Cryptic promoter activity in the DNA sequence corresponding to the *pim-1* 5′-UTR

Zeping Wang, Matt Weaver and Nancy S. Magnuson*

School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4234, USA

ABSTRACT

The serine/threonine kinase *pim-1* mRNA contains a long and G/C rich 5′-untranslated region (5′-UTR). Previous work suggested that the *pim-1* 5′-UTR harbors an internal ribosomal entry site (IRES) allowing for internal initiation of translation. However, several previously reported eukaryotic IRES elements actually contain cryptic promoter activity. To test whether an IRES or a cryptic promoter is present in the *pim-1* 5′-UTR, the 5′-UTR was re-examined using stringent test procedures. Our results show the presence of strong promoter activity in the DNA sequence corresponding to the *pim-1* 5′-UTR. Both promoterless dicistronic test and northern blot analysis show transcripts being derived from the cryptic promoter in the *pim-1* 5′-UTR sequence. This cryptic promoter is active in all cell types tested, including Cos-7, NIH3T3, HEK293, Jurkat and K562 cells. When a dicistronic mRNA containing the *pim-1* 5′-UTR was translated *in vitro* or *in vivo*, no IRES activity could be detected. However, the control IRESs from both human rhinovirus and encephalomyocarditis virus exhibited strong IRES activities. In addition, both the RNase protection assay and the 5′-RACE assay detected endogenous *pim-1* transcripts with shorter 5′-UTRs. Our data strongly suggest that the IRES activity reported earlier for the *pim-1* 5′-UTR sequence is due to cryptic promoter activity.

INTRODUCTION

The proto-oncogene *pim-1* was originally identified as a preferential integration site of the moloney murine leukemia virus, which induces T-lymphomas in mice (1). Oncogenicity of *pim-1* has been well documented in both transgenic and retroviral models (2,3). By itself, *pim-1* has low oncogenic potential but cooperates strongly with other oncogenes, such as *c-myc*, *n-myc*, *bcl-2* and *gfi-1*, in T-lymphomagenesis (4–7). The *pim-1* gene encodes a serine/threonine kinase (8), and a recent report on its crystal structure indicates that it is a constitutively active kinase (9).

In addition to functioning in tumorigenesis, Pim-1 kinase also plays a role in cell survival, cell differentiation and cell proliferation [reviewed in (10)]. Recent studies show that Pim-1 protects hematopoietic cells from cell death caused by cytokine withdrawal, glucocorticoids or genotoxins (11–13). While the anti-apoptotic mechanisms of Pim-1 remain largely unknown, the finding of phosphorylation and inactivation of the pro-apoptotic protein Bad might provide a partial explanation for the Pim-1's role in cell survival (14). Early studies on the developmentally regulated expression of Pim-1 (15) and its association with the germ cell maturation (16) indicate an involvement of Pim-1 kinase in the differentiation of hematopoietic cells and germ cells. Pim-1 expression was also found to be clearly correlated with the increased differentiation of keratinocytes (17). A recent study by Zippo *et al.* (18) shows that Pim-1 kinase is required for endothelial and mural cell differentiation. Expression of Pim-1 kinase in hematopoietic cells is induced by a variety of cytokines, growth factors and mitogens (19–21). Cell cycle regulated expression of Pim-1 (22) and identification of several cell cycle regulators, including cdc25A (23), p21$^{cip1/waf1}$ (24) and NuMA (25) as substrates suggest that Pim-1 is important in cell proliferation, which has been confirmed by the recent study of Pim-1's role in smooth muscle cell proliferation (26).

Expression of the Pim-1 kinase is highly regulated and several mechanisms by which Pim-1 expression is controlled have been characterized. The levels of *pim-1* mRNA are regulated in part by transcriptional attenuation (27) as well as by the induction of *pim-1* transcripts upon mitogenic stimulation (28). The level of *pim-1* mRNAs is also controlled post-transcriptionally by modulation of mRNA stability (27,29). In addition, the total level of Pim-1 protein has been shown to be regulated post-translationally with heat shock protein, Hsp90,

*To whom correspondence should be addressed. Tel: +1 509 335 0966; Fax: +1 509 335 1907; Email: magnuson@wsu.edu
Present address:
Matt Weaver, Benarova Research Institute, Seattle, WA 98101-2795, USA

increasing the stability of Pim-1 (30) while overexpression of phosphatase PP2A reduces the level of Pim-1 protein (31).

Pim-1 expression is also regulated by its 5′-untranslated region (5′-UTR), which is long and G/C-rich (32). Our previous study showed that the 5′-UTR of *pim-1* mediates the inhibition of cap-dependent translation (33). Another report also indicated that *pim-1*'s 5′-UTR appears to contain an internal ribosome entry site (IRES) element, which allows for the internal initiation of the translation of *pim-1* mRNA under conditions that repress cap-dependent translation, such as viral infection (34). However, the notion of IRES-mediated translation in eukaryotes has recently been challenged on the basis of the methods typically used for the identification of IRES elements in eukaryotic mRNAs (35). It was proposed that IRES activity in cells transiently transfected with dicistronic DNA constructs may result from aberrant RNA cleavage, RNA splicing and/or from the presence of a cryptic promoter within the DNA construct itself (36). This could contribute to the formation of low amounts of monocistronic message that might be translated via conventional ribosomal scanning mechanisms. Several recent reports have also shown that previously claimed IRES elements actually contain cryptic promoter activities (37–40). Therefore, despite the initial finding indicating a putative IRES element in the *pim-1* 5′-UTR, further rigorous testing is required for the positive identification of real eukaryotic IRESs (41).

In this study, we tested whether an IRES element or a cryptic promoter is present in the *pim-1* 5′-UTR using more comprehensive and rigorous methods of analysis. Our results showed that cryptic promoter activity is present in the *pim-1* 5′-UTR sequence although we found that it is very difficult to disprove the presence of IRES. We found that DNA sequence corresponding to the *pim-1* 5′-UTR could regulate the expression of Pim-1. Thus, our data strongly suggest that the IRES activity reported earlier for the *pim-1* 5′-UTR sequence might be due to largely the cryptic promoter activity.

MATERIALS AND METHODS

Materials

Restriction enzymes, Lipofectamine 2000, DMRIE-C and GeneRacer kit were purchased from Invitrogen. T7 RiboMAX large-scale RNA production system, rabbit reticulocyte lysate (RRL) system, m⁷GpppG cap analog and Dual luciferase reporter assay system were from Promega. HeLa cell cytosol extract S100 and nuclear extract were from Protein One (college park, MD). Galactolight plus assay system was from Applied Biosystem. Midi plasmid purification kit, RNeasy mini kit and Oligotex mRNA mini kit were obtained from Qiagen. [α-^{32}P]dCTP and [α-^{32}P]CTP were from Perkin Elmer. Hybond N$^+$ membrane was from Amersham Biosciences. ULTRAhyb hybridization buffer, MEGA clear RNA purification kit, MAXIscript *in vitro* transcription kit and RPA III RNase Protection Assay kit were obtained from Ambion.

Plasmids constructs

The following plasmids were kindly provided by Dr A. Willis (University of Leicester, UK) and Dr J.-T. Zhang (Indiana

University, USA): pF (formerly pGL3) (42), pHpF (formerly pHpL) (43), pRF (formerly pGL3R) (42), pGL3RutrH (42), pR-HRV-F (38) and pR-EMCV-F (43).

The 5′-UTR of *pim-1* was amplified by PCR with the following two primers containing SpeI and NcoI sites: 5′-ATACTAGTGCTGCAGCGGCCGCGGTGGCTGA-3′ and 5′-AACCATGGCCCAACCTCCAGGATGTCGGCGCA-3′. The PCR product was cloned into the SpeI and NcoI sites of the plasmids pF and pHpF to make plasmids pF-PIM and pHpF-PIM, respectively. The 5′-UTR of *pim-1* was then introduced into the SpeI and NcoI sites of pRF to create pR-PIM-F. Dicistronic plasmid pGL3RutrH, containing a stable hairpin at the 5′ end of *Renilla* luciferase, was used to make pHpR-PIM-F by replacing the 5′-UTR of *c-myc* with the 5′-UTR of *pim-1* in the SpeI and NcoI sites. The DNA sequence of this hairpin structure was originally reported by Stoneley *et al.* (42). pHpRF was created by removing the 5′-UTR of *c-myc* from pGL3RutrH through digesting with SpeI and NcoI.

To generate promoterless dicistronic constructs that allow analysis of promoter activity of the DNA insert in the intergenic region, the Simian virus 40 (SV40) promoter sequence was removed by SmaI and EcoRV digestion from pRF, pR-HRV-F, pR-EMCV-F and pR-PIM-F, resulting in RF, R-HRV-F, R-EMCV-F and R-PIM-F, respectively. The integrity of all plasmid constructs were confirmed by DNA sequencing.

In vitro transcription and translation

The plasmids pRF, pR-HRV-F, pR-EMCV-F and pR-PIM-F were linearized with BamHI. The capped and uncapped transcripts were synthesized with T7 RiboMAX large-scale RNA production system according to the manufacturer's protocol. The mRNAs were purified with Ambion's MEGA clear kit, quantified spectrophotometrically and their qualities were verified on a denaturing agarose gel. Equal amounts of RNA (typical final concentration of 20 ng/µl) were used in a 20 µl *in vitro* translation reaction mixture comprising 70% RRL. The translation mixture contained either 4 µl of extract buffer, 4 µl of HeLa cytosol extract S100 (8 µg/µl) or 2 µl of HeLa cytosol extract plus 2 µl of HeLa nuclear extract (8 µg/µl). An aliquot of 2 µl of each reaction mixtures were used in a luciferase activity assay (described below).

Cell culture, DNA and RNA transfection

HeLa, HEK293, NIH3T3 and Cos-7 cells were cultured in DMEM, while DU145, K562 and Jurkat cells were cultured in RPMI 1640, at 37°C with 5% CO$_2$ supplemented with 10% fetal bovine serum and containing penicillin/streptomycin. All cells were purchased originally from ATCC. DNA transfection of adherent cells was performed with Lipofectamine 2000 according to the manufacturer's protocol. For a typical transient transfection, cells were grown to 90–95% confluent in 24-well plates and then co-transfected with 0.8 µg of luciferase plasmid and 0.2 µg of pSV-β-galactosidase plasmid. Cells were harvested 30 h after transfection for luciferase and β-galactosidase activities assay. DNA transfection of suspension cells was performed with DMRIE-C according to the manufacturer's protocol. Cells (4 × 10^5) were co-transfected with 0.8 µg of luciferase plasmid and 0.2 µg

of pSV-β-galactosidase plasmid. At 30 h post-transfection, activities of luciferase and β-galactosidase were measured.

RNA transfection was performed with DMRIE-C according to the manufacturer's suggestion. An aliquot of 2 µg of capped mRNAs and 4 µl of DMRIE-C were used to transfect 80% confluent cells grown in 6-well plates. At 8 h after transfection, cells were harvested and lysed for luciferase activity assay.

Luciferase and β-galactosidase assay

Transfected cells were washed once with 1× PBS and then lysed in 120 µl of 1× passive lysis buffer. Firefly and *Renilla* luciferase activities were measured using a Dual luciferase reporter assay system according to the manufacturer's protocol with the exception that only 50 µl of each reagent was used. Light emission was quantified using a luminometer (Wallac Victor 2). Activity of β-galactosidase in cell lysates was determined using a Galacto-light plus assay system.

Northern blot analysis

Subconfluent Cos-7 cells in 10 cm plates were transfected with 4 µg/plate constructs using Lipofectamine 2000. At 48 h after transfection, the total RNAs were extracted using RNeasy mini kit. Residual plasmid DNA in the total RNAs was digested with RNase-free DNase. The poly(A) mRNAs were then isolated from 250 µg of total RNAs using an oligotex mRNA mini kit. One-tenth of the mRNAs were separated on a 1% formaldehyde-denatured agarose gel and transferred onto Hybond N$^+$ membranes. The blots were hybridized with a ^{32}P-labeled firefly luciferase probe. After stripping, the membranes were subsequently hybridized with a ^{32}P-labeled *Renilla* luciferase probe in ULTRAhyb hybridization buffer.

Rapid amplification of 5′ cDNA end

The 5′ end of the *pim-1* transcript was analyzed using the 5′-RACE method according to the protocol provided with GeneRacer kit. Briefly, total RNA (4 µg) was dephosphorylated with 10 U of calf intestinal phosphatase and precipitated by ethanol after phenol/chloroform treatment. The cap structure was subsequently removed with 0.5 U of tobacco acid pyrophosphatase to produce a phosphorylated RNA at the 5′ end, to which the RNA oligonucleotide (5′-CGACUGGGAG-CACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) was ligated. The resulting RNA was reverse-transcribed using a *pim-1* gene specific primer (5′-CGGGCGCCA-GCTTGGTG-3′) and 200 U of SuperScript III reverse transcriptase, and then treated with 2 U of RNase H. Tailed cDNA was amplified by PCR using the GeneRacer 5′ primer (CGACTGGAGCACGAGGACACTGA), the *pim-1* specific primer (5′-CTTGGTGGCGTGCAGGTCGTTGCA-3′) and 2.5 U of *Pfu* Turbo Hotstart DNA polymerase. The reaction condition was at 96°C for 2 min followed by 35 cycles at 96°C for 45 s, 56°C for 30 s and 72°C for 45 s. The reaction was then extended for 10 min at 72°C. PCR products were purified from 2% agarose gel and cloned using the PCR Zero Blunt Cloning kit. Individual clones were sequenced for the determination of the transcription start sites.

RNase protection assay

RNase protection assay was performed using the RPA III kit according to the supplier's instruction. Briefly, the template DNA (−435 to −1 of the human *pim-1* gene) was first cloned into the EcoRI and KpnI sites of the plasmid pBK/CMV. The resulting plasmid was linearized with EcoRI and transcribed with T7 RNA polymerase in the presence of 0.5 mM each of ATP, GTP, UTP and 0.02 mM CTP supplemented with 3.12 µM [α-^{32}P]CTP. The ^{32}P-labeled probe was digested with DNase and gel-purified after separation on a 6% acrylamide/8 M urea gel. About 3×10^5 c.p.m. of probe was hybridized with 10 µg total RNA at 45°C overnight, followed by digestion with RNase T1/A at 37°C. The protected RNA fragments were precipitated and then separated on a 6% acrylamide/8 M urea gel (from Invitrogen) for autoradiograghy.

RESULTS

The 5′-UTR of *pim-1* enhances translation when cap-dependent ribosomal scanning is inhibited

Structural prediction algorithms imply that the 5′-UTR of *pim-1* contains extensive RNA secondary structure which could reduce translational efficiency. Our previous study indicated that the 5′-UTR of *pim-1* inhibits translation of *pim-1* mRNA *in vitro* (33). To examine the effect of the 5′-UTR of *pim-1* on the expression of the downstream gene *in vivo*, we introduced this sequence into the reporter construct pF immediately upstream of the luciferase-coding region, to create pF-PIM (Figure 1A). In addition, we wanted to determine how the 5′-UTR sequence of *pim-1* affected translation when the classic cap-dependent mechanism was severely reduced. To achieve this, we inserted the 5′-UTR sequence of *pim-1* into pHpF between the inverted repeat sequence and firefly luciferase to create pHpF-PIM (Figure 1A). The inverted repeat sequence forms a very stable RNA hairpin structure ($\Delta G = -55$ kcal/mol) at the 5′ end of the mRNA and has been shown to inhibit cap-dependent translations (43).

Cos-7 cells were transfected with plasmids pF, pHpF, pF-PIM and pHpF-PIM, and the luciferase activity from each construct was determined. Interestingly, despite the *pim-1* 5′-UTR-mediated inhibition of translation *in vitro* (33), the expression of luciferase from pF-PIM is higher than that from pF. The 5′-UTR sequence of *pim-1* appears to enhance translation initiation *in vivo* (Figure 1B). As expected, the RNA hairpin sequence in the construct pHpF reduced luciferase expression by ~95% when compared with control construct pF. However, when the 5′-UTR sequence of *pim-1* was introduced between the hairpin and the reporter gene, luciferase expression was stimulated to a level above that observed with pF (Figure 1B). Because the hairpin structure efficiently inhibits cap-dependent translation initiation, it was concluded that an alternative mechanism must be responsible for the increased synthesis of luciferase.

5′-UTR sequence of *pim-1* enhances expression of the second cistron in dicistronic test

A previous study indicated that the 5′-UTR of *pim-1* might contain an IRES element (34), which could allow for internal translation of *pim-1* mRNA when cap-dependent translation is inhibited. To test whether it could function as an IRES element *in vivo*, the 5′-UTR of *pim-1* was subcloned into the intergenic region of a dicistronic vector pRF, to obtain pR-PIM-F
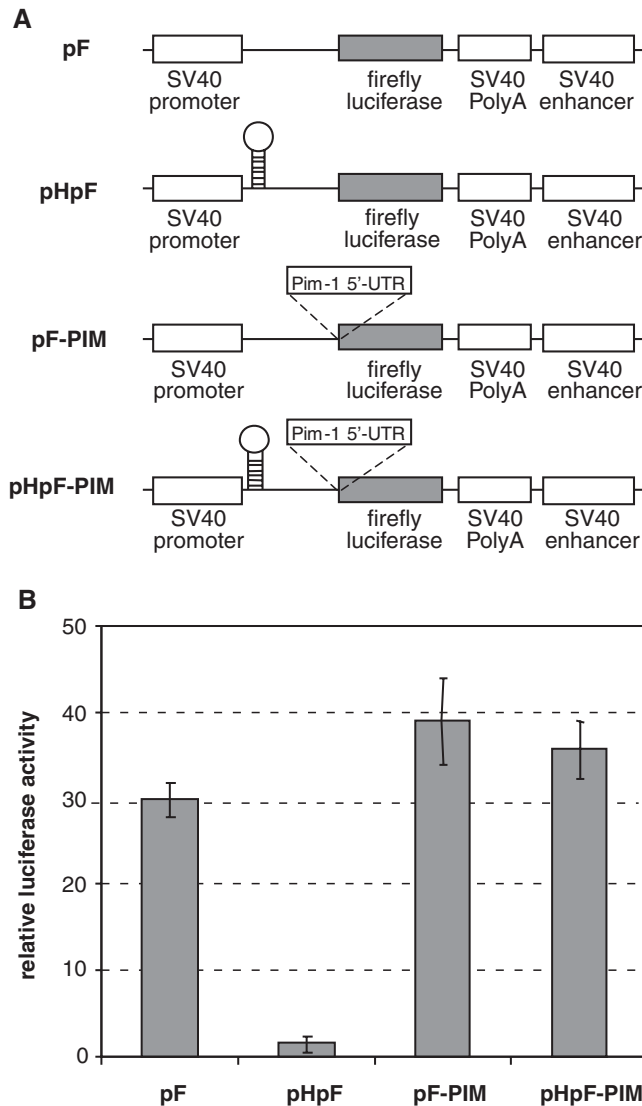
Figure 1. The 5′-UTR of *pim-1* enhances translation when cap-dependent scanning is inhibited in a monocistronic construct. (**A**) Schematic diagram of the constructs used. The 5′-UTR of *pim-1* was introduced immediately upstream of the luciferase open reading frame in the control plasmid pF to create pF-PIM. The stable hairpin structure with a free energy (ΔG) of -55 kcal/mol was introduced upstream of the firefly luciferase start site in pF to create pHpF. The 5′-UTR of *pim-1* was introduced between the hairpin and the luciferase start site to create pHpF-PIM. (**B**) Relative luciferase activities of the monocistronic reporter constructs. Cos-7 cells were transfected with constructs pF, pF-PIM, pHpF and pHpF-PIM in combination with pSV-β-gal plasmid. Cell lysates were prepared 30 h post-transfection, and the activity of firefly luciferase was measured and normalized to that of β-galactosidase. The data were from four independent assays.

Figure 2. Stimulation of the second-cistron expression by the 5′-UTR sequence of the *pim-1*. (**A**) Schematic diagram of the dicistronic constructs without inserts (pRF), or with the IRES of EMCV (pR-EMCV-F), the IRES of HRV (pR-HRV-F) and the 5′-UTR of *pim-1* (pR-PIM-F). (**B**) Relative luciferase activity generated from the dicistronic constructs. Cos-7 cells were transfected with dicistronic constructs together with plasmid pSV-β-gal. Lysates were prepared from cells 30 h post-transfection, the *Renilla* and firefly luciferase activities were measured and the relative ratios were calculated and normalized to that of the vector-transfected cells (pRF). The data were from four independent assays performed in triplicates.

(Figure 2A). The IRES sequences of HRV and EMCV were also engineered in the same way and used as positive controls. The pRF-based constructs contain a SV40 promoter to direct the transcription of dicistronic RNA encoding *Renilla* luciferase as the first cistron and firefly luciferase as the second cistron. Translation of the first cistron (*Renilla* luciferase) serves as an indication of cap-dependent translation, while translation of the second cistron (firefly luciferase) reflects the IRES activity of the inserted intergenic sequence. This approach is considered as a 'gold standard' for characterizing
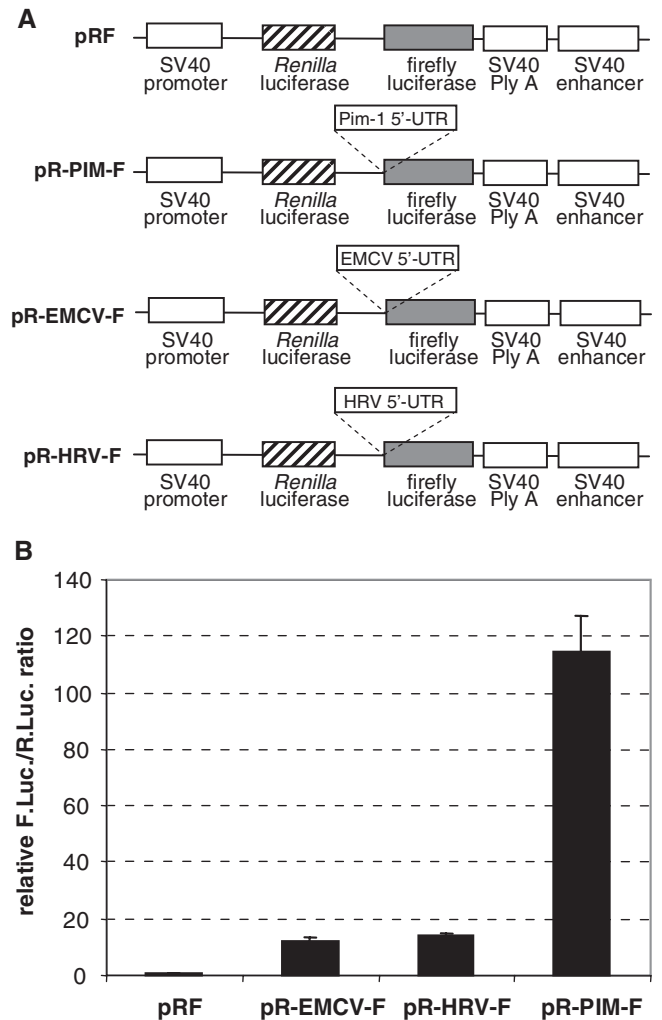
cellular IRES (44). Dicistronic constructs were transfected into Cos-7 cells and both *Renilla* and firefly luciferase activities were measured. As shown in Figure 2B, the 5′-UTR sequence of *pim-1* stimulated the expression of firefly luciferase by ~115-fold over negative control pRF and ~7–8-fold over positive controls pR-EMCV-F and pR-HRV-F. Similar data were also obtained from HeLa cells (data not shown). These results suggest that either (i) the 5′-UTR of *pim-1* may enhance read-through of ribosome through the intergenic region; or (ii) the 5′-UTR of *pim-1* contains IRES activity that enhances the translation of firefly luciferase from the dicistronic mRNA by internal initiation, as suggested previously (34); or (iii) the 5′-UTR of *pim-1* may contain a cryptic promoter or splicing acceptor site that creates a monocistronic transcript of firefly luciferase.

The 5′-UTR of *pim-1* does not promote ribosome read-through in the dicistronic construct

To determine whether the effect of the 5′-UTR of *pim-1* on the translation of the second cistron is due to enhanced read-through by the ribosome through the intergenic region, we inserted a synthetic hairpin upstream of the *Renilla* luciferase to create the dicistronic constructs, pHpRF and pHpR-PIM-F (Figure 3A). This hairpin structure has a free energy of −55 kcal/mol and has been shown to significantly inhibit the cap-dependent translation of the first cistron (42). As shown in Figure 3B, the insertion of hairpin resulted in ∼90% decrease in the *Renilla* activity (first cistron) in both pHpRF and pHpR-PIM-F, as compared with pRF and pR-PIM-F, respectively, suggesting that the hairpin structure inhibits the cap-dependent translation. However, the activity
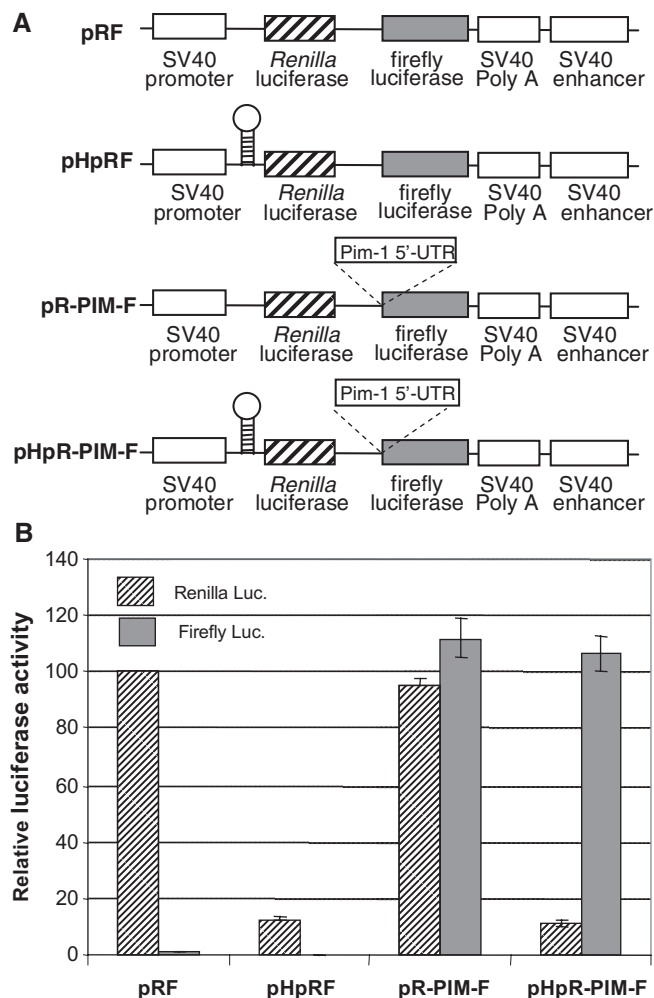

Figure 3. The 5′-UTR is still able to direct second cistron expression in the presence of a stable hairpin. (**A**) Schematic diagram of the dicistronic constructs. The stable hairpin structure with a free energy of −55 kcal/mol was introduced upstream of the *Renilla* open reading frame in the vectors pHpRF and pHpR-PIM-F. (**B**) Relative luciferase activity conferred by the dicistronic constructs. Cos-7 cells were transfected with pRF, pHpRF, pR-PIM-F or pHpR-PIM-F in combination with plasmid pSV-β-gal. Cells were harvested 30 h post-transfection, and *Renilla* and firefly luciferase activities were measured and normalized to those of the control plasmid (pRF). The data were from three independent experiments performed in triplicates.

of firefly luciferase (second cistron) produced by pHpR-PIM-F was almost not affected when compared with pR-PIM-F. This result demonstrates that the enhanced translation of the second cistron by the 5′-UTR of *pim-1* is independent of the cap-dependent translation initiation of the first cistron. Therefore, the increased activity of firefly luciferase is not a result of the read-through by ribosomes from the first cistron (*Renilla* luciferase).

5′-UTR of *pim-1* does not display an IRES activity in dicistronic mRNA assay

To determine whether the effect of the 5′-UTR of *pim-1* on the translation of the second cistron is due to the presence of IRES activity, we generated dicistronic mRNAs *in vitro* from the dicistronic constructs (Figure 2A) and used them to program translation both *in vitro* and *in vivo*. First, the translation efficiencies of both capped and uncapped dicistronic mRNAs in RRL were compared. As shown in Figure 4A, the first cistron (*Renilla* luciferase) of uncapped dicistronic mRNAs were translated much less efficiently than that of capped mRNAs. As expected, the translation of firefly luciferase (second cistron) in negative control pRF was significantly less than that of *Renilla* luciferase (first cistron). In addition, IRES of EMCV supported strong expression of the firefly luciferase (second cistron) from both the capped and uncapped dicistronic mRNAs, confirming that the function of EMCV IRES is independent of cap-structure of the dicistronic mRNAs (45). However, the 5′-UTR of *pim-1* did not promote the expression of the firefly luciferase from either capped or uncapped mRNAs. Actually, the expression of firefly luciferase (second cistron) from pR-PIM-F transcripts was significantly less than that of vector control (pRF). Second, RRL has been shown to lack factors necessary for IRES-dependent translation initiation for some cellular and viral IRESs (43,46). To test whether it is the case for the putative IRES element of the 5′-UTR of *pim-1*, HeLa cytoplasmic and nuclear extract were added into the RRL for *in vitro* translation. As shown in Figure 4B, when IRES of the HRV was used as positive control, the addition of HeLa cytoplasmic extract S100 stimulated expression of firefly luciferase (second cistron) ∼12-fold above that of the negative control. Further enhancement of HRV IRES activity (∼25-fold) was observed with the additional supplement of HeLa nuclear extract. However, no stimulation of firefly expression (second cistron) was observed by the 5′-UTR of *pim-1* in the presence of either cytoplasmic extract alone or cytoplasmic plus nuclear extract.

RNA transfection is one of the major methods for characterizing translation efficiency and identifying eukaryotic regulatory factors influencing IRES activity (41). This method allows a direct analysis of whether the 5′-UTR of *pim-1* in the intergenic region of a dicistronic mRNA can enhance the translation of the second cistron without transcriptional interference. The *in vitro* transcribed dicistronic mRNAs were transfected into Cos-7 cells and the expression of both *Renilla* and firefly luciferases were measured. As expected, the firefly luciferase from the vector control (pRF) was very poorly translated and its activity (arbitrary units) represented only ∼0.16% of the *Renilla* luciferase (data not shown). The activity of IRES was expressed as relative ratio of firefly/*Renilla* activities
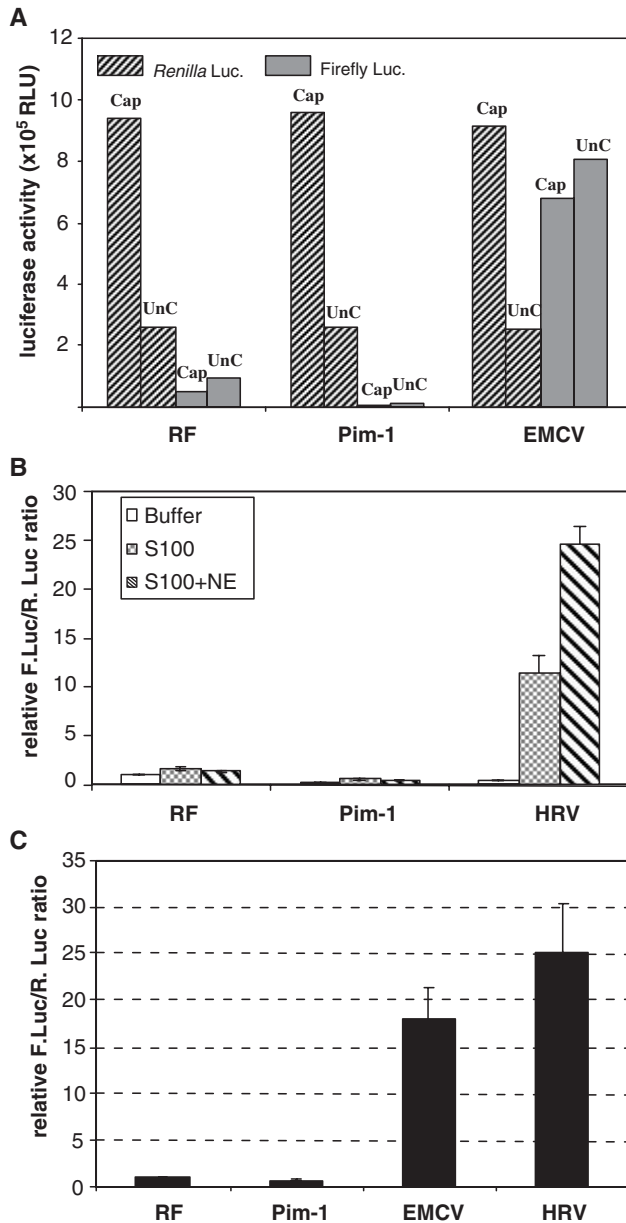
Figure 4. Translation of dicistronic mRNAs *in vitro* and *in vivo*. *In vitro* transcribed dicistronic mRNAs were synthesized with T7 RNA polymerase from linearized constructs pRF, pR-EMCV-F, pR-HRV-F and pR-PIM-F (Figure 2A). (**A**) *In vitro* translation of capped and uncapped dicistronic mRNAs. *In vitro* transcribed capped and uncapped dicistronic transcripts were translated in RRL. After incubating at 30°C for 90 min, *Renilla* and firefly luciferase activities were measured and are displayed as relative light units (RLUs). Cap and UnC indicate capped and uncapped mRNAs, respectively. The result represents one of three independent experiments. (**B**) Relative luciferase activity from dicistronic mRNAs in RRL. *In vitro* transcribed capped dicistronic transcripts were translated in RRL in the presence of buffer alone (Buffer), the HeLa cytosolic extract (S100) or HeLa cytosolic extract plus nuclear extract (S100+NE). After 90 min incubation at 30°C, *Renilla* and firefly luciferase activities were measured and the ratio of firefly to *Renilla* activity was determined and normalized to the vector control in the presence of buffer. The data show the results from three independent assays performed in triplicates. (**C**) Relative luciferase activity from dicistronic mRNAs *in vivo*. Cos-7 cells were transfected with the capped dicistronic mRNAs, and 8 h following transfection, *Renilla* and firefly luciferase activities were measured and the relative ratios were calculated and normalized to that of the vector-transfected cells (pRF). The data show the results from four independent assays performed in triplicates.

normalized to that of vector control. As shown in Figure 4C, the EMCV and HRV IRES significantly stimulated the translation of firefly luciferase ∼18- and 25-fold over vector control. However, no stimulation of firefly luciferase expression was observed with the 5′-UTR of *pim-1*, the expression of firefly luciferase (second cistron) from pR-PIM-F transcripts was ∼0.06% of *Renilla* luciferase activity, significantly less than that observed with vector control. Together, these data suggest that the 5′-UTR of *pim-1* does not contain an IRES element to mediate internal ribosome entry.

5′-UTR sequence of *pim-1* contains a ubiquitously functional promoter

The above results prompted us to explore whether the 5′-UTR sequence of *pim-1* contains a promoter. For this purpose, we simply removed the unique SV40 promoter together with the intron sequence from the pRF-based dicistronic constructs. These promoterless dicistronic constructs (Figure 5A) were then transfected into Cos-7 cells and the activities of both *Renilla* and firefly luciferases were measured (Figure 5B). As shown previously (38), both the *Renilla* and the firefly luciferase activities were minimal, but detectable for RF vector control. Only about a 2-fold increase in firefly luciferase activity was observed with both R-EMCV-F and R-HRV-F constructs. This small increase was in contrast with the 15-fold increase associated with the pR-EMCV-F or the pR-HRV-F constructs (Figure 2B). Thus, the enhanced expression of firefly luciferase from pR-EMCV or pR-HRV-F construct was not due to production of monocistronic transcripts by a promoter present in the EMCV or HRV IRES. However, the R-PIM-F construct generated >120-fold firefly luciferase activity over vector control. This increase was similar to that generated by the pR-PIM-F construct in Figure 2B. This significant increase in the expression of firefly luciferase by the 5′-UTR of *pim-1* is probably due to the presence of a strong promoter in this sequence.

The existence of a constitutively active promoter in the 5′-UTR sequence of cellular mRNAs was reported previously (47). To determine whether a promoter present in the 5′-UTR of *pim-1* is ubiquitously utilized, we measured its activity in various cell lines including NIH3T3 (mouse fiberblast cells), Jurkat (human T cells), HEK293 (transformed primary embryonic kidney cells) and K562 (erythroleukemia cells). As shown in Figure 5C, the 5′-UTR of *pim-1* displays strong promoter activity in all cell lines tested albeit to different degrees, suggesting that the promoter located in the 5′-UTR of *pim-1* is ubiquitously active.

Northern blot analysis

To determine whether the transcript derived from the promoter located in the 5′-UTR of *pim-1* can be detected in cells, poly(A) mRNAs were isolated from Cos-7 cells after transfection with constructs pRF, pR-PIM-F, R-PIM-F, pF, pR-EMCV-F and pR-HRV-F. The construct pF (Figure 1A) was used as a monocistronic control, this vector is expected to generate transcript containing firefly luciferase alone. The construct pRF (Figure 2A) was used as a dicistronic control, which will produce a dicistronic transcript of both firefly and *Renilla* luciferase sequences. As shown in Figure 6, the dicistronic transcript from control pRF was detected as expected by
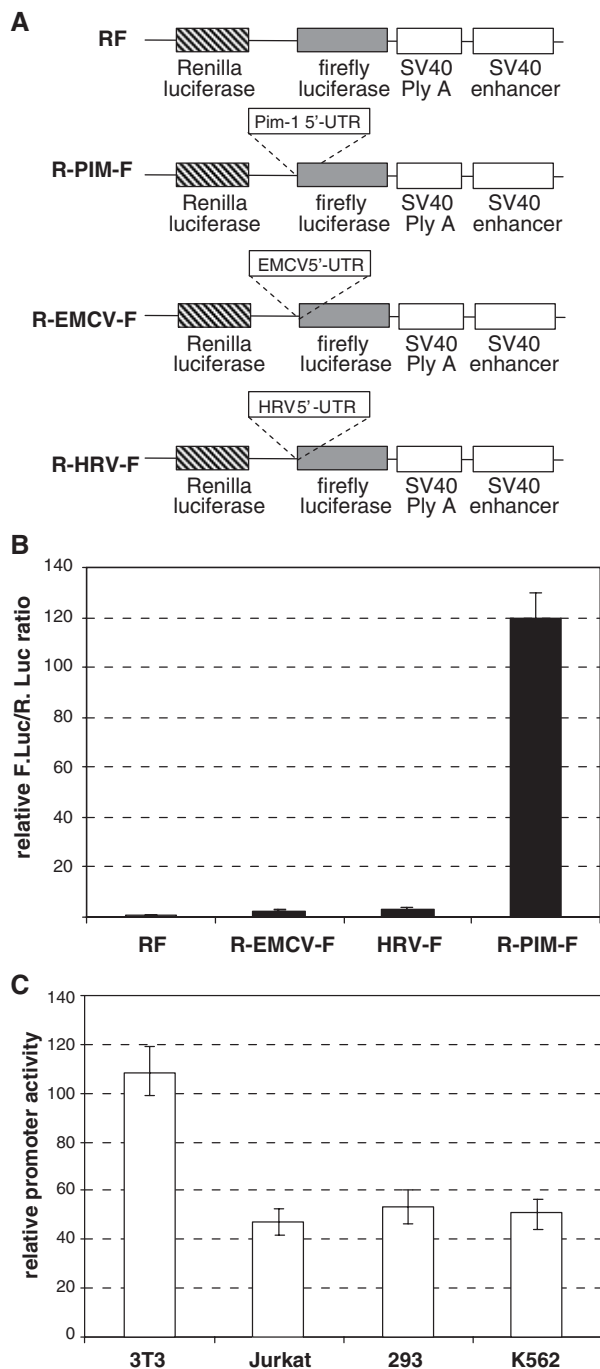
Figure 5. Cryptic promoter activity of the 5′-UTR of *pim-1*. (**A**) Schematic diagram of the promoterless dicistronic constructs of RF, R-EMCV-F, R-HRV-F and R-PIM-F. The sequence of the SV40 promoter and the chimeric intron were deleted from the parental dicistronic constructs shown in Figure 2A. (**B**) Relative luciferase activities generated from the promoterless dicistronic constructs. Cos-7 cells were transfected with the promoterless constructs. Cells were harvested 30 h post-transfection, and *Renilla* and firefly luciferase activities were measured. The relative ratios of firefly to *Renilla* luciferase activities were calculated and normalized to that of control plasmid (RF). The data were from four independent experiments performed in triplicates. (**C**) Promoter activity of the 5′-UTR sequence of *pim-1* in various cell lines. K562, Jurkat, 293 and 3T3 cells were transfected with promoterless constructs RF and R-PIM-F. Cells lysates were made 30 h post-transfection, *Renilla* and firefly luciferase activities were measured. Relative promoter activity was calculated as the ratio of firefly luciferase activity of R-PIM-F to that of the control RF. The result shown was derived from three independent experiments.

both firefly and *Renilla* luciferase probes (cf. lane 1 in Figure 6A and B, indicated by an arrowhead), while the monocistronic transcript from control pF was only detected with the firefly luciferase probe (cf. lane 4 in Figure 6A and B, indicated by an arrow). Intact dicistronic mRNAs were generated from both pR-EMCV-F and pR-HRV-F constructs with the expected sizes, and no monocistronic transcripts were detected with these constructs (lanes 5 and 6 in Figure 6A and B). These observations are consistent with the conclusion that the EMCV and HRV sequences are IRES elements, which do not contain cryptic promoters. However, both dicistronic and monocistronic transcripts were generated from pR-PIM-F and this monocistronic transcript had a similar size to that from monocistronic control pF (cf. lanes 2 and 4 in Figure 6A), suggesting that it is a monocistronic mRNA from the promoter in the 5′-UTR of *pim-1*. In addition, the same monocistronic transcript was also detected with the promoterless dicistronic construct R-PIM-F (lane 3 in Figure 6A). These observations confirmed that the 5′-UTR of *pim-1* has strong promoter activity to derive the transcript of the second cistron (firefly luciferase).

Our northern blot result is different from what was reported previously for the *pim-1* 5′-UTR (34). This discrepancy is probably due to the different kind of dicistronic vector used by these investigators. Their dicistronic vector contained an inactive EMCV IRES fragment of ∼440 nt, which was inserted into the intercistronic region. Although there is a small deletion in the EMCV insert that could prevent it from functioning independently as an IRES, it might still bind protein factors without which the appended 5′-UTR of *pim-1* would not be active (35). Furthermore, the 5′-UTR of *pim-1* that they used was only 354 nt long which was 47 nt shorter than the full-length *pim-1* 5′-UTR we used. It is possible that those 47 nt missing from their 5′-UTR might have been necessary for the cryptic promoter activity. It is also possible that the 440 nt of the EMCV fragment in their dicistronic vector inhibited the cryptic promoter activity of the shorter *pim-1* 5′-UTR. In addition, the HeLa cells they used for transfection, might only produce limiting levels of the transcription factor(s) necessary for cryptic promoter activity. Therefore, in their experimental system they did not detect monocistronic transcripts generated from the cryptic promoter in the *pim-1* 5′-UTR.

Detection of endogenous transcripts derived from the 5′-UTR promoter in human cancer cell lines

The above results showed that the promoter located in the 5′-UTR of *pim-1* is active in transfected cell lines. It is of great interest to know whether this cryptic promoter is used to generate endogenous *pim-1* transcripts with a short 5′-UTR sequence. To determine whether those shorter *pim-1* transcripts exist, we first performed an RNase protection assay with the RNAs isolated from both K562 and DU145 cells, which are known to express high level of Pim-1 protein. Using a probe covering the full-length of the *pim-1* 5′-UTR (Figure 7A), three protected bands with estimated sizes of 53, 33 and 21 bases, respectively, were observed (Figure 7B). Interestingly, a large protected band of ∼421 bases was only detected with RNAs isolated from the K562 cells. However, no protected RNAs were found with yeast tRNA control. This result indicates that *pim-1* transcripts from K562 cells and DU145 cells have various 5′-UTRs and most probably, multiple transcriptional start sites.
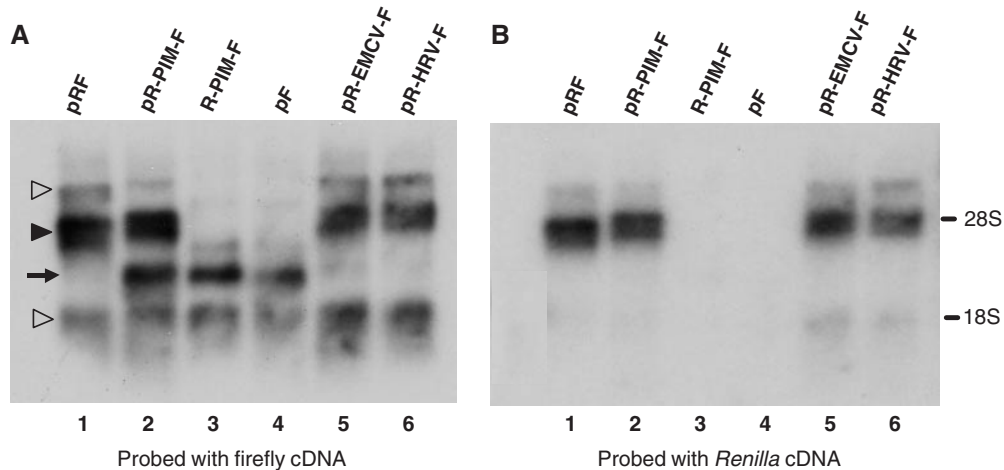
Figure 6. Northern blot analysis of RNA transcripts derived from the *pim-1* 5′-UTR promoter. Cos-7 cells were transfected with pRF (lane 1), pR-PIM-F (lane 2), R-PIM-F (lane 3), pF (lane 4), pR-EMCV-F (lane 5) and pR-HRV-F (lane 6). Poly(A) mRNAs were isolated from transfected cells 48 h later, electrophoresed in the presence of formaldehyde, transferred onto Hybond N$^+$ nylon membrane and probed with ^{32}P-labeled firefly luciferase probe (**A**). After stripping, the membrane was subsequently re-probed with ^{32}P-labeled *Renilla* luciferase specific probe (**B**). The arrowhead and arrow indicate the dicistronic and monocistronic mRNA transcripts, respectively. The open arrowhead shows unknown RNAs hybridized with luciferase probes, which have been consistently observed in previous studies (38,47,60). The migrations of 28S and 18S rRNA are indicated.
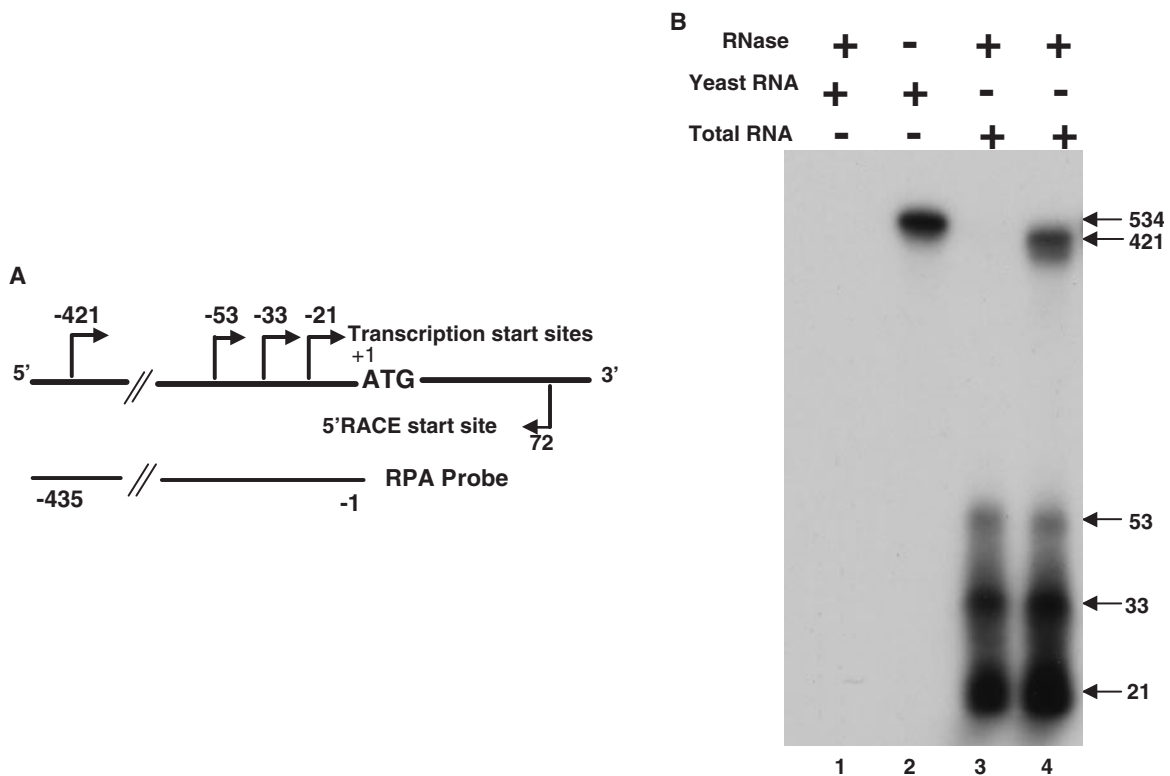

Figure 7. Endogenous *pim-1* transcripts with shorter 5-UTRs exist in human cancer cells. (**A**) Schematic diagram showing relative locations of the transcription start sites, primer used in the 5′-RACE and the anti-sense RNA probe used for RNase protection assay. (**B**) Mapping the 5′-UTRs of the endogenous *pim-1* transcripts by RNase protection assay. A 534 nt RNA probe covering the full-length of the *pim-1* 5′-UTR was used in RNase protection assay as described in Materials and Methods. Lane 1, yeast RNA (10 μg); lane 2, undigested RNA probe; lane 3, total RNA from DU145 cells (10 μg); and lane 4, total RNA from K562 cells (10 μg). The four distinctively protected bands with estimated sizes of 421, 53, 33 and 21 bases are shown.

To further demonstrate the existence of endogenous *pim-1* transcripts with shorter 5′-UTRs, we also carried out 5′-RACE with total RNAs isolated from K562 cells. After one round PCR of 35 cycles with gene specific primer, multiple products with various lengths ranging from 100 to 550 bp were amplified. Those products were then excised from the gel, cloned and sequenced. The sequencing data indicate that three small transcripts were initiated from nucleotide −21, −33 and −53 upstream of the translation start codon, respectively, which are consistent with the protected bands of 53, 33

and 21 bases shown by RNase protection assay (data not shown). In addition, our DNA sequencing data also show that *pim-1* transcripts with the full-length 5′-UTR were generated, which most probably accounts for the longest band (421 bases) shown in RNase protection assay (data not shown). Together, these results show that endogenous *pim-1* transcripts with shorter 5′-UTRs exist in human cell lines and that they are probably derived from the promoter located in the 5′-UTR of *pim-1* sequence.

DISCUSSION

The expression of Pim-1 is subject to complicated regulation at multiple levels: transcriptional, post-transcriptional, translational and post-translational (10). This complexity underlines the importance for the appropriate control of Pim-1 kinase to cell survival and function. Pim-1 kinase, as indicated by a recent study of its crystal structure, is constitutively active (9). This is in sharp contrast to other serine/threonine kinases, such as protein kinase A (PKA), Akt and PKC, whose activities need to be activated through phosphorylation, and/or other modes of post-translational modification. The activity of Pim-1 kinase, however, appears to be controlled by its total amount. This means that the regulation of Pim-1 expression is crucial for its activity and cellular functions. In this study, we report for the first time that the expression of Pim-1 kinase can also be regulated by the cryptic promoter in the 5′-UTR sequence.

Most vertebrate transcripts contain 5′-UTRs of 20–100 nt long that are unlikely to impede translation initiation from the AUG codon (48). The 5′-UTR of *pim-1* is 391 nt long and 76% G/C-rich. The predicted secondary structure has a free energy of $\Delta G = -153$ kcal/mol and could severely inhibit the translation of *pim-1* mRNA *in vitro* (33). A previous study showed that the *pim-1* transcript was found associated with polysomes during polioviral infection and a dicistronic test indicated that the 5′-UTR of *pim-1* might contain an IRES element (34). Here, we demonstrated that the 5′-UTR of *pim-1* could enhance downstream gene expression in a monocistronic construct when cap-dependent initiation is blocked by the introduction of a stable hairpin structure (Figure 1). In the conventional dicistronic DNA assay, the 5′-UTR of *pim-1* enhanced the expression of the second cistron by ∼120-fold (Figure 2) and translation of the second cistron persisted even when a stable hairpin structure was used to block translation of the first cistron (Figure 3). When dicistronic RNA transcripts were used to perform *in vitro* translation and RNA transfection, the 5′-UTR of *pim-1* failed to display any enhanced activity for the second cistron. In contrast, both EMCV and HRV IRESs in dicistronic RNAs can direct the translation of the second cistron both *in vitro* and *in vivo* (Figure 4). This strongly suggests that the 5′-UTR of *pim-1* does not have an IRES. This conclusion is supported by our promoterless dicistronic test, which revealed that the enhanced expression of the second cistron was due to the existence of promoter activities in the 5′-UTR of *pim-1* (Figure 5). In addition, northern blot analysis clearly demonstrated that the monocistronic transcripts of firefly luciferase were generated from both the conventional dicistronic and the promoterless dicistronic constructs (Figure 6). Finally, using the RNase protection

assay and 5′-RACE, we identified three endogenous *pim-1* transcripts with shorter 5′-UTRs of 53, 33 and 21 bases in both human cancer cell lines K562 and DU145 (Figure 7).

Despite the previous claim of IRES presence in the 5′-UTR of *pim-1*, the existence of such cellular IRES has been challenged recently due to the potential uncertainty with the methods typically used for IRES identification (35,36). Recent studies stress the importance of using alternative test procedures (e.g. direct RNA transfection) in conjunction with a combination of sensitive RNA analysis for discerning IRES-containing sequences in eukaryotic mRNAs (41,49). In fact, several previously claimed IRESs activities were discovered to be due to promoter activity present in the same region of the 5′-UTR (37–40). Our finding is reminiscent of this situation, about a eukaryotic translation initiation factor eIF4G, where a strong promoter was found in the putative IRES of the transcripts encoding eIF4G. This promoter also lies in the 5′-UTR believed to harbor an IRES (38). As in our case, no evidence for IRES could be found when a dicistronic mRNA containing the 5′-UTR sequence was translated *in vitro* or *in vivo*. The promoterless dicistronic test and northern blot analysis, however, clearly demonstrated the presence of a cryptic promoter in the 5′-UTR sequence.

It is recognized that the long 5′-UTRs present in the majority of the proto-oncogenes inhibit cap-dependent translation (50) and that an IRES-mediated translation initiation may be used for these mRNAs (51). However, it has not been well appreciated that these long 5′-UTR DNA sequences may contain promoters generating significantly shorter 5′-UTRs. Our RPA and 5′-RACE results (Figure 7) demonstrated that the 5′-UTR of endogenous *pim-1* transcripts in human cancer cell lines are heterogeneous, three of them are significantly shorter than the full-length 5′-UTR. Most cellular mRNAs possess 5′-UTRs of <100 nt and are likely to allow efficient initiation of translation by cap-dependent ribosomal scanning (52). Therefore, these alternative transcripts with shorter 5′-UTRs are most probably quite suitable for efficient cap-dependent translation initiation and do not require an IRES-mediated translation mechanism.

Although the existence of a cryptic promoter in the 5′-UTR of a gene is not common, alternative promoter usage and heterogeneity of transcription initiation have been observed for many genes, including oncogenes and protein kinases. For example, transcription of *c-myc* gene involves P0, P1, P2, P3 and P4 promoters (53) and c-Abl kinase gene has two promoters (54). Alternative promoters are often responsible for tissue-specific or developmental stage-specific gene expression [reviewed in (55)]. The usage of alternative promoters is also very important in regulating the level of gene expression. This can be achieved at the transcriptional level by (i) using promoters of different strengths (56); (ii) responding to different extracellular signals through different promoters (57); or (iii) generating mRNA isoforms that differ in their 5′-UTR, tissue and subcellular distribution and stability (58). The wild-type promoter of the *pim-1* gene was previously reported to have the structural features of a house-keeping gene (59). The cryptic promoter in the 5′-UTR of *pim-1* would contribute additional diversity and flexibility to the complicated regulation of Pim-1 expression and might provide partial explanation for its tissue-specific and/or developmental differential expression.

REFERENCES

1. Cuypers,H.T., Selten,G., Quint,W., Zijlstra,M., Maandag,E.R., Boelens,W., van Wezenbeek,P., Melief,C. and Berns,A. (1984) Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell*, **37**, 141–150.
2. Breuer,M.L., Cuypers,H.T. and Berns,A. (1989) Evidence for the involvement of pim-2, a new common proviral insertion site, in progression of lymphomas. *EMBO J.*, **8**, 743–748.
3. Selten,G., Cuypers,H.T., Boelens,W., Robanus-Maandag,E., Verbeek,J., Domen,J., van Beveren,C. and Berns,A. (1986) The primary structure of the putative oncogene pim-1 shows extensive homology with protein kinases. *Cell*, **46**, 603–611.
4. van Lohuizen,M., Verbeek,S., Krimpenfort,P., Domen,J., Saris,C., Radaszkiewicz,T. and Berns,A. (1989) Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. *Cell*, **56**, 673–682.
5. Moroy,T., Verbeek,S., Ma,A., Achacoso,P., Berns,A. and Alt,F. (1991) E mu N- and E mu L-myc cooperate with E mu pim-1 to generate lymphoid tumors at high frequency in double-transgenic mice. *Oncogene*, **6**, 1941–1948.
6. Acton,D., Domen,J., Jacobs,H., Vlaar,M., Korsmeyer,S. and Berns,A. (1992) Collaboration of pim-1 and bcl-2 in lymphomagenesis. *Curr. Top. Microbiol. Immunol.*, **182**, 293–298.
7. Schmidt,T., Karsunky,H., Gau,E., Zevnik,B., Elsasser,H.P. and Moroy,T. (1998) Zinc finger protein GFI-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis. *Oncogene*, **17**, 2661–2667.
8. Hoover,D., Friedmann,M., Reeves,R. and Magnuson,N.S. (1991) Recombinant human pim-1 protein exhibits serine/threonine kinase activity. *J. Biol. Chem.*, **266**, 14018–14023.
9. Qian,K.C., Wang,L., Hickey,E.R., Studts,J., Barringer,K., Peng,C., Kronkaitis,A., Li,J., White,A., Mische,S. *et al.* (2005) Structural basis of constitutive activity and a unique nucleotide binding mode of human Pim-1 kinase. *J. Biol. Chem.*, **280**, 6130–6137.
10. Wang,Z., Bhattacharya,N., Weaver,M., Petersen,K., Meyer,M., Gapter,L. and Magnuson,N.S. (2001) Pim-1: a serine/threonine kinase with a role in cell survival, proliferation, differentiation and tumorigenesis. *J. Vet. Sci.*, **2**, 167–179.
11. Moroy,T., Grzeschiczek,A., Petzold,S. and Hartmann,K.U. (1993) Expression of a Pim-1 transgene accelerates lymphoproliferation and inhibits apoptosis in lpr/lpr mice. *Proc. Natl Acad. Sci. USA*, **90**, 10734–10738.
12. Lilly,M. and Kraft,A. (1997) Enforced expression of the Mr 33,000 Pim-1 kinase enhances factor-independent survival and inhibits apoptosis in murine myeloid cells. *Cancer Res.*, **57**, 5348–5355.
13. Pircher,T.J., Zhao,S., Geiger,J.N., Joneja,B. and Wojchowski,D.M. (2000) Pim-1 kinase protects hematopoietic FDC cells from genotoxin-induced death. *Oncogene*, **19**, 3684–3692.
14. Aho,T.L., Sandholm,J., Peltola,K.J., Mankonen,H.P., Lilly,M. and Koskinen,P.J. (2004) Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site. *FEBS Lett.*, **571**, 43–49.
15. Amson,R., Sigaux,F., Przedborski,S., Flandrin,G., Givol,D. and Telerman,A. (1989) The human protooncogene product p33pim is expressed during fetal hematopoiesis and in diverse leukemias. *Proc. Natl Acad. Sci. USA*, **86**, 8857–8861.
16. Sorrentino,V., McKinney,M.D., Giorgi,M., Geremia,R. and Fleissner,E. (1988) Expression of cellular protooncogenes in the mouse male germ line: a distinctive 2.4-kilobase pim-1 transcript is expressed in haploid postmeiotic cells. *Proc. Natl Acad. Sci. USA*, **85**, 2191–2195.
17. Stewart,B.E. and Rice,R.H. (1995) Differentiation-associated expression of the proto-oncogene pim-1 in cultured human keratinocytes. *J. Invest. Dermatol.*, **105**, 699–703.
18. Zippo,A., De Robertis,A., Bardelli,M., Galvagni,F. and Oliviero,S. (2004) Identification of Flk-1 target genes in vasculogenesis: Pim-1 is required for endothelial and mural cell differentiation *in vitro*. *Blood*, **103**, 4536–4544.
19. Lilly,M., Le,T., Holland,P. and Hendrickson,S.L. (1992) Sustained expression of the pim-1 kinase is specifically induced in myeloid cells by cytokines whose receptors are structurally related. *Oncogene*, **7**, 727–732.
20. Dautry,F., Weil,D., Yu,J. and Dautry-Varsat,A. (1988) Regulation of pim and myb mRNA accumulation by interleukin 2 and interleukin 3 in murine hematopoietic cell lines. *J. Biol. Chem.*, **263**, 17615–17620.
21. Matikainen,S., Sareneva,T., Ronni,T., Lehtonen,A., Koskinen,P.J. and Julkunen,I. (1999) Interferon-alpha activates multiple STAT proteins and upregulates proliferation-associated IL-2Ralpha, c-myc, and pim-1 genes in human T cells. *Blood*, **93**, 1980–1991.
22. Liang,H., Hittelman,W. and Nagarajan,L. (1996) Ubiquitous expression and cell cycle regulation of the protein kinase PIM-1. *Arch. Biochem. Biophys.*, **330**, 259–265.
23. Mochizuki,T., Kitanaka,C., Noguchi,K., Muramatsu,T., Asai,A. and Kuchino,Y. (1999) Physical and functional interactions between Pim-1 kinase and Cdc25A phosphatase. Implications for the Pim-1-mediated activation of the c-Myc signaling pathway. *J. Biol. Chem.*, **274**, 18659–18666.
24. Wang,Z., Bhattacharya,N., Mixter,P.F., Wei,W., Sedivy,J. and Magnuson,N.S. (2002) Phosphorylation of the cell cycle inhibitor p21Cip1/WAF1 by Pim-1 kinase. *Biochim. Biophys. Acta*, **1593**, 45–55.
25. Bhattacharya,N., Wang,Z., Davitt,C., McKenzie,I.F., Xing,P.X. and Magnuson,N.S. (2002) Pim-1 associates with protein complexes necessary for mitosis. *Chromosoma*, **111**, 80–95.
26. Katakami,N., Kaneto,H., Hao,H., Umayahara,Y., Fujitani,Y., Sakamoto,K., Gorogawa,S.I., Yasuda,T., Kawamori,D., Kajimoto,Y. *et al.* (2004) Role of Pim-1 in smooth muscle cell proliferation. *J. Biol. Chem.*, **279**, 54742–54749.
27. Nagarajan,L. and Narayana,L. (1993) Transcriptional attenuation of PIM-1 gene. *Biochem. Biophys. Res. Commun.*, **190**, 435–439.
28. Yip-Schneider,M.T., Horie,M. and Broxmeyer,H.E. (1995) Transcriptional induction of pim-1 protein kinase gene expression by interferon gamma and posttranscriptional effects on costimulation with steel factor. *Blood*, **85**, 3494–3502.
29. Wingett,D., Reeves,R. and Magnuson,N.S. (1991) Stability changes in pim-1 proto-oncogene mRNA after mitogen stimulation of normal lymphocytes. *J. Immunol.*, **147**, 3653–3659.
30. Mizuno,K., Shirogane,T., Shinohara,A., Iwamatsu,A., Hibi,M. and Hirano,T. (2001) Regulation of Pim-1 by Hsp90. *Biochem. Biophys. Res. Commun.*, **281**, 663–669.
31. Losman,J.A., Chen,X.P., Vuong,B.Q., Fay,S. and Rothman,P.B. (2003) Protein phosphatase 2A regulates the stability of Pim protein kinases. *J. Biol. Chem.*, **278**, 4800–4805.
32. Wang,Z., Petersen,K., Weaver,M.S. and Magnuson,N.S. (2001) cDNA cloning, sequencing and characterization of bovine pim-1. *Vet. Immunol. Immunopathol.*, **78**, 177–195.
33. Hoover,D.S., Wingett,D.G., Zhang,J., Reeves,R. and Magnuson,N.S. (1997) Pim-1 protein expression is regulated by its 5′-untranslated region and translation initiation factor elF-4E. *Cell Growth Differ.*, **8**, 1371–1380.
34. Johannes,G., Carter,M.S., Eisen,M.B., Brown,P.O. and Sarnow,P. (1999) Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. *Proc. Natl Acad. Sci. USA*, **96**, 13118–13123.
35. Kozak,M. (2001) New ways of initiating translation in eukaryotes? *Mol. Cell. Biol.*, **21**, 1899–1907.
36. Kozak,M. (2003) Alternative ways to think about mRNA sequences and proteins that appear to promote internal initiation of translation. *Gene*, **318**, 1–23.
37. Han,B., Dong,Z. and Zhang,J.T. (2003) Tight control of platelet-derived growth factor B/c-sis expression by interplay between the 5′-untranslated region sequence and the major upstream promoter. *J. Biol. Chem.*, **278**, 46983–46993.
38. Han,B. and Zhang,J.T. (2002) Regulation of gene expression by internal ribosome entry sites or cryptic promoters: the eIF4G story. *Mol. Cell. Biol.*, **22**, 7372–7384.

39. Verge,V., Vonlanthen,M., Masson,J.M., Trachsel,H. and Altmann,M. (2004) Localization of a promoter in the putative internal ribosome entry site of the Saccharomyces cerevisiae TIF4631 gene. *RNA*, **10**, 277–286.

40. Dumas,E., Staedel,C., Colombat,M., Reigadas,S., Chabas,S., Astier-Gin,T., Cahour,A., Litvak,S. and Ventura,M. (2003) A promoter activity is present in the DNA sequence corresponding to the hepatitis C virus 5′ UTR. *Nucleic Acids Res.*, **31**, 1275–1281.

41. Van Eden,M.E., Byrd,M.P., Sherrill,K.W. and Lloyd,R.E. (2004) Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedures. *RNA*, **10**, 720–730.

42. Stoneley,M., Paulin,F.E., Le Quesne,J.P., Chappell,S.A. and Willis,A.E. (1998) C-Myc 5′ untranslated region contains an internal ribosome entry segment. *Oncogene*, **16**, 423–428.

43. Stoneley,M., Subkhankulova,T., Le Quesne,J.P., Coldwell,M.J., Jopling,C.L., Belsham,G.J. and Willis,A.E. (2000) Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the nuclear compartment. *Nucleic Acids Res.*, **28**, 687–694.

44. Sachs,A.B. (2000) Cell cycle-dependent translation initiation: IRES elements prevail. *Cell*, **101**, 243–245.

45. Borman,A.M., Bailly,J.L., Girard,M. and Kean,K.M. (1995) Picornavirus internal ribosome entry segments: comparison of translation efficiency and the requirements for optimal internal initiation of translation *in vitro*. *Nucleic Acids Res.*, **23**, 3656–3663.

46. Paulous,S., Malnou,C.E., Michel,Y.M., Kean,K.M. and Borman,A.M. (2003) Comparison of the capacity of different viral internal ribosome entry segments to direct translation initiation in poly(A)-dependent reticulocyte lysates. *Nucleic Acids Res.*, **31**, 722–733.

47. Han,B., Dong,Z., Liu,Y., Chen,Q., Hashimoto,K. and Zhang,J.T. (2003) Regulation of constitutive expression of mouse PTEN by the 5′-untranslated region. *Oncogene*, **22**, 5325–5337.

48. Kozak,M. (1991) Effects of long 5′ leader sequences on initiation by eukaryotic ribosomes *in vitro*. *Gene Expr.*, **1**, 117–125.

49. Van Eden,M.E., Byrd,M.P., Sherrill,K.W. and Lloyd,R.E. (2004) Translation of cellular inhibitor of apoptosis protein 1 (c-IAP1) mRNA is IRES mediated and regulated during cell stress. *RNA*, **10**, 469–481.

50. Willis,A.E. (1999) Translational control of growth factor and proto-oncogene expression. *Int. J. Biochem. Cell Biol.*, **31**, 73–86.

51. Stoneley,M. and Willis,A.E. (2004) Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene*, **23**, 3200–3207.

52. Kozak,M. (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.*, **15**, 8125–8148.

53. Marcu,K.B., Bossone,S.A. and Patel,A.J. (1992) myc Function and regulation. *Annu. Rev. Biochem.*, **61**, 809–860.

54. Bernards,A., Paskind,M. and Baltimore,D. (1988) Four murine c-abl mRNAs arise by usage of two transcriptional promoters and alternative splicing. *Oncogene*, **2**, 297–304.

55. Schibler,U. and Sierra,F. (1987) Alternative promoters in developmental gene expression. *Annu. Rev. Genet.*, **21**, 237–257.

56. Schibler,U., Hagenbuchle,O., Wellauer,P.K. and Pittet,A.C. (1983) Two promoters of different strengths control the transcription of the mouse alpha-amylase gene Amy-1a in the parotid gland and the liver. *Cell*, **33**, 501–508.

57. Iynedjian,P.B. (1993) Mammalian glucokinase and its gene. *Biochem. J.*, **293**, 1–13.

58. Cianetti,L., Di Cristofaro,A., Zappavigna,V., Bottero,L., Boccoli,G., Testa,U., Russo,G., Boncinelli,E. and Peschle,C. (1990) Molecular mechanisms underlying the expression of the human HOX-5.1 gene. *Nucleic Acids Res.*, **18**, 4361–4368.

59. Meeker,T.C., Loeb,J., Ayres,M. and Sellers,W. (1990) The human Pim-1 gene is selectively transcribed in different hemato-lymphoid cell lines in spite of a G + C-rich housekeeping promoter. *Mol. Cell. Biol.*, **10**, 1680–1688.

60. Coldwell,M.J., Mitchell,S.A., Stoneley,M., MacFarlane,M. and Willis,A.E. (2000) Initiation of Apaf-1 translation by internal ribosome entry. *Oncogene*, **19**, 899–905.