Tight Control of Platelet-derived Growth Factor B/c-sis Expression by Interplay between the 5′-Untranslated Region Sequence and the Major Upstream Promoter*

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The long and GC-rich 5′-untranslated region (5′-UTR) of the known 3.8-kb platelet-derived growth factor B (PDGF-B)/c-sis mRNA is highly conserved and inhibits its own translation. It has been thought that this 5′-UTR functions by regulating translation possibly using an internal ribosome entry site (IRES)-mediated mechanism. However, in the present study we found no evidence that the 5′-UTR sequence of PDGF-B mRNA contains any IRES activity. Instead, we found that the 5′-UTR sequence of PDGF-B functions as a promoter both constitutively and upon induction in a variety of cell lines. The 5′-UTR sequence contains two promoters (termed P1 and P2) when only the 5′-UTR sequence is integrated in the upstream TATA-box-containing promoter (P0), P1 and P0 promoters are integrated into one promoter, whereas the P2 promoter still functions. The full promoter with combined P0, P1, and P2 produced two transcripts, with the major one having the full-length 5′-UTR and the minor one the short 5′-UTR. The integrated P0/P1 promoter and P2 promoter are likely responsible for producing the endogenous 3.8- and 2.8-kb PDGF-B mRNAs that are detected in cultured human renal microvascular endothelial cells, a few tumor cells, and rat brain tissues. Furthermore, we detected the 2.8-kb PDGF-B mRNA in erythroleukemia K562 cells upon 12-O-tetradecanoylphorbol-13-acetate-induced differentiation. Considering that the 5′-UTR in the 3.8-kb mRNA contains no IRES activity and inhibits cap-dependent translation, we believe that the endogenous 2.8-kb mRNA produced from the 5′-UTR promoter is likely the major template responsible for protein production both constitutively and upon induction. We also found that the transcription from the 5′-UTR P2 promoter might be coordinated by the major upstream P0 promoter upon stimulation. Based on these observations, we propose that the TATA-containing P0 promoter and the 5′-UTR promoter work together to tightly control the expression of PDGF-B.

Platelet-derived growth factor B (PDGF-B)/c-sis belongs to a family of proteins consisting of four gene products, PDGF-A, PDGF-B, PDGF-C, and PDGF-D. They function as homodimers or heterodimers to selectively signal through cell surface tyrosine kinase receptors (PDGFR-α and PDGFR-β) and regulate diverse cellular functions (1–3). PDGF in general has been implicated to play an important role in embryogenesis, wound healing, as well as in the development of several serious disorders, including certain malignancies, atherosclerosis, and various fibrotic conditions. Understanding the mechanism of regulation of PDGF expression is, thus, important for designing strategies to control the expression of PDGF in such disorders.

The regulation of human PDGF-B expression is complex. The human PDGF-B gene contains seven exons spanning 24 kb of the genomic DNA on human chromosome 22. Transcription is normally driven by a short basal TATA-containing promoter that is responsible for production of the regular 3.8-kb transcript (4–7). However, this mRNA contains a GC-rich 5′-untranslated region (5′-UTR) of 1022 bases with three AUG codons and a highly stable secondary structure. This type of 5′-UTR sequences normally have an inhibitory effect on translation. Indeed, it has been found that the high level of the 3.8-kb PDGF-B mRNA is not accompanied by detectable proteins in many cell lines (8–10). In contrast, the PDGF-B protein is detected in some cell lines despite the presence of low levels of the 3.8-kb mRNAs (11). An alternative 2.8-kb transcript that lacks the long 5′-UTR sequence was detected in a few tumor cell lines and was shown to be associated with high level of PDGF-B protein (12). The 2.8-kb mRNA was also reported in cultured human renal microvascular endothelial cells (HRMECs) upon stimulation with transforming growth factor β (TGF-β) or phorbol 12-myristate 13-acetate (13). Interestingly, the 2.8-kb mRNA is degraded in a protein synthesis-dependent pathway and can be selectively enriched by cycloheximide treatment. The 2.8-kb mRNAs have also been detected in rat brain at certain stages of brain development, and its detection was associated with the increased level of PDGF-B protein (12). Thus, it has been suggested that the expression of PDGF-B mRNA with short 5′-UTR sequences possibly exists widely in non-transformed tissues in vivo (12).

One of the major sites of PDGF synthesis is within bone marrow megakaryocytes, the platelets progenitor cells. Be* This work was supported in part by National Institutes of Health Grants CA64539 and GM59475, and by Department of Defense Grant DAMD17-02-1-0073. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: PDGF, platelet-derived growth factor; SI2, Schneider’s Drosophila cell line 2; 5′-UTR, 5′-untranslated region; IRES, internal ribosome entry site; HRV, human rhinovirus; LUC, luciferase; TPA, 12-O-tetradecanoylphorbol-13-acetate; HRMEC, human renal microvascular endothelial cell; TGF-β, transforming growth factor β; RACE, rapid amplification of cDNA ends; CMV, cytomegalovirus; Mops, 4-morpholinepropanesulfonic acid; RPA, ribonuclease protection assay; CRD, coding region instability determinant.
cause human erythroblastemia K562 cells are differentiated into megakaryocytes upon TPA stimulation, regulation of PDGF-B expression in K562 cells has been extensively studied. The 3.8-kb species of PDGF-B mRNA was dramatically increased upon TPA-induced differentiation (14). cis-elements and transcription factors, which are responsible for such induction, were also identified for the TATA-containing promoter (15, 16). Recently, the 5′-UTR of the 3.8-kb transcript has been reported to contain an internal ribosome entry site (IRES), which became more active in K562 cells upon differentiation works by directly recruiting the translational efficiency of proteins, and it has been reported to contain an internal ribosome entry site (IRES), which became more active in K562 cells upon differentiation.

Regulation of PDGF Expression

Herein, we report our surprising finding that the 2.8-kb human PDGF-B mRNA, previously detected in HRMECs (13), is also produced in K562 cells upon TPA-induced differentiation. This transcript represents a minor species in Northern blot analysis. However, treatment of cycloheximide stabilizes the 2.8-kb transcript and enables its clear detection by Northern blot analysis. Using luciferase reporter promoter assay and Northern blot analysis, we demonstrated that the DNA sequence encoding the 5′-UTR of the long PDGF-B mRNA contains promoters that function in various cell lines and produces two mRNA species with a medium and a short 5′-UTR, respectively. However, in the presence of the upstream TATA-containing promoter, only the transcript with a short 5′-UTR was produced in addition to the transcript with the full-length 5′-UTR, which may explain why only the 3.8-kb and the 2.8-kb endogenous mRNAs are produced. As the other hand, the 5′-UTR sequence of the long PDGF-B transcript severely inhibits translation and does not display any IRES activity when tested using more stringent methods such as RNA transfection and promoterless dicistronic assays. Therefore, the 3.8-kb mRNA may contribute little, if any, to the production of PDGF-B protein. Based on the above observations, we conclude that the major TATA-containing promoter and the promoters in the 5′-UTR work together to control the expression of the 2.8-kb PDGF-B transcript in a variety of cells as an effective source of mRNA for protein production. Tight control of the production of the 2.8-kb mRNA and its stability may be used widely to control PDGF-B expression, both constitutively and upon stimulation.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from New England Biolabs. Site-directed mutagenesis QuikChange XL mutagenesis kit was from Stratagene. SP6 RNA polymerase, RNasin, Rnasase-free DNase, rabbit reticulocyte lysate, Luciferase assay ‘Stop & Glo’ kit, and pSP64 Poly(A) plasmid were from Promega. RNeasy Mini Kit and Oligotex mRNA Mini Kit were from Qiagen. The mGpppG cap analogue, Rediprime II Random Prime Labeling System, [32P]dCTP and [32P]TP were from Amersham Biosciences. The Sephadex G-25 Quick Spin Columns for purification of labeled DNA were from Roche Applied Science. MAGNA nylon transfer membrane was from Osmonics Inc. The 5′-RACE system for rapid amplification of cDNA ends and Lipo- fectin transfection reagents were purchased from Invitrogen. Oligonucleotides were synthesized by Sigma-Genosys. TaKaRa LA Taq polymerase was purchased from Takara Bio Inc. The Galacto-Light Plus chemiluminescent reporter assay kit for β-galactosidase was purchased from Tropix. The pTR-GAPDH-Human (pTR-glyceraldehyde-3-phosphate dehydrogenase-Human), the RNA Century Marker, the MAXIscript in vitro transcription kit, and RPA III ribonuclease protection assay kit were products of Ambion. Cycloheximide and TPA were from Sigma. The plasmid pSM1 (24) containing PDGF-B DNA insert was obtained from ATCC. The human IMAGE cDNA (ID 5174750 and GenBank TM accession number BC029822) containing the partial 5′-UTR and the entire coding region of PDGF-B were purchased from Open Biosystems.

Construction of Plasmids—The plasmid pBLHHCAT was a gift from Dr. William E. Fah (McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, WI). The plasmid contains a 5.5-kb PDGF-B DNA fragment (HindIII-HindIII), which includes the full-length 5′-UTR and the proximal promoter (5). The DNA sequence for the long 5′-UTR was amplified using the following primers: JB7, 5′-CCCCACTATGTCGCACTCTCCCATCTTCCTTCCTCC and OS35, 5′-CCCCCATG-GCCACTCCGGCGCGCGCCCC (18). The purified PCR product was cloned into pRF, and pRF(+P) vector (25), resulting in pR-PDGF-F and pR-PDGF-F(-P), respectively. To construct the plasmid containing the 5′ region, −807 to +1022, of PDGF-B (numbered relative to the known transcription start site in the remaining text unless otherwise specified), pHHCAT was first digested with HpaI and XhoI to isolate a 1.2-kb fragment that includes −807 to +475, which was then used to replace the HpaI/XhoI fragment in pR-PDGF-F(+P). To obtain the new construct that contains −807 to +82 of PDGF-B 5′ region, a Hpal/AvrII fragment isolated from the −807 to +1022 construct was cloned into pRF(+P) at the Hpal and NcoI sites.

Mutagenesis of the TATA box was performed using the Stratagene QuikChange XL mutagenesis kit according to the manufacturer’s protocol. The sense primer for mutagenesis was 5′-CCCATGTCGCACTCTCCTTCTCCCTCTCTAGATGCGCACTCTCCCTCC and OS35, 5′-CCCCCATG-GCCACTCCGGCGCGCGCCCC (18). The purified PCR product was cloned into pRF, and pRF(+P) vector (25), resulting in pR-PDGF-F and pR-PDGF-F(-P), respectively. To construct the plasmid containing the 5′ region, −807 to +1022, of PDGF-B (numbered relative to the known transcription start site in the remaining text unless otherwise specified), pHHCAT was first digested with HpaI and XhoI to isolate a 1.2-kb fragment that includes −807 to +475, which was then used to replace the HpaI/XhoI fragment in pR-PDGF-F(+P). To obtain the new construct that contains −807 to +82 of PDGF-B 5′ region, a Hpal/AvrII fragment isolated from the −807 to +1022 construct was cloned into pRF(+P) at the Hpal and NcoI sites.

Dicistronic constructs containing poly(A) for in vitro transcription (Fig. 4) were engineered using the vector pSP64 Poly(A), which has 36-base 5′UTR. The EcoRI/XhoI fragment of the pRSV-Luciferase Renilla luciferase gene was first cloned into pSP64 Poly(A) vector at the XbaI and blunt HindIII sites to generate the plasmid pSP-BHLuc. The XbaI fragment of pR-HRV-F (25) containing the IRES of HRV and the firefly luciferase gene was then isolated and cloned into pSP-RALuc at the XbaI site to generate pSP-R-HRV-F A5. The pSP-R F A5 plasmid was obtained by removing the IRES sequence of HRV from the pSP-R-HRV- F A5 by digestion with SpeI and NcoI. To engineer pSP-R-PDGF-F A5, the SpeI-NcoI fragment from pR-PDGF-F, which contains the +1 to +1022 sequence, was used to replace the HRV IRES fragment in pR-HRV-F A5 construct.

Monocistronic constructs containing poly(A) were also obtained from pSP64 Poly(A). Further, the XbaI fragment containing the 5′-UTR sequence of PDGF-B and the firefly luciferase-encoding sequence from pR-PDGF-F was cloned into pSP64 Poly(A), resulting in pSP-PDGF-F A5. Deletion of SpeI/BamHI, SpeI/SmaI, and SpeI/MluI regions from pSP-PDGF-F A5 resulted in pSP-PDGF-F A50, pSP-PDGF-F A50-50, and pSP-PDGF-F A50-100, which contain the +769 to +1022, +802 to +1022, and +937 to +1022 regions, respectively. To obtain the full-length 5′-UTR of the pSP-R-HRV-F A5 construct, which contains the +375 to +1022 region of the 5′-UTR, was constructed by cloning a PCR fragment into the SpeI and NcoI sites of pSP-R-HRV-F A50 construct.
transcripts were purified using a Qiagen RNasey Mini kit and quantified. For in vitro translation, 25 ng of the capped RNA transcripts was used to program translation in rabbit reticulocyte lysate in a final volume of 10 μl. Translation products were measured for firefly luciferase activity.

Cell Culture, DNA, and RNA Transfection—HeLa cells were maintained in Dulbecco's modified Eagle's medium, whereas H1299 and K562 cells were maintained in RPMI 1640 media both supplemented with 10% fetal bovine serum at 37 °C with 5% CO₂. DNA transfection in both HeLa and H1299 cells were performed with LipofectAMINE Plus reagents according to the manufacturer's protocol. In a 24-well plate, ~1×10⁶ cells/well were plated and transfected with 0.4 μg of DNA. Cells were harvested 24 h following transfection for luciferase assay.

RNA transfection was performed using the cationic liposome-mediated method as previously described (25). Briefly, ~2×10⁶ cells/well were seeded onto 6-well plates on the day before transfection. Cells were washed once with Opti-MEM I-reduced serum medium (Invitrogen) and left in the incubator with some medium during preparation of the liposome-polynucleotide complexes. One milliliter of Opti-MEM I medium in a 12 × 75-mm polystyrene snap-cap tube was mixed with 12.5 μg of Lipofectin reagent and 5 μg of capped mRNA. The liposome/RNA/medium mixture was immediately added to cells. Eight hours following transfection, cells were harvested and processed for luciferase analysis.

Transient Transfection of K562 Cells—Transient transfections of K562 cells were performed using electroporation. K562 cells were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in the same buffer at 10⁶ cells per 0.4 ml. For each electroporation, 0.4 ml of cell suspension was mixed with 20 μg of constructs and 1.0 μg of pmCMV-gal in a final volume of 0.5 ml. Each electroporation pool received an electric pulse of 240 V and 1025 microfarads. After electroporation, the cells were incubated in 20 ml of prewarmed medium containing 20% serum for 24 h. The cells were then divided into two dishes with one supplemented with TPA (2 ng/ml) and the other supplemented with ethanol as a control. Forty-eight hours after TPA addition, the cells were collected by centrifugation, washed with phosphate-buffered saline, and lysed in passive lysis buffer and dual-luciferase, and β-galactosidase activity were determined.

Northern Blot Analysis—Subconfluent H1299 cells in 10-cm plates were transfected with 4 μg/plate constructs using LipofectAMINE Plus. Twenty-four hours following transfection, the total RNAs were extracted using an RNeasy Mini kit and digested with RNase-free DNase to remove residual plasmid DNA. The poly(A) RNAs were then isolated from 250 μg of total RNAs using an Oligotex mRNA Mini kit. One-fifth of the mRNAs were separated in 1% agarose gels in the presence of formaldehyde and MOPS buffer and blotted onto MAGNA nylon membranes. The blots were then hybridized with a 32P-labeled firepolished DNA probe (1656 bp), which was isolated by cleaving pCR2.1 with EcoRI and XbaI and labeled using the Rediprime II random primer labeling system. The glyceraldehyde-3-phosphate dehydrogenase mRNA was also detected using specific probes as a control.

For Northern blot analysis of PDGF-B expression in K562 cells, the total RNA was prepared from normal and differentiated K562 cells. The differentiation of K562 cells into megakaryocytes was induced by TPA (2 ng/ml) for 2 days. Cycloheximide (10 μg/ml) was added to the medium on the third day following TPA treatment to stabilize the 2.8-kb mRNA. The poly(A) mRNA was isolated, and ~2 μg of mRNA of each sample was used for Northern blot analysis as described above. The probe used for Northern blot was a 2-kb fragment isolated from pCMV-gal DNA by BamHI digestion.

PCR Analysis—Determination of the 5′-end of the 2.8-kb species was performed with the 5′ RACE system for rapid amplification of cDNA ends using the method provided by the supplier. Briefly, the first cDNA strand was synthesized using 5 μg of total RNA isolated from K562 cells that was cultured with TPA for 2 days and subsequently cultured with both TPA and cycloheximide for another 16 h. The primer used for cDNA synthesis is human PDGF-B-specific antisense primer, 5′-CCACGTGTCCACCTGG-3′, located at 548 bases downstream of the translation start codon. The primers used for first-round PCR were 5′ RACE Abridged Anchor Primer from the supplier and PDGF-B-specific primer, 5′-CTGACCAGACGGAGTA-3′, located at 56 bases downstream of the translation start codon. Takara Taq polymerase and GC buffer II were used for PCR amplification. The reaction conditions were 94 °C for 2 min followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. The PCR products were then purified, blunted with Klenow, and then cloned using the PCR Zero Blunt Cloning kit. Individual clones were isolated and sequenced to determine the transcription start sites.

Determination of the 5′-end of the longer transcript from the pR-PDGFB-P-P transcript was performed by RT-PCR. About 5 μg of total RNA isolated from pR-PDGFB-F and pR-PDGFB-P-P-transfected H1299 cells was used. The first strand of cDNA was synthesized using an antisense primer targeting firefly luciferase mRNA located 78 bases downstream of the translation start codon. PCR amplification was performed using antisense primer located at +675 and sense primer located at +150, +225, +300, and +575 of the 5′-UTR of PDGF-B, respectively.

Ribonuclease Protection Assay—RPA was performed using the RNA probe according to the supplier's instructions. Briefly, the RNA probe was produced by first cloning into gEM-4Z at the EcoRI and HindIII site the region ~118 to ~241 (numbered relative to the translation start site) of PDGF-B derived from the Image 5174750 (National Institutes of Health) cDNA clone by PCR. The resulting plasmid was linearized with EcoRI and transcribed using T7 RNA polymerase in the presence of 0.5 mM each of ATP, GTP, UTP, and 0.01 mM CTP supplemented with 3.12 μg [α-32P]CTP. The 32P-labeled probe was digested with DNase and purified using a Sephadex G-25 Quick Spin column. About 0.5 × 10⁶ cpm of the probe was hybridized to 2 μg of mRNA at 45 °C overnight followed by digestion with RNase T1/α at 37 °C. The reaction was then stopped, and the protected RNAs were precipitated before separation by electrophoresis on a 6% acrylamide/8% urea gel for autoradiography.

Results

Production of the 2.8-kb mRNA of PDGF-B in K562 Cells upon TPA-induced Differentiation—Fen and Daniel (13) first described that the 2.8 kb PDGF-B mRNA with truncation at the 5′-UTR (it has a 5′-UTR with only 15 bases) was produced in HRMECs upon either TGF-β or phorbol 12-myristate 13-acetate stimulation. It was thought that the 2.8-kb mRNA was degraded through a protein synthesis-dependent pathway, because treatment with cycloheximide selectively stabilized the 2.8-kb mRNA but not the 3.8-kb mRNA. Fen and Daniel also demonstrated that the 2.8-kb mRNA did not arise from post-transcriptional processing of the 3.8-kb mRNA but rather possibly from internal promoter within the 5′-UTR. The cycloheximide treatment did not enhance transcription of PDGF-B but rather increased the half-life of the 2.8-kb mRNA. The 5′-truncated PDGF-B mRNA was also observed in a few tumor cell lines and increased in rat brain at a certain stage of brain development, and the level of the 5′-truncated mRNA was shown to be associated with PDGF-B protein level (12).

We hypothesize that the 2.8-kb mRNA of PDGF-B may also be produced in K562 cells upon TPA-induced differentiation. To test this hypothesis, we first performed Northern blot analysis of the endogenous PDGF-B transcript in K562 cells and demonstrated that a major transcript of 3.8 kb was detected when K562 cells were treated with TPA for 2–3 days (Fig. 1A, lane 3). A minor transcript of 2.8 kb was also evident (lane 3) and more pronounced with longer exposure (data not shown). When cells were treated with cycloheximide for 6 or 16 h following TPA treatment, the 2.8-kb mRNA increased significantly (Fig. 1A, lanes 4 and 5). Treatment of K562 cells with cycloheximide alone, however, did not enhance expression of either mRNA species (Fig. 1A, lane 2), suggesting that cycloheximide alone did not affect PDGF-B transcription. Therefore, the TPA-stimulated K562 cells displayed a similar profile of PDGF-B transcription as HRMECs.

To map the 5′-end of the 2.8-kb PDGF-B mRNAs induced in differentiated K562 cells, we performed 5′ RACE using RNAs isolated from K562 cells following TPA and cycloheximide treatment. After two rounds of PCR using nested PDGF-B-specific primers, we obtained a single PCR product (Fig. 1B,
The PCR product was cloned, and 10 individual clones were selected for sequencing. Three of the clones were identical and start at 15 bases upstream of the translation start codon ATG, whereas the other seven start at 27 bases upstream of the translation start codon (Fig. 1B). This observation suggests that there are at least two transcription start sites for the short RNA species of PDGF-B in differentiated K562 cells.

Because the results in 5'-RACE analysis could also be due to premature termination of polymerase reaction by the secondary structures in the 5'-UTR sequence, we performed a ribonuclease protection assay (RPA) using a PDGF-B probe spanning −118 and +241 (numbered relative to the translation start codon) (Fig. 1C). As shown in Fig. 1D, two fragments were found to be protected, and their sizes were estimated to be 289 and 212 bases, respectively (lane 3). While the longer protected fragment is possibly derived from the 3.8-kb transcript, the smaller fragment with −77 bases less is likely derived from the 2.8-kb transcript with shorter 5'-UTRs. It is noteworthy that the smaller fragment represents a significant species of protected fragments in samples treated either without (lane 3) or with cycloheximide (lane 4), suggesting the 2.8-kb transcript appears to be more efficiently protected. It also appears that the 2.8-kb transcript (the smaller fragment) is relatively more abundant upon cycloheximide treatment (compare lanes 3 and 4). Thus, these findings support the conclusion derived from the results shown in Fig. 1 (A and B) and confirm that the cycloheximide treatment selectively stabilizes the 2.8-kb mRNA.

The Long 5'-UTR Sequence of PDGF-B Inhibits Cap-dependent Translation—Although the 2.8-kb mRNA represents a minor species in TPA-induced PDGF-B transcripts, it may serve as the primary template for PDGF-B protein production, because the full-length PDGF-B transcript may not be efficiently translated due to translation inhibition by the long 5'-UTR. To test this concept, we determined the effect of the long 5'-UTR on translation and the primary template for PDGF-B protein production. For this purpose, we constructed a series of plasmids that were used for generating in vitro transcripts with different lengths of 5'-UTR. These deletion mutants contain putative secondary structures with different free energies (ΔG) ranging from −421 to −15 kcal/mol as predicted by Zuker's mfold program (26) (Fig. 2A). The ΔG value is an
scripts were then generated (Fig. 2B, schematic diagram of sequential deletions in the 5'-UTR sequence of in vitro were engineered into pSP64 Poly(A) for production of translation (27). The higher the indicator of the stability of the secondary structure that may range from 375 to 5'-UTR with full-length (1022) increased the translation by 1022) or 160 bases 5'-UTR sequence (1022) is about 20-fold more efficiently translated than the one with a full-length 5'-UTR sequence contains promoter activity in both HeLa and H1299 cells.

Previously, it has been suggested that the long 5'-UTR sequence of PDGF-B contains an internal ribosome entry site (IRES), which can mediate cap-independent translation (17, 18). However, the data shown in Fig. 3 strongly suggest that the previously claimed IRES activity of PDGF-B is likely due to the promoter activity present in the 5'-UTR sequence. To further determine whether the long 5'-UTR sequence of PDGF-B contains IRES activity, dicistronic mRNAs with m7GpppG cap and polyadenylated tail were generated from dicistronic constructs (pR-PDG-F-A30) (Fig. 4A). Equal amounts of in vitro transcripts were then transfected into H1299 cells by Lipofectin encapsulation. Transfections using dicistronic RNAs from pRF-A30 vector and HRV-IRES-containing plasmid pHRV-F-A30 were performed as negative and positive controls, respectively.

Eight hours following transfection, cell lysates were prepared for luciferase activity measurement. The efficiency of transfection was similar for all three RNAs as suggested by Renilla luciferase activity (with arbitrary units of 3360, 1183, and 1876, respectively, for RF-A30, R-PDG-F-A30, and R-HRV-F-A30 in one typical experiment). As expected, RA30 displayed a very low level of firefly luciferase activity, which only represents about 0.06% of Renilla luciferase activity. The firefly luciferase activity increased to about 2.7% of Renilla luciferase activity in R-HRV-F-A30-transfected cells. As shown in Fig. 4B, the IRES of HRV enhanced the expression of the second cistron by about 40-fold as compared with vector control. However, the 5'-UTR sequence of PDGF-B did not enhance the expression of the second cistron. In fact, it reduced the basal level expression by about 40-fold as compared with vector control. Therefore, it was concluded that the IRES activity of PDGF-B is likely due to the promoter activity present in the 5'-UTR sequence.
and firefly luciferase activities were measured and normalized against pRF luciferase activity of pRF. The data shown represent one of the four independent experiments.

erase gene, was used as a monocistronic control (25). It is expected to use the SV40 promoter to produce a transcript with a 5'-UTR of ~100 bases and the firefly luciferase-encoding sequence. The pRF vector was used as a dicistronic control, which produces only a dicistronic mRNA using the SV40 promoter. As shown in Fig. 5A, a dicistronic transcript from control pRF (lane 2, indicated by an asterisk) and a monocistronic transcript from control pRF(-R) (lane 3, indicated by an arrowhead) were detected as expected. The dicistronic transcript derived from pR-PDG-F (lane 1, indicated by an asterisk) has a slower mobility than the one from pRF (lane 2), consistent with the presence of the 5'-UTR sequence of PDGF-B in the intergenic region. Two additional transcripts were generated from pR-PDG-F (lane 1, indicated by A and B), which have similar sizes to the monocistronic transcript derived from pRF(-R) (lane 3), suggesting that they may be monocistronic mRNA transcribed from the 5'-UTR sequence of PDGF-B located in the intergenic region. This conclusion was further confirmed by the production of the same two transcripts from pR-PDG-F(-P) construct (lane 4), which lacks the vector SV40 promoter and thus the dicistronic product (lane 4). The bigger transcript (labeled as A) may have a longer 5'-UTR than the smaller one (labeled as B). The 5'-UTR sequence of the transcript B may be very short, because it was smaller than the monocistronic mRNA transcript generated from pRF(-R) (compare lanes 4 and 3). Based on the above observations, we conclude that the 5'-UTR sequence of PDGF-B may contain two discrete promoters (designated as P1 and P2) that mediate the production of two types of transcripts with medium and short 5'-UTR sequences, respectively. The transcripts with the short 5'-UTR is likely the one that can be much more efficiently translated than the one with medium and the original long 5'-UTRs as shown in Fig. 2.

**Graphical Abstract**

**A** Schematic diagram of traditional and promoterless dicistronic constructs. The 5'-UTR sequence of human PDGF-B and the IRES element of HRV are cloned into the intergenic region of the dicistronic vectors. The locations of several relevant restriction enzyme sites are shown by arrows. In the promoterless constructs, the SV40 promoter and the chimeric intron sequence were deleted. **B** Relative luciferase activities generated by the dicistronic constructs in H1299 cells. H1299 cells were transfected with dicistronic constructs together with β-galactosidase plasmid. Twenty-four hours following transfection, cells were harvested and the Renilla and firefly luciferase activities were measured and normalized against β-galactosidase activity followed by normalization against the firefly luciferase activity of pRF. The data shown represent one of the four independent experiments.

**Fig. 3. Dicistronic DNA test of the 5'-UTR sequence of human PDGF-B.** A, schematic diagram of traditional and promoterless dicistronic constructs. The 5'-UTR sequence of human PDGF-B and the IRES element of HRV are cloned into the intergenic region of the dicistronic vectors. The locations of several relevant restriction enzyme sites are shown by arrows. In the promoterless constructs, the SV40 promoter and the chimeric intron sequence were deleted. B, relative luciferase activities generated by the dicistronic constructs in H1299 cells. H1299 cells were transfected with dicistronic constructs together with β-galactosidase plasmid. Twenty-four hours following transfection, cells were harvested and the Renilla and firefly luciferase activities were measured and normalized against β-galactosidase activity followed by normalization against the firefly luciferase activity of pRF. The data shown represent one of the four independent experiments.

To further delineate the boundaries of the DNA region that are responsible for promoter activities, systematic deletion mutants were created from either 5'- or 3'-end of the 5'-UTR of PDGF-B (Fig. 6A). These 5'-UTRs with deletions were engineered into the promoterless dicistronic vector and used to determine their ability to direct firefly luciferase expression in both H1299 and HeLa cells (Fig. 6B). It should be noted, however, that the results of the luciferase reporter assay may be subject to both transcriptional and translational control when mRNAs with different lengths of 5'-UTRs are produced (27).
Thus, the data in Fig. 6 should be analyzed together with the Northern blot and RT-PCR result shown in Fig. 5B. The data in Fig. 6 clearly demonstrated that two discrete regions, i.e. the +1 to +395 (P1) and the +769 to +1022 (P2) sequence, contain promoter activity. This result confirmed the data in Fig. 5 where we detected two discrete transcripts (Fig. 5A, labeled as A and B in lane 4). The P1 promoter activity is about 3- to 4-fold of that of the P2 promoter. Because transcripts from the P1 promoter have a relatively long 5'-UTR, they can not be efficiently translated (see Fig. 2). The +1 to +769 construct does not contain the P2 sequence. However, because it contains the full P1 promoter, the +1 to +769 construct can produce a transcript with a shorter 5'-UTR that lacks the downstream major GC-rich region. As shown in Fig. 2A, the computed free energy for the +769 to +1022 is about −119 kcal/mol. Removal of this region is expected to result in an enhanced translation efficiency and, consequently, an increase in luciferase activity (Fig. 6B). Further deletion from the 3'-end up to 675 bases resulted in further increase in luciferase activity (+675 to +1022). The luciferase activity started to drop in the +1 to +303 construct in both cell lines, indicating that the +303 to +395 region may contain enhancing elements for P1 promoter.

Deletion of 150 bases from the 5'-end did not affect luciferase activity (+150 to +1022) as compared with the +1 to +1022 construct. However, deletion of 225, 300, and 375 bases from the 5'-end significantly decreased the luciferase activity. Combined with the 3'-end deletion construct data, these results indicate that the P1 promoter sequence is located roughly in the +150 to +395 region. The P1 transcript is thus expected to be poorly translated, because it contains a significant secondary structure with an approximate free energy of −280 kcal/mol (Fig. 2). Nevertheless, the integrity of the P1 promoter is important for the activity of the full-length 5'-UTR promoter, because deletion of the P1 promoter from the 5'-UTR (+375 to +1022) significantly decreased the luciferase activity. It is likely that the P1 promoter enhances transcription from the P2 promoter in the intact 5'-UTR construct. The deletion mutants with the 5'-end from +375 to +769 displayed similar luciferase activities, although a slight variation in luciferase activity was observed, arguing for the P2 promoter being located roughly between +769 and +1022. The transcript from the P2 promoter would contain significantly shorter 5'-UTRs than that from the P1 promoter. This is consistent with our observation of the transcript with a short 5'-UTR in Northern blot analysis (labeled as B in Fig. 5A, lane 4). The P2 promoter is likely responsible for production of the endogenous 2.8-kb mRNA with short 5'-UTRs of 15–27 bases.
A

To address this issue, we generated a series of constructs as the presence of the upstream TATA-containing P0 promoter. The positions of the 5'- and 3'-ends of each deletion are indicated on the left and right, respectively. These mutant 5'-UTRs were engineered into the promoterless dicistronic vector pRF(-P) at the intergenic region. B and C, relative luciferase activity from H1229 (B) and HeLa (C) cells transfected with the wild type and mutant PDGF-B 5'-UTR constructs. Twenty-four hours following transfection, cells were harvested for Renilla and firefly luciferase activity determination. The ratio of firefly to Renilla luciferase was calculated and normalized to the full-length 5'-UTR construct. The data were from three independent experiments.

Function of the 5'-UTR Promoter in the Presence of the Upstream TATA-containing Promoter (P0)—The above promoter analysis focused on the 5'-UTR sequence only. However, the promoter activity of the 5'-UTR sequence may be different in the presence of the upstream TATA-containing P0 promoter. To address this issue, we generated a series of constructs as shown in Fig. 7A and analyzed the promoter activity using both luciferase assay (Fig. 7B) and Northern blot (Fig. 7C). The longest construct, from −807 to +1022, contained both the TATA-containing promoter (P0) and the full-length 5'-UTR sequence. It has been known that the TATA-box is critical for constitutive promoter activity for P0 promoter and, thus, a TATA-box mutant construct (from −807 to +1022 mTATA with mutation from TTTATAAA to CTCTAGAT, which has no homology with any known transcription factor binding sites) was also generated to determine the effect of P0 promoter on expression from the −807 to +1022 sequence. Two other constructs (those from −807 to +87 and −807 to +87 mTATA) were also generated to determine the P0 activity in the absence of the 5'-UTR. These plasmids were transfected into HeLa, H1229, and HEK293 cells for promoter analysis. As shown in Fig. 7B, the −807 to +1022 construct has about a 3-, 4-, and 2-fold increase in luciferase expression in HeLa, H1229, and HEK293 cells, respectively, as compared with the control +1 to +1022 construct (pR-PDGF-F(-P)) that has only the 5'-UTR sequence. Mutation of the TATA-box almost eliminated the enhancement, suggesting that the integrity of the P0 promoter affects transcription from the −807 to +1022 construct. On the other hand, removal of the 5'-UTR sequence (−807 to +87 construct) enhanced the reporter expression about 4- to 5-fold compared with the full-length construct (−807 to +1022), indicating that the 5'-UTR caused an inhibitory effect on luciferase expression in the −807 to +1022 construct. This observation is consistent with the data shown in Fig. 2B. The −807 to +87mTATA construct had about 20–30% of luciferase activity compared with the −807 to +87 construct, which again demonstrate that the TATA-box is critical for the activity of the P0 promoter.

The constructs in Fig. 7A are expected to produce mRNAs with different lengths of 5'-UTRs and, thus, likely affect the results of the luciferase reporter assay (Fig. 7B) due to the possible effect of 5'-UTR sequence on translation of the reporter. To address this issue, we performed Northern blot analysis. As shown in Fig. 7C, cells transfected with the +1 to +1022 construct but not the vector pRF(-P) control produced two transcripts labeled B and C (compare lanes 1 and 6), derived from P1 and P2 promoters, respectively. The −807 to +1022 construct produced both a major product labeled A and the other minor diffused band labeled C (lane 2). Transcript A is the largest transcript derived from P0 promoter and has a 5'-UTR longer than that of the transcript C, which is derived from promoter P2. Interestingly, the −807 to +1022 mTATA-transfected cells generated three distinct bands labeled as A, B, and C, derived from P0, P1, and P2, respectively (lane 3). It appears that the TATA-box mutation dramatically reduced the transcription from P0 promoter and, thus, reduced production of the transcript A from the P0 promoter. Such an effect was also observed with the TATA-box mutation in the −807 to +87 construct (compare lane 4 and lane 5). However, the transcript B derived from the P1 promoter was not produced from the −807 to +1022 construct (lane 2). It is possible that the P0 and P1 promoter are integrated into one promoter due to their nearness to each other. Because the P0 promoter is about 10-fold more active than the P1 promoter, it is possible that the stronger promoter (P0) may dominate the weaker one (P1). This is consistent with the observation that the reduction of the P0 promoter activity by TATA-box mutation is accompanied by the appearance of transcript B from P1 promoter at a similar level to that produced from the +1 to +1022 construct (compare lanes 1 and 3). Although the level of transcript A produced from the −807 to +1022 construct is much higher, the luciferase activity produced is only about 2- to 4-fold over that of the +1 to +1022 construct (Fig. 7B). Furthermore, despite the production of the additional transcript A in cells transfected with the −807 to +1022 mTATA construct, the luciferase activity in these cells is almost equal to that of cells transfected with the +1 to +1022 construct (see Fig. 7B). Thus, we conclude that the transcript A produced from the −807 to +1022 and the −807 to +1022 mTATA constructs contributes little if any to the luciferase activity. The fact that the transcription
from the P1 promoter occurs only upon significant reduction of the P0 promoter activity suggests that a competition of transcription between the P0 and P1 promoter may exist. Thus, it is possible that the P1 promoter does not function in vivo in the presence of the P0 promoter. This possibility is consistent with our observation that only the 3.8- and 2.8-kb endogenous PDGF-B mRNA were detected.

Induction of the P0, P1, and P2 Promoter during Megakaryocytic Differentiation—We next examined the induced activity of the 5′-UTR promoter and the major P0 promoter in K562 cells upon TPA stimulation. For this purpose, K562 cells were transiently transfected with various constructs (Fig. 5A) by electroporation and were then treated without (Fig. 8B) or with (Fig. 8C) TPA for 2 days followed by measuring luciferase activities in cell lysates. The vector control was used to normalize the luciferase activities. As shown in Fig. 8B, the 5′-UTR sequence (+1 to +1022) alone stimulated about 10-fold expression compared with the vector control in K562 cells. In the presence of both P0 promoter and the 5′-UTR sequence (−807 to +1022), the activity increased another 2.6-fold. However, mutation of the P0 promoter eliminated this increase (−807 to +1022 mTATA). Deletion of the 5′-UTR sequence dramatically increased the expression of luciferase in K562 cells (−11-fold increase) (compare the −807 to +82 range with that from −807 to +1022), suggesting that the long 5′-UTR sequence inhibits translation. These observations are consistent with the results derived from 293, HeLa, and H1299 cells shown in Fig. 7.

Upon TPA-induced megakaryocytic differentiation, the 5′-UTR promoter (+1 to +1022) activity increased ∼2.7-fold (Fig. 8C). Interestingly, this result is in agreement with a 2–3-fold increase of “IRES” activity by induction observed previously (29), suggesting that the promoter activity of the 5′-UTR sequence might be misinterpreted as IRES activity. In the presence of the P0 promoter, however, the production of the luciferase reporter increased ∼10-fold by TPA stimulation (compare −807 to +1022 with +1 to +1022). The induction of luciferase expression of P0 promoter without the 5′-UTR sequence was ∼20-fold (−807 to +87), demonstrating that the P0 promoter is the major promoter that responds to TPA stimulation as compared with the 5′-UTR promoter.

The luciferase activity from the construct containing P0 and 5′-UTR (the P1 and P2 promoter) increased about 10-fold, which is higher than the increase observed with the 5′-UTR promoter (P1 and P2) alone (2.7-fold) but lower than that by the P0 promoter alone (20-fold). Disruption of the integrity of the upstream promoter by mutation of the TATA box significantly decreased the production of luciferase protein from 20- to 5-fold (Fig. 8C). However, the -fold increase in luciferase activity with the construct containing mutated TATA box was still higher than that with the 5′-UTR sequence alone. This is consistent with results previously reported by others (6, 16) that cis-elements other than TATA signals in the P0 promoter also contribute to TPA induction. Because the transcript derived from the P0 promoter contains the full-length 5′-UTR that inhibits translation and only the transcripts derived from P2 can be efficiently translated, we propose that the transcription from the 5′-UTR promoter is greatly enhanced in the presence of the upstream promoter by TPA stimulation.

DISCUSSION

In this study, using firefly luciferase as a heterologous reporter gene, we found that the full-length 5′-UTR of PDGF-B inhibits translation of the luciferase gene in a rabbit reticulocyte lysate cell-free system (Fig. 2). We also found that the mRNA lacking 5′-UTRs or containing shorter 5′-UTRs are 30- to 70-fold more efficiently translated. These observations are consistent with previous studies, which showed that the long 5′-UTR inhibits the translation of PDGF-B mRNA (4, 28). However, we found no evidence that the long 5′-UTR of PDGF-B contains any IRES activity that has been suggested to bypass the observed translational inhibition of PDGF-B mRNA (17, 18). Instead, we found that the long 5′-UTR sequence of PDGF-B contains two promoters (P1 and P2) that can produce mRNAs with shorter 5′-UTRs. These two promoters were demonstrated to work coordinately with the upstream TATA-box containing promoter.

By using RNA transfection, we demonstrated that dicistronic mRNAs bearing a full-length 5′-UTR of PDGF-B in the intergenic region does not enhance the translation of the downstream cistron (Fig. 4). Although in a conventional dicistronic DNA transfection assays, cells transfected with a dicistronic construct, which contains the full-length 5′-UTR of PDGF-B in the intergenic region, showed an enhanced expression of the second cistron, no significant decrease in the expression of the
Fig. 8. Promoter analysis in K562 cells upon differentiation. A, schematic diagrams of the constructs with or without the TATA-containing promoter the same as shown in Fig. 7A. B, relative promoter activity from K562 cells transfected with different constructs. Cells were harvested 48 h following transfection by electroporation and the firefly luciferase activity was measured and was normalized against that of the pRF(-P) vector control. C, fold induction of promoter activity by TPA in K562 cells. Twenty-four hours following transfection by electroporation, cells were treated with or without 2 ng/ml TPA for 48 h and collected for determination of luciferase activity. The -fold induction was calculated by dividing the luciferase activity of cells treated with TPA by that of cells not treated with TPA. The data are derived from four independent experiments.

second cistron was observed after the SV40 promoter of the vector was removed (Fig. 3). These observations suggest that the 5'-UTR sequence of PDGF-B contains promoters that underlie the enhanced expression of the second cistron observed in the traditional dicistronic DNA assay. We also found that the 5'-UTR promoter activity increased by about 2- to 3-fold in K562 cells undergoing megakaryocytic differentiation following TPA treatment (Fig. 8). This increase is surprisingly similar to the reported differentiation-induced IRES activity (18, 29). Thus, it is likely that the previously claimed IRES activity of PDGF-B was misinterpreted from the promoter activity present in the 5'-UTR sequence of PDGF-B. In light of these findings, we propose that the effective production of PDGF-B protein mainly relies on the production of the transcripts with shorter 5'-UTRs generated from transcription using the 5'-UTR promoter.

Although the existence of promoter activities in the 5'-UTR sequence of PDGF-B has been proposed previously (10, 12, 13), we, for the first time, characterized the 5'-UTR promoter and demonstrated that they are inducible and may be responsible for the previously thought IRES activity. The 5'-UTR promoter activity showed about 5- to 10% of the activity of the major TATA-box containing P0 promoter in various cell lines tested when analyzed by the luciferase reporter promoter assay (Fig. 7B). As shown by Northern blot analysis, the 5'-UTR promoter of PDGF-B can by itself initiate transcription from discrete locations, with one located around 800 bases upstream (Fig. 5B), and the other is very close to the translation start codon (Figs. 5 and 7). Detailed promoter analysis revealed two distinctive promoter regions, located approximately in position +150 to +303 (P1) and +769 to +1022 (P2) of the 5'-UTR sequence, respectively (Fig. 6). The P1 promoter in the 5'-UTR is about 3- to 4-fold more active than P2 in both HeLa and H1299 cells (Fig. 6).

We further investigated the function of the 5'-UTR promoter in the presence of the major TATA-box containing promoter (P0) (Fig. 7). TATA-box has previously been shown to be critical for proper function of the P0 promoter activity (5). This is confirmed in our promoter assay using both reporter luciferase activity assay and Northern blot analysis (compare the result of the −807 to +87 construct with that of the −807 to +87 mTATA construct). Although the major promoter P0 alone is about 7- to 20-fold more active than the 5'-UTR promoter (compare the result of the −807 to +87 construct with that of the +1 to +1022 construct), the luciferase activity increased only 2- to 4-fold when they coexist. Mutation of the TATA-box in the −807 to +1022 construct caused the luciferase activity to drop to the similar level to that of the +1 to +1022 construct. Although the P2 promoter functions in all of the constructs, the P1 promoter functions only in the absence of the P0 promoter or in the presence of the P0 promoter with a mutated TATA box (see Fig. 7C). Furthermore, the transcript generated from the P0 promoter with the −807 to +1022 mTATA construct did not cause any increase in the luciferase activity produced. Thus, it is likely that the P0 and P1 promoter are integrated into one promoter and drives transcription of the 3.8-kb full-length transcript that can be poorly translated in vivo.

We also analyzed the induction of the promoter activities in K562 cells upon TPA-induced megakaryocytic differentiation (Fig. 8) and demonstrated that the P0 promoter is a major player to respond to TPA stimulation, whereas the P1 and P2 promoters in the absence of P0 are induced only in a minor fashion. However, when P0 and the 5'-UTR promoter coexist, the overall protein production increased about 10-fold upon induction in transient transfection assays. Stable clones transfected with the −807 to +1022 construct with all three promoters displayed a similar induction profile. Based on these observations, we propose that transcriptions from the 5'-UTR promoters are greatly induced by TPA in the presence of the P0 promoter, and P0 and the 5'-UTR promoters work in a highly coordinated manner to tightly control the production of transcripts both with the long and short 5'-UTRs and, therefore, the constitutive and induced production of PDGF-B protein. Although cis-elements and transcription factors has been well characterized for the P0 promoter (5, 7, 15, 16, 30, 31), further

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*B. Han, Z. Dong, and J.-T. Zhang, unpublished observation.*
Regulation of PDGF Expression

46993

studies is clearly needed to characterize the 5′-UTR promoter. Nevertheless, our experiments using Drosophila SL2 cells transfected with 5′-UTR promoter constructs demonstrated that Sp1 might be one of the transcription factors that can trans-activate the 5′-UTR promoter.2

Our promoter analysis strongly supports the argument that the 2.8-kb PDGF-B mRNA with short 5′-UTR is likely derived from transcription from the P2 promoter of the 5′-UTR sequence as previously suggested (10, 12, 13), although there is no evidence available for the function of the natural P2 promoter of PDGF-B on human chromosome 22. The integrated P0/P1 promoter likely drives the production of the 3.8-kb full-length PDGF-B mRNAs. The finding that the dramatic increase of 2.8-kb mRNA species in K562 cells following TPA treatment (Fig. 1A) contradicts with only a 2-fold induction of the 5′-UTR promoter activity in the absence of P0 (Fig. 5C) and argues for a coordination between the P0/P1 promoter and the P2 promoter.

In the analysis of PDGF-B mRNA in K562 cells upon TPA-induced differentiation, we clearly detected a 2.8-kb mRNA that has a 5′-UTR of about 15–27 bases. The 2.8-kb mRNA was not reported for K562 cells in previous studies, possibly because it represents only a minor species and is not evident in Northern blot with short exposures. We, however, noticed that a Northern blot performed by Colominci et al. (14) clearly showed a 2.8-kb PDGF-B mRNA in K562 cells that were treated for 3 or 4 days (see Fig. 2 in Ref. 14), although it is not discussed in this report. Similarly, Fen and Daniel (13) reported the detection of a 2.8-kb mRNA species in human renal microvascular endothelial cells (HRMECs) upon treatment with either TGF-β or TPA. The 5′-truncated mRNAs were also detected in a few tumor cells and in rat brain tissue at a certain stage of development, and its level correlates the level of PDGF-B protein (12). Based on these observations, we propose that the production of the 2.8-kb mRNA may be tightly regulated and widely used for effective protein production of PDGF-B, both constitutively and upon induction by biological stimuli. However, it remains unknown what is the role of the 3.8-kb mRNA of PDGF-B if it cannot be used as template for efficient protein synthesis.

Consistent with Fen and Daniel (13), the 2.8-kb mRNA in TPA-induced K562 cells is selectively enriched by cycloheximide treatment. Although cycloheximide likely does not affect the transcription of PDGF-B (see Fig. 1, lanes 1 and 2), we cannot rule out the possibility that cycloheximide treatment may inhibit the synthesis of a suppressor for transcription and translation inhibitor cycloheximide. The CRD-BP protein specifically binds to the CRD region and prevented c-myc mRNA from degradation (34). CRD-BP expression parallels c-myc expression during liver development. Interestingly, the abundance of the 2.8-kb mRNA of PDGF-B is also developmentally regulated in rat brain (12). Because the level of the 2.8-kb mRNA species is a major determinant of PDGF-B protein level (10, 12), alteration of the stability may be another level of control in PDGF-B expression regulation. How the stability of the 2.8-kb mRNA is regulated remains to be an intriguing question for further studies.

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Tight Control of Platelet-derived Growth Factor B/c-sis Expression by Interplay between the 5′-Untranslated Region Sequence and the Major Upstream Promoter
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