PDGF2/c-sis mRNA Leader Contains a Differentiation-linked Internal Ribosomal Entry Site (D-IRES)*

Jeanne Bernstein‡, Onsart Sella‡, Shu-Yun Le§, and Orna Elroy-Stein‡

From the Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel and the Laboratory of Mathematical Biology, DCBDC, NCI, NIH, Frederick, Maryland 21702

It has become clear that a given cell type can qualitatively and quantitatively affect the expression of the platelet-derived growth factor B (PDGF2/c-sis) gene at multiple levels. In a previous report, we showed that PDGF2/c-sis 5'-untranslated region has a translational modulating activity during megalakaryocytic differentiation of K562 cells. This study points to the mechanism used for this translational modulation. The unusual mRNA leader, which imposes a major barrier to conventional ribosomal scanning, was found to contain an internal ribosomal entry site that becomes more potent in differentiating cells and was termed differentiation-linked internal ribosomal entry site (D-IRES). The D-IRES element defines a functional role for the cumber- some 1022-nucleotide-long mRNA leader and accounts for its uncommon, evolutionary conserved architecture. The differentiation-linked enhancement of internal translation, which provides an additional step to the fine tuning of PDGF2/c-sis gene expression, might be employed by numerous critical regulatory genes with unusual mRNA leaders and might have widespread implications for cellular growth and development.

The mechanisms that control the expression of genes operate at a variety of levels that include all the transcriptional and post-transcriptional stages. In a rapidly growing number of examples, gene expression is regulated also at the level of translation, usually involving the translational initiation step. mRNA leaders that mediate efficient translation initiation are short (50–70 nt),1 contain 5'-m7G cap structure, and lack stable secondary structures as well as upstream AUG codons (reviewed in Ref. 1). Interestingly, genes encoding for regulatory proteins involved in growth and development often do not meet these criteria and produce long, structured, and upstream AUG-burdened mRNA leaders. These features are incompatible with efficient translation by the ribosomal scanning mech- anism which is considered valid for the translation of the vast majority of cellular and viral mRNAs (2).

Platelet-derived growth factor (PDGF) is a potent mitogen of all cells of mesenchymal origin and has a major role in wound healing as well as in embryogenesis and development. PDGF consists of homo- or heterodimers of two protein chains, PDGF-A and PDGF-B. The PDGF-B chain is the product of the PDGF2/c-sis proto-oncogene, which has a neoplastic transformation potential and is expressed in various types of tumor tissues. Normally, the expression of PDGF2/c-sis is tightly regulated due to multiple control mechanisms at the transcriptional and post-transcriptional levels. PDGF2/c-sis mRNA has a striking architecture: it has a 723-nt coding region and exceptionally long 5'- and 3'-untranslated regions (UTRs) of 1022 and 1625 nt, respectively. The extraordinarily long 5'-UTR contains stable secondary structures and three mini-open reading frames upstream of the translation initiator AUG codon. As such, it imposes a significant barrier to the linear ribosomal scanning from the 5'-m7G cap structure toward the fourth AUG codon, thereby serving as a potent translational inhibitor (reviewed in Ref. 3).

One of the major sites of PDGF synthesis is within bone marrow megakaryocytes, the platelets progenitor cells. As shown in our earlier work, the 5'-UTR-mediated translational inhibition was relieved at a certain time window during megalakaryocytic differentiation (4). The translation inhibitory effect of PDGF2/c-sis 5'-UTR was not relieved by elevated levels of the cap-binding protein eIF4E (4), nor by mutations of the upstream AUG codons (5, 6). These observations prompted us to examine whether during the differentiation process PDGF2/c-sis mRNA is translated by an alternative cap-inde- pendent initiation mechanism.

A cap-independent mechanism for translation initiation has been demonstrated for picornaviruses, which lack 5'-m7G cap and have long, structured, AUG-burdened mRNA leaders. An internal ribosomal entry site (IRES) was found in members of the cardio-, aphyto-, entero-, rhino-, and hepatoviruses, as well as in pestiviruses. Although there is no similarity in the primary sequences of the different IRES elements, there are similarities in their secondary structural features (reviewed in Refs. 7 and 8). Interestingly, internal translation has been found also for the cellular genes of mammalian Bip (9), FGF2 (10), IGF-II (11), eIF4G (12), and Antennapedia of Drosophila melanogaster (13). In this study we demonstrate that the PDGF2/c-sis 5'-UTR contains an IRES element that responds to changes in the cellular milieu and facilitates translation of the mRNA during megalakaryocytic differentiation. Computer analysis involving inspection of compensatory base changes among divergent PDGF2/c-sis sequences from human, cat, and mouse predicts structural features within the 5'-UTR that are common to known IRES elements.

This study provides the first example for a differentiation-
linked IRES (D-IRES) element, which might be one of many inducible IRES elements in extraordinary mRNA leaders of cellular genes encoding critical regulatory proteins. 

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**

pT7CAT-LUC obtained from Dr. Sarnow (9) was digested with SalI, filled in, and ligated with 1-kb NotI-MulI filled in fragment of pPD1 (4), to create pT7CPL. pT7CEL was constructed by three-part ligation of the following fragments: (i) 0.5-kb SalI/filled-in-NotI fragment from pT7CAT-LUC, (ii) 0.5-kb ApaI/filled-in-NotI fragment of pEMCV-CAT (14), and (iii) the backbone NotI fragment of pT7CAT-LUC. To construct pT7CHL, 0.7-kb SalI fragment was generated from pHAVT template (15) by polymerase chain reaction using oligonucleotide primers 5’-GGGGTTGACGCATTACCGCGCTG-3’ and 5’-GGGGGGTCGCATTTAAATGCCCC-3’, followed by SalI digestion and ligation into the SalI site of pT7CAT-LUC. The bi-cistronic plasmids under the CMV promoter were generated using plBI-FC1 or pH-FC1 from Dr. A.C. Prats as backbones (10). The following cloning steps were performed: 1.8-kb NotI-SacI fragment harboring the LUC gene was generated by polymerase chain reaction using pT7CAT-LUC (9) as template and oligonucleotide primers 5’-GGGTGCAAGCGGCCCCGATGGAACTGTA-3’ and 5’-GCCTCAGCTTTCCCCATCTCATTTTG-3’. The NotI-SacI fragment was cloned into NotI-SacI digested pT7CEL or pT7CHL, respectively. In addition, the 0.5-kb SacI-NcoI fragment harboring the LUC gene was ligated with the 4.1-kb SacI-NcoI fragment of pBI-FC1 to create the bi-cistronic pCL plasmid. Intact or truncated c-sis 5’-UTRs were inserted into pBI-LUC, upstream to LUC. This was done by ligating pBS-LUC/SacI-NcoI fragment with polymerase chain reaction fragments which were generated by using pisa4.0 (5) as template and oligonucleotide primers homologous to specific c-sis 5’-UTR sequences. The following oligonucleotides, which bear synthetic SpeI or NcoI sites, were used: 5’-CCCCCCTAGTGAAAATCCTTCCTC-3’ (JB7) and 5’-CCCCCATGGACGGTGCCCAGGCCC-3’ (OS35) to amplify the ATM region; 5’-GGGCTCAGCTTTCCCCCATCTCATTTTG-3’ (OS68) and 5’-CCACCATGGAATCCTACCGCCGCGGCGGCGG-3’ (OS69) to amplify region 215–488; and 5’-GGGTGCAAGCGGCCCCGATGGAACTGTA-3’ (OS72) and 5’-CTACCCAGCTTTCCCCCATCTCATTTTG-3’ (OS73) to amplify region 475–870. The ATM of the NcoI site was designed to match exactly with the ATM of LUC. The SpeI-SacI fragment containing the full-length 5’-UTR fused to LUC was ligated with the 4.1-kb SpeI-SacI fragment of pH-FC1 or pH-FC1 to create pCPL or pHCPL, respectively. The SpeI-SacI fragments containing the truncated 5’-UTR fused to LUC were ligated with the 4.1-kb SpeI-SacI fragment of pSCT Bil8 to create pC(215–488)L and pC(475–870)L.

**Meggakaryocytic Differentiation and Plasmid Expression**

**Infection-Transfection**—Meggakaryocytic differentiation was induced by treatment of exponentially growing K562 cells with TPA (Sigma) at multiplicity of infection of 4 to 105 g of supercoiled plasmid DNA per a total of 2 × 107 cells using Tri Reagent procedure (Tri Reagent®, MRC, Inc.), which is based on the phenol and guanidine thiocyanate in a monophase solution. Poly(A)⁺ RNA was purified from 150 μg of total RNA using oligo(dT) magnetic beads (Dynatech, Inc.). Poly(A)⁺ RNA was subjected to Northern blot analysis according to standard procedures (17), using 32P-labeled cDNA probes specific for the CAT, PDGF2/c-sis, LUC, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. To quantify the RNA, the intensities of specific bands were determined using the phosphorimager (Fuji Bas 100, Fuji).

**RESULTS**

**Effect of Megakaryocytic Differentiation on Activity of IRES Elements**—The leader of PDGF2/c-sis mRNA is highly complex and imposes a major difficulty to linear ribosomal scanning. However, as demonstrated previously, megakaryocytic differentiation of K562 cells alleviates its translational inhibitory effect (4). We therefore wished to examine whether PDGF2/c-sis mRNA leader harbor a weak IRES element which is activated upon differentiation. Since internal translation activity is known to be dependent on trans-acting factors (reviewed in Refs. 7 and 18), the performance of IRES elements is assumed to be affected by the cellular availability of such factors. Hence, the first objective was to determine whether the cellular environment created by the TPA-induced differentiation is more supportive for internal translation. Internal ribosomal entry is traditionally demonstrated using bi-cistronic expression plasmids. In these plasmids, the second cistron is not translated unless it is located immediately downstream to an active IRES which is downstream to the stop codon of the first cistron. Choromaphenicol acetyltransferase (CAT) and luciferase (LUC) reporter genes were used as the first or second cistrons, respectively, under control of the T7 promoter. The well characterized IRES elements of encephalomyocarditis virus (EMCV) or hepatitis A virus (HAV) were inserted between the two cistrons, downstream to the CAT translational stop codon, to create plasmids pT7CEL or pT7CHL, respectively. In addition, the 5’-UTR of PDGF2/c-sis was inserted between the two cistrons to create pT7CPL. Plasmid pT7CL, in which LUC is located immediately downstream to the stop codon of CAT (Fig. 1A), served as the control plasmid. The hybrid T7/vaccinia expression system was employed to express bi-cistronic mRNA from T7 promoter in the cytoplasm of control or TPA-treated K562 cells.

Control or TPA-treated K562 cells were infected with vTF7-3, a recombinant vaccinia virus that expresses the T7 RNA polymerase in the cytoplasm, and transfected with one of the above bi-cistronic plasmids. 24 h after the infection-transfection experiment, CAT and LUC enzymatic activities were measured. Fig. 1B illustrates the LUC/CAT ratio obtained in the differentiated cells relative to the control cells for each of the plasmids. As shown, the differentiation process generated a cellular milieu that facilitated the internal translation conferred by the viral IRES elements. The enhancement of EMCV IRES activity was about 3-fold whereas that of HAV was less prominent (Fig. 1B). Interestingly, differentiation-linked activation of the second cistron translation was also observed upon insertion of a PDGF2/c-sis 5’-UTR (Fig. 1B, pT7CPL). This phenomenon led us to speculate that the mRNA leader of PDGF2/c-sis might contain an IRES element that gains activity upon certain cellular changes.

**Computerized Prediction of c-sis 5’-UTR mRNA Folding**—From studies on picornaviruses, IRES elements are known to have structural features that are important for their role as mediators of internal ribosomal entry (7, 18). The RNA structural analysis of PDGF2/c-sis 5’-UTR was performed by theo-
Two pseudoknot structures, denoted by the letter K in Fig. 2, were predicted. One (K1) is formed by interaction between sequences in loop B5b of Y-type structure B5 with sequences in the loop of structure B7. The potential pseudoknot is present in the human, mouse, and cat structures, although the interacting sequences are not phylogenetically conserved. The second pseudoknot (K2) is formed by interaction of CCCG in the Y-type structure F loop (F′, Fig. 2B), with CGGG just downstream of it. The evolutionary conservation of this interaction suggests that the structure has a functional relevance.

A sequence complementary to the conserved 3′-end of 18 S rRNA was found at the 3′-end of the 5′-UTR, just upstream of the fourth AUG codon, from which the PDGF2/c-sis open reading frame begins. The region of complementarity was predicted to be single-stranded (G, Fig. 2, A and B). It is noteworthy that picornavirus IRES elements share a conserved complementary sequence to the 3′-end of human 18 S rRNA (23, 24).

All the above features are incompatible with the notion that cis-elements within the 5′-UTR of PDGF2/c-sis confer internal translation initiation. A Y-type structure, followed by a pseudoknot interaction and a sequence complementary to 18 S rRNA, immediately upstream to the start codon of the internal translation, is a common structural feature observed in all IRES elements of picornaviruses, hepatitis C virus, and pestiviruses RNAs (23–25). All the Y-type structures predicted for human PDGF2/c-sis mRNA leader were found to be conserved in the feline and murine PDGF2 mRNA leaders. The evolutionary conservation of these distinct structures suggests a role in the IRES-dependent translation.

Effect of Differentiation on Internal Translation Conferred by Truncated mRNA Leader—The relevance of the results shown in Fig. 1B to a cellular setup in which transcription takes place in the nucleus was verified by determining the behavior of the bi-cistronic mRNA in the absence of vaccinia virus infection. To that end, another set of bi-cistronic plasmids was constructed employing the CMV promoter (Fig. 3A). In pCL plasmid, the LUC reporter gene was located immediately downstream to the translational stop codon of CAT, whereas in pCPL the entire PDGF2/c-sis 5′-UTR was located between the two cistrons. To determine the ability of specific structural motifs to mediate the TPA-linked IRES activity, two additional plasmids, pC(205–488)L and pC(475–870)L, were constructed according to the structural model of the 5′-UTR presented in Fig. 2A. In these plasmids, the regions spanning nucleotides 215–488 or 475–870 of the 5′-UTR were located between the two cistrons, respectively. Each of these plasmids was introduced into K562 cells by electroporation, followed by incubation of the cells in medium with or without TPA for 48 h. Following TPA treatment, CAT and LUC enzymatic activities and mRNA levels were detected. The integrity of the bi-cistronic mRNA was verified by Northern blot analysis using 32P-labeled cDNA probes specific for the CAT, LUC, or GAPDH genes. The endogenous GAPDH mRNA level served as internal control to monitor the plasmid-derived mRNA level present in each of the different samples. As observed in Fig. 3B, the expected 3.8-kb bi-cistronic mRNA was detected by both CAT and LUC probes. The anticipated reductions in the bi-cistronic mRNAs length were observed, as expected, from plasmids that contained the truncated 5′-UTR. An additional, unexpected, smaller mRNA picked up by the CAT probe probably reflects cross-hybridization with cellular mRNA, as seen previously by others (9). However, the LUC probe picked exclusively the bi-cistronic mRNA, indicating its integrity. Longer exposure of the blot after its hybridization with 32P-labeled LUC cDNA clearly demonstrated the absence of smaller LUC mRNA molecules (Fig. 3B), implying that LUC protein synthesis results from translation of the second cistron. Unexpectedly, TPA treatment

Fig. 1. Effect of differentiation on activity of IRES elements. A, schematic drawing of the T7 transcription unit of pT7CL, pT7CHL, pT7CEL, and pT7CPL plasmids. Triangles, T7 promoter (P-T7); dark boxes, the CAT coding sequence; open boxes, 5′-UTR of HAV, EMCV, or c-sis as indicated; light boxes, the LUC coding sequence, circles, T7 terminator (T-T7). B, control or TPA-treated cells were infected with vTF7-3 and transfected with each of the indicated plasmids (see “Experimental Procedures”). 24 h later, CAT and LUC enzymatic activities were determined. The data represent the LUC/CAT ratio obtained in the TPA-treated cells relative to control cells. The bars represent the average ± S.E. of three independent transfection experiments.

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FIG. 2. Secondary structure model of the 5′-UTR of human PDGF. A, conserved structure of PDGF2/c-sis 5′-UTR, which is composed of six domains, A–F. All stem-loop structures are marked and refer to specific nt regions of the 5′-UTR as follows: A, 4–187; A2a, 20–73; A2b, 74–128; A2c, 133–164; B, 192–685; B2, 202–226; B3, 232–495; B3a, 272–412; B3ab, 299–336; B3b, 337–382; B4, 498–511; B5, 512–581; B5a, 520–549; B5b, 552–570; B6, 587–596; B7, 600–632; B8, 633–672; C, 689–781; D, 784–860; D2a, 793–817; D2b, 819–853; E, 866–932; F, 934–1005; F2a, 946–969; F2b, 970–990. Nucleotide numbering refers to the human PDGF2/c-sis 5′-UTR (GenBank™ accession number M19719).

RNA pseudoknot interactions are denoted by K1 and K2. The three upstream AUG codons are denoted by dark squares 1–3, and the corresponding in-frame stop codons are denoted by dark squares 1′–3′. AUG4, the translational initiator codon, is positioned at nt 1023–1025 and denoted by dark square 4. Sequence homologous to 18 S rRNA is marked as a heavier line and denoted by the letter G. A, sequence alignment of the 3′ portion of PDGF2/c-sis 5′-UTR. Sequences of human, mouse, and cat (GenBank™ accession numbers M19719, M64844, X05112, respectively) are shown. Dots indicate deletions. Conserved and nonconserved nt in the alignment are indicated by upper and lowercase letters, respectively. Conserved base pairing illustrated in A are marked by boxes and labeled by the letters B′-B′, C′-C′, D′-D′, E′-E′, F′-F′. A nt in a box enclosed by another box failed to form an appropriate base pair. Positions of AUG codons are indicated. Box G shows the complementary sequence (5′-GCCggaGUGGC-3′) to the 3′-end sequence (5′-GUCGuaACaaGGU-3′, nt 1826–1838) of human 18 S rRNA. Pseudoknot interactions are denoted by K1 and K2.
led to elevation in the steady-state level of all the CMV promoter-driven mRNAs. However, in view of the integrity of the bi-cistronic mRNA molecules, both CAT and LUC proteins are believed to be translated from an intact bi-cistronic mRNA. Thus, the LUC/CAT ratio deduced from LUC and CAT enzymatic activities in the different samples reflects the relative translation efficiencies of both cistrons, regardless of the absolute mRNA level in the samples.

Fig. 3C shows the LUC/CAT ratio obtained in the differentiated cells relative to control cells for each of the plasmids. The pCL-derived LUC activity, which represents second cistron translation from an IRES-less mRNA, probably reflects some level of read-through, leaky scanning, re-initiation, or translation of undetectable degradation products of the bi-cistronic mRNA. However, such pCL-derived second cistron translation was hardly affected by TPA treatment (Fig. 3C, pCL). Insertion of region spanning nucleotides 215–488, which harbor structure B3 of the 5′-UTR (Fig. 2A) upstream of LUC, decreased LUC translation level about 2-fold compared with pCL (not shown), but did not change the relative LUC/CAT upon differentiation (Fig. 3C, pC(215–488)L). Insertion of the full-length (1022 nt) or a 395-nt segment spanning nucleotides 475–870, which harbor structures B4–D of the 5′-UTR (Fig. 2A), also decreased the second cistron translation by 5- or 2-fold, respectively, compared with pCL (not shown). However, these two segments were able to confer differentiation-linked LUC translation enhancement (Fig. 3C, pCPL and pC(475–870)L). In summary, the LUC/CAT ratio was not affected by the TPA treatment unless the full-length or a specific region of the 5′-UTR was inserted between the cistrons. These specific segments conferred about 2-fold enhancement in translation efficiency of the second cistron over the first cistron due to the TPA treatment. These observations suggest the presence of a differentiation-linked internal ribosomal binding site within the 5′-UTR of PDGFB/c-sis.

**The Effect of 5′ Hairpin on Translation Efficiency**—To verify the ability of the 5′-UTR to confer internal translation, we tested its ability to confer 5′-end-independent translation to the second cistron. For that purpose, a hairpin structure with stability of \( \Delta G = -40 \text{ kcal mol}^{-1} \) upstream to CAT (see "Experimental Procedures"). Triangles, CMV promoter; dark boxes, the CAT coding sequence; open boxes, intact or truncated 5′UTR of c-sis, as indicated; light boxes, the LUC coding sequence. B, Northern blot analysis. K562 cells were mock-transfected (lane 1) or transfected with pCMV-CAT (lanes 2 and 3), pCL (lanes 4 and 5), pCPL (lanes 6 and 7), pHCPL (lanes 8 and 9), pC(215–488)L (lanes 10 and 11), or pC(475–870)L (lanes 12 and 13), followed by incubation in medium with or without TPA (see "Experimental Procedures"). 72 h after the electroporation, poly(A)+ RNA from 150 \( \mu \text{g} \) of total RNA were used for Northern hybridization analysis using sequentially \( ^{32} \text{P} \)-labeled cDNA probes specific for CAT, LUC, or GAPDH, as indicated. Following each hybridization, the blot was exposed for 15 h, except for the LUC blot, which was also subjected to longer exposure for 4 days to rule out the presence of shorter luciferase transcripts. C, K562 cells were transfected with each of the indicated plasmids as detailed in B. 72 h after the electroporation, CAT and LUC enzymatic activities were determined. The data represent the LUC/CAT ratio obtained in the TPA-treated cells relative to control cells. The bars represent the average ± S.E. of three independent transfection experiments.
A. Effect of 5'-hairpin on CAT and LUC expression

B. Effect of 5'hp on differentiation-enhanced IRES potency.

**Fig. 4. Effect of 5' hairpin.** K562 cells were transfected with pCL (open bar), pCPL (dark bar), or pHcPL (striped bars) which are shown in Fig. 3A, followed by incubation in medium with (+TPA) or without TPA (-TPA). 72 h after electroporation, CAT and LUC enzymatic activities were determined. A. The pHcPL-derived LUC and CAT activities were compared with the pCPL-derived CAT or LUC activities, which were arbitrarily set as 1. The bars represent the average ± S.E. of three independent transfection experiments. B. The data represent the LUC/CAT ratio obtained in the TPA-treated cells relative to control cells for each of the plasmids, as indicated.

**DISCUSSION**

Structural elements that determine the efficiency of translation initiation include the 5'-m7G cap structure, the context surrounding the initiator codon, 5'-UTR length, stability of secondary structures in the 5'-UTR, and presence or absence of open reading frames upstream of the initiator codon (uORFs) (1). A hairpin structure with predicted stability of ΔG = -50 kcal mol⁻¹ in the 5'-UTR was found to significantly inhibit protein synthesis (26). In addition, uORFs, which in some cases have a regulatory role (27), were found to decrease the frequency of initiation of the major ORF. Thus, it is not surprising that the extraordinarily long PDGF2/c-sis 5'-UTR, which contains stable secondary structures and three uORFs acts as a strong translational inhibitor (4-6, 28). Mutations of the uORFs did not relieve the inhibitory effect (5-6, 28). This observation, together with the highly stable secondary structures (ΔG = -270 kcal mol⁻¹) within the PDGF2/c-sis 5'-UTR, raised a question about the ability of ribosomes to linearly scan along this mRNA leader from its 5'-end according to the conventional scanning model (29).

As demonstrated in this study, when the PDGF2/c-sis 5'-UTR was placed upstream of the second cistron in a bi-cistronic vector, it conferred a differentiation-linked enhancement of translation with preference for the second cistron (Figs. 3C and 4B). However, regardless of the cellular conditions, the 5'-hairpin inhibitory effect on the first cistron was 3-4-fold stronger than on the second cistron, indicating the inherent ability of PDGF2/c-sis 5'-UTR to confer internal translation to the second cistron even before differentiation (Fig. 4A). These observations indicate the presence of an IRES element within PDGF2/c-sis 5'-UTR which has low or high activity depending on the cellular milieu. As shown in Figs. 1B, 3C, and 4B, the PDGF2/c-sis IRES potency was enhanced about 2-fold by TPA treatment. These data do not match our previous experiments with monocistronic vectors showing 8-fold 5'-UTR-mediated translational enhancement upon TPA treatment (4). We strongly believe that the stronger effect observed using monocistronic vectors reflects the behavior of the authentic PDGF2/c-sis mRNA. Sequences flanking the 5'-UTR in the bi-cistronic vector may dramatically affect its folding and consequently its IRES activity. This discrepancy is analogous to the discrepancies reported previously for other IRES elements studied in the context of mono- and bi-cistronic vectors (11, 30-31). In addition, since luciferase protein is very unstable compared with CAT (32), LUC activity reflects the level of newly synthesized LUC molecules, whereas CAT activity represents the total accumulated CAT enzyme. Thus, the relative LUC/CAT ratio, which in this study was used to measure IRES potency, can detect only the differentiation-linked enhancement phenomenon and not its actual intensity.

The evolutionary conservation of the stable secondary structures predicted for the PDGF2/c-sis mRNA leader, and the conserved complementarity to 18 S rRNA immediately upstream of the major ORF (Fig. 2), strongly suggest a role in mediating internal translation. The D-IRES activity was conferred by the full-length (1022 nt) 5'-UTR or by a 395 nt segment spanning nucleotides 475-870 of the 5'-UTR. In contrast, the 273-nt segment spanning nucleotides 215-488 of the 5'-UTR did not confer D-IRES activity and thereby served as a negative control in our experiments (Fig. 3). Interestingly, this non-IRES segment, harboring structure B3 (Fig. 2), is composed of highly stable stem-loop structures and is located upstream of the D-IRES-conferring segment. Hence, we suggest that the role of structure B3 is to inhibit PDGF2/c-sis mRNA translation in case it is present at the wrong tissue or time, by imposing a barrier to ribosomal scanning. As shown previously, the 5'-UTR-mediated translation inhibition is relieved during megakaryocytic differentiation of K562 cells (4). The present study demonstrates that translational inhibition relief is achieved by enhanced activity of an IRES element that resides downstream of structure B3, the scanning barrier.

It can be argued that expression of the major ORF reflects translation of undetectable shorter transcripts that are transcribed from a cryptic promoter and therefore lack most of the inhibitory 5'-UTR. Our data rule out this possibility for PDGF2/c-sis, since we used both cytoplasmic and nuclear expression systems. The cytoplasmic system employed the bacteriophage T7 promoter in the cytoplasm of vaccinia virus-infected cells, in which the host protein synthesis is shut off by...
the virus. The fact that the D-IRES phenomenon was observed in that cytoplasmic expression system (Fig. 1B) proves that a cryptic promoter activity within the PDGF2/c-sis 5'-UTR is not responsible for the differentiation-linked enhanced expression effect. We cannot rule out possible translation of degradation products that contain only the second cistron, but such a phenomenon does not negate our interpretation, since the small amount of potential degradation products is probably similar in all the samples, including the control plasmid pC (Fig. 3B).

It has become clear that a given cell type can qualitatively and quantitatively affect the expression of the PDGF A and B chains at the levels of transcription, RNA processing, translation, and post-translation modifications (3). The presence of an IRES element within PDGF2/c-sis 5'-UTR defines a functional role to the cumbersome mRNA leader and justifies its conserved unusual architecture. The evolutionary conservation of the secondary structures hints that the IRES activity is mediated by evolutionary conserved RNA-binding proteins. We speculate that the availability of such trans-acting factors differs in response to specific signals according to the cell’s need. The differentiation-linked enhancement of PDGF2/c-sis IRES activity might involve post-translational modifications of existing proteins or synthesis of new proteins with RNA binding activity. Apparently, the profile of proteins that bind to PDGF2/c-sis 5'-UTR dramatically changes due to differentiation of K562 cells. It has become evident that viral IRES elements require cellular trans-acting RNA binding proteins for their function (reviewed in Ref. 7). Thus, some of the differentiation-induced trans-acting factors might also mediate the IRES activity of certain viruses. The enhanced IRES activity of EMCV in the TPA-treated K562 cells (Fig. 1) supports this notion and underscores the importance of cell type to virulence potential. Additionally, the variable potency of different known IRES elements might result from variable binding affinities to common factors and/or different requirements for additional tissue-specific factors. In contrast to viral IRES elements, cellular IRES elements are expected to be much less potent. Indeed, we found that PDGF2/c-sis IRES is about 10-fold less active in TPA-treated K562 cells than EMCV IRES (not shown).

PDGF belongs to a subclass of genes that encode for regulatory proteins with a major role in cell proliferation. Among these are several proto-oncogenes as well as growth factors, cytokines, receptors, and transcription factors (2). A typical property of these genes is their tight expression regulation to guarantee correct protein level at the appropriate time and location. An associated feature is their complex architecture, that is, cis-regulatory elements that mediate expression level in response to signals. The stringent regulation is a result of various mechanisms that simultaneously control expression at multiple levels. One of the cis-elements shared by the above subclass of genes is their extraordinarily long, structured and AUG-burdened mRNA leader, which provides a built-in blockade against efficient ribosomal scanning. Although leaky scanning and reinitiation are the mechanisms responsible for some translation modulation phenomena (reviewed in Ref. 27), a conditional internal ribosomal entry may also provide a widespread mechanism for translation regulation. First, it gives certain mRNA molecules cap-independent translation ability in response to viral infection or stress conditions, as was shown for immunoglobulin heavy-chain binding protein (BiP) IRES (9) and recently proposed for eIF4G IRES (12). Second, it can enforce an alternative translational start site, resulting in translation of different proteins from the same mRNA molecule, as mediated by the IRES element of FGF2 (10). Third, it may provide a developmentally regulated protein synthesis like that proposed for the homeotic gene Antennapedia of D. melanogaster and for human IFG-II leader 1 IRES elements (13, 11). The present study provides evidence for a differentiation-linked IRES element residing within a cumbersome mRNA leader. In the absence of appropriate trans-acting factors, it may provide a double safety control mechanism, since it prevents efficient translation of the mRNA in cases of uncontrolled transcription. Upon differentiation, the IRES becomes more active, leading to efficient protein synthesis. The translational enhancement has an additive effect with other levels of control to achieve significant expression enhancement in the appropriate time window during differentiation. This mechanism, which provides an additional step to the fine tuning of PDGF2/c-sis gene expression, might be employed by numerous critical regulatory genes with unusual 5'-UTRs and might have widespread implications for cellular growth and development.

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Addendum—After completion of the study, we have noticed that TPA from Calbiochem is more potent than that of Sigma with regards to the effects measured in this research.

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Jeanne Bernstein, Osnat Sella, Shu-Yun Le and Orna Elroy-Stein

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