untranslated region of α and β isoforms of the human thromboxane A_2 receptor (TP)

Differential promoter utilization by the TP isoforms

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In humans, thromboxane (TX) A_2 signals through two TXA_2 receptor (TP) isoforms, TPα and TPβ, that diverge within their carboxyl terminal cytoplasmic (C) tail regions and arise by differential splicing. The human TP gene contains three exons E1–E3; while E1 exclusively encodes 5′ untranslated region (UTR) sequence, E2 and E3 represent the main coding exons. An additional noncoding exon, E1b was identified within intron 1. Additionally, the TP gene contains two promoters P1 and P2 located 5′ of E1 and E1b, respectively.

Herein, we investigated the molecular basis of the differential expression of the TP isoforms by characterizing the 5′ UTR of the TP transcripts. While E1 and E1b were found associated with TP transcript(s), their expression was mutually exclusive. 5′ rapid amplification of cDNA ends (5′ RACE) established that the major transcription initiation (TI) sites were clustered between −115 and −92 within E1 and at −99 within E1b. While E1 and E1b sequences were identified on TPα transcript(s), neither existed on TPβ transcript(s). More specifically, TPα and TPβ transcripts diverged within E2 and the major TI sites for TPβ transcripts mapped to −12/−15 therein. Through genetic reporter assays, a previously unrecognized promoter, termed P3, was identified on the TP gene located immediately 5′ of −12. The proximity of P3 to the TI site of TPβ suggests a role for P3 in the control of TPβ expression and implies that TPα and TPβ, in addition to being products of differential splicing, are under the transcriptional control of distinct promoters.

Keywords: thromboxane receptor; isoforms; splicing; promoter; 5′ untranslated region.

Thromboxane (TX) A_2, generated through the sequential metabolism of arachidonic acid by cyclooxygenases 1/2 and TXA_2 synthase, acts as a potent agonist of platelet activation and aggregation and mediates a diversity of actions in a number of other target cell or tissue types [1]. TXA_2 signals through interaction with its specific cell surface TXA_2 receptor, also termed TP, a member of the G-protein coupled receptor (GPCR) superfamily [2,3]. In humans, but not in other species thus far investigated, the TP exists as two isoforms, referred to as TPα and TPβ [2,3] that are encoded by a single TP gene located on chromosome 19p13.3 and arise by a novel differential splicing mechanism [3,4]. TPα and TPβ are identical for their N-terminal 328 amino acid residues but differ exclusively in their carboxyl terminal cytoplasmic (C) tail sequences [2,3] such that TPα (343 amino acids) and TPβ (407 amino acids) have some 15 and 79 amino acids within their divergent C-tail sequences, respectively. In humans, the single TP gene is composed of three major exons, E1–E3, and two intervening introns I1 and I2 [4]. Through primer extension analysis, an additional exon, referred to as E1b, was also identified within I1 [4]. Two individual promoters (P), designated P1 and P2, each with distinct signature transcription factor binding sites, located 5′ of E1 and E1b sequences, respectively, were identified [4] and a number of independent studies have indicated that P1 may be the major promoter [5–7]. While E1 and E1b each exclusively encode 5′ untranslated region (UTR) sequences, E2 encodes some 83 nucleotides of 5′ UTR and, along with E3, represents the major coding exons of both TPα and TPβ receptors [4]. The coding sequence of TPα and TPβ mRNAs are identical from nucleotide +1 to +983 nucleotides (where the initiation codon is designated +1), but diverge within E3 whereby excision of nucleotides +984 to +1642, representing intron 3b sequences, creates a mRNA transcript with a new open reading frame encoding TPβ divergent sequences [3,4]. Retention of these potential intron 3b sequences within E3 of the TPα mRNA encodes TPα divergent sequences [3].

While the biologic relevance for the existence of two TP receptors in humans is currently unknown, there is substantial evidence that they mediate differential signalling [8–11] and are subject to differential regulation [12–16], providing compelling evidence that the individual TP isoforms have distinct physiologic/pathophysiologic roles. Consistent with this view, it appears that the TPα and TPβ are also subject to differential expression [17,18]. In a study

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Abbreviations: E, exon; HEK, human embryonic kidney; HEL, human erythroleukemia; I, intron; P, promoter; RLU, relative luciferase units; 5′ RACE, 5′ rapid amplification of cDNA ends; TP, thromboxane receptor; TI, transcription initiation; TXA_2, thromboxane A_2; UTR, untranslated region.

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investigating the expression of the mRNAs encoding the TPs throughout a range of cell and tissues of particular relevance to TXA_2 biology, most cell/tissue types examined were found to express mRNAs for both the TPα and TPβ isoforms [17]. While TPα mRNA expression was constant and predominated, levels of TPβ mRNA expression varied enormously and, hence, extensive differences in the relative ratios of TPα : TPβ mRNA expression were identified [17]. Additionally, whilst isoform specific antibodies permitted the detection of TPα, but not TPβ, expression in human platelets [18], both receptors were detected in cultured vascular smooth muscle cells [19]. The molecular basis of this differential TP expression is currently unknown but suggests that the TP receptors may not only be the products of differential splicing but may also be subject to differential transcriptional regulation. Moreover, the identification of two putative promoters (P), P1 and P2, on the single TP gene raises the possibility that the TPα and TPβ isoforms may be under the transcriptional control of distinct promoters [4].

In the current study, we sought to investigate the molecular basis of the differential expression of TPα and TPβ isoforms. Our initial aim was to map the patterns of exon usage in the 5′ UTR of the major TP transcripts expressed in megakaryocytic human erythroleukemic (HEL) 92.1.7 cells and in trophoblast TM-1 cells [17] and, through the 5′ rapid amplification of cDNA ends (5′ RACE), to identify the major transcription initiation (TI) site(s) within the TP gene in both cell types. Moreover, we also sought to identify the patterns of exon usage within the 5′ UTR(s) of the individual TPα and TPβ mRNA transcripts and to map their major TI site(s), in both HEL cells and TM-1 cells. Whilst sequences corresponding to E1 and E1b sequences were found to exist within the TPα mRNA transcripts, neither E1 nor E1b sequences were found associated with TPβ mRNAs and, more specifically, TPα and TPβ mRNA sequences were found to diverge at nucleotide −12 within E2, representing the site of transcription initiation for TPβ mRNA sequences. Moreover, through genetic reporter assays, a previously unidentified promoter, herein designated P3, located 5′ of the −12 region on the human TP gene has been uncovered. The location of P3 close to the transcription initiation site of TPβ suggests a role for this promoter in the control of TPβ expression and indeed implies that the TPα and TPβ are under the transcriptional control of distinct promoters.

EXPERIMENTAL PROCEDURES

Materials

Ultraspec™ total RNA isolation system was obtained from Biotecx Laboratories, Houston, TX, USA. Perfectly Blunt Cloning kit, and Pellet-paint coprecipitant, was obtained from Calbiochem-Novabiochem, Nottingham, UK. Random hexamers, 5′ RACE system and eLONGase enzyme mix were purchased from Life Technologies Inc., Gaithersburg, MD, USA. Mouse moloney leukemia virus (MMLV) reverse transcriptase (RT), recombinant RNasin® ribonuclease inhibitor, pGEM DNA molecular weight markers, DNA polymerase I Large (Klenow) fragment, deoxynucleotide triphosphates, restriction endonucleases, RQ DNase I, pGL3 Basic, pGL3 Enhancer and pRL Thymidine Kinase reporter vectors and Dual Luciferase® Reporter Assay System were obtained from Promega Corporation, Madison, WI, USA. Taq DNA polymerase, T4 DNA ligase and calf intestinal alkaline phosphatase were obtained from Roche Molecular Biochemicals, Sussex, UK. Nytran supercharge membrane (0.45 μm) was from Schleicher and Schuell. T7 Sequenase Version 2.0 DNA sequencing kit was obtained from US Biochemical Corp. Oligonucleotides were synthesized by Genosys Biotechnologies, Cambridgeshire, UK. RNeasy Mini Kit and Effectene Transfection Reagent was purchased from Qiagen Ltd, Crawley, West Sussex, UK. DMRIE-C Reagent was purchased from Life Technologies. All other reagents were of molecular biology grade.

Cell culture

All mammalian cells were grown at 37 °C in a humid environment with 5% CO_2. Human erythroleukemic (HEL) 92.1.7 cells [20] and human embryonic kidney (HEK) 293 cells were cultured in RPMI 1640, 10% fetal bovine serum and in Eagle's minimal essential medium, 10% fetal bovine serum, respectively. The primary trophoblast cell line TM-1 [17] was grown in Dulbecco's minimal essential medium, 10% fetal bovine serum.

RT-PCR

Total RNA was isolated using the Ultraspec® RNA isolation procedure and aliquots (25 μg) were treated with 6.25 U RQ DNase I in the presence of 40 U RNasin® ribonuclease inhibitor using standard methodology [21].

DNase I treated total RNA (1.4 μg) was converted to first strand cDNA with mouse moloney leukemia virus (MMLV) RT in the presence of random hexamers (100 μM), essentially as previously described [17].

Thereafter, aliquots (3.5 μL) of first strand cDNA were used as templates in each PCR reaction (25 μL) in the presence of 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 6.7% glycerol, 1 μM sense primer, 1 μM antisense primer, 1 U *Taq* DNA polymerase. The oligonucleotide primers used include Kin2: 5′-dCAGCCGTCTCTCCTCCAGGGT-3′ (+72 to +52, Exon 2); Kin3: 5′-dTGTGGGCCGGAAACAGGGC-3′ (+45 to +27, Exon 2); Kin6: 5′-dGTGGCCCAACGG CAGTTC-3′ (+3 to +20, Exon 2); Kin16: 5′-dGAGAT GATGGCTCAGCTCCT-3′ (+724 to +743, Exon 2); Kin45: 5′-dCCTGATGGGGTGGTGAC-3′ (−40 to −24, Exon 2); Kin46: 5′-dGGCTCCGGAGCCATGTG-3′ (−12 to +5, Exon 2); Kin47: 5′-dTGACTGATCCCTCAGGG -3′ (−27 to −11, Exon 2); Kin60: 5′-dCGAGGCCGCAG AGGAAGGTGA-3′ (+211 to +191, Exon 2); Kin129: 5′-dCCCTCGCCCCACCCTCGG-3′ (−196 to −180, Exon 1); Kin130: 5′-dGTTCAGTGGCACGATCTT-3′ (−198 to −181, Exon 1b); G*f*: 5′-dTGAAGGTCGGAGTCAACG -3′ (+71 to +89, glyceraldehyde-3-phosphate dehydrogenase GAPDH mRNA) and G*r*: 5′-dCATGTGGGC CATGAGGTC-3′ (+1053–1035, GAPDH mRNA), where corresponding nucleotide positions within the TP mRNA or GAPDH mRNA sequences are indicated in parentheses. In order to specifically amplify TPα mRNA sequences, the primer Kin75, with the sequence 5′-dCCAGCCCCT

GAATCCTCA 3′ complementary to nucleotides +1121 to +1106 found on TPα, but not TPβ mRNA sequences, was used as antisense primer throughout. Similarly, in order to specifically amplify TPβ mRNA transcripts, the primer Kin21, with the sequence 5′-dAGACTCCGTCTGG GCCG-3′ complementary to nucleotides +992 to +976, and traversing a splice site at +983/1693 within the TPα mRNA, was used as antisense primer throughout.

To ascertain their identities, Kin130/2 and the Kin129/2 RT-PCR amplification products, were subcloned into pBluescript II SK(–) using standard methodology [21] and then subjected to DNA sequence analysis. Where indicated, densitometic analysis of RT-PCR products were performed essentially as previously described [17].

5′ Rapid amplification of cDNA ends (5′ RACE)

The 5′ RACE System, Version 2™ (Life Technologies, Inc.) was used essentially as described by supplier. Three independent 5′ RACE experiments were carried out and are designated (i) (ii) or (iii). Briefly, total RNA was converted to first strand cDNA using Superscript reverse transcriptase and, for 5′ RACE experiment (i), using the TP-specific antisense primer Kin60 (+ 211 to +191, Exon 2); for 5′ RACE experiment (ii), using the TP-specific antisense primer Kin58: 5′-dCAGAGTGAGACTCCG TCTG-3′(+999 to +981, TP_β specific primer; Exon 2); and for 5′ RACE experiment (iii) using the TP-specific antisense primer Kin51: 5′-dGGGACAGGCCGAAGAA GATCATGAC-3′ (+355 to +331, Exon 2). Thereafter, following dC-tailing by terminal deoxynucleotidyl transferase, first strand cDNA was used as templates in the first round of PCR amplifications using the sense anchor primer, AAP (5′-dGGCCACGCGTCGACTAGTACGGGIIGG GIIGGGIIG-3′) and for 5′ RACE experiments (i) (ii) and (iii), using the TP-specific antisense primers Kin2, Kin21 and Kin60, respectively. Thereafter, second round or nested PCR amplifications were performed with the sense abridged universal anchor primer, AUAP (5′-dGGCCACGCGTC GACTAGTAC-3′) and for 5′ RACE experiments (i) (ii) and (iii), using the TP-specific primers Kin3, Kin60 and Kin2, respectively.

The nested 5′ RACE amplification products were cloned into pSTBlue-1, using the Perfectly Blunt Cloning kit, as described by the manufacturer (Novagen). In the case of the TPβ-specific 5′ RACE amplifications (i.e 5′ RACE experiment (ii) using Kin58 during the first strand cDNA synthesis), AUAP/Kin60 products were blunt end subcloned into pBluescript II SK(–). All resulting recombinant plasmids were subject to DNA sequence analysis.

Southern blot analysis

Southern blot analysis of the RT-PCR and 5′ RACE amplification products was carried out using standard methodology [21]. Oligonucleotide primers Kin2, Kin46, Kin47 or Kin119 (5′-dCAGAAGACTGTGGATGGC-3′, corresponding to nucleotides +552 to +570 of GAPDH mRNA) were each 5′ end labelled with T4 polynucleotide kinase and were used as hybridization probes as previously described [17]. Radioactive images were captured by autoradiography on Fuji New RX Film.

Construction of luciferase-based genetic reporter plasmids

To investigate the relative strengths of the putative promoters located within the TP gene, gene fragments encoding the two previously identified promoters Promoter 1 and 2 [4] and a third novel promoter, herein designated Promoter 3, were subcloned into two genetic reporter plasmids pGL3Basic and pGL3Enhancer, containing a SV40 enhancer located downstream of the luciferase gene (Promega). Gene fragments encoding each promoter were amplified by PCR using as template a recombinant cosmid pWE15: TXR containing the entire human TP gene [22]. Promoter 1 fragment was amplified with the sense primer Kin108 (5′-dGAGAGGTACCGAGGGCGCGTGAGCT GGGGAG-3′, corresponding to nucleotides −8500 to −8479, where the −designation indicates nucleotides 5′ relative to the translational initiation codon ATG, which is designated +1) and the antisense primer Kin109 (5′-dAGAGACGCGTCTTCAGAGACCTCATCTGCG GGG-3′, corresponding to nucleotides −5922 to −5895). Promoter 2 fragment was amplified using the sense primer Kin110 (5′-dGAGAGGTACCGTGCTGCTCTACTGC CCCACC-3′, corresponding to nucleotides −3308 to −3287) and the antisense primer Kin111 (5′-dAGAGAC GCGTCTGTAATCCAGCTACTCGGGAG-3′, corresponding to nucleotides −2003 to −1980). Promoter 3 fragment using the sense primer Kin112 (5′-dGA GAGGTACCCAGGATGGTCTCGATCTCCTGAC-3′, corresponding to nucleotides −1394 to −1373) and the antisense primer Kin113 (5′-AGAGACGCGTGGCT CCGGAGCCCTGAGGGATC-3′, corresponding to nucleotides −21 to −1). In each case, sequences underlined in the sense and antisense primers correspond to *Kpn*I and *Mlu*I sites, respectively. The amplified gene fragments containing Promoter 1–3 sequences were digested with *Kpn*I and *Mlu*I and were subcloned into pGL3 Basic and pGL3 Enhancer vectors to generate the recombinant plasmids pGL3b:Prm1; pGL3b:Prm2 and pGL3b:Prm3, each in pGL3Basic and pGL3e:Prm1; pGL3e:Prm2 and pGL3e:Prm3, each in pGL3Enhancer. The fidelity of all recombinant plasmids was verified by restriction endonuclease mapping and by DNA sequence analysis.

Assay of luciferase activity

HEK 293 cells were plated in Eagle's minimum essential medium, 10% fetal bovine serum in six well dishes at 1×10^5 cells per well. At 70–80% confluence, cells were cotransfected with recombinant pGL3 Basic or pGL3 Enhancer control vector, encoding firefly luciferase, or their recombinant derivatives (0.4 μg per well) along with pRL TK (50 ng per well) using Effectene (Qiagen), encoding Renilla luciferase, as recommended by the supplier. Forty-eight hours after transfection, cells were washed in phosphate buffered saline ($NaCl/P_i$), harvested in 350 μL Reporter Lysis Buffer (Promega) and centrifuged at 14 000 ***g*** for 1 min at room temperature.

HEL 92.1.7 cells were transfected using the DMRIE-C transfection reagent, essentially as described by the supplier (Life Technologies, Inc). Briefly, 0.5 mL of serum free RPMI 1640 medium was dispensed into a six-well dish and 6 μL of DMRIE-C reagent was added. Thereafter, 0.5 mL

of serum free RPMI 1640 medium containing 2 μg of recombinant pGL3 Basic or pGL3 Enhancer vectors and 200 ng of pRL-TK was added and DNA/DMRIE-C reagent was complexed by incubation at room temperature for 30 min. Thereafter, 0.2 mL of serum free RPMI 1640 medium containing 2×10^6 HEL 92.1.7 cells were added to the complex followed by incubation for 4 h (37 °C in a CO_2 incubator) after which 2 mL of RPMI 1640 medium containing 15% fetal bovine serum was added. Forty-eight hour after transfection, the cells were washed in NaCl/Pi, harvested in 100 μL Reporter Lysis Buffer (Promega) and were centrifuged at 14 000 ***g*** for 1 min at room temperature.

HEK 293 and HEL 92.1.7 cell supernatants were assayed for both firefly and renilla luciferase activity using the reagents from the Dual Luciferase Assay System™. Briefly, 100 μL of firefly luciferase assay reagent was predispensed into the required number of luminometer tubes, to these, 20 μL of cell lysate was added and luminescence measured for 10 s following a 2-s premeasurement delay in a Turner luminometer (TD-20/20). Subsequently 100 μL of the Stop/Glo Reagent ™ was added and luminescence due to renilla luciferase was measured. Relative firefly to renilla luciferase activities were calculated as a ratio and were expressed in relative luciferase units (RLU).

Bioinformatic analysis

The complete nucleotide sequence of the TP gene and flanking sequences on human chromosome 19 is available at *http://www.ncbi.nlm.nih.gov*, accession no. AC005175. The nucleotide sequence corresponding to accession no. AC005175 is 41 303 bp and contains the reverse complement of the TP coding sequence from nucleotides 5′ 11127–24481 3′. For bioinformatic analysis, the reverse complement of nucleotides 5′ 11127–24481 3′ were obtained to generate the sequence 5′ −9500 to +3854 3′ (i.e complementary to 5′ 11127–24481 3′) where the translational start site (ATG) corresponds to nucleotide +1. Thereafter, sequences immediately 5′ of the ATG, i.e. nucleotides −1399 to +1 were analyzed for putative transcription factor binding sites and regulatory elements using the Matinspector Professional™ program [23] available at http://genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl. All programs were used at the default settings.

Statistical analysis

Statistical analysis of differences were analyzed using the two-tailed students unpaired *t*-test. All values are expressed as mean ± standard error of the mean (SEM). *P* values ≤ 0.05 were considered to indicate statistically significance differences.

RESULTS

Identification of the major TP mRNA transcripts and determination of their transcription initiation sites

The organization and the exon-intron boundaries of the human thromboxane (TX) A_2 receptor (TP) gene and the theoretical range of putative TP mRNA transcripts are illustrated in Fig. 1. In this study, in view of the reported discrepancies in the patterns of exon usage and the presence of multiple transcription initiation (TI) sites within the human TP gene [4,6,7], the initial aim was to characterize the major TP mRNA transcripts with respect to their 5′ UTR sequences and to identify the major TI sites in the megakaryocyte HEL 92.1.7 and in the trophoblast TM-1 cell lines, both of which have been confirmed to express TP mRNA at high levels [17].

To identify the major TP mRNA transcripts, a RT-PCR based approach was utilized employing total RNA isolated from HEL 92.1.7 cells as a template. The strategy adopted and relative positioning of the primers used are illustrated in Fig. 2A. Following RT-PCR analysis with the E1 primer Kin129 and the antisense primer Kin2, a single 268 bp product was amplified (Fig. 2B, lane 2) and characterized by nucleic acid sequence analysis (data not shown) and was confirmed to represent sequences due to splicing between E1-E2 sequences. No product containing E1-E1b-E2 sequences was amplified. Similarly, following RT-PCR analysis with the sense primer Kin130 and the antisense primer Kin2, a 270 bp product was amplified (Fig. 2B, lane 1) and subjected to nucleic acid sequence analysis (data not shown) and was confirmed to represent sequences due to splicing between E1b-E2 sequences. In addition to the correct amplification product corresponding to predicted E1b-E2 sequences, a minor RT-PCR product of higher molecular weight (~700 bp) was also amplified but was confirmed to represent nonspecific artifactual sequence. Thus, these data demonstrate that whereas the major TP mRNA transcripts contain either E1-E2 or E1b-E2 sequences, there was no evidence of a TP transcript containing E1-E1b-E2 sequences (Fig. 2C,D) and these data correlate with findings in the trophoblast TM-1 cell line (data not shown)

Thereafter, a 5′ RACE approach was adopted to identify the TI sites of the TP mRNA transcripts in HEL 92.1.7 and TM-1 cells, as outlined in Fig. 3A. Following subcloning of the resultant nested 5′ RACE products and their subsequent nucleotide sequence analysis, multiple TI sites clustered around −92 to −115 within E1 were identified in HEL 92.1.7 cells (Fig. 3B). Furthermore, two additional transcription initiation sites were located at −99 within E1b (Fig. 3B). Consistent with this, in TM-1 trophoblast cells, the major TI sites were identified around −94 and −114, within E1, and a TI site was also identified within E1b at −99 (Fig. 3C). Thus, taken together, these data indicate that there are two major types of TP transcripts that are distinguishable on the basis of their differential utilization of either E1 or E1b sequences; moreover, 5′ RACE confirmed that there are multiple TI sites within both HEL 92.1.7 cells and TM-1 cells with the major TI sites clustered at sites within E1 and E1b.

Analysis of 5′ UTR of TPα and TPβ

Previous studies have demonstrated substantial variations in the relative levels of expression of TPα and TPβ mRNAs in a variety of human tissues; whereas TPα mRNA levels remains constant between cell types, the levels of TPβ vary considerably indicating that their expression may be independently regulated [17]. Additionally, the presence of two putative promoters, P1 and P2, raised the possibility that

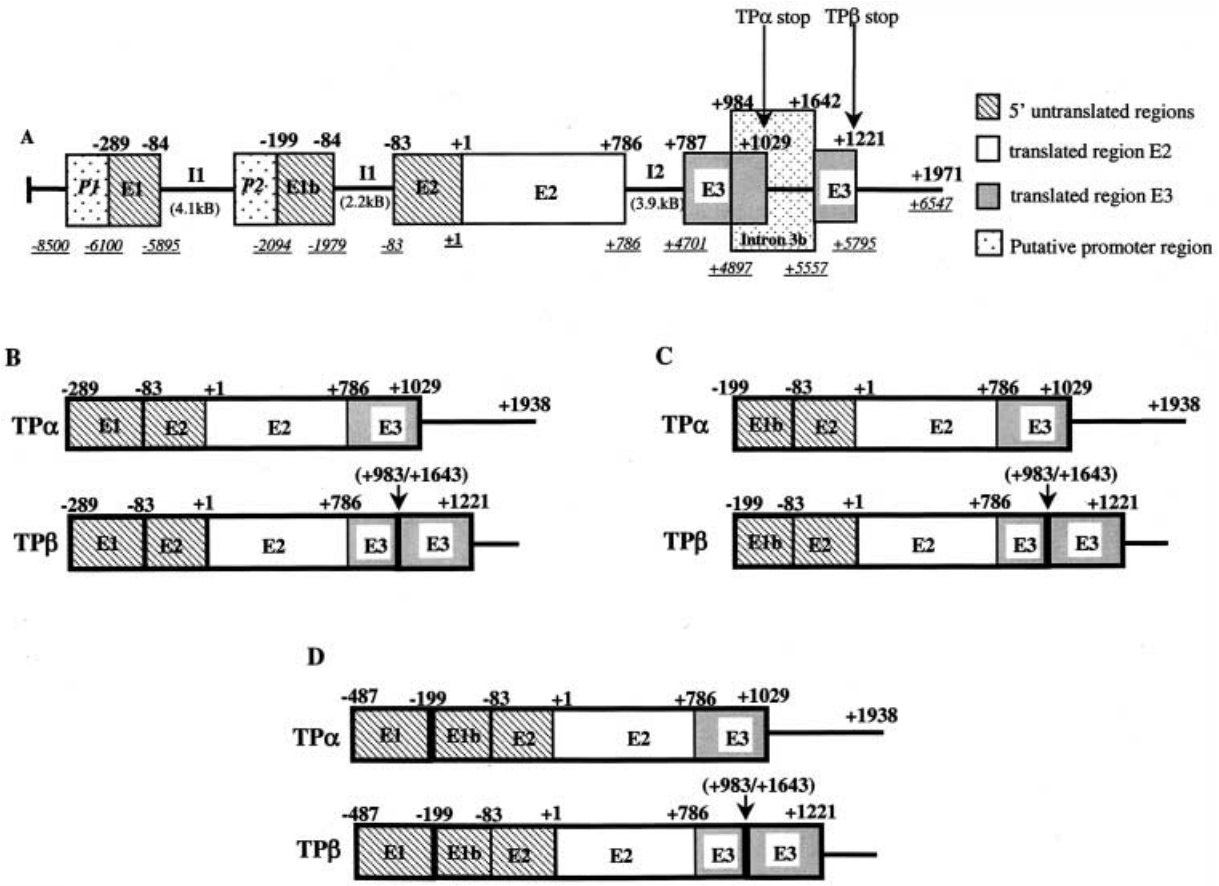


Fig. 1. Organization of the human TP gene including potential TP mRNA transcripts. (A) The human TP gene contains three exons E1, E2 and E3 separated by two introns I1 and I2. An additional exon, E1b, is located within I1 and there are two putative promoters P1 and P2, located 5′ of E1 and E1b sequences, respectively. The lower numbering system indicates the position of those sequences within the TP gene, spanning from −8500 to +6547 (italics, underlined) while the upper numbering system indicates the position of the exons sequences within the TP mRNA(s) (bold). All nucleotide numbers are assigned relative to the translation start site, ATG designated +1 and all sequences 5′ of +1 are given a – designation and all numbers 3′ of +1 are given a + designation. E1, encodes nucleotides −289 to −84 of 5′ untranslated region (UTR) of the TP mRNA; alternatively, exon E1b, of 115 bp, located within I1 encodes −199 to −84 of 5′ UTR sequence. E2 contains nucleotides −83 to −1 of 5′ UTR sequence and +1 to +786 of coding sequence, encoding amino acids 1–261. E3 contains nucleotides +787 to +1029, coding for amino acids 262–343 of TPα, and nucleotides +1030 to +1938, representing 3′ UTR sequences. Nucleotides +984 to +1642 behave as a potential intron (Intron 3b) on the TP mRNA; splicing of nucleotides +983/+1643 generates a mRNA which has a novel open reading frame, encoding TPβ of 407 amino acids, whereby nucleotides +983 to +1221 encode amino acids 328–407 that are unique to TPβ. (B–D) In theory, depending on the differential utilization of E1 and/or E1b sequences, the TP gene may be transcribed to generate three putative, alternatively spliced mature mRNAs, namely E1-E2-E3 (Panel B), E1b-E2-E3 (Panel C) and E1-E1b-E2-E3 (Panel D), that differ within their 5′ UTR sequences. Additionally, further alternative splicing of the latter TP transcripts within E3 may potentially double the number of TP transcripts to six, depending on the presence (TPα transcripts) or absence (TPβ transcripts) of intron 3b (+ 983/+1643) sequences.

TPα and TPβ expression may be regulated by alternative promoter utilization. Hence, to ascertain whether the TP isoforms may be subject to alternative promoter utilization, we sought to initially identify the 5′ UTR sequences associated with the individual TPα and TPβ mRNAs and thereafter, to identify the major TI sites of those isoform specific transcripts.

Initially, to identify the 5′ UTR sequences found on TPα and TPβ mRNAs, an RT-PCR approach was employed using 5′ sense primers based on E1 or E1b sequences and 3′ antisense primers that distinguish TPα from TPβ sequences using RNA isolated from HEL 92.1.7 cells as a specific template (Fig. 4A). RT-PCR analysis with the E1 primer Kin129 and the TPα specific primer Kin75 generated a 1319 bp product (Fig. 4B, lane 1) that was confirmed, by Southern blot analysis (Fig. 4C, lane 1) and nucleotide sequence analysis (data not shown), to represent sequences due to splicing between E1:E2:E3 sequences. Additionally, RT-PCR analysis with the E1b primer Kin130 and the TPα specific primer Kin75 generated a 1321 bp product (Fig. 4B, lane 3) that was confirmed, by Southern blot analysis (Fig. 4C, lane 3) and nucleotide sequence analysis (data not shown), to correspond to sequences due to E1b:E2:E3 splicing. In striking contrast, RT-PCR analysis with either Kin129 or Kin130 vs. the TPβ specific antisense primer Kin21 failed to result in the amplification of the predicted 1188 bp product (Fig. 4B,C, lane 2) or of the predicted 1190 bp product (Fig. 4B,C, lane 4), respectively. Hence, it

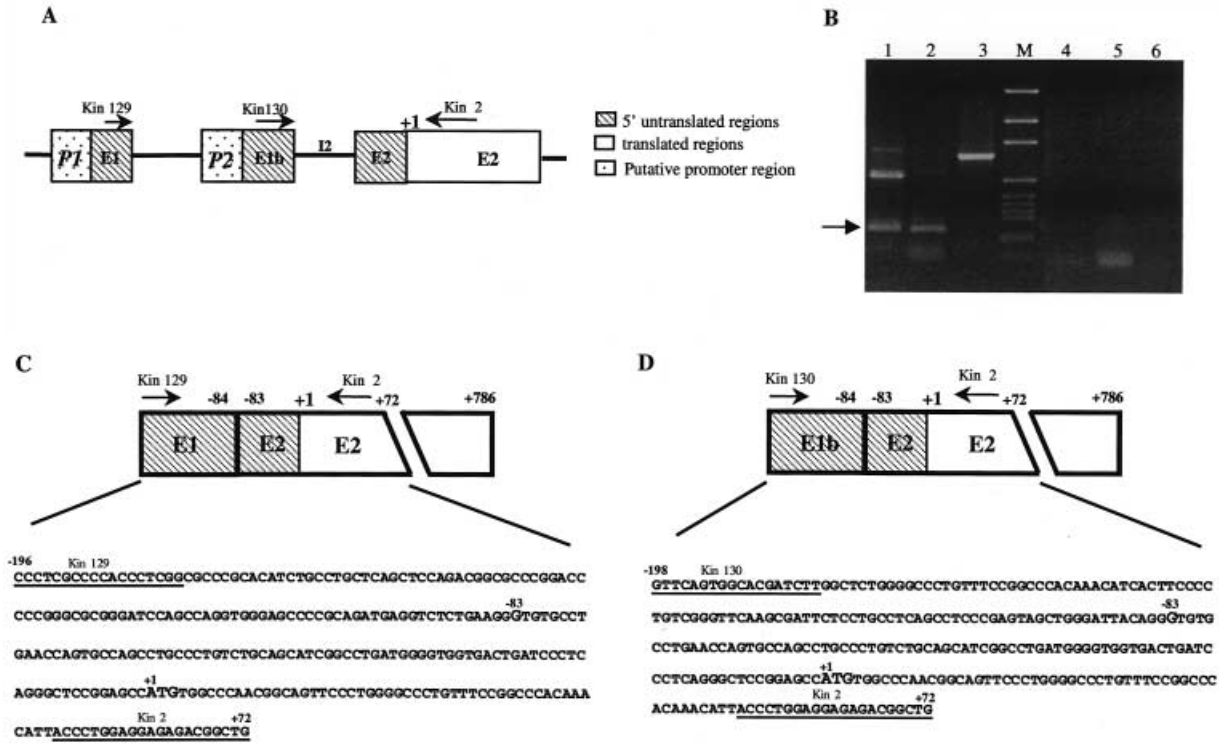
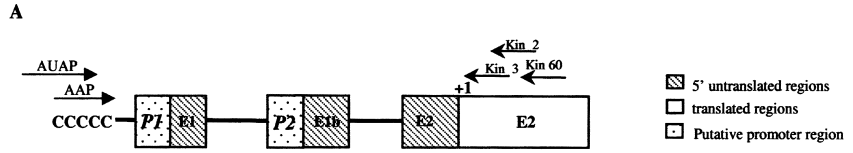


Fig. 2. Arrangement of noncoding exons in the 5′ UTR of the TP transcripts. (A) Relative positioning of the two sense oligonucleotide primers Kin129 and Kin130 and the antisense primer Kin2 used for the analysis of the 5′ UTR sequences of TP mRNAs. (B) Agarose gel electrophoresis of RT-PCR products (7 μL per lane) derived from HEL 92.1.7 first strand cDNA templates: lane 1, E1b-E2, 270 bp product vs. primers Kin130/Kin2; lane 2, E1-E2, 268 bp product vs. primers Kin129/Kin2; lane 3, GAPDH, 983 bp product vs. GAPDH forward (*Gf*) and GAPDH reverse (*Gr*) primers; lanes 4–6, negative control PCR reactions carried out in the absence of template first strand cDNA in the presence of primers Kin130/Kin2, Kin129/Kin2 and *Gf*/*Gr*, respectively; lane M, pGEM DNA markers. The correctly sized Kin130/Kin2 and Kin129/Kin2 generated PCR fragments are indicated by the arrow. (C and D) Schematic representation of the RT-PCR products, and their corresponding nucleotide sequences, generated using primers Kin129/Kin2 and Kin130/2, respectively. The gap 3′ of nucleotide +72 within E2 indicates the position at which the nucleotide sequence of the RT-PCR product generated with Kin2 terminates.

appears that the TPα, but not the TPβ, mRNA contains E1 and E1b sequences.

To further characterize the 5′ UTR of the TP mRNA transcripts, RT-PCR analysis was performed using the sense primer Kin45 (Fig. 4A) in combination with the TPα specific antisense primer Kin75 or the TPβ specific antisense primer Kin21. While RT-PCR analysis with the E2 sense primer Kin45 and the TPα specific primer Kin75 produced a fragment of 1163 bp (Fig. 4B, lane 5) that was confirmed, by Southern blot analysis (Fig. 4C, lane 5) and nucleotide sequence analysis (data not shown), to represent TPα specific sequences, RT-PCR analysis with Kin45 and the TPβ specific primer Kin21 failed to result in the amplification of the expected 1032 bp product (Fig. 4B,C, lane 6). Thus, it appears that while nucleotides 5′ of −40 to −24 within E2 were present on the TPα mRNA transcript, this region was actually not present on the TPβ mRNA transcript (Fig. 4C) indicating that TPα and TPβ mRNA sequences diverge at 5′ UTR sequences within E2. However, despite the latter finding, consistent with our previous reports [17], the positive expression of mRNA encoding TPβ sequences in HEL 92.1.7 cells was indeed confirmed by RT-PCR analysis using a sense primer Kin6 and the TPβ-specific antisense primer Kin21 whereby a product of 990 bp was amplified and confirmed by nucleotide sequence (Fig. 5B,C, lane 10).

Thereafter, to ascertain the precise point of divergence between TPα and TPβ mRNAs within their 5′ UTR regions, two additional sense primers, Kin47, corresponding to nucleotides −27 to −11 within E2, and Kin46 corresponding to nucleotides −12 to +5 within E2 were analyzed in combination with TPα (Kin75) and TPβ (Kin21) specific antisense primers (Fig. 5A). RT-PCR analysis using the sense primers Kin46 or Kin47 vs. the TPα specific antisense primer Kin75 resulted in the amplification of TPα specific products of 1134 bp and 1150 bp, respectively (Fig. 5B, lane 1 & 3) that were confirmed by Southern blot analysis (Fig. 5C, lane 1 & 3) and nucleotide sequence analysis (data not shown) to represent TPα specific sequences. Using the TPβ specific primer, Kin21, while RT-PCR analysis using the primer Kin47 did not result in the amplification of the predicted TPβ specific product of 1019 bp (Fig. 5B,C, lane 4), RT-PCR analysis using the primer Kin46 did result in the amplification of a TPβ specific product of 1003 bp (Fig. 5B, lane 2) that was confirmed by Southern blot analysis (Fig. 5C, lane 2) and by nucleotide sequence analysis (data not shown) to represent TPβ specific sequences. Similar findings were also shown to occur in TM-1 trophoblast cells and in vascular smooth muscle (data not shown), thus ruling out the possibility of tissue specific splicing events. To establish that the primer pairs Kin47/21



B

Transcription Initiation Sites- HEL92.1.7	Region
-92, -95 (2 clones), -96 (3 clones), -100, -102, -111, -115	Exon 1
-99 (2 clones)	Exon 1b

C

Transcription Initiation Sites- TM-1	Region
-94, -96, -114, -95	Exon 1
-99	Exon 1b

Fig. 3. Determination of the transcription initiation sites for the TP gene. (A) Relative positioning of the oligonucleotide primers used for the amplification of the 5′ UTR of TP mRNA transcripts. The TP specific primer Kin60 was used to direct first strand cDNA synthesis. Following the addition of a homopolymeric dC tail to the first strand cDNA using deoxynucleotidyl transferase, primary PCR amplifications were performed using the anchor primer, AAP, in conjunction with the TP-specific primer, Kin2. Thereafter, nested (2°) amplifications were performed with the sense abridged universal anchor primer, AUAP, in combination with the TP-specific antisense primer Kin3. Following subcloning of the secondary amplification products, nucleotide sequence analysis of the amplification products was performed. (B) Location of the major transcription initiation (TI) sites within E1 and E1b using RNA isolated from HEL 92.1.7 cells as a template. (C) Location of the major TI sites detected in trophoblast TM-1 cells. The TI number assigned to each transcript is indicated relative to the translation start site, designated +1, and are consistent with splicing of either E1 (−84) or E1b (−84) to E2 (−83).

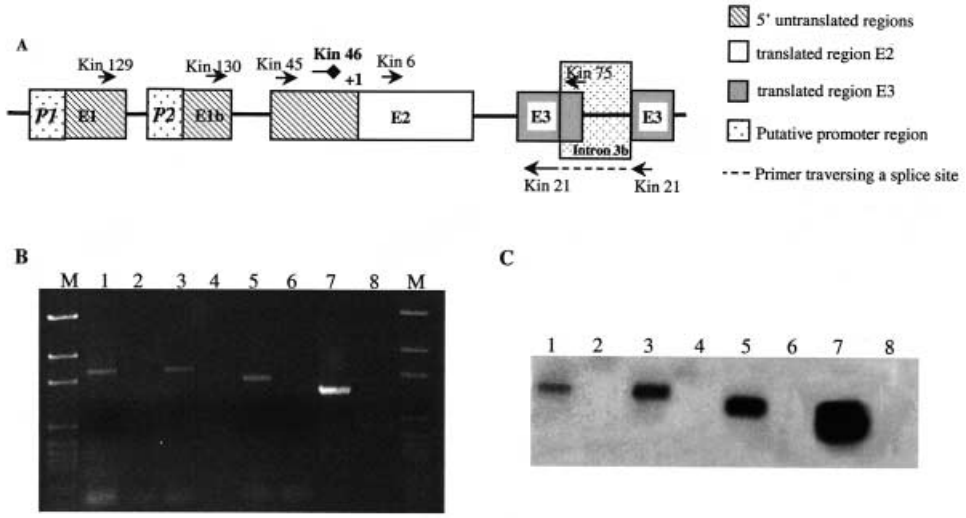


Fig. 4. Analysis of differential 5′ UTR utilization by TP mRNA transcripts. (Panel A) Relative positioning of the oligonucleotides primers (→) and radiolabelled probes (–◆) used to characterize the 5′ UTR of the TPα and TPβ mRNA transcripts. To specifically amplify TPα mRNA transcripts, the antisense primer Kin75 was used in conjunction with either the E1-specific sense primer, Kin129, the E1b-specific sense primer, Kin130 or the E2-specific primer, Kin45. Similarly, to specifically amplify TPβ mRNA transcripts, the TPβ-specific antisense primer Kin21 was used in conjunction with either Kin129, Kin130 or Kin45. (B) Agarose gel electrophoresis of RT-PCR products (7 μL per lane) derived from HEL 92.1.7. First strand cDNA templates: lane 1, Kin129/Kin75 predicted to amplify 1310 bp TPα fragment containing E1 and E2; lane 2, Kin129/Kin21 predicted to amplify 1188 bp TPβ fragment containing E1 & E2; lane 3, Kin130/Kin75 predicted to amplify 1321 bp TPα fragment containing E1b & E2; lane 4, Kin130/Kin21 predicted to amplify 1190 bp TPβ fragment containing E1b & E2; lane 5, Kin45/Kin75 predicted to amplify 1040 bp TPα fragment containing E2; lane 6, Kin45/Kin21, predicted to amplify 899 bp TPβ fragment, containing E2; lane 7, GAPDH, 983 bp product vs. primers *Gf*/*Gr*; lane 8, RT-PCR negative control in the absence of RT for the primer pairs Kin129/Kin75; lane M, pGEM DNA markers. (C) Southern blot analysis of the RT-PCR products (B, lanes 1–8) using a P^{32} radiolabelled TP specific probe, Kin46 and a GAPDH probe, Kin119 (specific for the +552 to +570 region of GAPDH).

were indeed functional, the previously described plasmid construct pBluescript II KS(–):TPβ [4] containing a 1.5 kb *Eco*RI insert encoding the full-length coding sequence (+1 to +1224) for TPβ plus an additional 5′ (212 bp), corresponding to TPα 5′ UTR sequences, and 3′ (69 bp) UTR was used as a positive control. Following PCR analysis

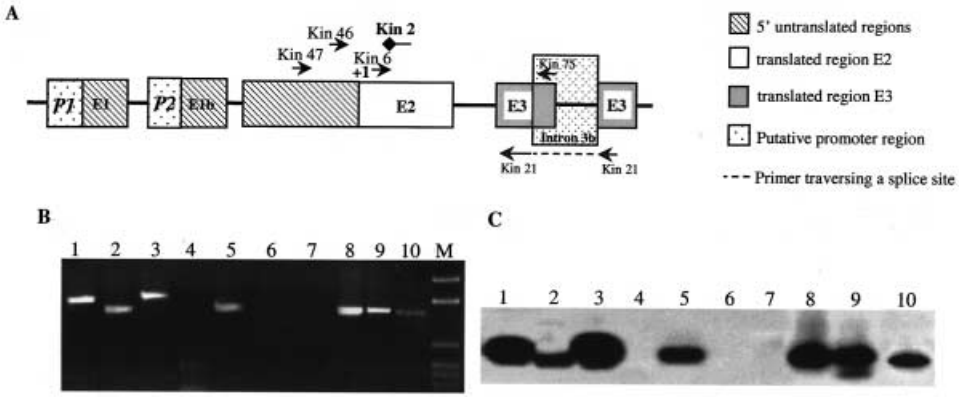


Fig. 5. Analysis of the differential 5′ UTR utilization of the TP mRNA transcripts within exon 2. (A) Relative positioning of the oligonucleotides primers (→) and radiolabelled probes (–◆) used for the analysis of the 5′ UTR of TPα and TPβ mRNAs. (B) Agarose gel electrophoresis of RT-PCR products (7 μL per lane) derived from HEL 92.1.7. First strand cDNA templates (lanes 1–7 and lane 10) and pBluescript II KS(–):TPβ [4] (lanes 8–9): lane 1, Kin46/Kin75 predicted to amplify a 1134 bp fragment; lane 2, Kin46/Kin21 predicted to amplify 1003 bp fragment; lane 3, Kin47/Kin75 predicted to amplify 1150 bp fragment; lane 4, Kin47/Kin21 predicted to amplify 1019 bp fragment; lane 5, GAPDH, 983 bp product vs. primers *Gf*/*Gr*; lanes 6–7, RT-PCR negative controls in the absence of RT in the presence of the primer pairs Kin46/Kin75 and Kin46/Kin21, lane 8, Kin47/21 predicted to amplify a 1019 bp fragment; lane 9, Kin46/21 predicted to amplify 1004 bp fragment, respectively; lane 10, Kin6/21 predicted to amplify 990 bp fragment; lane M, pGEM DNA markers. (C) Southern blot analysis of the RT- PCR products (B, lanes 1–10) using a P^{32} radiolabelled TP specific probe (Kin2) and a GAPDH probe (Kin119).

using the primer pair Kin47/21 or positive control primers Kin46/21, correct size amplification products of 1019 and 1004 bp, respectively, were amplified (Fig. 5, panels C and D, lanes 8 and 9, respectively), thus ruling out the possibility that the primer pair may be nonfunctional.

Taken together these data suggest that in addition to their widely recognized sequence differences due to the presence (TPα mRNA) or absence (TPβ mRNA) of Intron 3b sequences within E3, the TPα and TPβ mRNA sequences also diverge at −12 within E2. Moreover, while E1 and/or E1b sequences in addition to E2 sequences 5′ of −12 are found associated with TPα mRNA transcripts, they are not found associated with TPβ mRNA sequences.

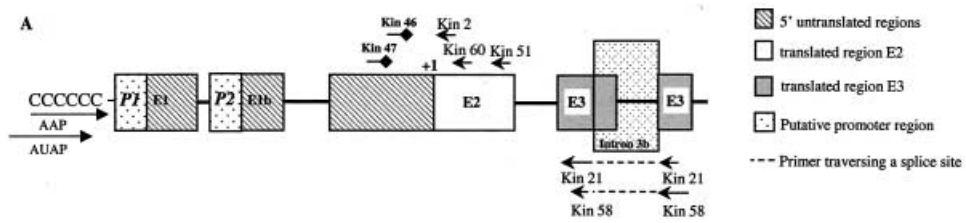
Identification of the TI site of the TPβ mRNA transcript

The sequence divergence of TPβ from TPα mRNAs at −12 within E2 could be explained by either the presence of a TI site for the TPβ mRNA transcript at −12 or due splicing of nucleotides elsewhere in the 5′ flanking region of the TP gene to nucleotides located within the −12 region of TPβ mRNA transcripts, giving rise to a novel 5′ UTR for the TPβ mRNA transcript. Therefore, to identify the TI site of TPβ, two alternative 5′ RACE approaches were employed, as outlined in Fig. 6A. In the first approach, two TPβ specific primers were utilized. Specifically, total HEL 92.1.7 cell RNA was converted to 1 °CDNA using the primer Kin58. Following dC-tailing, primary amplification was performed using the primer AAP in combination with the TPβ-specific antisense primer, Kin21. Nested amplification was then performed using the sense primer AUAP in combination with the TP-specific primer, Kin60 (Fig. 6A). Following subcloning and nucleotide sequence analysis of the 5′ RACE products, a number of independent TPβ specific transcripts with a TI site at −12 were identified (Fig. 6B). No transcripts with TP sequences 5′ of −12 were identified. However, a number of transcripts containing TI sites within the coding region of E2 were also noted; these transcripts are most likely the result of incomplete reverse transcription as a consequence of the large distance, ∼1000 bp, between the first strand cDNA primer, Kin58 and the putative 5′ terminus of the TPβ mRNA transcript (data not shown).

Thereafter, employing a second 5′ RACE approach to confirm our data and also to eliminate the problem of premature termination of first strand cDNA synthesis, nested amplification products were subjected to differential hybridization using the primers Kin46 and Kin47, corresponding to sequences 3′ and 5′ of the −12 divergent region within TPα/TPβ mRNAs, respectively, as discriminatory hybridization probes (Fig. 6A). Following nucleotide sequence analysis of $Kin46^{+}Kin47^{-}$transcripts, a number of independent clones with TI sites at −12 (two clones) and −15 (three clones) were identified (Fig. 6B). No transcripts with TP sequences 5′ of −12 were identified. These results demonstrate that whereas the TI site of TPα occurs within E1 and/or E1b, the TI site of TPβ occurs at −15 to −12. As neither E1 nor E1b were present on the TPβ mRNA transcript, this also suggests that neither P1 nor P2 are regulating the expression of TPβ. Moreover, the identification of a TI site for the TPβ mRNA transcript occurring at −12 to −15 within E2 suggests that TPβ mRNA expression may be regulated by a novel putative promoter, arbitrarily termed P3, most likely located 5′ of −12.

Functional analysis of the 5′ flanking region of the TPβ mRNA transcript

Thereafter, we sought to assess the ability of a gene fragment encoding the nucleotides −1394 to −1, immediately flanking the divergent −12 region between TPα/TPβ transcripts, to direct the basal transcription of luciferase activity in genetic reporter assays. As comparative controls, gene fragments encoding the previously described P1, 5′ of



B

Transcription Initiation sites	Region
-12 (2 clones) -15 (3 clones)	Exon 2

Fig. 6. Identification of the transcription initiation (TI) sites of the TPβ transcripts using 5′ RACE analysis. (A) Relative positioning of the oligonucleotides (→) used for 5′ RACE amplification and radiolabelled probes (–◆) used for Southern blot analysis. The TPβ-specific primer Kin58 (spanning across the splice site) was used to direct first strand cDNA synthesis using RNA from HEL 92.1.7 cells as template. Following the addition of a homopolymeric dC tail to the first strand cDNA template, primary PCR amplifications were performed using the AAP primer in conjunction with the TPβ-specific primer, Kin21. Thereafter, secondary amplifications were performed with AUAP in combination with the TP-specific antisense primer Kin60. Following subcloning of the secondary amplification products, nucleotide sequence analysis of the amplification products was performed to identify the major TI sites (B). As an alternative, the TP primer Kin51 was used to direct first strand cDNA synthesis. Following the addition of a homopolymeric dC tail to the first strand cDNA template, primary PCR amplifications were performed using the AAP primer in conjunction with the TP-specific primer, Kin60. Thereafter, secondary amplifications were performed with AUAP in combination with the TP-specific primer Kin2. Using the latter approach, a library of secondary amplification products was generated and screened with P^{32} labelled Kin46 and Kin47 probes. (B) TI sites of Kin46$^+$, Kin47$^-$ clones following nucleotide sequence analysis.

E1 [6] and P2, 5′ of E1b [4] were also assessed for their ability to direct transcription and expression of luciferase (Fig. 7A). Additionally, as negative controls, the promoterless pGL3b and pGL3e vectors were assessed for their ability to direct transcription and expression of luciferase. Routinely, 0.09 ± 0.01 and 0.31 ± 0.10 RLU were obtained for the empty pGL3b and pGL3e vectors, respectively. The plasmid pGL3b:Prm1 directed high level of luciferase activity in both HEL and HEK 293 cells (Fig. 7B) consistent with high basal transcription from P1 sequences in both cell types. Additionally, the presence of the SV40 enhancer sequence located downstream of the luciferase gene in the recombinant plasmid pGL3e:Prm1 greatly increased the level of basal luciferase transcription under the control of P1 in both HEL 92.1.7 and HEK 293 cells (Fig. 7B). It was noteworthy that while the level of basal P1 transcriptional activity was significantly lower in HEK 293 cells than in HEL 92.1.7 cells ($P = 0.0035$) when expressed in pGL3b:Prm1, this effect was negated when P3 was expressed in pGL3e:Prm1 (Fig. 7B).

The ability of P2 to direct basal transcription of the luciferase gene was also investigated. Transfection of the plasmid pGL3b:Prm2 directed low levels of luciferase expression in both HEL cells or in HEK 293 cells (Fig. 7C), consistent with the low basal transcriptional activity associated with P2. However, in the presence of the SV40 enhancer, the recombinant plasmid pGL3e:Prm2 did mediate significant increases in basal luciferase expression confirming that P2 may indeed function as a promoter in both HEL 92.1.7 cells and also in HEK 293 cells (Fig. 7C), albeit with substantially weaker activity than P1 in both cell types (Fig. 7B). While there was no difference in the level of basal P2-directed transcription when expressed in pGL3b:Prm2, the level of P2 activity in HEK 293 cells was significantly lower than in HEL cells when P2 was expressed in pGL3e:Prm2 ($P = 0.0166$).

Thereafter, we examined the ability of the gene fragment (−1394 to +1) encoding a possible putative promoter, arbitrarily termed P3, located immediately 5′ of the translational start site/divergent −12 region between TPα and TPβ to direct expression of luciferase activity. Transfection of the plasmid pGL3b:Prm3 into both HEL 92.1.7 cells and HEK 293 cells did lead to significant increases in the basal expression of luciferase activity in both cell types (Fig. 7D). Moreover, in the presence of the SV40 enhancer, transfection of the plasmid pGL3e:Prm3 did result in substantial increases in luciferase expression in both HEL cells and HEK 293 cells (Fig. 7D). It was noteworthy that there were no significant cell-dependent differences in the basal transcriptional activity of P3 between either HEL 92.1.7 or HEK 293 cells when expressed in either pGL3b:Prm3 or in pGL3e:Prm3. Taken together, these data not only confirm the functional activity associated with the well characterized P1, but also indeed confirm activity associated with P2 in both HEL 92.1.7 and HEK 293 cells. Moreover, they strongly support the existence of a novel promoter, P3, immediately 5′ of the translational start site and, in view of our previous data presented herein, suggest that P3 may direct expression of TPβ.

Effect of PMA on TP mRNA expression

Thereafter, in order to identify regions that may be important for promoter activity, the nucleotide sequence encoding the P3 gene fragment (spanning −1394 to +1) was analyzed for both transcriptional and regulatory sequence elements using the Matinspector Professional^{TM} bioinformatics programme [23]. P3 sequences were found to lack typical TATA or CAAT boxes (Fig. 8), similar to that

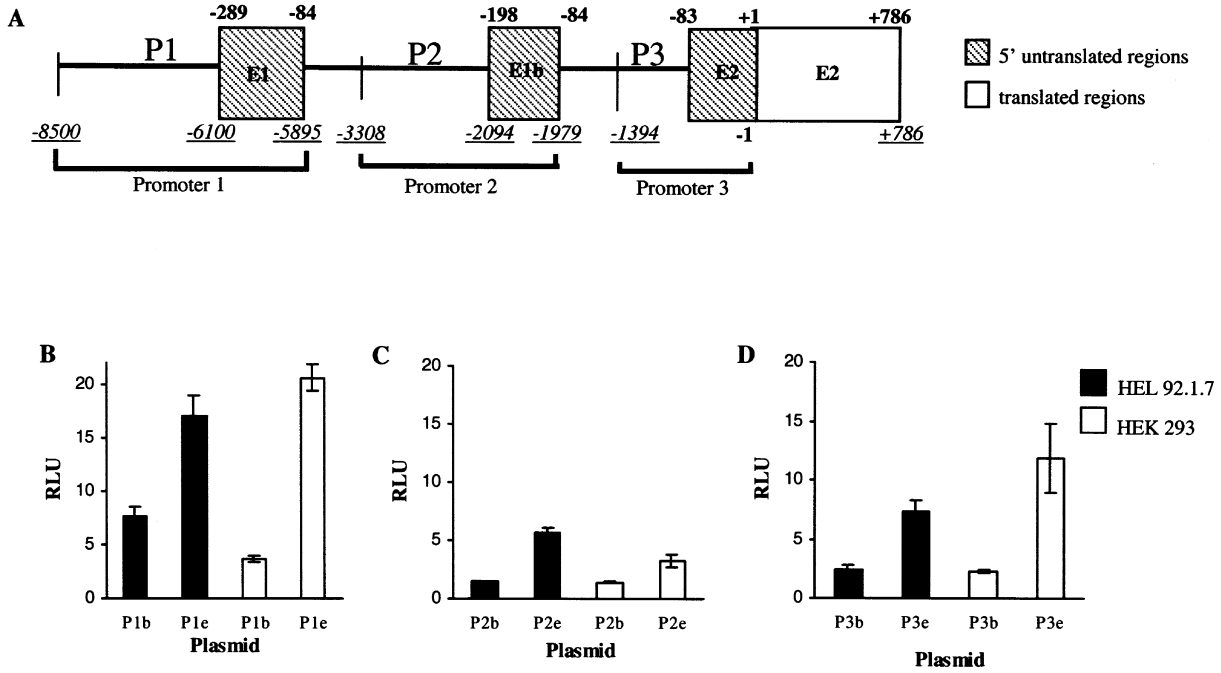


Fig. 7. Functional analysis of the 5′ flanking region of the TP receptor gene. (A) Schematic of the TP genomic region spanning nucleotides −8500 to +786, encoding promoter P1, P2 and P3 in addition to E1, E1b and E2 sequences and intronic sequences. The lower and upper coordinates are used to compare the genomic position of E1, E1b and E2 with their location within the TP transcripts, respectively. Three major gene fragments encoding Promoters 1 (−8500 to −5895), Promoter 2 (−3308 to −1979) and Promoter 3 (−1394 to −1) sequences were subcloned into the luciferase reporter vectors pGL3 Basic (pGL3b) and pGL3 Enhancer (pGL3e) to generate the recombinant plasmids pGL3b:Prm1, pGL3b:Prm2, pGL3b:Prm3, pGL3e:Prm1, pGL3e:Prm2 and pGL3e:Prm3. (B) The plasmids pGL3b:Prm1 (P1b), pGL3e:Prm1 (P1e), encoding firefly luciferase under the control of P1, were cotransfected with pRL TK into HEL 92.1.7 cells and HEK 293 cells. (C) The plasmids pGL3b:Prm2 (P2b), pGL3e:Prm2 (P2e), encoding firefly luciferase under the control of P2 were cotransfected with pRL TK into HEL 92.1.7 cells and HEK 293 cells. (D) The plasmids pGL3b:Prm3 (P3b), pGL3e:Prm3 (P3e), encoding firefly luciferase under the control of P3 were cotransfected with pRL TK into HEL 92.1.7 cells and HEK 293 cells. Thereafer, firefly and renilla luciferase activity was assayed and results are expressed as mean relative luciferase activity, in arbitrary units (RLU), ± SEM ($n = 6$).

previously identified for P1 and P2 sequences [4]. Consensus sequences for serum response element binding protein (SREB) and Oct-1 transcription factor binding sites were identified at −675 and −107, respectively (Fig. 8). Additionally, a number of consensus sequences were predicted for phorbol myristic acid (PMA)-responsive transcription factors, including an AP-4 site located at −1005, two AP-1 sites at −236 and −27 and an SP-1 site at −670 (Fig. 8).

Previous studies have established that exposure of HEL 92.1.7 cells to PMA resulted in an increase in TP mRNA expression and yielded an overall increase in TP protein expression [5], possibly mediated through a phorbol response element located within P1, but not in P2. D'Angelo *et al.* [7] later proposed that the major PMA response element within the TP gene is actually located within a *Sp*1 site 5′ to E1. However, these studies did not discriminate between TPα and/or TPβ mRNA expression. Thus, to investigate whether P3 transcriptional activity may be responsive to PMA treatment, we investigated the effect of PMA stimulation in HEL 92.1.7 cells transiently transfected with pGL3b:Prm3/pGL3e:Prm3 comparing it to that which occurred with corresponding reporter vectors encoding the well characterized PMA-responsive P1 [7] and the non-PMA responsive P2 (Fig. 9A–C). Pretreatment of HEL 92.1.7 cells with PMA resulted in 1.7- and 1.6-fold augmentations in luciferase expression in cells transfected with pGL3b:Prm3 and pGL3e:Prm3, respectively (Fig. 9C; $P \leq 0.05$). As a positive control, pretreatment of HEL 92.1.7 cells with PMA resulted in a 2.75- and 2.66-fold augmentation in cells transfected with pGL3b:Prm1 and pGL3e:Prm1, respectively (Fig. 9A; $P \leq 0.003$). In contrast, pretreatment of HEL 92.1.7 cells transfected with either pGL3b:Prm2 and pGL3e:Prm2 with PMA did not alter P2-mediated luciferase activity relative to vehicle-treated cells (Fig. 9B). As an additional control, the effect of PMA on the ability of the promoterless pGL3b and pGL3e vectors to direct transcription and expression of luciferase was assessed. Routinely, 0.10 ± 0.02 and 0.29 ± 0.10 RLU were obtained for the empty pGL3b and pGL3e vectors, respectively, in the presence of PMA. When compared to non-PMA treated vectors, no significant alteration in basal RLU levels was detected ($P > 0.70$).

To establish whether PMA has an effect on TPβ mRNA expression levels, a previously described semiquantitative RT-PCR based approach was utilized [17], whereby TPα mRNA levels were used as a reference control [5]. RT-PCR analysis was performed using a common TP sense primer (Kin16) in combination with discriminatory TPα (Kin75) and TPβ (Kin21) based antisense primers, essentially as previously described [17]. Correlating with previous findings [17], both TPα and TPβ mRNAs were expressed in HEL 92.1.7 cells (Fig. 9D). Exposure of HEL 92.1.7 cells to PMA

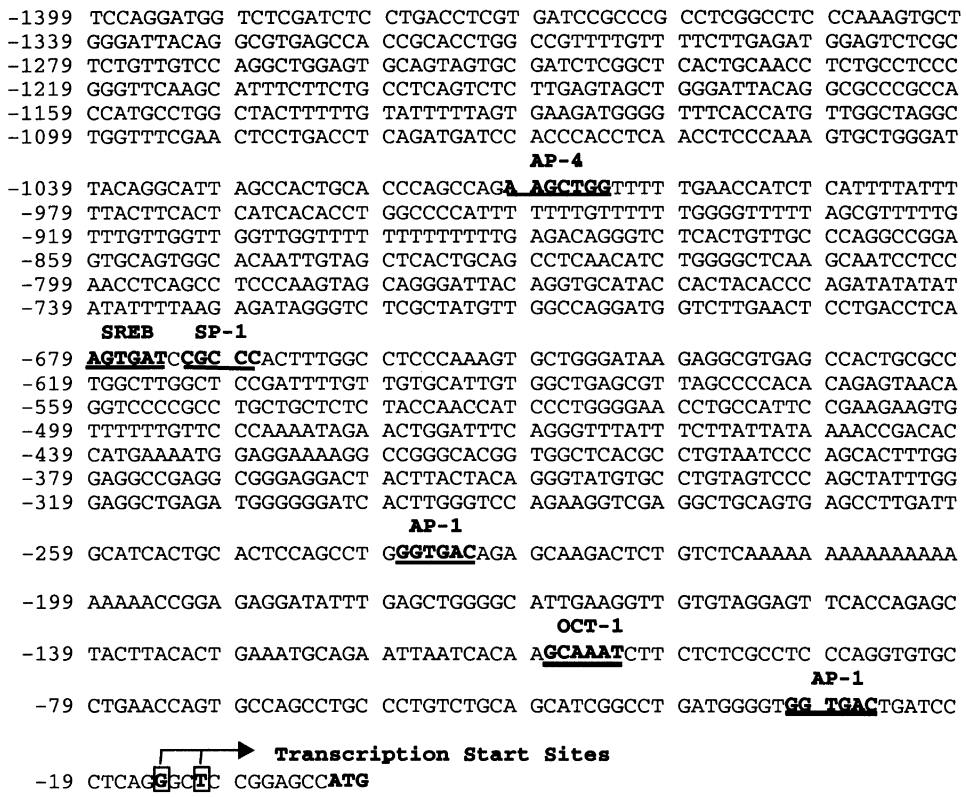


Fig. 8. Bioinformatic analysis of the genomic sequence containing Promoter 3 and the 5′ UTR within E2. Bioinformatic analysis of the genomic sequence of the TP gene, spanning from +1 to −1399, encoding the promoter 3 gene fragment, was performed as described under Experimental procedures. Using Matinspector Professional™, bioinformatics programme [23], no typical TATA or CAAT boxes were predicted. A number of consensus transcription factor binding sites were predicted, namely an AP-4 site located at −1005, two AP-1 sites located at −236 and −27, a SP-1 site located at −670, a serum response element binding protein (SREB) located at −675 and an Oct-1 transcription factor binding site predicted at −107. The arrow indicates the position of the transcription initiation (TI) sites of the TPβ mRNA transcript as predicted by 5′ RACE analysis where the translation start site, designated +1, is highlighted in bold.

(100 nM; 6 h) resulted in a 1.41-fold increase in TPβ mRNA expression when compared to vehicle-treated HEL 92.1.7 cells (TPβ/GAPDH densitometric ratios of 0.29 ± 0.07 and 0.41 ± 0.07 in normal and PMA-treated HEL 92.1.7 cells, respectively). Expression of PMA-responsive control TPα mRNA expression was increased by 1.3-fold compared to control vehicle-treated HEL 92.1.7 cells (TPα/GAPDH densitometric ratios of 0.52 ± 0.10 and 0.67 ± 0.06 in control non-PMA treated and PMA-treated HEL 92.1.7 cells, respectively). Taken together, these data suggest that a novel promoter region, termed P3, exists 5′ of the TI site of TPβ, within intron 1. Bioinformatic analysis suggests the presence of key signature transcription factor binding site, including a number of potential PMA-responsive elements, such as AP-1, AP-4 and SP-1 transcription factor binding sites. Genetic reporter assay confirm that P3 can direct basal expression of luciferase activity in HEL and in HEK 293 cells and that P3 is responsive to PMA-treatment in HEL cells. Coincident with this, expression of TPβ mRNA is up-regulated in response to PMA treatment.

DISCUSSION

Whereas the main physiologic actions attributed to TXA_2 is its role in vascular hemostasis [24], it can induce diverse cellular responses including constriction of vascular and bronchial smooth muscle cells [25], potentiation of the mitogenic and hypertrophic growth of vascular smooth muscle cells [26–28], contraction of glomerular mesangial cells and intrarenal vascular tissue decreasing glomerular filtration rates [29], stimulation of apoptosis of immature thymocytes [30]. TXA_2 has been implicated as a mediator of a number of vascular disorders including thrombosis, unstable angina, myocardial infarction, pregnancy-induced hypertension, bronchial asthma and glomerulonephritis [1,29,29,30]. Patients with acute myocardial infarction have increased numbers of platelet TXA_2 receptors (TPs) and receptor number is greatest in patients with prolonged ischemic chest pain [33]. Mice deficient in TXA_2 receptors display mild bleeding dysfunction and exhibit diminished responses to vascular stimuli [34]. Moreover, there is substantial evidence that TXA_2 may mediate the actions of the isoprostane 8-epi prostaglandin (PG) $F_{2\alpha}$, generated in abundance in situations of oxidative stress [35,36].

In humans, but not in other species thus far investigated, TXA_2 signals through two TP isoforms, TPα and TPβ [2,3], that arise by differential splicing [3,4]. Whereas both TPα and TPβ display indistinguishable coupling to phospholipase (PL) Cβ members, the main signalling pathway of TP [1,11,12], they oppositely regulate adenylyl cyclase activity [8] and TPα, but not TPβ, mediates-agonist activation of Gh leading to PLC activation [9]. Additionally, TPβ, but not

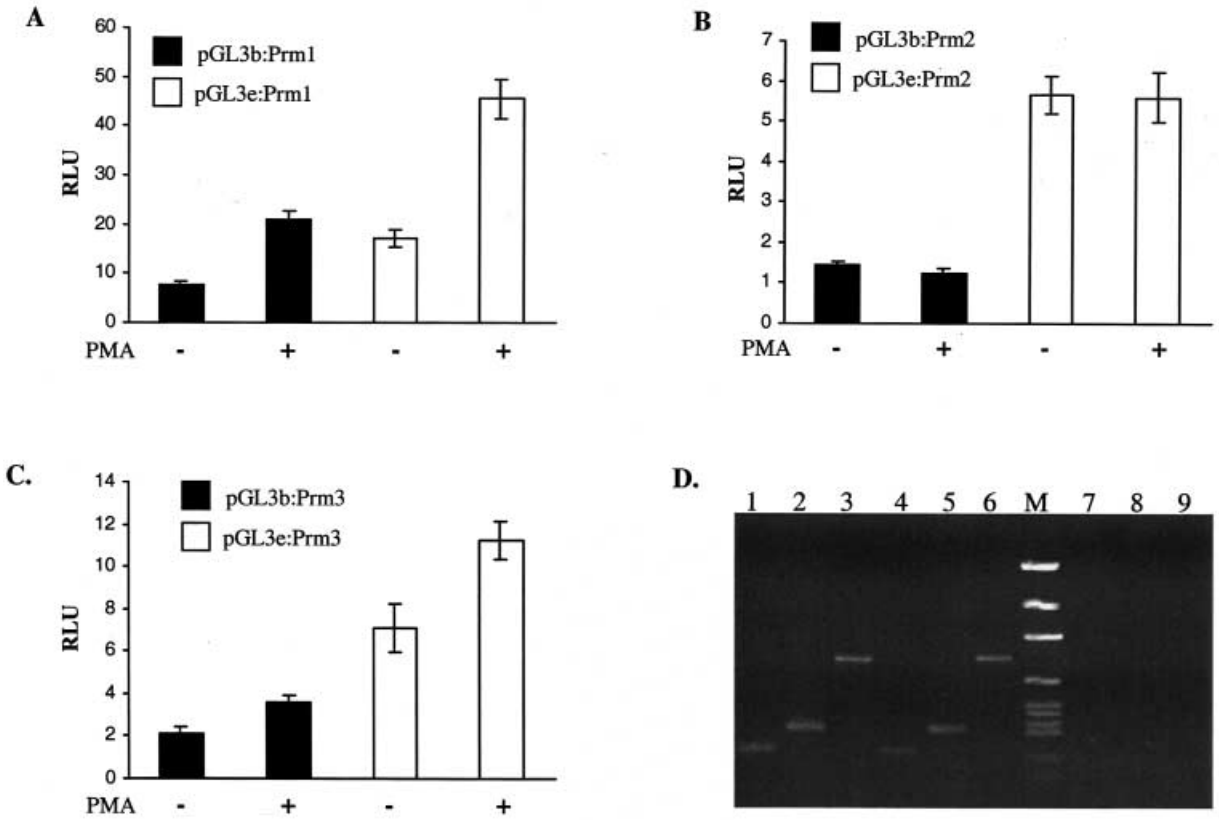


Fig. 9. Effect of PMA on promoter 3 activity and TPβ mRNA expression levels. pGL3b:Prm1 or pGL3e:Prm1 (A), pGL3b:Prm2 or pGL3e:Prm2 (B) pGL3b:Prm3, or pGL3e:Prm3 (C) were transfected into HEL 92.1.7 cells. After 36 h, the cells were treated with PMA (100 nM; 12 h) with vehicle/nonstimulated cells serving as a control. Thereafter, firefly and renilla luciferase activity was assayed and results are expressed as mean relative luciferase activity, in arbitrary units (RLU) ± SEM ($n = 6$). (D) As a complementary approach, total RNA was isolated from HEL 92.1.7 cells treated with PMA (100 nM; 6 h), with vehicle only treated cells serving as a control. Following first strand cDNA synthesis, PCR amplifications were performed using the TP sense primer Kin16 in combination with the TPα specific antisense primer Kin75 or in combination with the TPβ-specific antisense primer, Kin21. As a positive control, amplification of GAPDH was performed using the primer pair G*f*/G*r*, as previously described. (D) Agarose gel electrophoresis of RT-PCR products derived from first strand cDNA templates; lane 1, TP_β, 269 bp product vs. primers Kin16/21 derived from PMA treated cells; lane 2, TPα, 400 bp product vs. primers Kin16/Kin75 derived from PMA treated cells; lane 3, GAPDH, 983 bp product vs. primers G*f*/G*r* derived from PMA treated cells; lane 4, TP_β, 269 bp product vs. primers Kin16/Kin21 derived from control cells; lane 5, TPα, 400 bp product vs. primers Kin16/Kin75 derived from control cells; lane 6, GAPDH, 983 bp product vs. primers G*f*/G*r* derived from control cells; lanes 7–9, negative control reactions performed in the absence of template first strand cDNA in the presence of the primer pairs Kin16/Kin21, Kin16/Kin75 and G*f*/G*r*, respectively; lane M, pGEM DNA markers.

TPα, is subject to agonist induced internalization leading to its down-regulation following prolonged exposure to the TP agonist, U46619 [15,16]. Both TP isoforms mediate activation of the extracellular signal regulated protein kinase and stress activated protein kinase families of the mitogen activated protein kinase cascades in response to U46619 and the isoprostane 8-epi $PGF_{2\alpha}$ [19,37]. In studies investigating cross-talk between TXA_2 and other prostanoids, we have established that signalling by TPα, but not TPβ, is subject to prostacyclin and PGD_2-mediated desensitization involving direct protein kinase A phosphorylation of TPα within its unique C-tail [12,13]. These studies imply an essential role for TPα in vascular hemostasis and, possibly, a redundant role of TPβ in this essential process. In addition, both TPα and TPβ are subject to protein kinase C-mediated desensitization by the EP_1 isoform of the PGE_2 receptor family; however, TPα is significantly more sensitive than TPβ to this desensitization [14].

Thus, while the biologic relevance for the existence of two TP receptors in humans is unknown, these data provide compelling evidence that the individual TP isoforms have distinct physiologic/pathophysiologic roles. Consistent with this view, it appears that the TPα and TPβ are also subject to differential expression [17,18]. The molecular basis of this differential TP expression is unknown but suggests that the TP receptors may not only be the products of differential splicing but are also subject to differential gene regulation. The presence of two promoters, P1 and P2, on the single TP gene raises the possibility that the TPα and TPβ isoforms may be under the transcriptional control of distinct promoters [4].

The structure of the human TP gene was first elucidated by Nüsing *et al.* [4] and was proposed to contain three major exons, E1, E2 and E3 with an additional 115 bp exon, designated E1b, identified between E1 and E2 within intron (I) 1 (Fig. 1A). The two individual promoters P1 and P2

identified 5′ of E1 and E1b sequences, respectively, raised the possibility for alternative promoter utilization within the TP gene. Nüsing *et al.* [4] proposed that the major TP mRNA transcripts contain E1, E2 and E3 sequences; however, in a minority of cases, transcripts containing either E1b, E2 and E3 or E1, E1b, E2 and E3 sequences were identified [4]. Additionally, multiple transcription initiation (TI) sites were identified for the human TP gene. Specifically, employing primer extension analysis, a major TI site was postulated at −289 within E1 in placental tissue [4]. In contrast, multiple TI sites clustered around −140, −161 and −173, within E1, were identified in placental tissue and MEG-01 cells [4]. Additionally, in MEG-01 cells, TI sites were also identified at −158 and −186, within E1b, and at −69 and −79, within E2 [4]. Moreover, in the human platelet-like K562 cell line and in human uterine tissue, transcription initiation sites were identified at −123 within E1 [6] and more recently, D'Angelo *et al.* [7] proposed that the major TI site for the human TP actually occurs at −553, upstream from the major TI, located at −289, previously identified by Nüsing *et al.* [4]. Thus, in view of the apparent discrepancies in the patterns of exon usage and the presence of multiple TI sites, the initial aim of this study was to characterize the major TP mRNA transcripts in HEL 92.1.7 cells and in trophoblast TM-1 cells and, thereafter, to establish the molecular basis of the differential expression of TPα and TPβ.

We initially sought to characterize the major TP transcripts, with respect to their possible divergent 5′ UTR sequences, and to map the TI sites therein. Using an RT-PCR approach, it was established that while E1 and E1b were found associated with TP transcripts in both HEL and TM-1 cells, their presence were mutually exclusive and transcripts containing both E1 along with E1b, as postulated by Nüsing *et al.* [4], were not identified. The exclusive nature of E1 and E1b expression suggests that the putative promoter P1 and P2 regions 5′ of E1 and E1b, identified by Nüsing *et al.* [4], may independently control the expression of transcripts containing E1 and E1b, respectively. Of course, our data cannot exclude the possibility that initiation of transcription at one promoter (e.g. P1) may serve to repress transcription at the other promoter (e.g. P2) and *vice versa*, accounting for the exclusive nature of E1 and E1b on the TP transcripts. Additionally, through 5′ RACE, the major TI sites of TP mRNAs identified in both HEL and TM-1 cells were found clustered between −115 and −92 within E1 and at −99 within E1b. The potential physiologic significance of mRNAs that differ within the 5′ UTR is currently unknown but may be related to the tissue dependent expression of the TPs.

Thereafter, to ascertain whether the TP isoforms may be subject to alternative promoter utilization, we sought to identify the 5′ UTR sequences associated with the individual TPα and TPβ mRNAs and to identify to the major TI sites associated with those isoform specific transcripts. Detailed RT-PCR analyses established that while both E1 or E1b sequences were associated with TPα transcripts, these sequences were not found associated with TPβ mRNA transcripts. More specifically, it was established, in addition to their defining differences within E3, that TPα and TPβ mRNA sequences also diverged at nucleotide −12 within E2, suggesting that sequences surrounding the −12 region may represent a novel splice acceptor site or, alternatively, may actually correspond to the site of TI of the TPβ mRNA. Thereafter, through a number of independent approaches employing 5′ RACE, it was established that the major TI of the TPβ transcripts in both HEL and TM-1 cells mapped to the −12/−15 region. Moreover, no additional TPβ transcripts were identified with sequences 5′ of −12/−15 suggesting that TPβ mRNA expression may be regulated by a novel promoter, P3, most likely located immediately 5′ of the −12 region, rather than the previously identified P1 or P2.

Consistent with this, through genetic reporter assays, it was established that a 1.39-kB DNA fragment corresponding to sequences immediately flanking the −12 region could direct the efficient basal transcription of luciferase in both HEL 92.1.7 and HEK 293 cells. Functional transcriptional activity was also confirmed for P1 and for P2 in both HEL 92.1.7 cells and in HEK 293 cells, consistent with previous findings by Nüsing *et al.* [4]. While P3 directed weaker basal expression of luciferase relative to the P1, it was substantially stronger than P2 in both HEL and HEK 293 cells. It was noteworthy that we observed no significant cell-dependent differences in the basal transcriptional activity of P3 between either HEL or HEK 293 cells; however, while the level of basal P1 transcriptional activity was significantly lower in HEK 293 cells than in HEL cells ($P = 0.0035$) when expressed in the pGL3basic vector (pGL3b:Prm1), this effect was negated when P3 was expressed in the pGL3enhancer vector (pGL3e:Prm1). Confirmation of functional transcriptional activity for P1 in the megakaryocyte HEL 92.1.7 cell line is consistent with the studies of D'Angelo *et al.* [6] in the megakaryocyte K562 cell line; however, they failed to detect functional activity for P1 in the fibroblast HEK 293 cells and failed to detect any functional activity for P2 in either K562 or HEK 293 cells. The most likely explanation for the apparent discrepancies between our studies and those reported by D'Angelo *et al.* [6] may be due to differences in the relative sensitivities of the genetic reporter assays used; while we employed the relatively sensitive, entirely quantitative, luciferase based assay, those studies by D'Angelo *et al.* [6] were based on the qualitative, relatively insensitive, chloramphenicol-based assay system. Thus, we have identified a novel promoter P3 immediately 5′ of the −12 divergent region that, from studies reported herein, most likely directs expression of TPβ.

Bioinformatic analyses of a 1.39-kb DNA fragment encoding P3 revealed that, similar to that previously established for P1 and P2 [4], it did not contain TATA or CAAT boxes or a consensus initiator element found on many common promoter elements [38,39]. It is widely recognized that the TATA box and initiator elements have an important role in directing transcription initiation from a specific site [40] and, thus, it appears that TATA-less genes contain multiple TI sites, consistent with that found for the TP gene through earlier reports [4,6,7] and through studies reported herein. Analysis of P3 sequences also identified the presence of a number of signature transcription factor binding sites including those for SREB and Oct-1 in addition to a number of sites that may be regulated by phorbol esters/protein kinase C signalling including sites for SP-1 AP-1 and AP-4. In the current study, we established that treatment of HEL cells with PMA led to significant increases in P3-directed luciferase expression and also led to

significant increases in TPβ mRNA expression. Consistent with that found by D'Angelo *et al.* [7], transcriptional activity of P1, but not P2, was also up-regulated by PMA in HEL cells. The importance of an SP-1 site in P1 has been highlighted by D'Angelo *et al.* [7] who reported that PMA up-regulation of P1 is mediated through an SP-1 site located at a site corresponding to the −8273 to −8261 region upstream of the +1 (ATG translational start site) within the TP gene. While, in the current study, we have indeed established that PMA up-regulates P3 transcriptional activity, further detailed molecular investigations are required to identify those specific transcription factor site(s) that mediate(s) this effect; moreover, additional detailed investigations are required to define the extent of P3 required for full promoter activity and to establish the importance of other transcription factor binding sites in directing P3 transcriptional activity.

Thus, through studies reported herein, it is evident that in addition to their established differences in E3, due to the presence (TPα) or absence (TPβ) of intron 3b sequences, the TPα and TPβ transcripts also differ extensively in their 5′ UTR sequences and are subject to differential regulation through the transcriptional activity of P1/P2 and P3, respectively. It is also evident that the 5′ UTR of TPβ (12–15 bp) is appreciably shorter than that determined by us and other investigators for TPα [4,6,7]. While it has been reported that the optimum 5′ UTR length for ribosomal binding is 40–50 bp, sequences of 12–15 bp, such as that found in the TPβ mRNA, are sufficient for ribosomal binding and to support efficient translation [41]. There are a number of genetic precedences whereby protein/receptor isoforms arise through differential splicing, being products of the same gene, but may be under the transcriptional control of separate or distinct promoters. Similar to the TP, the human progesterone receptor (PR) exists as two isoforms, referred to as PR-A and PR-B and, whilst they arise through differential splicing, they are transcribed from two distinct promoters within the single PR gene [42,43]. *In vivo*, the two PR isoforms are usually coexpressed in normal tissues, yet the ratio of their expression varies dramatically in different tissues, physiological states, and in disease [43–46]. Similarly, alternative promoter usage by the estrogen receptor (ER) α and ERβ isoforms has been shown to be responsible for the disparate distribution of both receptors [47,48]. In the case of the murine calcitonin receptor, three individual promoters direct the expression of at least seven murine calcitonin receptor isoforms that exhibit differences in their 5′ UTR sequences and in their profiles of tissue expression [49].

In keeping with these diverse examples, there is a growing awareness that transcription and splicing might not be independent events as previously held, but on the contrary, may be highly co-ordinated processes both at the functional and structural levels [50–52]. It is thought that distinct promoters within a given gene may direct the transcription and pattern of splicing events, and indeed 5′ capping and polyadenylation, of distinct transcripts leading to the generation of distinct promoter-driven, differentially spliced mRNA end products. Alternative mRNA splicing is a common feature within a large number of the diverse superfamily of GPCRs, not least among the prostanoid receptor subfamily of GPCRs [53]. The EP_3 subtype of the PGE_2 receptors is one of the most heavily spliced among all of the GPCRs, with eight splice variants or isoforms identified in human that differ exclusively in their C-tail sequences [53]. Similarly, alternative spliced isoforms of the human EP_1 and ovine FP receptors have also been reported, where similar to the TPs, isoform differences are confined to their respective C-tail domains [54,55]. The discovery that the expression of human TPα and TPβ isoforms is controlled by differential promoter utilization, in additional to differential splicing, may have major implications for how the expression of other prostanoid receptor isoforms are regulated. Thus, further investigation of those genes encoding prostanoid receptor isoforms and indeed those genes encoding GPCRs that undergo differential splicing may uncover similar patterns of transcriptional regulation to that identified in the current study for the human TP.

In summary, we have shown that the 5′ UTRs of TPα and TPβ diverge at −12 within E2 and that transcription of TPβ initiates at −12/−15 and is controlled by a novel promoter, P3, located 5′ of E2. Further characterization of the functional elements within P3 will provide a more complete understanding of the factors regulating TPβ expression and, indeed, are likely to shed further insight on the specific role of TPβ and, more specifically, on the physiologic requirement for two distinct TP receptors, namely TPα and TPβ, in human tissues.

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REFERENCES

1. Narumiya, S., Sugimoto, Y. & Ushikubi, F. (1999) Prostanoid Receptors: Structures, properties, and functions. *Physiol. Rev.* **79**, 1193–1226.
2. Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, R., Kageyama, S., Nakanishi, S. & Narumiya, S. (1991) Cloning and expression of the cDNA for a human thromboxane A_2 receptor. *Nature*. **349**, 617–620.
3. Raychowdhury, M.K., Yukawa, M., Collins, L.J., McGrail, S.H., Kent, K.C. & Ware, J.A. (1994) Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A_2 receptor. *J. Biol. Chem.* **269**, 19256–19261.
4. Nüsing, R.M., Hirata, M., Kakizuka, A., Eki, T., Ozawa, K. & Narumiya, S.J. (1993) Characterization and chromosomal mapping of the human thromboxane A_2 receptor gene. *J. Biol. Chem.* **268**, 25253–25259.
5. Kinsella, B.T., O'Mahony, D.J. & FitzGerald, G.A. (1994a) Phosphorylation and regulated expression of the human thromboxane A_2 receptor. *J. Biol. Chem.* **269**, 29914–29919.
6. D'Angelo, D.D., Davis, M.G., Houser, W.A., Eubank, J.J., Ritchie, M.E. & Dorn, G.W. 2nd. (1995) Characterization of 5′ end of human thromboxane receptor gene. Organizational analysis and mapping of protein kinase C – responsive elements regulating expression in platelets. *Circ. Res.* **77**, 466–474.
7. D'Angelo, D.D., Oliver, B.G., Davis, M.G., McCluskey, T.S. & Dorn, G.W. (1996) Novel role for Sp1 in phorbol ester enhancement of human platelet thromboxane receptor gene expression. *J. Biol. Chem.* **271**, 19696–19704.
8. Hirata, T., Ushikubi, F., Kakizuka, A., Okuma, M. & Narumiya, S. (1996) Two thromboxane A_2 receptor isoforms in human platelets – Opposite coupling to adenylyl cyclase with different sensitivity to Arg^{60} to Leu mutation. *J. Clin. Invest.* **97**, 949–956.

9. Vezza, R., Habib, A. & FitzGerald, G.A. (1999) Differential signaling by the thromboxane receptor isoforms via the novel GTP-binding protein, Gh. *J. Biol. Chem.* **274**, 12774–12779.
10. Walsh, M.-T., Foley, J.F. & Kinsella, B.T. (2000) Investigation of the role of the carboxyl terminal tails of the α and β isoforms of the human thromboxane A_2 receptor (TP) in mediating receptor: effector coupling. *Biochem. Biophys. Acta* **1496**, 164–182.
11. Kinsella, B.T. (2001) Thromboxane A_2 signalling in humans: a 'tail' of two receptors. *Biochem. Soc. Transactions* **29**, 641–654.
12. Walsh, M.-T., Foley, J.F. & Kinsella, B.T. (2000) The α, but not the β, isoform of the human thromboxane A2 receptor (TP) is a target for prostacyclin mediated desensitization. *J. Biol. Chem.* **275**, 20412–20423.
13. Foley, J.F., Kelley, L.P. & Kinsella, B.T. (2001) Prostaglandin D(2) receptor-mediated desensitization of the alpha isoform of the human thromboxane A(2) receptor. *Biochem. Pharmacol.* **62**, 229–239.
14. Walsh, M.T. & Kinsella, B.T. (2000) Regulation of the human prostanoid TPalpha and TPbeta receptor isoforms mediated through activation of the EP(1) and IP receptors. *Br. J. Pharmacol.* **131**, 601–609.
15. Parent, J.-L., Labrecrue, P., Orsini, M.J. & Benovic, J.L. (1999) Internalization of the TXA_2 receptor α and β isoforms. *J. Biol. Chem.* **274**, 8941–8948.
16. Parent, J.L., Labrecque, P., Driss Rochdi, M. & Benovic, J.L. (2001) Role of the differentially spliced carboxyl terminus in thromboxane A_2 receptor trafficking: identification of a distinct motif for tonic internalization. *J. Biol. Chem.* **276**, 7079–7085.
17. Miggin, S.M. & Kinsella, B.T. (1998) Expression and tissue distribution of the mRNAs encoding the human thromboxane A_2 receptor (TP) alpha and beta isoforms. *Biochim. Biophys. Acta.* **1425**, 543–559.
18. Habib, A., FitzGerald, G.A. & Maclouf, J. (1999) Phosphorylation of the thromboxane receptor, the predominant isoform expressed in human platelets. *J. Biol. Chem.* **274**, 2645–2651.
19. Miggin, S.M. & Kinsella, B.T. (2001) Thromboxane A_2 receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells. *Biochem. Biophys. Acta* **1539**, 147–162.
20. Papayannopoulou, T., Nakamoto, B., Yokochi, T., Chait, A. & Kannagi, R. (1983) Human erythroleukemia cell line (HEL) undergoes a drastic macrophage-like shift with TPA. *Blood.* **62**, 832–845.
21. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring. Harbor Laboratory Press, Cold Spring Harbor, NY.
22. Kinsella, B.T., O'Mahony, D.J., Lawson, J.A., Pratico, D. & FitzGerald, G.A. (1994b) Cellular activation of thromboxane receptors. *Ann. NY Acad. Sciences* **714**, 270–278.
23. Quandt, K., Frech, K., Karas, H., Wingender, E. & Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**, 4878–4884.
24. Armstrong, R.A. (1996) Platelet prostanoid receptors. *Pharmacol. Ther.* **72**, 171–191.
25. Dorn, G.W.I.I. & Becker, M.W. (1992) Thromboxane A_2 stimulated signal transduction in vascular smooth muscle. *J. Pharmacol. Exp. Ther.* **265**, 447–456.
26. Hanasaki, K., Nakano, K. & Arita, H. (1988) Receptor-mediated mitogenic effect of thromboxane A_2 in vascular smooth muscle cells. *Biochem. Pharmacol.* **40**, 2535–2542.
27. Dorn II, G.W., Becker, M.W. & Davis, M.G. (1992) Dissociation of the contractile and hypertrophic effects of vasoconstrictor prostanoids in vascular smooth muscle. *J. Biol. Chem.* **267**, 24897–24905.
28. Ali, S., Davis, M.G., Becker, M.W. & Dorn, 11, G.W. (1993) Thromboxane A_2 stimulates vascular smooth muscle hypertrophy by up-regulating the synthesis and release of endogenous basic fibroblast growth factor. *J. Biol. Chem.* **268**, 17397–17403.
29. Lianos, E.A. & Bresnahan, B.A. (1999) Effect of thromboxane A_2 inhibition and antagonism on prostaglandin and leukotriene synthesis in glomerular immune injury. *J. Laboratory Clin. Med.* **134**, 478–482.
30. Ushikubi, F., Aiba, Y., Nakamura, K., Namba, T., Hirata, M., Mazda, O., Katsura, Y. & Narumiya, S. (1993) Thromboxane A_2 receptor is highly expressed in mouse immature thymocytes and mediates DNA fragmentation and apoptosis. *J. Exp. Med.* **178**, 1825–1830.
31. Oates, J.A., FitzGerald, G.A., Branch, R.A., Jackson, E.K., Knapp, H.R. & Roberts, L.J. 2nd (1988) Clinical implications of prostaglandin and thromboxane A_2 formation. *N. Engl. J. Med.* **319**, 689–698.
32. Fitzgerald, D.J., Rocki, W., Murray, R., Mayo, G. & FitzGerald, G.A. (1990) Thromboxane A_2 synthesis in pregnancy-induced hypertension. *Lancet* **335**, 751–754.
33. Dorn, G.W., 2nd, Liel, N., Trask, J.L., Mais, D.E., Assey, M.E. & Halushka, P.V. (1990) Increased platelet thromboxane A_2/prostaglandin H_2 receptors in patients with acute myocardial infarction. *Circulation* **81**, 212–218.
34. Thomas, D.W., Mannon, R.B., Mannon, P.J., Latour, A., Oliver, J.A., Hoffman, M., Smithies, O., Koller, B.H. & Coffman, T.M. (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. *J. Clin. Invest.* **102**, 1994–2001.
35. Kinsella, B.T., O'Mahony, D.J. & Fitzgerald, G.A. (1997) The human thromboxane A_2 receptor alpha isoform (TPalpha) functionally couples to the G proteins G_q and G_{11} *in vivo* and is activated by the isoprostane 8-epi prostaglandin F2 alpha. *J. Pharmacol. Exp. Ther.* **281**, 957–964.
36. Janssen, L.J. (2001) Isoprostanes: an overview and putative roles in pulmonary pathophysiology. *Am. J. Physiol. Lung Mol. Physiol.* **280**, L1067– L1082.
37. Miggin, S.M. & Kinsella, B.T. (2002) Regulation of the extracellular signal regulated protein kinase cascades by the α and β isoforms of the human thromboxane A_2 receptor. *Mol. Pharmacol.* **61**, 817–831.
38. Hu, S.L. & Manley, J.L. (1981) DNA sequence required for initiation of transcription *in vitro* from the major late promoter of adenovirus 2. *Proc. Natl Acad. Sci. USA* **78**, 820–824.
39. Tan, S. & Richmond, T.J. (1998) Eukaryotic transcription factors. *Curr. Opin. Struct. Biol.* **8**, 41–48.
40. Smale, S.T. (1997) Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochim. Biophys. Acta* **1351**, 73–88.
41. Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B. & Smale, S.T. (1994) DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol. Cell* **14**, 116–127.
42. Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. & Chambon, P. (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J.* **9**, 1603–1014.
43. Richer, J.K., Jacobsen, B.M., Manning, N.G., Abel, M.G., Wolf, D.M. & Horwitz, K.B. (2002) Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J. Biol. Chem.* **277**, 5209–5218.
44. Boyd-Leinen, P.A., Fournier, D. & Spelsberg, T.C. (1982) Non-functioning progesterone receptors in the developed oviducts from estrogen-withdrawn immature chicks and in aged nonlaying hens. *Endocrinology* **111**, 30–36.
45. Spelsberg, T.C. & Halberg, F. (1980) Circannual rhythms in steroid receptor concentration and nuclear binding in the chick oviduct. *Endocrinology* **107**, 1234–1244.

46. Kato, J., Hirata, S., Nozawa, A. & Mouri, N. (1993) The ontogeny of gene expression of progestin receptors in the female rat brain. *J. Steroid Biochem. Mol. Biol.* **47**, 173–182.
47. Donaghue, C., Westley, B.R. & May, F.E. (1999) Selective promoter usage of the human estrogen receptor-alpha gene and its regulation by estrogen. *Mol. Endocrinol.* **13**, 1934–1950.
48. Li, L.C., Yeh, C.C., Nojima, D. & Dahiya, R. (2000) Cloning and characterization of human estrogen receptor beta promoter. *Biochem. Biophys. Res. Commun.* **275**, 682–689.
49. Anusaksathien, O., Laplace, C., Li, X., Ren, Y., Peng, L., Goldring, S.R. & Galson, D.L. (2001) Tissue-specific and ubiquitous promoters direct the expression of alternatively spliced transcripts from the calcitonin receptor gene. *J. Biol. Chem.* **276**, 22663–22674.
50. Xing, Y., Johnson, C.V., Dobner, P.R. & Lawrence, J.B. (1993) Higher level organization of individual gene transcription and RNA splicing. *Science* **259**, 1326–1330.
51. Cramer, P., Pesce, C.G., Baralle, F.E. & Kornblihtt, A.R. (1997) Functional association between promoter structure and transcript alternative splicing. *Proc. Natl Acad. Sci. USA* **94**, 11456–11460.
52. Caceres, J.F. & Kornblihtt, A.R. (2002) Alternative splicing: multiple control mechanisms and involvement in human diseases. *Trends Genet.* **18**, 186–193.
53. Kilpatrick, G.J., Dautzenberg, F.M., Martin, G.R. & Eglen, R.M. (1999) 7TM receptors: the splicing on the cake. *Trends Pharmacol. Sci.* **20**, 294–301.
54. Okuda-Ashitaka, E., Sakamoto, K., Ezashi, T., Miwa, K., Ito, S. & Hayaishi, O. (1996) Suppression of prostaglandin E receptor signaling by the variant form of EP1 subtype. *J. Biol. Chem.* **271**, 31255–31261.
55. Anthony, T.L., Pierce, K.L., Stamer, W.D. & Regan, J.W. (1998) Prostaglandin F2 alpha receptors in the human trabecular meshwork. *Invest. Ophthalmol. Vis. Sci.* **39**, 315–321.