Nucleic Acids Research, 1997, Vol. 25, No. 14 **2869–2876**

The conserved 5′-untranslated leader of Spi-1 (PU.1) mRNA is highly structured and potently inhibits translation *in vitro* but not *in vivo*

Jack O. Hensold[1,2,*], Carl A. Stratton[2] and Diane Barth[1]

[1]The University/Ireland Cancer Center, Department of Medicine and Case Western Reserve University, Cleveland, OH 44106, USA and [2]Department of Veteran's Affairs Medical Center, Cleveland, OH 44106, USA

Received March 4, 1997; Revised and Accepted May 6, 1997

ABSTRACT

The transcription factor Spi-1 (PU.1) has a central role in regulating myeloid gene expression during hematopoietic development and its overexpression has been implicated in erythroleukemic transformation. Thus regulation of Spi-1 expression has broad significance for hematopoietic development. A comparison of human and murine cDNA sequences demonstrates that the 5′-untranslated region (5′-UTR) of Spi-1 mRNA is as highly conserved as the coding region (87% identical), suggesting that this sequence may be involved in regulating expression of this protein. The experiments presented in this manuscript provide evidence that the 5′-UTR of Spi-1 contains extensive secondary structure, including three stem–loops that precede the AUG codon. Analysis of the *in vitro* transcribed Spi-1 5′-UTR by partial nuclease digestion sensitivity is consistent with the existence of two of these stem–loops. The 5′-UTR decreased translation of Spi-1 transcripts in reticuloctye lysates 8- to 10-fold. A series of partial deletions of the 5′-UTR identified the sequence corresponding to the stem–loop most proximal to the initiating AUG codon as sufficient for inhibition of translation. However, the effect of the 5′-UTR on translation *in vivo* was negligible and resulted in only a slight reduction in the number of ribosomes that became associated with the mRNA. Further, this sequence had no affect on expression of luciferase. The disparity between *in vivo* and *in vitro* effects, coupled with the observation that endogenous Spi-1 mRNA is wholly associated with polysomes in MEL cells, suggests that additional cellular mechanisms contribute to regulation of Spi-1 expression in these cells or that conservation of these sequences serves a function that is independent of translation.

INTRODUCTION

Spi-1 (PU.1) is a member of the ETS family of transcription factors that is restricted in expression to hematopoietic cells (excluding T cells) and testis (1). Normal hematopoietic development is dependent upon Spi-1, since mice lacking this gene are inviable and demonstrate a generalized defect in hematopoiesis (2,3). This transcription factor plays a role in tissue-specific activation of a number of myeloid, monocytic and B lymphocytic genes, including the CD11b (4), IgGκ (5), M-CSF receptor (c-*fms*) (6) and prointerleukin 1β genes (7), and is also expressed in erythroid progenitors (CFU-E) and more differentiated erythroid cells (1). While Spi-1 has been demonstrated to bind to a conserved sequence in an erythroid-specific DNase I hypersensitive site in the second intron of β-globin, the functional significance of this site is undefined (8). However, like other ETS family members (9,10), abnormal expression of Spi-1 transforms erythroid cells. Increased expression of Spi-1 is found in virtually all Friend spleen focus-forming virus (SFFV)-transformed murine erythroleukemia (MEL) cells, due to retroviral insertion 5′ of this gene (11,12). Further evidence that overexpression of this protein interferes with normal erythroid development is provided by the demonstration that infection of long-term bone marrow cultures with Spi-1 retroviral constructs selectively immortalizes erythroid cells (13). Inducers of MEL cell differentiation cause a 4- to 8-fold decrease in Spi-1 expression (1,14), due to a decrease in the stability of this mRNA (15).

The nucleotide sequence of the 5′-untranslated region (UTR) of Spi-1 is as highly conserved as the coding region, being 87% identical between mouse and human (12,16), suggesting that this sequence may serve a regulatory function. While investigating expression of recombinant Spi-1 transcripts in differentiating MEL cells, we noted that several premature terminations occurred during reverse transcription of the 5′-UTR. In this manuscript we demonstrate that this is likely due to stable secondary structure that includes three stem–loops that precede the initiating AUG codon. The 5′-UTR inhibited translation 8- to 10-fold in reticulocyte lysates and deletion analysis determined that the RNA sequence that corresponded to the stem–loop most proximal to the initial AUG codon was sufficient for this effect. However, the effect of the 5′-UTR on translation *in vivo* was negligible since: (i) endogenous Spi-1 mRNA appears to be efficiently tranlsated in MEL cells; (ii) excision of the 5′-UTR from recombinant Spi-1 transcripts only results in a minimal increase in the number of ribosomes associated with the mRNA; (iii) ligation of the Spi-1 5′-UTR to a luciferase cDNA did not significantly affect luciferase expression in murine cell lines. The disparity between *in vivo* and *in vitro* effects suggests either that additional mechanisms contribute to regulation of Spi-1 expression in cells

*To whom correspondence should be addressed at: BRB 333, 10900 Euclid Avenue, Cleveland, OH 44106-4937, USA. Tel: +1 216 844 8245; Fax: +1 216 844 8230; Email: joh2@po.cwru.edu

or that conservation of the 5′-UTR does not serve a translational function.

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Biochemicals (St Louis, MO) and Boehringer Mannheim Biochemicals (Indianapolis, IN). Enzymes and molecular biological reagents were obtained from New England BioLabs (Beverly, MA) and Promega (Madison, WI), except for Superscript II reverse transcriptase, which was from Life Science Inc. (Gaithersburg, MD). Ribonuclease V1 (sequencing grade) was obtained from Pharmacia Biotech (Piscataway, NJ) and RNase A (analytical grade) from Boehringer Mannheim Biochemicals. Radiochemicals were obtained from New England Nuclear (Boston, MA). Tissue culture supplies were from Gibco (Grand Island, NY) and fetal calf serum from Intergen (Purchase, NY). Reagents for luciferase assays were from Analytical Luminscence Laboratory (San Diego, CA) and for β-galactosidase assays from Tropix Inc. (Bedford, MA).

Cell culture

MEL cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 2 mM glutamine and 12% fetal bovine serum. Da-3 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% WEHI-3 conditioned medium. Cells were split every 2–3 days to maintain densities that ensured logarithmic growth.

Primer extension analysis of mRNAs

Cytoplasmic RNA was purified from MEL cells as previously described (17). Primer extensions on cellular RNA were performed utilizing standard techniques. In brief, a primer complementary to nucleotides –7 to +15 of the coding sequence was labeled with ^{32}P at the 5′-terminus with polynucleotide kinase. The RNA and labeled primer were hybridized for 18 h at 37°C in 0.4 M sodium chloride, 40 mM PIPES, pH 6.4, 1 mM EDTA, 80% formamide, precipitated with ethanol and resuspended in buffer for reverse transcription. Reverse transcriptions were carried out for 2 h at 42°C, except where indicated in the text. The reactions were terminated by adding a 1/10 vol 2 M sodium hydroxide and incubation for 15 min at 65°C to degrade RNA. Following neutralization with a 1/10 vol 3 M sodium acetate, pH 5.4, the cDNA was precipitated with 2 vol ethanol. The pellets were resuspended in sample buffer containing 80% formamide and analyzed by electrophoresis in 6% polyacryamide gels under denaturing conditions. The extended products were visualized by autoradiography.

DNA constructs

A Spi-1 cDNA extending to within 19 nt of the longest published cDNA sequence (designated –132) and a cDNA containing only the 17 nt of 5′-UTR (–17) immediately proximal to the initial AUG codon were obtained from R.Maki (18). These cDNAs were ligated into the *Eco*RI site of the pBlueScript II SK(+) plasmid, oriented to utilize the T7 promoter for *in vitro* transcription of Spi-1 RNA. A Spi-1 cDNA that included the 49 nt of 5′-UTR proximal to the initial AUG codon (–49) was obtained from D.Galson (1). This cDNA was cloned into the pBlueScript II SK(+) *Xho*I site, also oriented to utilize the T7 promoter for transcription. A Spi-1 cDNA which retained only 10 nt of 5′- and 3′-untranslated sequences (designated –10/Δ3′) was synthesized by PCR from the full-length Spi-1 cDNA and ligated into pBlueScript II SK(+). Primers and PCR conditions have been previously described (15). A Spi-1 cDNA that included the 5′-UTR but lacked the 3′-UTR (designated Δ3′) was created by excising the 3′-terminus of the full-length cDNA with *Nco*I and *Cla*I and replacing it with the corresponding sequences 3′ of the *Nco*I site from the above described (–10/Δ3′) vector. For analysis of Spi-1 expression *in vivo*, the 3′-truncated (Δ3′) and the 5′- and 3′-truncated (–10/Δ3′) cDNAs were cloned into the polylinker of the eukaryotic expression vector pRC/RSV (Invitrogen, San Diego, CA). The RSV promoter in this plasmid has been previously determined to be constitutively active in MEL cells (15).

Luciferase coding sequences were inserted into the pRC/RSV vector by excising the Spi-1 coding sequences and contiguous bovine growth hormone (BGH) 3′-UTR (including polyadenylation signal) from the Δ3′ plasmid described above. The Spi-1 5′-UTR was left intact in this vector by digesting with *Sac*I and *Ava*I. *Sac*I cleaves immediately 5′ of the initiating AUG codon of Spi-1 mRNA and thus leaves all but the 6 nt that immediately precede the initial AUG codon present in the vector. *Ava*I cuts in the vector backbone, resulting in excision of the remaining Spi-1 sequences and BGH 3′-UTR from this plasmid. Complete excision of the Spi-1 and BGH 3′-UTR was achieved by digestion with *Hin*dIII and *Bam*HI. *Hin*dIII cuts the plasmid in the polylinker 5′ of the inserted Spi-1 sequences and *Bam*HI cuts in the plasmid backbone in proximity to the *Ava*I site. The luciferase coding sequence and the contiguous SV40 3′-UTR (including polyadenylation signal) were excised from the pGL2-Basic vector (Promega, Madison, WI) with *Sac*I and *Sal*I for ligation into the *Sac*I/*Ava*I-digested Δ3′ plasmid or with *Hin*dIII and *Bam*HI for ligation into the *Hin*dIII/*Bam*HI-digested Δ3′ plasmid.

In vitro transcriptions

Plasmid DNAs were linearized by restriction endonuclease digestion at a site in the polylinker 3′ of the cloned Spi-1 sequences. The linearized fragment was gel purified and RNA transcripts were synthesized with T7 polymerase from 500 ng plasmid DNA. Two μCuries of [α-^{32}P]UTP were added to the transcription reactions to allow for standarization of the molar amounts of RNA added to the translation reactions, described below. Cap analog, m^{7}G(5′)ppp(5′)G, was added to the reactions at a 10-fold molar excess with respect to GTP to 'cap' the transcripts, except where indicated. Transcriptions were performed under standard conditions and the transcripts purified with r-Elutips (Schleicher & Schuell). Transcript integrity was confirmed by gel electrophoresis and autoradiography. Confirmation that the transcripts had been 'capped' was assessed by determining their sensitivity to translation in wheatgerm lysates, under standard conditions.

In vitro translations

The labeled transcripts were added to rabbit reticulocyte lysates in equimolar amounts (as determined by incorporated radioactivity, correcting for the uridine content of each transcript). Translations were carried out at 30°C for 1 h with the addition of [^{35}S]methionine, as described by the manufacturer (Promega). The reactions were terminated by digestion with RNase A (0.4 mg/ml

final concentration) and prepared for gel electrophoresis by addition of an equal volume of 2× Laemmli sample buffer and boiling. Equal aliquots of the extracts were analyzed by electrophoresis in 12.5% Laemmli gels and visualized by fluorography. The results were quantitated by densitometry.

Analysis of translation *in vivo*

The pRC/(–10/Δ3′) and pRC/(Δ3′) expression plasmids were linearized and 30 µg plasmid DNA introduced into 1×10^7 MEL cells by electroporation, as previously described (15). Stably expressing cells were selected by growth in medium containing G418 (0.6 mg/ml). Cell pools (arising from one to three clonogenic cells) were analyzed for expression of the recombinant transcripts by electroblotting of RNAs separated by denaturing polyacrylamide gel electrophoresis and blot hybridization, as described by Stoeckle and Guan (19).

Translational efficiency of the recombinant and endogenous Spi-1 transcripts was assessed by determining the distribution of the transcripts in polysome fractions. Cytosolic lysates were prepared as previously described (20) and layered onto 10–50% sucrose gradients containing 100 mM potassium chloride, 5 mM magnesium chloride, 20 mM HEPES, 2 mM dithiothreitol, pH 7.5. Gradients were centrifuged in an Sw28.1 rotor at 27 000 r.p.m. for 4 h at 4°C and collected with continous monitoring of UV absorbance (254 nm) using an ISCO 640 gradient fractionator and UA-5 absorbance detector. Gradient fractions were collected directly into phenol and RNA isolated by phenol and chloroform extraction and ethanol precipitation. Volumes were maintained constant throughout and the precipitated pellets run in their entirety on denaturing urea gels and electroblotted to membranes, as described above. The blots were hybridized with a Spi-1 cDNA fragment which had been labeled with ^{32}P by random priming (21) and were developed by autoradiography.

For experiments analyzing the effect of the Spi-1 5′-UTR on transient expression of luciferase, plasmid DNAs were not linearized prior to their introduction into cells. Parameters for electorporation of MEL cells have been previously described (15), except that the amount of plasmid DNA used was increased to 50 µg. Electroporation of Da-3 cells was performed with 1×10^7 cells in 1 ml growth medium at room temperature utilizing a Cell-Porator (Life Sciences Inc., Gaithersburg, MD) set to the following parameters: 300 V and 1980 µF capacitance at a low resistance setting. Electroporations were performed in quadruplicate and cell extracts prepared 6 h following electroporation, using the Triton X-100 lysis protocol described by the manufacturer and light units measured in a Monolight 2010 luminometer (Analytical Luminometer). The results were standardized to total extract protein (MEL and Da-3 cells) or to the results obtained from a simultaneously introduced β-galactosidase reporter construct (Da-3 cells). Galactosidase activity of the extracts was determined as described by the manufacturer and results measured in a luminometer, as above. Similar results were obtained with both methods of standardization.

Secondary structure analysis

The initial 169 nt of the longest published sequence of a Spi-1 cDNA clone (22) was analyzed for formation of secondary structure using the RNAdraw (23) and MulFold (24–26) computer programs. To analyze secondary structure in the Spi-1 5′-UTR, unlabeled transcripts were synthesized with T7 RNA polymerase as described above. The amount of transcript utilized for nuclease digestion was empirically determined (~1/50 of the total product of a transcription reaction from 500 ng linearized plasmid reaction was used per digestion). The RNA was suspended in 100 µl 100 mM potassium chloride, 5 mM magnesium chloride, 20 mM HEPES, 2 mM dithiothreitol, pH 7.4, heated to 65°C and allowed to cool to room temperature. Fifteen micrograms of tRNA were added to the digestions with Cobra venom V1 nuclease and 30 µg tRNA were added to the digestions with RNase A. The amount of nuclease added and the times of digestion were determined empirically: V1 nuclease digestions were performed at 37°C for 15 min with 0.1 and 0.01 U nuclease/100 µl and RNase A digestions were performed on ice for 10 min with 10 and 1 ng nuclease/100 µl reaction. Digestions were terminated by extraction with phenol and chloroform, the RNA precipitated with 2 vol ethanol and analyzed by primer extension as described above. For accurate determination of the sites of nuclease cleavage, a sequence ladder was created by sequencing the undigested RNA with reverse transcriptase in the presence of dideoxy-NTP at a final concentration of 50 µM ddNTP, 500 µM dNTP. The cDNA products were analyzed by gel electrophoresis and autoradigraphy.

RESULTS

Following exposure of MEL cells to inducers of differentiation, expression of Spi-1 decreases ~5-fold, due to a decrease in mRNA stability (15). During analysis of expression of Spi-1 transgenes in MEL cells we utilized primer extension in an attempt to distinguish the 5′-termini of recombinant from endogenous Spi-1 mRNAs. However, in repeated experiments reverse transcription to the predicted 5′-terminus of the endogenous mRNA was interrupted by premature termination. Utilizing a primer complementary to nucleotides –7 to +15 (relative to the initial in-frame AUG codon), most transcripts were seen to terminate following polymerization of 71 nt (Fig. 1), corresponding to nucleotide –56, which was ~95 nt short of the predicted 5′-terminus of the longest published cDNA clone (22) and of the transcriptional start sites mapped in MEL cells (12,16). Additional premature terminations were also noted following transcription of ~90 and 140 nt. Only a small percentage of the transcripts extended to the predicted 5′-terminus. Similar results were obtained when transcriptions were performed either at an elevated temperature (50°C) or in the presence of 10% Me_2SO, as indicated at the top of Figure 1. These results suggested that either the 5′-UTR of Spi-1 contained stable secondary structure that impeded the passage of reverse transcriptase (27,28) or that distinct species of Spi-1 mRNAs with different 5′-termini existed within the cells.

Since overexpression of Spi-1 mRNA in MEL cells is due to genomic insertion of proviral sequences 5′ of the Spi-1 genomic sequences (11), the premature terminations in these cells could represent 5′-truncations that occured due to retroviral insertion. This could be significant for expression of Spi-1, since 5′-truncation of the *lck* proto-oncogene has previously been demonstrated to increase its expression and to transform lymphocytes via a translational mechanism (29). To determine if the previous results were due to secondary structure, primer extension was performed on an *in vitro* transcribed Spi-1 RNA that contained all but the initial 19 nt of the 5′-UTR. Similar to the results obtained on endogenous mRNA, premature terminations were observed after

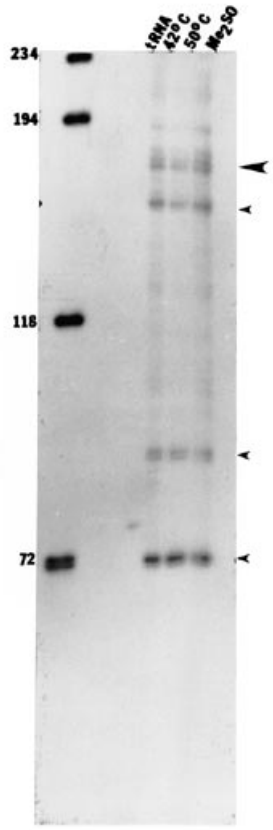

Figure 1. Primer extensions on Spi-1 mRNA prematurely terminate in the 5′-UTR. RNA was extracted from MEL cells and hybridized to a 5′-^{32}P-labeled primer complementary to the sequence –7 to +15 (relative to the first in-frame AUG codon). Reverse transcriptions were performed under standard conditions (42°C), at 50°C and with the addition of 10% DMSO, as indicated at the top of each lane. DNA size markers are displayed on the left. The position of the predicted 5′-terminus of the mRNA is indicated by the large arrowhead and the premature terminations are indicated by the small arrowheads.

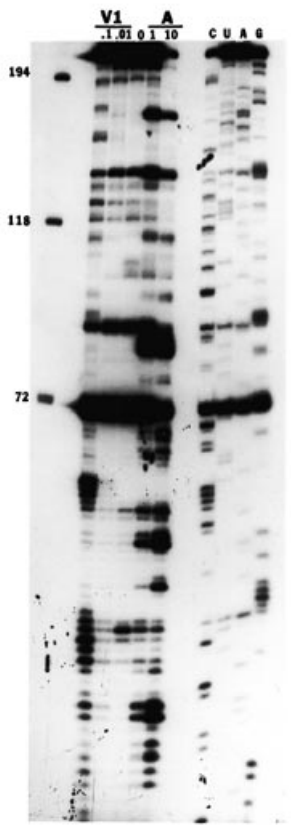

Figure 2. Sensitivity of the Spi-1 5′-UTR to ribonuclease digestion. An *in vitro* transcribed Spi-1 RNA was incubated with V1 nuclease to cleave at base paired nucleotides or RNase A to cleave at single-stranded pyrimidine nucleotides, as described in the text. Sites of nuclease sensitivity were identified as premature terminations of reverse transcription that initiated in an oligonucleotide primer complementary to nucleotides –7 to +15 (relative to the first in-frame AUG codon) of Spi-1. Precise sites of nuclease cleavage were identified by comparison with an RNA sequence ladder that had been created by reverse transcription of the RNA in the presence of dideoxynucleotides, as described in the text. To facilitate reading of the sequence, the indicated nucleotide above the four lanes on the right is the complement of the dideoxynucleotide used in the transcription reaction. The results of the nuclease digestions are shown on the left, with the sequencing reactions on the right. An undigested sample of the RNA (labeled 0) was run between the samples that had been digested with V1 nuclease or RNase A, as indicated above the lanes on the autoradiograph. Nucleotide size markers are shown on the far left. The sites of cleavage with RNase A or ribonuclease V1 are indicated on the predicted secondary structure in Figure 3.

polymerization of 71, 90 and 140 nt of the *in vitro* transcribed RNA (see Fig. 2, lane 0).

The preceding results suggested that sequences within the 5′-UTR of Spi-1 formed stable secondary structure that caused premature termination of reverse transcription. To further assess this sequence for secondary structure, limited ribonuclease digestion of the *in vitro* transcribed Spi-1 RNA was performed. Ribonuclease V1 was used to detect base paired nucleotides, while RNase A was used to detect unpaired pyrimidine nucleotides (30). The partially digested RNA was analyzed by primer extension and the positions of nuclease cleavage assigned by comparison with a reverse transcribed sequence ladder run on the same gel. Of note, when the products of reverse transcription of the undigested RNA (lane 0) were compared with the sequenced transcript, the previously identified premature terminations were all noted to occur on the 3′ side of a run of four to five guanosine nucleotides. The sites of susceptibility to double strand (V1)- and single strand (RNase A)-specific ribonucleases shown in Figure 2 were compared with the secondary structure determined for the initial 169 nt of Spi-1 mRNA sequence (Fig. 3) predicted by the RNAdraw and MulFold programs. The calculated ΔG for the structure shown in the figure was –55.4 kcal/mol. While the two programs predicted slight differences in base pairing in stem–loop I, all of the predicted structures contained identical base pairing in stem–loops II and III.

The results of the nuclease digestions are consistent with the existence of stem–loops II and III shown in Figure 3. While several sites produced ambiguous results, exhibiting sensitivity to cutting with both enzymes, these were found primarily in the region between stem–loops II and III, which would be predicted to be only weakly base paired. The premature termination that had been previously identified at position –56 corresponds to the 3′-side of the base of stem–loop II, at the beginning of the run of five guanosines that was predicted to be base paired with a corresponding sequence of five cytosines. Due to the strong termination of reverse transcription that occurred at this location, we were unable to identify a pattern of nuclease digestion on the 3′-side of this helix. However, sensitivity to V1 ribonuclease was

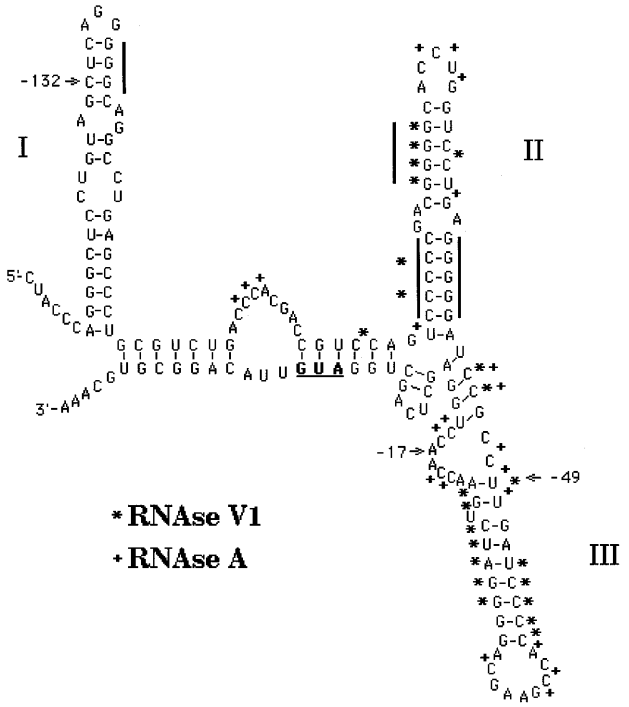

Figure 3. Predicted secondary structure of the 5′-UTR of Spi-1 mRNA. The initial 169 nt of the longest published cDNA sequence for Spi-1 were analyzed for formation of secondary structure using the RNAdraw and MulFold programs. Base paired nucleotides are indicated by a solid line connecting the nucleotides. The initial nucleotide of Spi-1 sequence in the transcripts analyzed in the subsequent experiments is indicated by an arrow and is numbered relative to the first in-frame AUG codon. The sites of premature termination of reverse transcription are indicated by the solid bars next to the sequence. The sites of cleavage with double strand (V1)- and single strand (RNase A)-specific ribonucleases are indicated (see also Fig. 2).

detected in the corresponding cytosines that were predicted to be base paired with guanosines in this helix. In addition, since the pattern of nuclease sensitivity in the surrounding sequence was consistent with the existence of stem–loops II and III, it is likely that these guanosines are paired as predicted in the model. Since the cDNA constructs used in these experiments lacked the initial 19 nt of cDNA sequence as described by Paul *et al*. (22), we were unable to directly confirm the existence of stem–loop I. However, these data support the existence of stable secondary structure within the 5′-UTR of Spi-1, including at least two stem–loops that precede the initial in-frame AUG codon.

The 5′-UTR of Spi-1 potently inhibits translation *in vitro*

Stable secondary structure in the 5′-UTR of mRNAs has been demonstrated to inhibit their translation (31). Therefore, to determine if this was the case for Spi-1 mRNA, *in vitro* transcribed RNAs were translated in reticulocyte lysates and synthesis of Spi-1 protein determined by incorporation of [^{35}S]methionine into protein. When equimolar amounts of Spi-1 transcripts were translated there was an 8- to 10-fold reduction in Spi-1 protein synthesized from the transcripts that included 5′-UTR sequences up to –132, when compared with transcripts that had been truncated at position –17 (Fig. 4A). This difference remained constant with incubation times ranging from 10 to 60 min (not shown) and was observed on both capped and uncapped mRNAs. Examination of two additional constructs that were truncated 10 nt 3′ of the termination codon demonstrated that the 3′-UTR did not have a detectable independent effect upon translation of this mRNA (Fig. 4B). Thus, as predicted by the existence of stable secondary structure, the 5′-UTR of Spi-1 mRNA inhibited its translation in reticulocyte lysates.

To determine if the sequences that were responsible for inhibiting translation overlapped stem–loop II, which caused the most pronounced premature termination of reverse transcription, a Spi-1 transcript that included sequences beginning at position –49 was analyzed. This transcript excluded the sequences that formed stem–loop II, but retained the sequences that formed stem–loop III (see Fig. 3). When translated in reticuloctyte lysate, the –49 transcript was inhibited to a similar extent as the transcript which extended to –132 (Fig. 4C). Thus, the Spi-1 5′-UTR which preceded –49 did not appear to contribute to the observed translational inhibition. Since the transcripts used in these experiments all initiated in similar vector sequences, the observed translational effect could not be attributed to the vector sequences. In addition, computer analyses of potential secondary structures within the 5′-UTR of these transcripts did not detect any vector sequence-dependent secondary structure uniquely present in the translationally inhibited transcripts. Thus, the observed translational inhibition was mediated by sequence located between –17 and –49 of the Spi-1 5′-UTR, which forms stem–loop III, as indicated in Figure 3.

The 5′-UTR of Spi-1 has minimal effects on translation *in vivo*

The previous experiments suggested that endogenous Spi-1 mRNA would be poorly translated in cells. In contrast, we had previously determined that this mRNA was found almost exclusively in MEL cell polysomes (20; see also Fig. 5). However, since expression of Spi-1 is regulated, at least in part, by mRNA stability (15), the near exclusive presence of this mRNA in polysomes might reflect the preferential stability of translating mRNAs, rather than their efficient translation. To assess the effect of the 5′-UTR on translation of this mRNA *in vivo*, sucrose density gradients were utilized to assess the translational efficiency of a Spi-1 transcript that had been truncated at position –10. As demonstrated above, this cDNA, –10/Δ3′ (which also contained the previously described 3′ truncation), was translated 8-fold more efficiently in the lysates than the Δ3′ transcript (which contained the 3′-truncation but retained the 5′-UTR sequences). The cDNA that lacked both 5′- and 3′-UTRs (–10/Δ3′) was ligated into the pRC/RSV expression vector and stably introduced into MEL cells by electroporation. Since MEL cells already express Spi-1 protein, translation of the endogenous and recombinant transcripts was assessed by their association with polysomes. Since these analyses required Northern blotting to identify the two different transcripts, the clones were selected for this analysis based on the ability to detect the truncated, recombinant transcript by blot hybridization. Two of the selected clones were analyzed in density gradients and gave identical results. A Northern blot from one of these clones is shown in Figure 5 (top panels). These results demonstrated that the 5′-truncated mRNA was associated with an increased number of ribosomes, relative to endogenous Spi-1 mRNA. The 5′-truncated mRNA peaked in fraction 11, as compared with endogenous Spi-1 mRNA, which peaked between fractions 9 and 10. From

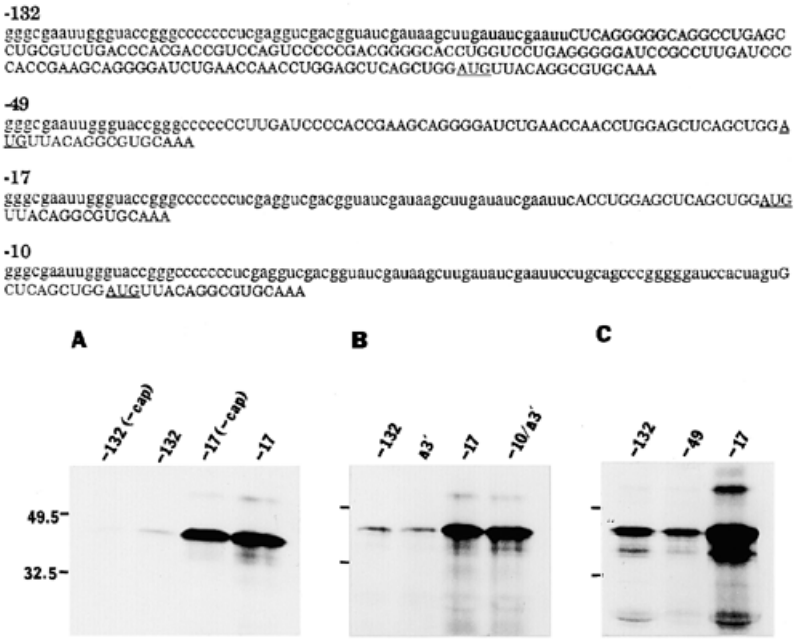

Figure 4. The 5′-UTR of Spi-1 mRNA inhibits its translation in reticulocyte lysates. Equimolar amounts of *in vitro* transcribed Spi-1 RNAs were incubated in reticulocyte lysates with the addition of [^{35}S]methionine. All transcripts were initiated with m^{7}G(5′)ppp(5′)G, except as indicated (–cap). The complete sequence of the 5′-UTRs of the transcripts are indicated at the top of the figure, with the initial AUG codon underlined. The vector-derived sequences are indicated in lower case and the Spi-1 sequences in upper case. The translations were terminated by addition of RNase A and the products analyzed by polyacrylamide gel electrophoresis and fluorography. (**A**) Spi-1 transcripts containing either 132 or 17 nt of the 5′-UTR proximal to the initial AUG codon (–132 and –17 respectively) were synthesized with and without a 5′-methylated cap structure and translated in the lysates. Both transcripts included the entire 3′-UTR. (**B**) Transcripts –132 and Δ3′ both contained the 132 nt of 5′-UTR proximal to the initial AUG codon. The –132 transcript also contained the entire 3′-UTR, while the Δ3′ transcript was truncated 10 nt 3′ of the termination codon. The transcripts –17 and –10/Δ3′ contained 17 and 10 nt of the 5′-UTR most proximal to the initial AUG. The –17 transcript included the entire 3′-UTR, while the –10/Δ3′ transcript had been truncated 10 nt 3′ of the termination codon. (**C**) The –132 and –17 transcripts have been previously described. The –49 transcript included the 49 nt of 5′-UTR proximal to the initial AUG and the entire 3′-UTR. The positions of the molecular weight markers (kDa) are indicated to the left of each gel.

UV tracings of the polysomes it was estimated that this difference would result in an increase from ~8 ribosomes/mRNA to ~14 ribosomes/mRNA.

To ensure that the increase in number of ribosomes associated with the 5′-truncated transcript was attributable to the 5′-UTR of Spi-1 and not an unexpected effect of the vector sequences contained within the –10/Δ3′ mRNA, a recombinant Spi-1 mRNA which included Spi-1 5′-UTR sequences up to –132 was similarly analyzed. As demonstrated in Figure 4, the 3′-truncation of this transcript (Δ3′) did not independently affect its translation in reticulocyte lysates. Stably expressing clones were isolated and translation of the Δ3′ transcript analyzed by density gradient as previously done for the –10/Δ3′ transcript. The results for one of these clones is displayed in Figure 5 (bottom panels). Similar results were seen on analysis of an additional clone. These analyses demonstrated that the recombinant transcript that included the 5′-UTR was distributed in the same gradient fractions as endogenous Spi-l mRNA. Thus, the difference in number of associated ribosomes observed in the preceding experiments was due to excision of the Spi-1 5′-UTR sequences. However, while these results demonstrate that the Spi-1 5′-UTR decreased the number of ribosomes that became associated with the mRNA and suggest that translation initiation had been inhibited (32), this *in vivo* effect appeared to be substantially less than that observed *in vitro*.

The decrease in Spi-1 expression in differentiating MEL cells is regulated by a decrease in mRNA stability (15). Since mRNA degradation and translation are inter-related (33), it is possible that in the preceding experiments a translational effect may have been masked by preferential stabilty of ribosome-associated mRNA. Therefore, experiments were performed to directly quantitate the effect of the 5′-UTR on expression of a linked reporter gene. A luciferase cDNA was ligated immediately 3′ of the Spi-1 5′-UTR present in the pRC/RSV(Δ3′) vector described above. The luciferase cDNA with contiguous SV40 3′-UTR and polyadenylation signal was ligated into a vector from which Spi-1 and contiguous BGH 3′-UTR sequences had been completely excised (designated –5′/luc) or which retained the 5′-UTR of Spi-1 intact through the *Sac*I site at position –6 (designated +5′/luc). This latter construct is capable of forming stem–loops II and III, although the duplex preceding stem–loop II is disrupted, since this requires the initial Spi-1 coding sequence (see Fig. 3). However, based on the results of the *in vitro* experiments, the loss of this duplex should not adversely affect the ability of the 5′-UTR to inhibit translation. The +5′/luc and –5′/luc plasmids were introduced into MEL cells as well as into Da-3 cells, an IL 3-dependent hematopoietic cell line. Translational efficiency was determined by assaying luciferase expression from the two vectors. The results are summarized in Table 1. As shown by comparing the expression of +5/luc relative to –5/luc (indicated at the bottom of Table 1) the 5′-UTR of Spi-1 did not significantly affect expression of luciferase in either of these cell lines. Similar results were obtained when the DNAs were introduced into 3T3 cells by calcium phosphate precipitation (data not shown). Thus, in contrast to the results obtained in reticulocyte lysates, the

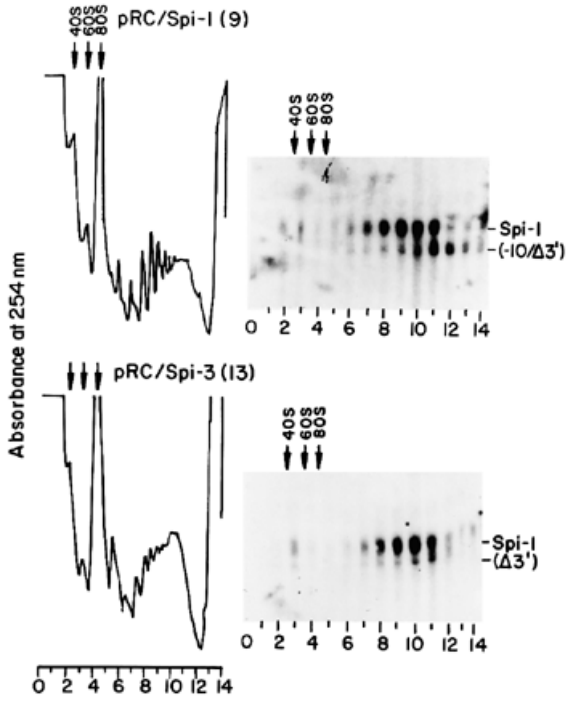

Figure 5. The 5′-UTR of Spi-1 decreases the number of ribosomes associated with Spi-1 mRNA in MEL cells. Recombinant Spi-1 cDNAs that included either a truncated (–10/Δ3′) or full-length (Δ3′) 5′-UTR were ligated into the pRC/RSV expression vector and introduced into MEL cells by electroporation. Stable clones were analyzed for expression of the recombinant transcript by Northern blotting and selected for analysis in sucrose density gradients, as described in the text. Gradient fractions were collected with continuous UV monitoring and the fractions indicated below the UV tracings for two of the clones as shown in the left panels. RNA extracted from the fractions was analyzed by electrophoresis in 3.5% polyacrylamide/urea gels, electroblotting and blot hybridization. Autoradiographs of the blots for two of the clones are shown in the right panels. The positions of the endogenous (Spi-1) and the recombinant mRNAs are indicated on each autoradiograph.

5′-UTR of Spi-1 mRNA did not have a demonstrable effect upon translation *in vivo*.

DISCUSSION

The data presented in this manuscript provide evidence that the conserved 5′-UTR of the hematopoietic transcription factor Spi-1 (Pu.1) forms stable secondary structure that includes three stem–loops preceding the open reading frame. The existence of the two stem–loops most proximal to the initial in-frame AUG codon is consistent with the data from limited digestion with ribonucleases. *In vitro* Spi-1 transcripts that included the 5′-UTR were translated 8- to 10-fold less efficiently than transcripts that lacked this sequence and this effect was retained in truncated transcripts that included only the stem–loop most proximal to the initial in-frame AUG codon. However, the translational effect of the 5′-UTR was not apparent in intact cells. This disparity between *in vivo* and *in vitro* effects, coupled with the observation that endogenous Spi-1 mRNA is found almost exclusively associated with polysomes in MEL cells, suggests that additional post-transcriptional mechanisms contribute to regulation of its expression in cells.

Table 1. The 5′-UTR of Spi-1 does not inhibit translation of a recombinant luciferase transcript

Plasmid	MEL (standardized light units[a])	Da-3 (standardized light units[b])
+5′/luc	237.9	0.354
	152.2	0.418
	154.8	0.375
	245.0	0.373
		0.308
Mean	197.5	0.366
5′/luc	225.0	0.412
	181.3	0.594
	94.0	0.439
	183.0	0.431
		0.455
Mean	170.8	0.466
(+5′/luc)/(–5′/luc)	1.16	0.79

[a]Per μg extract protein.
[b]Per U galactosidase activity.
Luciferase cDNAs were cloned into the pRC/RSV eukaryotic expression plasmid and introduced into cells by electroporation, using the parameters described in the text. The plasmid DNA labeled +5′/luc included the Spi-1 5′-UTR preceding the luciferase coding region, while the –5/luc plasmid lacked this sequence. Cell extracts were prepared 6 h following electroporation and assays for luciferase and β-galactosidase performed.

The observation that reverse transcription prematurely terminated within the 5′-UTR of Spi-1 suggested the existence of stable secondary structure within this sequence. Secondary structure modeling of 151 nt of the Spi-1 5′-UTR and the adjoining 18 nt of coding sequence predicted the existence of stable secondary structure (ΔG = –55.4 kcal/mol) that included three stem–loops. Mapping of *in vitro* transcribed Spi-1 mRNA by limited ribonuclease digestion was consistent with the existence of the two stem–loops most proximal to the initial in-frame AUG codon. While the ionic conditions for the digestions were similar to that expected *in vivo*, it remains to be demonstrated that this structure exists in intact cells.

Consistent with the existence of the predicted secondary structure, the 5′-UTR of Spi-1 inhibited translation in reticulocyte lysates 8- to 10-fold. Analysis of deletion mutants demonstrated that the sequence between nucleotides –17 and –49, which formed the stem–loop most proximal to the coding region, was sufficient for this effect. However, it cannot be excluded that the excised sequences affect translation of this mRNA, since the constructs tested did not include sufficient 5′-sequence to determine if stem–loop I played an additional role in inhibiting translation. Further, the ability of stem–loop II to independently inhibit translation was not directly tested and it is possible that a translational effect of this structure was obscured by the dominant effect of the stem–loop more proximal to the initial AUG codon.

Translational inhibition by RNA stem–loops has been suggested to be dependent upon their positioning within the 5′-UTR. Initial observations in reticulocyte lysates suggested that translational inhibition increased with proximity to the 5′-cap (34), while more recent results in reticulocyte and yeast lysates, as well as in live yeast, determined that inhibition increased with proximity to the initiating AUG (35). While our results appear most consistent with the latter observations, it is noteworthy that the degree of translational inhibition observed with the –49 transcript appeared

to be slightly greater than that observed with the –132 transcript (see Fig. 4C). Since the –132 transcript lacked the full sequences necessary for formation of stem–loop I, the –49 truncation would be predicted to position a stem–loop more closely to the 5′-cap. Thus, although these experiments were not designed to directly test this hypothesis, this result suggests that proximity of a stem–loop to both the cap and the initiating AUG may be important determinants of translational efficiency in reticulocyte lysates.

In contrast to the pronounced effect of Spi-1 5′-UTR sequences on translation *in vitro*, minimal effects were observed upon translation in intact cells. While determination of the number of ribosomes associated with recombinant Spi-1 mRNAs demonstrated that the 5′-UTR did decrease translation, a direct effect of this sequence on translation of a linked luciferase construct could not be demonstrated. Further, given the 8- to 10-fold inhibition of translation observed in the reticulocyte lysates, which are derived from terminally differentiated erythrocytes, it is paradoxical that in the leukemic erythroblasts Spi-1 mRNA is fully associated with polysomes and thus appears to be more efficiently translated than the average mRNA in these cells (20). While cell transformation has been demonstrated to increase the efficiency of translation initiation (36), this does not explain the translational advantage that Spi-1 mRNA appears to have over the remaining 50–80% of mRNAs in these cells that are not associated with polysomes (20). Thus, other mechanisms must account for the preferential association of Spi-1 mRNA with polysomes in these cells.

While the reason(s) for the differential effect of the 5′-UTR on translation *in vitro* and *in vivo* is not evident, similar results have been noted for c-*myc* mRNA (37,38). While 5′-UTRs have been demonstrated to affect mRNA stability, presumptively through an effect on mRNA translation (39), the 5′-UTR of Spi-1 does not appear to play a significant role in the regulated decrease in mRNA stability that follows inducer exposure (15). Further, the lack of effect on expression of luciferase demonstrates that the Spi-1 5′-UTR does not significantly effect either translation or stability of the luciferase transcript. Thus, the functional significance of the conservation of these 5′-UTR sequences remains unexplained.

Although these studies were unable to demonstrate a role for the 5′-UTR in regulating translation of Spi-1 mRNA, this sequence is 87% identical between mouse and human (16,17), suggesting that this sequence has a regulatory function. Similarly, analysis of genes in different vertebrate species has demonstrated blocks of highly conserved regions (HCRs) located in both the 5′- and 3′-UTRs of a number of mRNAs (40). These HCRs are not a common feature of mRNAs, since only 10% of mRNAs have been observed to have 5′-HCRs (40). For several of these 5′-HCRs the mRNAs have previously been identified to be subject to translational regulation (40; for examples see 41,42). Thus, while our experiments failed to demonstrate a translational function for the 5′-UTR of Spi-1 in these cells, it seems likely that conservation of this sequence indicates a role in an as yet unidentified regulatory process.

ACKNOWLEDGEMENTS

The authors would like to acknowledge William Merrick and David McPheeters for helpful suggestions and for manuscript review and Deb Galson and Rick Maki for the Spi-1 cDNA clones. This work was supported by the Office of Research and Development, Medical Research Service, Department of Veteran's Affairs and grant DK43414 from the National Institutes of Health.

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