

# Characterization of an Internal Ribosomal Entry Segment in the 5' Leader of Murine Leukemia Virus *env* RNA

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**The 5' untranslated region, also called the leader, of oncoretroviruses and lentiviruses is long and is formed of several structured domains critically important in virus replication. The 5' leader of murine leukemia virus (MLV) RNA contains an internal ribosomal entry segment (IRES) which promotes synthesis of Gag and glyco-Gag polyprotein precursors. In the present study we investigated the translational features of the 5' leader of MLV subgenomic RNA (*env* RNA) encoding the Env polyprotein precursor. When the *env* leader was inserted between two genes, such as *lacZ* and the neomycin resistance cassette, in a dicistronic vector, it allowed IRES-dependent translation of the 3' cistron in the rabbit reticulocyte lysate system and in murine cells. The drug rapamycin and the foot-and-mouth disease virus L protease, known to inhibit cap-dependent translation, caused an enhancement of the translation driven by the *env* leader sequence, consistent with an IRES activity promoting Env expression. Analysis of several deletion mutants led us to localize the minimal *env* IRES between the splice junction and the *env* AUG start codon.**

The murine leukemia viruses (MLV) belong to the simple retrovirus family bearing in common a genomic RNA with the *gag*, *pol*, and *env* genes. The structural proteins and enzymes of the virion core encoded by the *gag* and *pol* genes, respectively, are translated by full-length viral RNA, whereas the envelope proteins, encoded by *env*, are translated by a spliced RNA (*env* RNA) (19, 32) (Fig. 1). In the Moloney strain of MLV, the splice donor site is located at position 204 of the genomic RNA and the splice acceptor site is in *pol* at position 5483 (27). As a consequence, (i) the dimerization and encapsidation sequences present in the 5' leader of the genomic RNA are absent in the spliced RNA, thus preventing its packaging into virions (16); (ii) the 5' *env* leader derives from the *pol* coding region from position 205 to the *env* initiation codon at position 5780; and (iii) the genomic RNA and the spliced RNA share the same 5' leader sequences from the 5' cap to the splice donor site, probably with the same stable secondary structures (18, 29). The presence of secondary structures between the cap and the initiation codon has been proposed to strongly interfere with the scanning mechanism of translation initiation (11). In this model, well established for most eukaryotic mRNAs, translation is initiated after a linear scanning of the 40S ribosomal subunit from the 5' end of the mRNA (the cap structure) to the initiation site (generally the first AUG codon) (12, 17). The features of a 5' leader that optimize the efficiency of translation initiation include the presence of a 5' cap, a short length, the absence of stable secondary structures as well as AUG codons upstream from the initiation site, and an initiation codon in a good context (A/GCCAUGG). A large number of eukaryotic mRNAs share these features, but a growing number of cellular and viral RNAs lack them. Studies of picornaviruses were the first to show that translation initiation can occur by a mechanism different from the canonical scanning. In these viruses, protein synthesis is initiated by internal ribosome

binding to the RNA (8, 22). This implies that a segment of the viral 5' untranslated region, now known as IRES (internal ribosomal entry segment), guides the ribosomes to the translation initiation site. IRESs are not restricted to viral RNAs but have also been identified in many cellular mRNAs (5, 7, 15, 20, 30). Recently, it has been shown that the 5' leader of MLV genomic RNA contains an IRES that promotes synthesis of Gag and glyco-Gag polyprotein precursors (2). When inserted between two genes in a dicistronic vector, the 5' leader of MLV genomic RNA can promote expression of a downstream cistron in vitro and in vivo. Interestingly, deletion of the 5' first 280 nucleotides of the leader results in a fourfold decrease of IRES activity, suggesting that this region plays an important role in the ribosome entry mechanism (2).

We wanted to investigate the translational features of the 5' leader of *env* RNA. Therefore, we analyzed the expression of dicistronic MLV-derived vectors in vitro and in murine NIH 3T3 cells under conditions that inhibit cap-dependent translation. In the present report we show that the *env* leader contains an IRES which most probably directs expression of the Env polyprotein precursor.

## MATERIALS AND METHODS

**General methods.** Standard procedures were used for restriction nuclease digestion and plasmid DNA construction (26). *Escherichia coli* HB101 strain 1035 (*recA* mutant) was used for plasmid DNA amplification. Details of plasmid construction are given below. The numbering is with respect to the *env* RNA cap site (position +1) unless otherwise stated.

**Reverse transcription-PCR amplification and cloning of the 5' leader of *env* RNA.** Reverse transcription of the 5' leader of *env* RNA was performed as follows. First-strand cDNA synthesis was performed on 100 ng of purified mRNA with the You-Prime First-Strand Beads kit (Pharmacia Biotech). By using Oligotex suspension (Qiagen), template mRNAs for the reverse transcription were extracted from total RNA purified from MLV-infected *Mus dunni* cells (9). Subsequently, a DNA fragment corresponding to the entire 5' leader of *env* RNA was generated by PCR with first-strand cDNA as a template and specific oligonucleotides with an *NheI* restriction site at their 5' ends. This PCR fragment was ligated into pBluescript in order to generate pBenv.

**DNA constructs for translation assays in the rabbit reticulocyte lysate (RRL) system.** A DNA fragment corresponding to the 5' leader of *env* RNA from position 1 to 500 was generated by digesting pBenv with *NheI*. DNA segments corresponding to the *env* leader from positions 204 to 500, 150 to 500, and 304 to 500 were generated by PCR with the pBenv plasmid as a template followed by digestion with *NheI* (PCR-added restriction site). To construct the dicistronic

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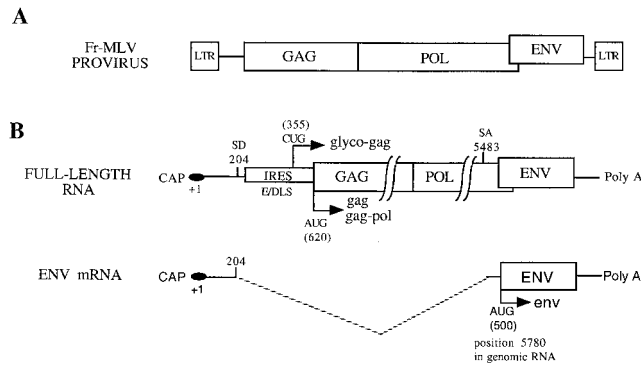


FIG. 1. Genetic organization and expression of Friend murine leukemia virus (Fr-MLV). (A) Fr-MLV provirus. LTR, long terminal repeat. (B) Viral RNAs: full-length viral RNA (used as genomic RNA, pre-mRNA, and mRNA) and splice RNA (used as messenger for *env*). SD, splice donor site; SA, splice acceptor site; E/DLS, dimerization and encapsidation sequence; Poly A, polyadenylate sequence. The numbering is with respect to the 5' cap (+1).

pENV 1 (1-500), pENV 2 (204-500), pENV 3 (304-500), and pENV 4 (500-204), each respective fragment was inserted between *neo* and *lacZ* of pEMCV-D 260-837 previously digested with *NheI* (2).

**In vitro RNA synthesis.** Capped and uncapped RNAs were synthesized with T7 RNA polymerase (mMessage; Ambion) with the linearized DNA template according to the manufacturer's instructions. A *SspI* site in the *lacZ* gene (position 1240) was used to linearize template DNA. RNAs were precipitated by lithium chloride, washed with 70% ethanol, and then resuspended in 50  $\mu$ l of water. RNA integrity was verified on a 1% agarose gel, and the concentration was determined spectrophotometrically.

**Translation in the RRL system.** Capped and uncapped dicistronic RNAs were translated in nuclease-treated RRL (Flexy; Promega) at 50% of the original concentration with 60 mg of RNA/ml (20 mg/ml was used for encephalomyocarditis virus [EMCV] RNA) and 0.6 mCi of [ $^{35}$ S]methionine (Amersham) per ml at 30°C for 1 h. All assay mixtures were supplemented with potassium chloride to a final concentration of 100 mM KCl and with 0.2 mM MgCl<sub>2</sub>. The effect of L protease on the translation of capped RNAs was assayed in the Flexy lysate as described above, but the lysate was pretreated with 1.2 mg of purified recombinant L protease (kindly provided by S. J. Morley, Department of Biochemistry, The University of Sussex, Sussex, United Kingdom)/ml. After translation, the reaction mixtures were treated at 70°C for 5 min in 62.5 mM Tris-HCl (pH 6.8)–2% sodium dodecyl sulfate (SDS)–10% glycerol–5%  $\beta$ -mercaptoethanol–0.002% bromophenol blue, and the  $^{35}$ S-labeled proteins were analyzed by electrophoresis on 15% (wt/vol) polyacrylamide gels. Bands were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

**Cell culture, DNA transfection, and selection.** Murine NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL) containing 10% newborn calf serum at 37°C in the presence of 5% CO<sub>2</sub>. *Mus dunni* cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C in the presence of 5% CO<sub>2</sub>. Transfections were performed by the calcium phosphate method (2a, 2b) with 20  $\mu$ g of plasmid DNA. Selection for neomycin resistance was performed with G418 at 0.8 mg/ml for 1 month.

**Histochemical staining.** For placental alkaline phosphatase (plap) histochemical staining, cells were fixed in phosphate-buffered saline (PBS) containing 2% formaldehyde and 0.2% glutaraldehyde. After two washes in 1 $\times$  PBS, the cells were incubated at 65°C for 30 min in 1 $\times$  PBS. The cells were washed twice with AP buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 50 mM MgCl<sub>2</sub> in 1 $\times$  PBS) and incubated for 5 h in staining solution (0.1 mg of 5-bromo-4-chloro-3-indolyl phosphate [BCIP], 1 mg of nitroblue tetrazolium salt/ml, and 1 mM levamisole in 1 $\times$  AP buffer).

**Effect of rapamycin on dicistronic plasmid expression.** Stably transfected murine NIH 3T3 cells were grown to 70% confluency before serum-free medium was added for 48 h. Then, complete medium containing rapamycin to a final concentration of 100 ng/ml was added for 20 h at 37°C, after which cellular proteins were extracted and enzymatic activities were determined.

**Protein extraction and enzymatic activities.** Cellular proteins were extracted with the  $\beta$ -galactosidase enzyme assay system (Promega). The protein concentration was determined with the Micro BSA kit (Pierce). Neomycin phosphotransferase (Neo) activity was measured by [ $^{32}$ P]ATP phosphate transfer to neomycin (25). In order to check for linearity of the assay and to quantify the neomycin activity in each cell extract, an internal standard curve was used by serial dilutions of the cell extract giving the strongest Neo activity. Alkaline phosphatase activity was determined spectrophotometrically (alkaline phosphatase substrate kit; Bio-Rad). Alkaline phosphatase (in units) was estimated by

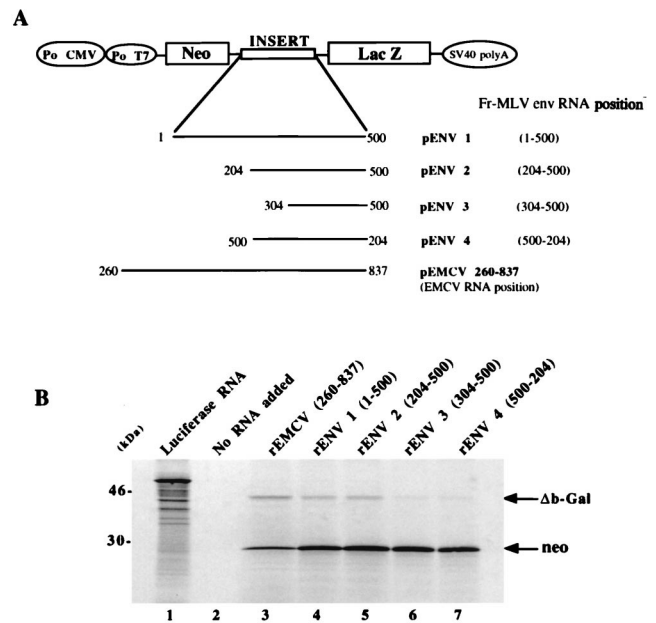


FIG. 2. Translation of capped dicistronic RNAs in the RRL system. (A) Schematic representation of the plasmid constructs used for in vitro RNA synthesis: DNA containing genetic elements of the MLV *env* RNA leader located between the neomycin phosphotransferase and *lacZ* genes under the control of the T7 promoter (Po T7). The numbering is with respect to the subgenomic *env* RNA 5' cap (+1). Po CMV, cytomegalovirus promoter. (B) Translation of cap dicistronic RNAs in the Flexy RRL system (Promega). After heat denaturation,  $^{35}$ S-labeled proteins were analyzed by SDS–15% PAGE. The positions of Neo (28 kDa) and of the carboxy-terminal-truncated  $\beta$ -Gal protein (46 kDa) are indicated. Luciferase RNA was used as the positive control (lane 1), and the negative control had no RNA added (lane 2).

using commercial calf intestine alkaline phosphatase (Boehringer Mannheim) as an activity standard.

## RESULTS

**Characterization of an IRES in the 5' leader of the *env* RNA.** Recent studies have shown that the 5' leader of MLV genomic RNA contains an IRES directing synthesis of Gag and glyco-Gag polyproteins (Fig. 1) (2). This finding prompted us to search for an IRES in the 5' leader of *env* RNA. Various fragments of the *env* leader were inserted between the neomycin phosphotransferase gene (*neo*) and *lacZ* sequences in dicistronic constructs (Fig. 2A). Subsequently, capped RNAs were generated in vitro and their translational activities were monitored in the RRL system. In this system, translation initiation of the 5' cistron (*neo*) is cap dependent (2, 14) while that of the 3' cistron (*lacZ*) can only occur via either (i) an internal ribosome binding to the intercistronic region (IRES-dependent translation) (22) or (ii) a cap-dependent mechanism, such as a reinitiation mechanism, and/or a shunt mechanism (10). As a positive control for IRES-dependent translation, we used pEMCV (260-837) containing the EMCV IRES from position 260 to 837 in the intercistronic region. In order to estimate the background level of  $\beta$ -galactosidase produced in the RRL by a non-IRES-dependent mechanism, we constructed a dicistronic plasmid, pENV 4 (500-204). In this plasmid, the intercistronic region contains the fragment of *env* leader from position 204 to 500 but inserted in the antisense orientation.

Figure 2 shows the protein products generated by translating the dicistronic RNAs and subsequently separated by 15% poly-

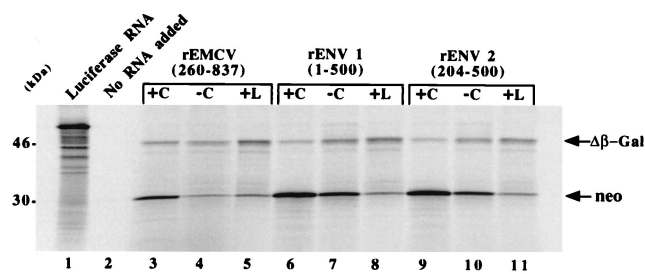


FIG. 3. Effect of cap and FMDV L protease on RNA translation in the RRL system. Translation of dicistronic uncapped RNAs (-C) and capped RNAs in the Flexy RRL system (Promega) with (+L) or without (+C) L protease. After heat denaturation,  $^{35}\text{S}$ -labeled proteins were analyzed by SDS-12% PAGE. The positions of Neo (28 kDa) and the carboxy-terminal-truncated  $\beta$ -Gal protein (46 kDa) are indicated. Luciferase RNA was used as the positive control (lane 1), and the negative control had no RNA added (lane 2).

acrylamide gel electrophoresis (PAGE). Translation of the 3' cistron,  $\Delta\text{LacZ}$ , with recombinant ENV1 (1-500) [rENV1 (1-500)] and rENV2 (204-500) RNAs was efficient in comparison with that of the positive control RNA containing the EMCV IRES (rEMCV) (lanes 4 and 5). In contrast, with the rENV3 (304-500) and rENV4 (500-204) RNAs, translation of the 3' cistron was very low (lanes 6 and 7). Translation of the 5' cistron, *neo*, was very efficient for all RNAs (Fig. 2). These results suggest that the 5' leader of *env* RNA contains an IRES and that the minimal IRES sequence should be located between nucleotide 204 and the *env* initiation codon.

**Effect of L protease and cap on activity of *env* IRES.** To confirm that translation of the 3' cistron in rENV1 (1-500) and rENV2 (204-500) is cap independent, we examined the effect of foot-and-mouth disease virus (FMDV) L protease on translation driven by the putative *env* IRES in the RRL system. The FMDV L protease cleaves eukaryotic initiation factor 4G (eIF4G), resulting in a partial inhibition of cap-dependent translation (4). IRES-dependent translation is not inhibited by L protease but can even be enhanced (33). After translation in RRL and separation of the protein products by gel electrophoresis (Fig. 3), the relative intensities of the  $\beta$ -Gal and Neo proteins were quantified by scanning densitometry and the effect of FMDV L protease was thus estimated. The results are presented as the percentage of protein synthesized in untreated RRL (summarized in Table 1). In addition, as complementary experiments, translation assays were performed with uncapped RNAs.

As shown in Fig. 3, translation of the 5' cistron, *neo*, was always severely reduced in the RRL system treated with L protease (compare lanes 3, 6, and 9 with 5, 8, and 11). In contrast, synthesis of  $\beta$ -galactosidase was enhanced with rEMCV (260-837), rENV 1 (1-500), and rENV 2 (204-500) RNAs (lanes 3, 5, 6, 8, and 11). Similar results were obtained

TABLE 1. Relative variation of translation caused by FMDV L protease in the RRL system<sup>a</sup>

RNA	Relative level of Neo (%)	Relative level of $\beta$ -Gal (%)
rEMCV (837-260)	44	175
rENV1 (1-500)	10	260
rENV2 (204-500)	8	220

<sup>a</sup> The gel shown in Fig. 3 was scanned, and the data were used to calculate the effect of FMDV L protease as a percentage of variation relative to translation in untreated RRL.

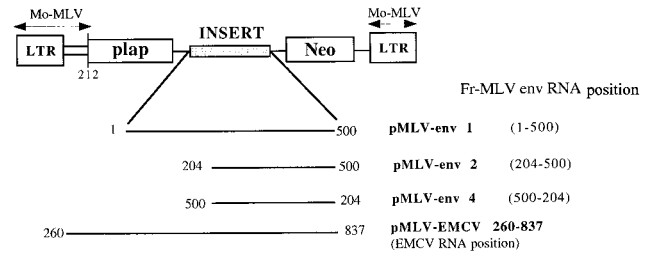


FIG. 4. Schematic representation of the DNA constructs used in cell culture experiments. The plasmids contain different genetic elements of the 5' *env* RNA leader located between the placental alkaline phosphatase gene (*plap*) and the neomycin gene (*Neo*). LTR, long terminal repeat. The numbering is with respect to the *env* mRNA 5' cap.

with uncapped RNAs in that translation of the 5' cistron was reduced while that of the 3' cistron was unchanged or enhanced (lanes 4, 7, and 10). These results strongly suggest that termination-reinitiation or another cap-dependent mechanism cannot account for the translation of the 3' cistron. In fact, in the RRL programmed with uncapped rather than capped RNA or in the presence of L protease, fewer ribosomes would be expected to reach the *lacZ* initiation codon and hence translation of the second cistron would be diminished. However translation of the 3' cistron was either slightly increased (RNAs minus cap) or clearly increased (addition of L protease) (Table 1). These data show that the 3' cistron is translated in a cap-independent fashion and thus indicate that the 5' leader of *env* RNA contains an IRES. These data also indicate that the minimal *env* IRES is probably located between nucleotide 204 and the *env* start codon.

**The *env* IRES directs gene expression in NIH 3T3 cells.** In order to examine the functionality of the *env* IRES in murine cells, dicistronic vectors were transfected in NIH 3T3 cells. The transfected constructs contained all or part of the *env* IRES inserted between the *plap* gene and *neo* (Fig. 4). Expression of the 5' cistron (*plap*) is cap dependent (2, 14), while that of the 3' cistron (*neo*) was IRES dependent (or cap dependent if a translation reinitiation mechanism could occur). We used pMLV-EMCV (260-837) with the EMCV IRES from position 260 to 837 between *plap* and *neo* as a positive IRES control. After 1 month of G418 selection, Neo<sup>r</sup> foci were histochemically stained for alkaline phosphatase and cellular proteins were extracted to determine the relative level of alkaline phosphatase and neomycin phosphotransferase expression in each cell population (the results are summarized in Table 2).

Vectors pMLV-EMCV (260-837), pMLV-env 1 (1-500), pMLV-env 2 (204-500), and pMLV-env 4 (500-204; antisense orientation) were able to confer G418 resistance. Based on histochemical staining, we determined that all Neo<sup>r</sup> foci were expressing *plap*. Enzymatic activities were measured on cellular extracts and showed that the level of *neo* expression was high in NIH 3T3 stably transfected with pMLV-EMCV (260-837) and pMLV env 1 (1-500), medium with pMLV-env 2 (204-500), and low with pMLV-env 4 (500-204). These data indicate that the *env* IRES is functional in NIH 3T3 cells and confirm results obtained in the RRL system. The negative control, pMLV-env 4 (500-204; antisense), was also able to confer G418 resistance, but the number of Neo<sup>r</sup> foci was at least 10 times less than with pMLV-env 2 (data not shown), thus indicating that *neo* expression was an inefficient process with pMLV-env 4 and probably results from a translation reinitiation of the ribosomes coming from the 5' cistron (compare rapamycin [Table 3]).

TABLE 2. Expression of dicistronic plasmids in cell culture<sup>a</sup>

Cell line or plasmid	Alkaline phosphatase sp act (U/ $\mu$ g of total protein)	Neomycin phosphotransferase sp act (arbitrary units)	neo/plap (%)
NIH 3T3	0	0	0
pMLV-EMCV (260-837)	$(1.0 \pm 0.4) \times 10^{-6}$	$100 \pm 35$	$100 \pm 35.0$
pMLV-env 1 (1-500)	$(7.2 \pm 0.5) \times 10^{-6}$	$86 \pm 19$	$12 \pm 2.6$
pMLV-env 2 (204-500)	$(5.7 \pm 0.7) \times 10^{-6}$	$38 \pm 14$	$7 \pm 2.5$
pMLV-env 4 (500-204)	$(2.6 \pm 0.4) \times 10^{-5}$	$28 \pm 10$	$1 \pm 0.4$

<sup>a</sup> Murine NIH 3T3 cells were transfected with the dicistronic plasmids described previously (Fig. 4A). After 3 weeks of selection with G418, total proteins were extracted and enzymatic activities were determined as described in Materials and Methods. The data shown correspond to the averages ( $\pm$  standard deviations) of three independent experiments. The ratio of neo to plap expression is shown as percentages in the third column. In the absence of a functional IRES between the two cistrons, there is clearly a large increase in plap (first cistron) expression (see pMLV-env 4).

In order to confirm the above data, we analyzed the variation of *plap* and *neo* expression caused by rapamycin, an inhibitor of cap-dependent translation.

**env IRES-dependent translation is enhanced in rapamycin-treated murine cells.** Rapamycin has been shown to partially inhibit cap-dependent translation. This inhibition results from the dephosphorylation and consequent activation of 4E-BP1, a repressor of the cap-binding protein eIF-4E (1, 6, 13). To further examine the translational properties of the *env* IRES in cells, we analyzed the effect of rapamycin on plap and neomycin expression in Neo<sup>r</sup> populations (the results are summarized in Table 3). As predicted, in the presence of rapamycin the expression of the 5' cistron (*plap*) was always decreased. This confirms the capacity of rapamycin to inhibit cap-dependent translation in murine cells. Similarly, in NIH 3T3 cells stably transfected with pMLV env 4 (500-204), the expression of the 3' cistron (*neo*) was inhibited in the presence of rapamycin. These results indicate that the fragment of the 5' leader *env* RNA from position 204 to 500 but in an inverted orientation does not possess an IRES activity. This supports the view that expression of the 3' cistron (*neo*) in pMLV env 4 (500-204) occurs via a cap-dependent and reinitiation mechanism. In contrast, in rapamycin-treated cells stably transfected with pMLV-EMCV (260-837), pMLV env 1 (1-500), pMLV env 2 (150-500), and pMLV env 3 (204-500), we observed a drastic increase of *neo* activity (Table 3). These results show that the full-length *env* leader, as well as *env* leader sequences from position 204 to 500, allows cap-independent translation of a downstream cistron in vivo. These data are in agreement with those obtained in vitro, indicating that a minimal *env* IRES is

present between the splice junction and the initiation codon of the *env* RNA.

## DISCUSSION

The genomic RNA of MLV and MLV-like type C retroviruses has a long 5' leader formed of structured domains involved in key functions of the viral life cycle, such as splicing, translation, dimerization and packaging, and reverse transcription (3, 31). Stable secondary structures in the 5' leader of MLV are thought to prevent translation initiation of the Gag precursor by the classical ribosome scanning mechanism. The presence of an IRES upstream of the *gag* initiation codon allows the local recruitment of ribosomes and synthesis of the Gag precursor (2). This prompted us to search for an IRES in the 5' leader of the *env* RNA, since the genomic and the subgenomic RNAs of MLV have the same sequence upstream of the splice junction. Therefore, we constructed dicistronic plasmids containing part or all of the *env* leader inserted between two reporter genes. The translational activity of these constructs was monitored in the RRL system and in NIH 3T3 cells. The results show that the 5' leader of *env* RNA contains an IRES directing translation of a downstream cistron independently of the first cistron. Furthermore, either the drug rapamycin or FMDV L protease, which are known to specifically shut off cap-dependent translation, caused an increase in translation driven by the *env* leader, whereas they both reduced expression of the 5' cap-dependent cistron present in the bicistronic RNA. Analysis of the expression of pENV 2 (204-500) and pENV 3 (304-500) constructs led us to identify a functional segment of the *env* IRES between the splice junction and the *env* initiation codon. Thus, the *gag* and *env* IRESes have common sequences at their 5' ends, and in agreement with this, the region from position 1 to 260 increases translation initiation directed by the *gag* IRES (2). Similarly, our data show that in murine cells the full-length *env* leader possesses a stronger IRES activity than the region extending from position 204 to 500 (Table 2). Therefore, it is tempting to speculate that the activity of both the *gag* and *env* IRESes might be coregulated by cellular factors binding to the same 5' sequence of the *gag* and *env* RNAs. Differential regulation of Gag and Env expression would then depend on 3' sequences unique to each IRES. This is presently under investigation.

IRES-dependent translation does not use the cap-binding initiation factor eIF4E required for cap-dependent translation. This protein is a key target in the regulation of protein expression (21, 23, 24). It has been proposed that internal entry, which avoids eIF4E regulation, may represent a viral strategy to favor viral protein synthesis at the expense of that of cellular proteins. Such an advantage would, for example, enable virus production during mitosis, when cap-dependent translation is

TABLE 3. Variation of dicistronic plasmid expression caused by rapamycin in cell culture<sup>a</sup>

Cell line (plasmid)	Variation (%) in alkaline phosphatase sp act	Variation (%) in neomycin phosphotransferase sp act
pMLV-EMCV (260-837)	-20	+826
pMLV env 1 (1-500)	-26	+316
pMLV env 2 (204-500)	-30	+800
pMLV env 4 (500-204)	-15	-16

<sup>a</sup> Murine NIH 3T3 cells were transfected with the dicistronic plasmids described previously (Fig. 4A). After 3 weeks of selection with G418, the cells were serum starved for 48 h and then serum was added as well as rapamycin to a final concentration of 50 ng/ml. Total proteins were extracted 20 h later from both rapamycin-treated and untreated cells, and enzymatic activities were determined. The data shown correspond to the means of three independent experiments. The level of the reporter gene, measured as enzymatic activity, in the presence or absence of rapamycin was used to calculate the effect of the drug as a percentage increase or decrease relative to the level in untreated cells.

significantly decreased (28). For such a strategy to be effective it is logical that, like Gag, Env polyprotein should possess a cap-independent translation mechanism.

Results obtained with dicistronic constructs containing portions of the *env* leader indicate that a functional part of the *env* IRES encompasses the splice junction and *env* initiator codon. This situation raises the possibility that some Env polyprotein precursors could be translated after internal binding on genomic RNAs. This hypothesis does not agree with early oocyte injection experiments which showed that genomic RNAs could only produce Gag-Pol proteins in oocyte cytoplasm (19, 32). One can argue that these experimental procedures do not recreate optimal cellular conditions for IRES-dependent translation. A more likely hypothesis is that the *env* IRES is not functional when present as part of the *pol* coding region. This would also explain the splicing of the genomic RNA. This is presently under investigation in vitro and in cell culture.

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