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The 5′ Untranslated Sequence of the c-*sis*/Platelet-Derived Growth Factor 2 Transcript Is a Potent Translational Inhibitor

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c-*sis*/platelet-derived growth factor 2 (PDGF-2) is a prototype growth factor with transforming potential. The c-*sis*/PDGF-2 transcript contains a long 5′ untranslated sequence (UTS) that is highly G · C rich. To examine the influence of this sequence on *sis*/PDGF-2 expression, we localized the c-*sis*/PDGF-2 promoter and used this promoter or the simian virus 40 early promoter to drive expression of the bacterial chloramphenicol acetyltransferase or *sis*/PDGF-2 gene. The 5′ UTS of c-*sis*/PDGF-2 mRNA had no effect on RNA expression but was shown to exert a potent inhibitory effect on translation. By deletion analysis, we demonstrated that the 5′ UTS inhibited protein expression by as much as 40-fold. The inhibitory effect was independent of reporter gene, cell type, or promoter used. A highly G · C-rich 140-base-pair sequence immediately preceding the c-*sis*/PDGF-2 initiation codon was shown to be nearly as effective as the entire 5′ UTS in translational inhibition. Transfection analysis demonstrated that the 5′ UTS significantly reduced the transforming efficiency of the *sis*/PDGF-2 gene as well. Thus, our findings raise the possibility that changes in regulation at the level of *sis*/PDGF-2 translation may play a role in development of the neoplastic phenotype.

Human platelet-derived growth factor (PDGF) is a potent mitogen for connective tissue cells (19, 38, 42). PDGF preparations contain two related but distinct polypeptides, one of which, PDGF-2, has been shown to be homologous to the transforming product of the retroviral v-*sis* oncogene (for a review, see reference 38). Normal sources of the *sis*/PDGF-2 mRNA include endothelial cells (2, 6), activated monocytes (31), and placental trophoblasts (14) as well as platelet precursor cells. However, even in those cells where the transcript is observed at reasonably high levels, c-*sis*/PDGF-2 proteins have been difficult to detect (unpublished results).

Connective tissue cell types that possess PDGF receptors are susceptible to growth alterations induced by the v-*sis* gene product (28). Moreover, expression of the normal human c-*sis*/PDGF-2 gene has been shown to induce malignant transformation of NIH 3T3 cells (10, 22). The c-*sis*/PDGF-2 transcript is frequently detected at reasonably high levels in human glioblastomas and fibrosarcomas (6, 9; unpublished results), yet even in such tumor cells, *sis*/PDGF-2 proteins have only been detected at relatively low abundance (20). These findings have suggested that *sis*/PDGF-2 expression may be inhibited at the level of translation. There is evidence that expression of a gene may be inhibited at a translational level by stable secondary structures upstream of the initiator codon (24). The *sis*/PDGF-2 mRNA contains an unusually long 5′ untranslated sequence (UTS) which is highly rich in G · C content (36). Thus, we undertook the present studies to determine whether the 5′ UTS of *sis*/PDGF-2 mRNA plays a role in regulating expression of this potent mitogen.

MATERIALS AND METHODS

Cells. Continuous mouse NIH 3T3 (21) and COS-1 (11) cell lines have been described. Bovine aortic endothelial cells were kindly provided by G. R. Grotendorst (17).

Materials. [^{14}C]chloramphenicol (40 to 60 mCi/mmol) and radionucleotides were purchased from New England Nuclear Corp. Enzymes used in plasmid constructions were obtained from New England BioLabs, Pharmacia Inc., Bethesda Research Laboratories, and Boehringer Mannheim Biochemicals. The pGEM series of plasmids and reagents for in vitro RNA transcription were from Promega Biotech. Plasmids pSV2Cat and pA10Cat$_2$ were kindly provided by Bruce Howard (13). Bacterial chloramphenicol acetyltransferase (CAT) and acetyl coenzyme A were from Pharmacia Inc.

Plasmid constructions. Standard protocols were used for all recombinant DNA techniques (30). The parental plasmid, pSHMCatP, was constructed by fusing a 5.0-kilobase-pair (kbp) genomic DNA fragment (*Hin*dIII-*Mlu*I), containing the entire 5′ UTS, except for 30 bp upstream of the c-*sis* ATG codon and 4.0 kbp flanking sequences upstream of the cap site, at the *Mlu*I site to the *cat* coding sequence containing the simian virus 40 (SV40) polyadenylation signals at the 3′ end (13). All deletion constructions were made from this plasmid, pSHMCatP. Deletions are represented by one or two letters of the restriction enzymes from the 5′ to 3′ end.

Plasmid pc-*sis*4.0 was constructed by joining genomic DNA sequences to those of cDNA sequences of clone psD1 (20, 37). The *Eco*RI-*Mlu*I genomic fragment, isolated from the 5.0-kbp *Hin*dIII-*Mlu*I fragment, was fused at the *Mlu*I site in the cDNA at the 5′ end, and a *Bam*HI-*Pst*I genomic fragment was fused at the *Bam*HI site of cDNA at the 3′ end. The resulting DNA fragment was inserted into the multiple cloning site of pGEM3 or pGEM4. Deletions were made on plasmid pc-*sis*4.0 to study the effect of the 5′ UTS on c-*sis* gene expression. The SV40 enhancer unit (*Pvu*II-*Nco*I fragment from pSV2Cat) was introduced at the *Bst*EII site upstream of the c-*sis*/PDGF-2 promoter by blunt-end ligation.

DNA sequence analysis. Sequence analysis was done by chemical cleavage (32) as well as dideoxy chain termination methods (41).

Electroporation and CAT assays. DNA was introduced into bovine endothelial cells by electroporation (34). Molar equivalent amounts of closed circular plasmid DNA were added to approximately 10^7 freshly prepared cells in a total

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volume of 300 μl of sterile phosphate-buffered saline in a 1.0-ml disposable cuvette and incubated on ice for 10 min. An electric pulse was then delivered from a model 494 Isco power supply at a setting of 1.5 kV and current at the maximum of 0.9 mA. The current and wattage were set to a value of 1 and 10, respectively, on a scale of 100. The cells and DNA were allowed to sit for 15 min at 0°C. Then the cells were pipetted into a 100-mm tissue culture dish to which 10 ml of culture medium was added. Twenty-four hours after plating, the medium was removed and fresh medium was added. Forty-eight hours after transfection, cells were harvested and cell extracts were assayed for CAT expression. CAT assays were performed by the method of Gorman et al. (13) with 0.5 μCi of [^{14}C]chloramphenicol and 30 μl of cell extract per reaction mix in a final volume of 150 μl.

Transfection assays. DNA transfection of NIH 3T3 and COS-1 cells was performed by the calcium phosphate precipitation technique (15) as modified by Wigler et al. (44). Transformed foci were scored at 2 to 3 weeks after transfection. Plasmid DNA (30 μg) was used for transfection of COS-1 cells for PDGF-2 polypeptide expression. Metabolic labeling of cells and immunoprecipitation of PDGF-2 proteins in transfected COS-1 cells were performed 48 h after transfection as described (20).

RNase A and RNase T_1 protection assay of RNAs. RNA was extracted by the method of Chirgwin et al. (5). The level of c-*sis*/PDGF-2 mRNA in transfected cells was measured by using highly radioactive riboprobes in S1-type analysis as described by Winter et al. (45). The 930-nucleotide-long riboprobe corresponds to the enhancer-minus pc-*sis*4.0 ΔF.M (see Fig. 3) that lacks the 5′ UTS and extends 400 nucleotides upstream (to *Eco*RI) and 530 nucleotides downstream (to *Pvu*II) of the c-*sis*/PDGF-2 cap site. Riboprobes were made by linearization of the recombinant pGEM3 at an *Eco*RI site and transcription from the SP6 promoter as described by the supplier. After digestion, samples were analyzed on 5% nondenaturing polyacrylamide gels. In some experiments, S1 nuclease was used. Similar results were obtained under both conditions.

RESULTS

Functional localization of the c-*sis*/PDGF-2 gene promoter region. To study the influence of 5′ UTS on c-*sis*/PDGF-2 promoter-driven CAT activity, we first attempted to characterize the promoter region of the gene. We isolated a 5-kbp *Hin*dIII-*Mlu*I DNA fragment from a human genomic clone, λ-c-*sis*10, including 4 kbp of genomic DNA upstream of the c-*sis*/PDGF-2 mRNA cap site (36). The composite structure of the c-*sis*/PDGF-2 transcribed sequence in relation to the upstream genomic DNA is shown in Fig. 1A. We fused the bacterial CAT coding sequence containing the SV40 polyadenylation signal at the unique *Fsp*I site located 50 bp downstream of the c-*sis*/PDGF-2 mRNA cap site. This chimeric fragment was then inserted into the multiple cloning site of plasmid pGEM3 or pGEM4. The resulting construct, pSHMCatPΔF.M, was transfected into endothelial cells, a cell type known to support c-*sis*/PDGF-2 transcription (2, 6).

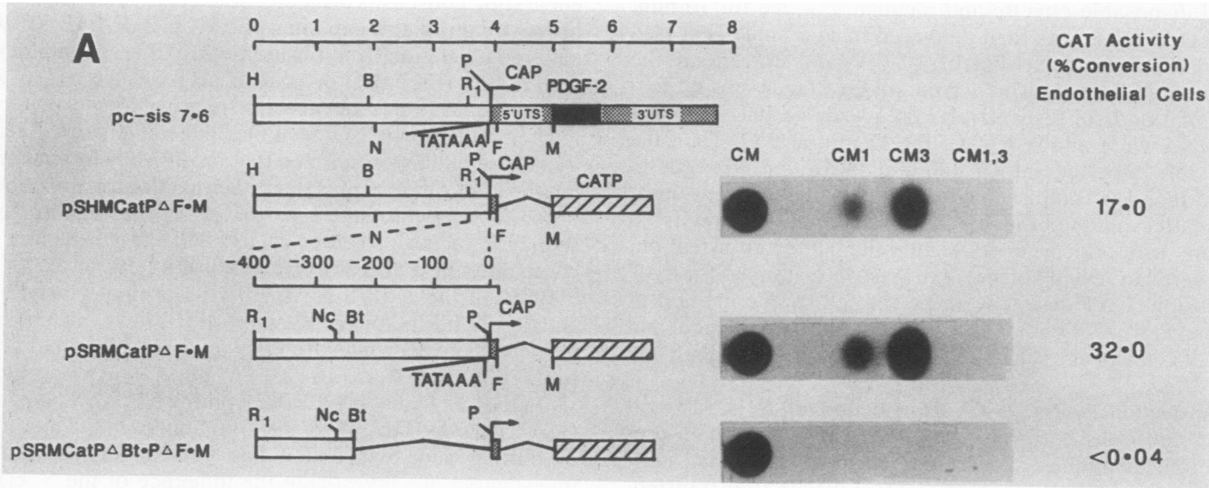
As shown in Fig. 1A, a significant level of CAT activity was observed when endothelial cells were transfected with pSHMCatPΔF.M, indicating that promoter activity was present in this DNA fragment. Deletion of a 3.6-kbp DNA fragment upstream of the *Eco*RI site in pSRMCatPΔF.M led to almost twofold-higher CAT activity. These findings localized the promoter region to a 397-bp fragment containing the eucaryotic consensus promoter sequence, the TATA box, 23 bp upstream of the cap site. pSRMCatPΔR.PΔF.M, which suffered a deletion from position −220 to −7, containing the TATA box (Fig. 1B), or pSRMCatPΔBt.PΔF.M, in which the entire 397-bp sequence upstream of the cap site was deleted (not shown), induced negligible levels of CAT activity. These findings confirmed that the 397-bp stretch containing the TATA box represented the functional transcriptional promoter region for the c-*sis*/PDGF-2 gene. The nucleotide sequence of the promoter region and the entire first exon containing the 5′ UTS are shown in Fig. 1B.

Effect of the 5′ UTS on *sis*/PDGF-2 promoter-driven CAT activity. Long and/or G · C-rich 5′ UTSs have been observed for several genes which are involved in regulation of cellular proliferation (1, 4, 7, 16, 23, 36, 40, 43). Among these is *sis*/PDGF-2, which has a 1,022-bp-long 5′ UTS and is highly G · C rich (36). The role of such sequences in the modulation of normal gene expression has yet to be systematically investigated. To investigate the influence of the 5′ UTS on c-*sis*/PDGF-2 promoter-driven CAT expression, we placed the CAT initiator codon at a position analogous to that of c-*sis*/PDGF-2 in pSRMCatP by substituting the CAT coding sequence for the *sis*/PDGF-2 coding sequence. Using convenient restriction sites, we also constructed a series of recombinants containing various portions of the 5′ UTS (Fig. 2). All constructs retained the sequence 50 bp downstream of the c-*sis*/PDGF-2 mRNA cap site, since deletion of the cap site sequences markedly reduced CAT activity (data not shown). The corresponding CAT activity induced by each plasmid was determined after transfection of endothelial cells.

As shown in Fig. 2, pSRMCatP, which contained the entire 5′ UTS, induced CAT activity but at a ca. 40-fold-lower level than pSRMCatPΔF.M, which lacked the 5′ UTS. Internal deletions in the 5′ UTS from positions 50 to 475 (pSRMCatPΔF.X) and 140 to 475 (pSRMCatPΔBs.X) each resulted in a four- to fivefold increase in expression of CAT activity compared with that of pSRMCatP. Deletion of a highly G · C-rich 220-bp sequence from position 770 to 990 in pSRMCatPΔB.M resulted in a fourfold increase in CAT expression as well. A further small increase in CAT activity was observed when the deletion in pSRMCatPΔB.M was extended further upstream to the *Xho*I site from positions 475 to 990. These results demonstrated that the 5′ UTS negatively influenced expression of the CAT gene.

Nonspecific enhancer elements can increase the level of transcription from a specific promoter (3, 12). To determine whether the inhibitory effects of the 5′ UTS could be overcome by increasing the level of transcription, we inserted various numbers of SV40 enhancer elements (18) upstream of the c-*sis*/PDGF-2 promoter in the pSRMCatP construct. As shown in Fig. 2, the addition of one, two, or three elements led to a proportional increase in the level of CAT activity observed. These findings indicated that by increasing the level of transcription, the inhibitory effect of the 5′ UTS could be partially overcome. With pSRM CatPE.6, in which six repeats of the SV40 enhancer unit were inserted, there was a dramatic reduction in the level of CAT activity induced. Structural changes in DNA induced by a larger number of enhancer copies may be unfavorable to the transcription of a linked gene or for binding of factors believed to mediate the transcriptional enhancement effect (27).

5′ UTS exerts potent translational inhibition of *sis*/PDGF-2 expression. To determine directly whether the 5′ UTS inhib-



B

↓RI -390 -380 -370 -360 -350 -340 -330 -320 -310 -300
GAATTCAGGCTCTCAGACTGCAGAGCCTGAGTCCCTCCCTGCCATGCCTGTGCCAGGGTGGAAATGTCTGGTCCTGGAGGGGAGCGTGGACTCCTGGCCT

-290 -280 -270 -260 ↓Nco I -240 -230 ↓Bst EII -210 -200
TGGCTCTGGAGACATCCCCCTAGACCACGTGGGCTCCTAACCTGTCCATGGTCACTGTGCTGAGGGGCGGGACGGTGGGTCACCCCTAGTTCTTCTTTCC

-190 -180 -170 -160 -150 -140 -130 -120 -110 -100
CCAGGGCCAGATTCATGGACTGAAGGGTTGCTCGGCTCTCAGAGACCCCCTAAGCGCCCCGCCCTGGCCCCAAGCCCTCCCCCAGCTCCCGCGTCCCCCC

-90 -80 -70 -60 -50 -40 -30 -20 ↓PvuII -1 CAPSITE
CCTCCTGGCGCTGACTCCGGGCCAGAAGAGGAAAGGCTGTCTCCACCCACCTCTCGCACTCTCCCTTCTCCTTTATAAAGGCCGGAACAGCTGAAAGGGT

↓Fsp I 100
GGCAACTTCTCCTCCTGCAGCCGGGAGCGGCCTGCCTGCCTCCCTGCGCACCCGCAGCCTCCCCCGCTGCCTCCCTAGGGCTCCCCTCCGGCCGCCAGCG

↓Bss HII 200
CCCATTTTTCATTCCCTAGATAGAGATACTTTGCGCGCACACACATACATACGCGCGCAAAAAGGAAAAAAAAAAAAAAAAGCCCACCCTCCAGCCTCGC

300
TGCAAAGAGAAAACCGGAGCAGCCGCAGCTCGCAGCTCGCAGCTCGCAGCCCGCAGCCCGCAGAGGACGCCCAGAGCGGCGAGCAGGCGGGCAGACGGAC

400
CGACGGACTCGCGCCGCGTCCACCTGTCGGCCGGGCCCAGCCGAGCGCGCAGCGGGCACGCCGCGCGCGCGGAGCAGCCGTGCCCGCCGCCCGGGCCCGC

↓Xho I 500
CGCCAGGGCGCACACGCTCCCGCCCCCCTACCCGGCCCGGGCGGGAGTTTGCACCTCTCCCTGCCCGGGTGCTCGAGCTGCCGTTGCAAAGCCAACTTTG

1 1 600
GAAAAAGTTTTTTGGGGGAGACTTGGGCCTTGAGGTGCCCAGCTCCGCGCTTTCCGATTTTGGGGGCCTTTCCAGAAAATGTTGCAAAAAAGCTAAGCCG

700
GCGGGCAGAGGAAAACGCCTGTAGCCGGCGAGTGAAGACGAACCATCGACTGCCGTGTTCCTTTTCCTCTTGGAGGTTGGAGTCCCCTGGGCGCCCCCAC

↓Nru I 2 ↓Bam HI 800
ACC[illegible]CTAGACGCCTCGGCTGGTTCGCGACGCAGCCCCCGGCCGTGGATGCTGCACTCGGGCTCGGGATCCGCCCAGGTAGCCGGCCTCGGACCCAGGTC

3 2 900
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3 ↓Mlu I 1000
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1022
GGCCGGGCCCGGAGTCGGC ATG AAT CGC TGC TGG GCG CTC TTC CTG TCT CTC TGC TGC TAC CTG CGT CTG GTC AGC GCC GAG
Met Asn Arg Cys Trp Ala Leu Phe Leu Ser Leu Cys Cys Tyr Leu Arg Leu Val Ser Ala Glu↓

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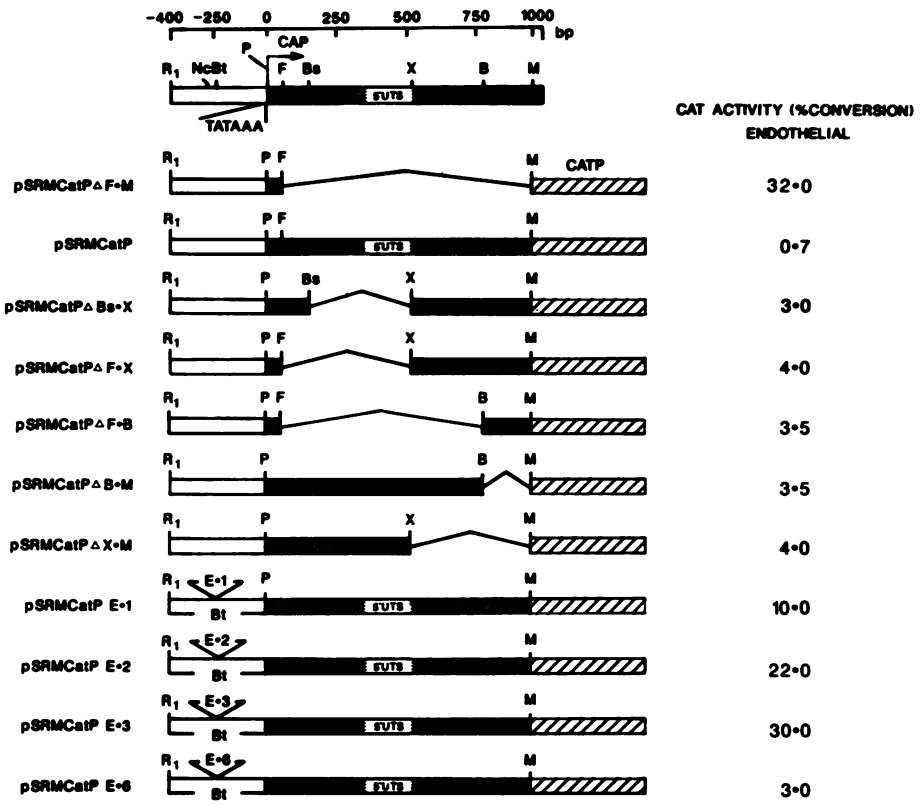

FIG. 2. Influence of 5′ UTS and SV40 enhancer element on the activity of c-*sis*/PDGF-2 promoter-*cat* hybrid genes. CAT activity was measured in extracts of endothelial cells transfected with the constructs shown. Molar amounts of plasmid DNAs equivalent to 30 μg of pSV2Cat were used. Percent conversion of [^{14}C]chloramphenicol to its acetylated forms is shown to the right. Abbreviations and symbols are as in Fig. 1, plus Bs, *Bss*HII. E.1, E.2, E.3, and E.6 represent the number of enhancer units inserted upstream of the c-*sis*/PDGF-2 promoter at the *Bst*EII site.

ited expression of *sis*/PDGF-2 at a translational level, we compared the levels of both RNA and protein expression in the presence and absence of the 5′ UTS. To do so, we constructed plasmids in which the c-*sis*/PDGF-2 coding sequence was placed under the control of c-*sis*/PDGF-2 transcriptional elements (Fig. 3A). Transfection with such a plasmid, pc-*sis*4.0, did not result in detectable c-*sis*/PDGF-2 RNA levels in any of several cell lines, including COS-1, tested under transient assay conditions (data not shown). Thus, we used constructs in which a single SV40 enhancer unit was introduced upstream of the c-*sis*/PDGF-2 promoter at the *Bst*EII site.

COS-1 cells were transfected with pc-*sis*4.0-E.1 or pc-*sis*4.0-E.1ΔF.M, and the levels of c-*sis*/PDGF-2 RNA and immunoprecipitable *sis*/PDGF-2 products were compared. The amount of c-*sis*/PDGF-2 mRNA in the transfected cells was determined by the RNase A and T_1 protection assay with riboprobes (45). The strategy for identifying the c-*sis*/PDGF-2 mRNA levels in these experiments is summarized in Fig. 3B. As shown in Fig. 3C, there were no significant differences in c-*sis*/PDGF-2 RNA levels either in the presence (pc-*sis*4.0-E.1) or absence (pc-*sis*4.0-E.1ΔF.M) of the 5′ UTS. Moreover, the RNAs exhibited the correct cap site for normal c-*sis*/PDGF-2 mRNA, as evidenced by the fact that two bands with a difference of 50 bp were observed when RNA containing the 5′ UTS was analyzed by S1-type analysis (Fig. 3C, left panel, lane 1). As expected, only the longer 530-bp fragment was observed for c-*sis*/PDGF-2 RNA lacking the 5′ UTS (Fig. 5C, left panel, lane 2). With cells from the same transfection experiment, immunoprecipitation with antiserum to human *sis*/PDGF-2 revealed expression of the 26,000- and 20,000-dalton c-*sis*/PDGF-2 products (20) in all transfectants analyzed. However, the level of *sis*/PDGF-2 products detected was at least

FIG. 1. (A) Localization of the *sis*/PDGF-2 promoter region. The structure of full-length c-*sis*/PDGF-2 cDNA and genomic sequences upstream (pc-*sis*7.6) are shown for reference. The PDGF-2 coding sequence is represented by a solid bar, and UTSs (5′ and 3′ UTSs) are represented by stipled bars. The CAT coding sequence with downstream SV40 polyadenylation signal sequences (CatP) is represented by a cross-hatched bar. Arrow indicates initiation and direction of transcription (36). The consensus promoter sequence (TATAAA) is indicated. An autoradiograph and values for conversion of [^{14}C]chloramphenicol (CM) to its acetylated forms (CM1, CM3, and CM1,3) are shown. Restriction sites: B, *Bam*HI; Bt, *Bst*EII; F, *Fsp*I; H, *Hin*dIII; M, *Mlu*I; N, *Nde*I; Nc, *Nco*I; R_1, *Eco*RI; P, *Pvu*II; X, *Xho*I. (B) Nucleotide sequence of the c-*sis*/PDGF-2 promoter region and first exon. The mRNA start site is nucleotide position 1. Upstream of the mRNA cap site, the consensus promoter sequence is boxed, and stretches homologous to the SV40 promoter are underlined. Within the 5′ UTS, the polypurine tract and terminator codons in-frame with the *sis*/PDGF-2 open reading frame are underlined. The locations of minicistrons upstream of the PDGF-2 open reading frame are indicated by their initiation codons (○) and their respective terminator codons (●). The heptanucleotide repeat sequence is boxed. Selected restriction enzyme sites are shown for purposes of reference.

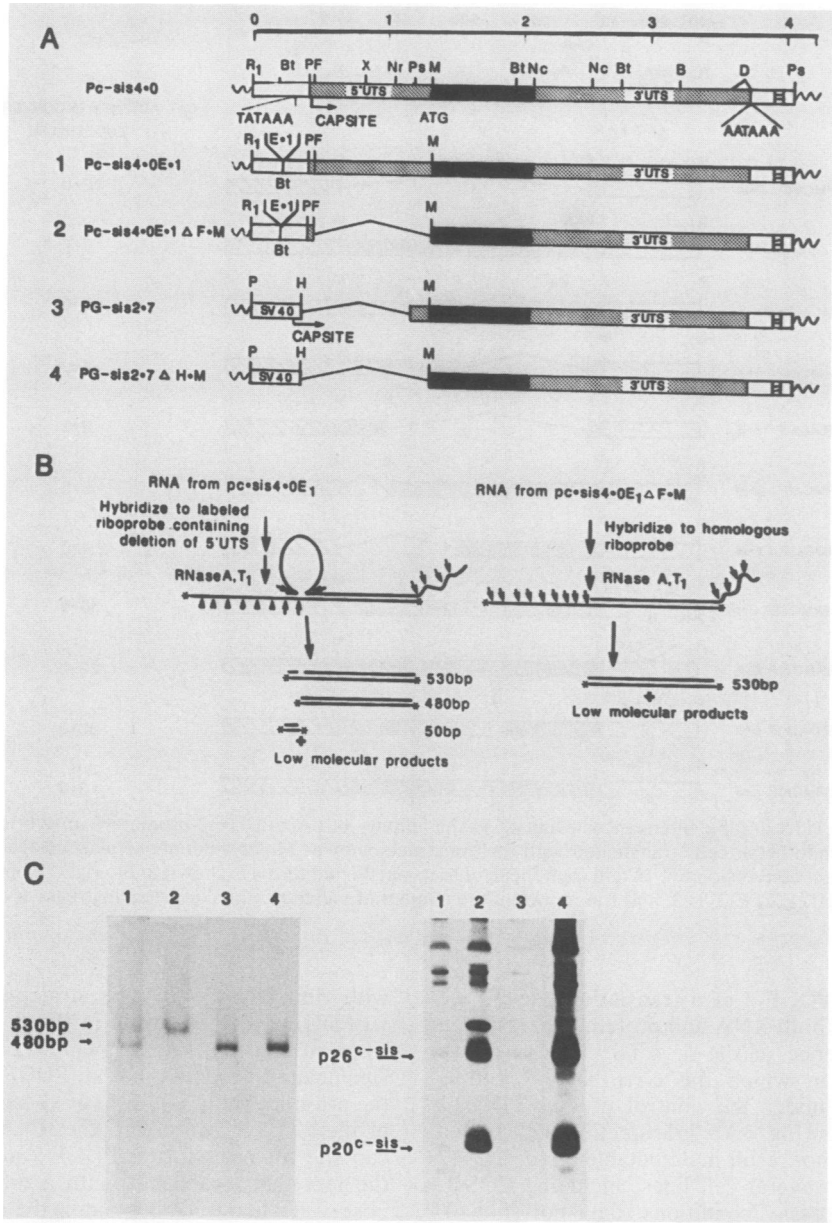

FIG. 3. Influence of 5′ UTS on transcription and translation of c-*sis*/PDGF-2. (A) Structural representation of constructs. The construction of pG-*sis*2.7 (PGSS2.7) and pG-*sis*2.7ΔH.M (PGSS) has been described earlier (20). Abbreviations and symbols are as in Fig. 1. (B) Schematic representation of strategy for measuring c-*sis* RNA levels in transfected COS-1 cells by using a riboprobe corresponding to pc-*sis*4.0ΔF.M. Both the level and site of transcriptional initiation could be observed for plasmids pc-*sis*4.0-E.1 and pc-*sis*4.0-E.1ΔF.M, as they contained c-*sis*/PDGF-2 mRNA cap site sequences. Thus, RNA transcribed from pc-*sis*4.0-E.1 gives two fragments of 530 and 480 bp, whereas only the longer 530-bp band was observed for RNA from pc-*sis*4.0-E.1ΔF.M. With RNAs from pG-*sis*2.7 and pG-*sis*2.7ΔH.M, only the smaller 480-bp band was observed, since these plasmids contained cap site sequences from the SV40 early promoter. (C) Comparison of c-*sis*/PDGF-2 transcriptional and translational levels in COS-1 cell transfectants. c-*sis*/PDGF-2 expressed in COS-1 cells transfected with pc-*sis*4.0-E.1 (lanes 1), pc-*sis*4.0-E.1ΔF.M (lanes 2), pG-*sis*2.7 (lanes 3), and pG-*sis*2.7ΔH.M (lanes 4). c-*sis*/PDGF-2 RNA and protein products were quantitated by the RNase A-T_1 protection assay and immunoprecipitation (left and right panels, respectively). Sizes of transcriptional products are shown in base pairs. Translational products were detected as described (20), and bands representing $p26^{c\text{-}sis}$ and $p20^{c\text{-}sis}$ are indicated.

30-fold higher in cells transfected with the construct lacking the 5′ UTS compared with that containing this sequence.

In a previous study (20), we described SV40 early-promoter-driven *sis*/PDGF-2 expression vectors, pG-*sis*2.7ΔH.M and pG-*sis*2.7. These constructs contained the *sis*/PDGF-2 cDNA either lacking the 5′ UTS or containing only the 140-bp G · C-rich sequence located immediately upstream of the *sis*/PDGF-2 open reading frame, respectively. Transfection of COS-1 cells with these constructs led to no significant difference in mRNA levels (Fig. 3C). However, immunoprecipitation analysis revealed a 30- to 40-fold-lower level of the *sis*/PDGF-2 proteins in COS-1 cells transfected with the plasmid containing the G · C-rich sequence. These results established that the 5′ UTS exerts potent translational inhibition of *sis*/PDGF-2 expression and that the 140-bp G · C-rich sequence immediately upstream of the *sis*/PDGF-2 open

reading frame was nearly as effective in this regard as the entire 5′ UTS.

Upstream minicistrons do not significantly influence translation of c-*sis*/PDGF-2 open reading frame. It has been hypothesized that upstream nonoverlapping minicistrons may facilitate translation of the downstream major open reading frame (26, 35). In the case of a highly G · C-rich 5′ UTS, initiation at the upstream minicistrons has been postulated to result in the destabilization of secondary structures downstream (26). Since we observed that the highly G · C-rich stretch immediately upstream of the *sis*/PDGF-2 open reading frame could inhibit translation nearly as effectively as the entire 5′ UTS, we investigated the influence of the three minicistrons located upstream of this G · C-rich sequence of the 5′ UTS on translation of the *sis*/PDGF-2 open reading frame. For these studies we used constructs in which the *sis*/PDGF-2 sequence containing various numbers of these minicistrons was expressed under the control of the *sis*/PDGF-2 promoter and SV40 enhancer. Following transfection of COS-1 cells, the levels of RNA and protein were compared. The presence of three (Fig. 4, lane 2), two (lane 3), or none (lane 4) of the upstream minicistrons had no observable effect on the efficiency of *sis*/PDGF-2 translation. The levels of *sis*/PDGF-2 mRNA in all of these transfectants were comparable (data not shown). These findings suggest that the minicistrons present in the 5′ UTS of *sis*/PDGF-2 mRNA do not play a major role in regulating the efficiency of translation.

Inhibitory effect of 5′ UTS on transforming ability of the *sis*/PDGF-2 coding sequence. To investigate the effect of the 5′ UTS on c-*sis*/PDGF-2 gene transforming ability, we transfected NIH 3T3 fibroblasts with constructs that either contained or lacked the 5′ UTS. As shown in Fig. 5, constructs which lacked the 5′ UTS demonstrated an ap-

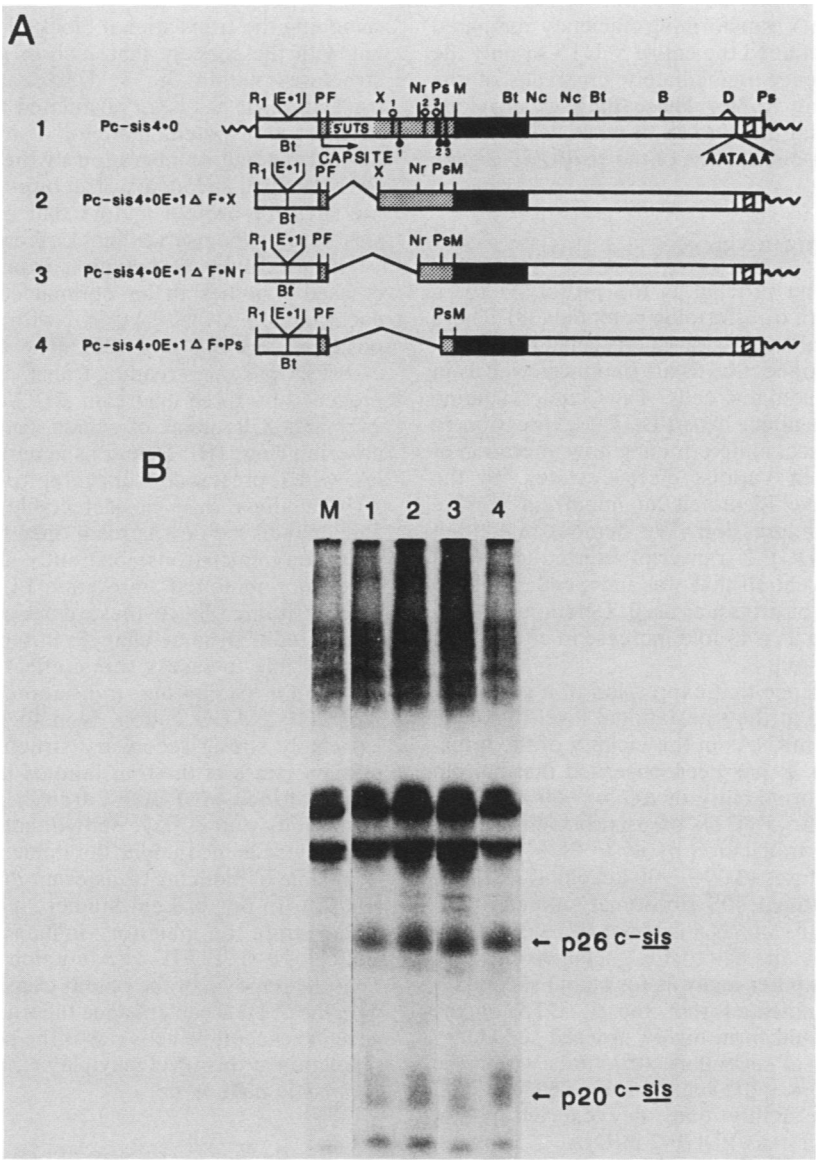

FIG. 4. Effects of upstream minicistrons on the translation of *sis*/PDGF-2. (A) Schematic representation of constructs containing three (pc-*sis*4.0-E.1ΔF.X), two (pc-*sis*4.0-E.1ΔF.Nr), or no (pc-*sis*4.0-E.1ΔF.Ps) minicistrons. Abbreviations and symbols are as in Fig. 1 and 2. (B) Expression of c-*sis*/PDGF-2 proteins in COS-1 cell transfectants. Translational products were immunoprecipitated as described (20). Bands representing p26 and $p20^{c\text{-}sis}$ are indicated. Lane M represents immunoprecipitation of mock-transfected COS-1 cells.

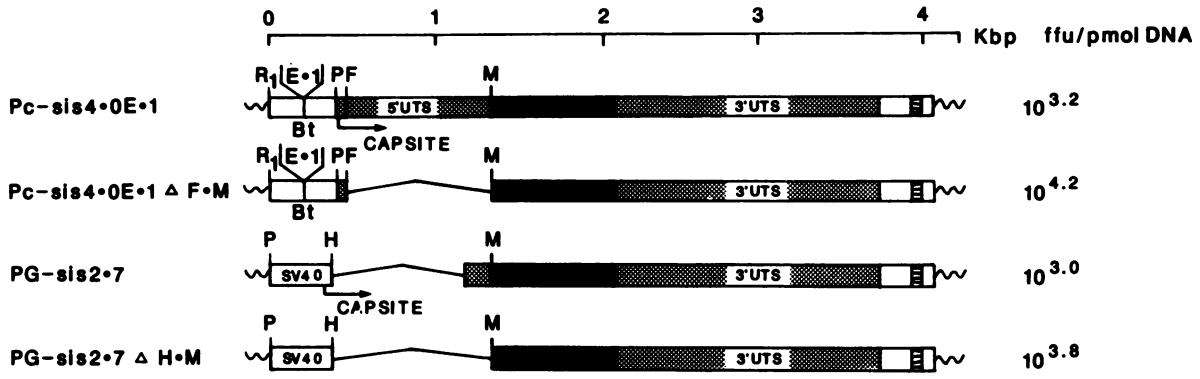

FIG. 5. 5′ UTS inhibits transformation by the *sis*/PDGF-2 gene. Constructs shown were used for transfection of NIH 3T3 cells as previously described (10). Foci were scored at 2 to 3 weeks. The specific transforming activity is expressed as focus-forming units per picomole of c-*sis*/PDGF-2 DNA. Abbreviations and symbols are as in Fig. 1 and 2.

proximately 10-fold-higher transforming efficiency compared with plasmids which contained the entire 5′ UTS or only the 140-bp G · C-rich sequence immediately upstream of the *sis*/PDGF-2 open reading frame. These findings provided independent evidence that the 5′ UTS of *sis*/PDGF-2 exerts a potent inhibitory effect on function of the *sis*/PDGF-2 gene as a transforming gene.

DISCUSSION

The c-*sis*/PDGF-2 gene product is the prototype for a normal growth factor with transforming potential (38). There is evidence that abnormal expression of this gene may play a role in development of connective tissue tumors as well as in other disorders of mesenchymal cells. Thus, understanding of the mechanisms that affect c-*sis*/PDGF-2 expression in normal cells may be critical in determining how alteration of this regulation results in various disease states. In the present studies, we have identified an important control affecting c-*sis*/PDGF-2 expression. We demonstrated that the 5′ UTS of the *sis*/PDGF-2 transcript exerted a striking inhibitory effect on translation that was independent of the reporter gene, cell type, or promoter used. Deletion of the 5′ UTS resulted in as much as a 40-fold increase in either CAT or c-*sis*/PDGF-2 expression.

There is growing evidence that expression of a gene may be negatively modulated at the translational level by stable secondary structures in mRNAs in the vicinity of the initiator ATG codon (24, 33). It has been observed that hairpin structures in the range of stability of $\Delta G = -50$ kcal/mol, when introduced into the 5′ UTS of preproinsulin, could reduce the efficiency of translation by 85 to 95%, whereas weaker secondary structures ($\Delta G = -30$ kcal/mol) could be readily melted by functional 40S ribosomal subunits (24). The 5′ UTS of c-*sis*/PDGF-2 mRNA is about 70% G+C, with stretches from 331 to 450 and 840 to 1022 being 90 and 85% G+C, respectively. Computer analysis for stable secondary RNA structures (46) indicated that the 5′ UTS of the c-*sis*/PDGF-2 mRNA could form highly ordered secondary structures. The stability of secondary structures within the 5′ UTS ranged from $\Delta G = -100$ kcal/mol to -250 kcal/mol, consistent with the possibility that they exerted potent translational inhibition of c-*sis*/PDGF-2 mRNA.

Each of several partial deletions within the 5′ UTS was shown to be associated with some abrogation of this inhibition. However, none of these individual deletions approached the effectiveness of deletion of the entire 5′ UTS in removing the translational block. These results are consistent with the concept that each of several stable secondary structures within the 5′ UTS can independently inhibit translation. It has been postulated that a G · C-rich 5′ UTS may act as a potent inhibitor of translation by preventing normal binding or migration of the 40S subunits along the mRNA. Such secondary structures may also serve as binding sites for protein factors that either stabilize the RNA structure or themselves block ribosome progression (24).

The efficiency of initiation from an AUG codon is increased if it lies in an optimal context, which in higher eucaryotes is $CC^{A}_{G}CCAUGG$, with purines being critical at positions -3 and $+4$ (25). The initiator codon for the c-*sis*/PDGF-2 open reading frame is in such a context and is preceded by three upstream AUG codons at positions 582, 751, and 870, none of which meet this favored-position criterion (Fig. 1B). Deletions generated in our present studies, which preserved from three to none of these upstream AUG codons, had no detectable effect on translational efficiency of the downstream open reading frame. Thus, the upstream minicistrons apparently neither significantly facilitated nor inhibited translation of the c-*sis*/PDGF-2 open reading frame. Since these deletions presumably are also associated with some changes in secondary structure, it was not possible to assess subtle effects of the upstream AUG codons in facilitating translation from the downstream *sis*/PDGF-2 AUG codon. Nonetheless, it appears that the extremely strong secondary structures of 5′ UTSs are the primary cause of the translational inhibition exerted by this region of the c-*sis*/PDGF-2 transcript.

Previous studies have shown that the normal c-*sis*/PDGF-2 coding sequence under the influence of a strong promoter is capable of inducing transformation of NIH 3T3 fibroblasts (10, 22). In our present studies, it was possible to directly demonstrate the inhibitory influence of the 5′ UTS on the ability of c-*sis*/PDGF-2 to function as a transforming gene. That the removal of the potent translational block associated with the 5′ UTS can enhance the transforming activity of this gene in susceptible cells raises the possibility that changes in regulation at this level may play a role in development of the neoplastic phenotype.

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