An Internal Ribosome Entry Segment Promotes Translation of the Simian Immunodeficiency Virus Genomic RNA*

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Theophile Ohlmann†, Marcelo Lopez-Lastra, and Jean-Luc Darlix
From LaboRetro, INSERM U412, Ecole Normale Superieure de Lyon, 46 Allee d’Italie, 69364 Lyon Cedex 07, France

The retroviral genomic RNA is the messenger for the synthesis of the group-specific antigen (gag) and polymerase precursors of the major structural proteins and enzymes of the virion. The 5′-untranslated leader of the simian immunodeficiency virus (SIV) genomic RNA is formed of highly structured domains involved in key steps of the viral life cycle. Thus, the presence of stable RNA structures between the 5′-cap and the gag start codon is thought to strongly inhibit scanning of a 43 S preinitiation ribosomal complex. This prompted us to look for an alternative to the canonical ribosome scanning. By using a standard bicistronic assay in the rabbit reticulocyte lysate, we show that the SIVmac 5′-leader contains an internal ribosome entry segment (IRES) and that gene expression driven by this IRES is stimulated upon cleavage of eukaryotic initiation factor 4G. Deletion analysis revealed that the sequence between the major splice donor and the gag AUG codon is required for IRES activity. DNA transfection and viral transduction experiments in both NIH-3T3 and COS-7 cells confirmed that translation driven by the SIV leader is IRES-dependent and thus insensitive to the immunosuppressant rapamycin. Identification of an IRES in SIV is of particular interest for the understanding of lentivirus replication and also for the design of novel lentiviral vectors suitable for gene transfer.

Translational control is a major contributor to the regulation of gene expression in eukaryotes. It is now recognized that the initiation step of translation is a determinant in the overall process of protein synthesis. For the vast majority of eukaryotic mRNAs, this occurs by a cap-dependent mechanism whereby the 40 S ribosomal subunit binds the m^GTP cap structure present at the 5′-terminus of virtually all eukaryotic mRNAs (except organelles). Subsequently, the ribosomal subunit migrates along the 5′-untranslated region until it encounters an AUG codon in a favorable context ((G/A)CCUGG). This process involves a number of initiation factors that have been described to allow both mRNA binding and ribosome scanning (1, 2). Among them, there are the cap-binding protein eIF4E,1 which specifically binds to the cap (3); eIF4A, which possesses RNA-dependent helicase activity (4); and eIF4G, which makes a bridge between the mRNA cap (via eIF4E) and the 40 S ribosomal subunit (via eIF3) (5).

An alternative mechanism of translation initiation has been described with the study of the picornavirus family. This mechanism, called internal initiation, is rendered possible by an internal ribosome entry segment (IRES), which is a 450-nucleotide RNA sequence with complex secondary structures that allows translation by direct ribosome binding to the 5′-untranslated region (6–8). This has first been described by inserting specific cis-acting sequences into the intercistronic spacer of a bicistronic construct coding for two proteins (9, 10). Expression of the 3′-cistron proved the ability of the inserted sequence to promote internal ribosome binding and translation.

Another major difference between cap-dependent and internal initiation of translation resides in the utilization of some of the canonical initiation factors. Whereas cap-dependent translation requires eIF4E, eIF4A, and eIF4G, internal initiation can proceed in the absence of the cap-binding protein eIF4E (11, 12). Moreover, after cleavage of eIF4E by picornavirus-encoded proteases, the carboxyl-terminal domain of eIF4G was shown to be sufficient to promote internal initiation (13, 14). The mechanism of internal initiation has been reported for many other viral and cellular mRNAs. More recently, IRESs have been identified in retro-elements such as mouse VL30 (15) and rat VL30 (16) and several members of the retrovirus family, including Friend murine leukemia virus (17), Moloney murine leukemia virus (MMLV) (18), human T-cell leukemia virus (19), and reticuloendotheliosis virus type A RNA (20). However and despite some attempt with human immunodeficiency virus type 1 (21), no IRES has yet been found in a lentivirus.

The 5′-leader of SIVmac is long (537 nucleotides up to the AUG codon of gag) and formed by extended RNA structures necessary for key steps of the viral life cycle. This includes the highly structured TAR element, which forms a stable RNA stem-loop required for the activation of transcription. The leader also comprises the polyadenylation loop and the primer-binding site responsible for primer RNA annealing. In addition, the leader of SIV also promotes the translation of gag and gag-polymerase precursor polyproteins.

Both the length of this leader and the high degree of RNA secondary structures prompted us to look for the presence of an IRES element. We inserted the SIV leader between two reporter genes, and this resulted in the expression of the downstream cistron both in the RRL and in cells. Moreover, as reported for other IRESs, this expression was stimulated by the cleavage of eIF4G in vitrō and upon treatment with the immunosuppressant rapamycin ex vitrō.

EXPERIMENTAL PROCEDURES

Plasmid Construction

Standard procedures were used for restriction nuclelease digestion and plasmid DNA construction and purification. All numberings are with respect to the genomic RNA cap site (position +1). Details of the
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Constructions are given below.

pSIV—The SIV genomic leader from positions 1 to 537 was amplified by PCR, digested with NheI (PCR-added restriction site), and inserted into pMLV-CB63 previously digested with NheI (17). pSIV-mo—The SIV leader in a monocistronic context was obtained by inserting the PCR product described above into pMLV-CB93 previously digested with NheI (17).

pSIV-unc—The SIVmac genomic leader from positions 1 to 537 was amplified by PCR, digested with NheI, and inserted in the reverse orientation into pMLV-CB63 previously digested with NheI (17). In this construct, β-galactosidase expression was promoted by an AUG codon in a good Kozak context (generated by PCR). pSIVJ3—The SIV leader from positions 1 to 466 was amplified by PCR. The oligonucleotides were designed to change the GCA codon (nucleotides 462–464), creating an AUG codon in the context GT-AU-GGG in frame with β-galactosidase protein. The resulting fragment was inserted into pMLV-CB63 previously digested with NheI (17).

pEMCV-D260–837—This was as described previously (16).

pMLV-SIV T1—SIV mac DNA (positions 1–537) was generated by PCR, digested with NheI (site added with the PCR primer), and cloned between PLAP and neo of pMLV-CB71 (17). pMLV-SIV T1 E+—The EcoRI fragment of pEMCV-CB74 (22) containing the MLV 5′-long terminal repeat and MLV E+IRES sequence was cloned into pMLV-SIV T1/EcoRI.

In vitro Transcription

Prior to in vitro transcription, the plasmids were linearized with SspI, truncating the lacZ gene at position 1240. Transcription reactions were carried out with the bacteriophage T7 RNA polymerase as described previously (23). For synthesis of capped transcripts, the GTP concentration was reduced to 0.48 mM, and the m7GppG cap analogue (New England Biolabs Inc.) was added at a concentration of 1.92 mM. At the end of the incubation period, the template was purified on a Microspin S-400 microcolumn (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and precipitated with LiCl (7.5 M). The integrity of the RNAs was checked by electrophoresis on agarose gels, and their concentration was measured by spectrophotometry.

In vitro Translation

Capped and uncapped RNAs were translated in nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of KCl (75 mM), MgCl2 (0.5 mM), 2-aminopurine (15 mM), and 20 μM each amino acid (except methionine). The mixture was incubated for 1 h at 30 °C in the presence of 0.6 μCi/ml [35S]methionine. Translation products were then separated on SDS-polyacrylamide gel, and the gel was dried and subjected to autoradiography for 12 h using Biomax films (Eastman Kodak Co.).

Oligonucleotides

2′-O-Methyloligoribonucleotides were annealed to RNAs in 20 mM Hepes/KCl (pH 7.6) and 100 mM KCl for 3 min at 65 °C, followed by a 20-min incubation at room temperature with a 100-fold molar excess of oligonucleotides over mRNA. The mixture was then kept on ice until the addition of the translation mixture. The translation reaction was carried out as described above.

Cell Culture

Murine NIH-3T3 cells and the ecotropic packaging cell line GP+E-86 (24) or the PG-13 helper cell line (25) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) at 37 °C with 10% fetal calf serum and 5% CO2 atmosphere. COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco’s modified Eagle’s medium at 37 °C with 10% fetal calf serum in a 5% CO2 atmosphere.

Transfection, Infection, and Titration

NIH-3T3 or ecotropic GP+E-86 cells were seeded at 5 × 105 cells/100-mm plate 24 h prior to transfection with 20 μg of plasmid DNA by the calcium phosphate method (26). 48 h after transfection, cells were selected using G418 at a final concentration of 0.8 mg/ml. The G418-resistant clones were mixed and expanded. The virus-containing medium of transfected cells was utilized for infection and titration of new cells. NIH-3T3 cells were seeded at 5 × 104 cells/100-mm plate 24 h prior to infection or at 2 × 104 cells/well in a 24-well plate for titration. Freshly harvested virus was filtered (0.45-μm pore size filter). Diluted virus-containing supernatants were overlaid onto cells in the presence of Polybrene, which was added at a concentration of 8 μg/ml. Cells were then incubated for 24 h, and the medium was replaced. Infected cells were grown for a total of 48 h and subjected to G418 selection at 0.8 mg/ml or stained for β-galactosidase or PLAP expression. The recombinant viral titer was determined by counting the number of LacZ- or PLAP-positive NIH-3T3 cells 48 h post-infection in limiting dilution assays. Titer, as transducing units/ml, was calculated by the following: (number of colonies) × (dilution of infecting retrovirus)/total volume in ml of diluted vector overlaid onto cells.

Histochemical Staining

Cells were fixed in phosphate-buffered saline containing 2% formaldehyde and 0.2% glutaraldehyde and washed with phosphate-buffered saline. Cells were either stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for LacZ activity or washed twice with AP buffer (100 mM NaCl, (pH 9.5), 0.05% Triton X-100, and 0.1 mM levamisole) and stained with 0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 1 mg/ml nitro blue tetrazolium salt, and 1 mM levamisole in 1× AP buffer for PLAP activity.

Enzymatic Activities

Cell extracts were used as substrate for subsequent enzymatic assays. Cells were washed twice with cold 1× phosphate-buffered saline, scraped using a rubber policeman, collected by centrifugation at 600 × g, and resuspended in Nonidet P-40 buffer (0.5% Nonidet P-40, 140 mM NaCl, and 30 mM Tris-HCl (pH 7.5)). Nuclei were removed by a 10-min centrifugation at 14,000 × g. Protein concentration was determined using the Micro BCA* protein assay reagent (Pierce). PLAP activity in cell extracts was determined spectrophotometrically (alkaline phosphatase substrate kit, Bio-Rad) using commercial calf intestine alkaline phosphatase (Roche Molecular Biochemicals) as a standard activity. The neomycin phosphotransferase activity (Neo) was measured by [γ-32P]ATP phosphate transfer to kanamycin.

Western Blotting

Cells were washed twice with phosphate-buffered saline, trypsinized, and collected by centrifugation at 600 × g. Cells were directly resuspended in Nonidet P-40 buffer, followed by a 10-min centrifugation at 14,000 × g. The supernatant was transferred to a new tube, and the protein concentration was determined using the Micro BCA* protein assay reagent. 10 μg of total protein were subjected to SDS-15% polyacrylamide electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane (Roche Molecular Biochemicals) by semidry transfer in a 30% methanol/Tris/glycine buffer. The filter was blocked with 5% fat-free dried milk in TBST (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20). The membranes were incubated for 1 h at room temperature in a 1:800 dilution of rabbit anti-neomycin phosphotransferase II antibody (5 Prime → 3 Prime, Inc.*, Boulder, CO) in blocking buffer. After two 15-min washes with TBST, the membrane was incubated as described above in a 1:800 dilution of biotinylated anti-rabbit IgG antibody (Bio Sys, Compiègne, France). After two washes with TBST, the membrane was incubated for 30 min in VECTRASTAIN® Elite* ABC avidin/peroxidase solution (Vector Labs, Inc., Burlingame, CA) and developed by ECL (Amersham Pharmacia Biotech) according to the manufacturer’s protocol.

Effect of Rapamycin on Protein Synthesis in Murine Cells

Cells were grown to 70–80% confluency and serum-starved for 48 h prior to the addition of Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum and 50 ng/ml rapamycin (Sigma) or vehicle alone for serum stimulation protein expression (see above). As described previously (20, 27), the level of reporter gene expression, measured by enzymatic activity or by Western blotting in the presence or absence of rapamycin, was used to calculate the effect of the drug as a percentage increase or decrease relative to untreated cells.

RESULTS

The 5′-Leader of SIV Promotes Gene Expression when Inserted into a Bicistronic Construct—The complete SIV leader (Fig. 1), from positions 1 to 537 (numbering is with respect to the genomic RNA cap site (position +1)), was inserted up-stream of the lacZ reporter gene. After in vitro transcription, the resulting mRNAs (capped and uncapped) were translated in the RRL system, and the results are reported in Fig. 2A. Interestingly, there was only a little difference in the yield of β-galactosidase arising from translation of the capped or uncapped transcripts. This low cap dependence of translation

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prompted us to look for the presence of an IRES within the SIV 5′-leader.

Thus, the 5′-leader of SIV (positions 1–537) was inserted into the intercistronic spacer of a bicistronic construct coding for neomycin (5′-cistron) and β-galactosidase (3′-cistron). The resulting RNAs were then translated in the reticulocyte lysate system (Fig. 2B). Preliminary experiments showed that the optimum salt concentrations for β-galactosidase expression were 75 mM KCl and 0.5 mM MgCl₂ (data not shown), and these conditions were used in all experiments described below. Fig. 2B shows the results from autoradiography of the translation products arising from the translation of capped or uncapped bicistronic SIV RNAs. As expected, the first cistron (neo) was expressed more efficiently from capped RNA, whereas higher expression of β-galactosidase was found when the first cistron was uncapped (compare lanes 1–4 with lanes 5–8). This suggests that translation of the second cistron occurs independently from that of the first one and not by a termination-reinitiation mechanism. This low level of β-galactosidase expression with the capped construct may be due to competition between the two cistrons. As a control, the uncapped bicistronic RNA containing the whole 5′-untranslated region of EMCV (from nucleotides 260 to 837) was translated, and the results are presented in lane 9. These results indicate that the SIV leader is capable of driving gene expression in a bicistronic context in the RRL.

**The SIV Leader in the Antisense Orientation Has No IRES Function**—The next step was to insert the SIVmac leader between the neo and lacZ genes, but in the antisense orientation. For this, it was necessary to recreate an AUG codon in a good Kozak context in frame with the lacZ coding region (see “Experimental Procedures”). As shown in Fig. 3B, translation of the SIVinv construct resulted in the production of a small amount of β-galactosidase compared with that obtained with SIV RNA in the sense orientation (compare lanes 1 and 2 with lanes 3 and 4). Moreover, the translational activity of SIV was not stimulated upon capping of the RNA transcript (lanes 5 and 6). This implies that the correct folding of the SIV leader is required to promote ribosome entry and cannot be replaced by a portion of an RNA of similar length.

**Addition of FMDV L Protease Does Not Inhibit Translation Driven by the 5′-Leader of SIV**—The L protease from FMDV cleaves the initiation factor eIF4G into an N-terminal domain (one-third of the molecule) and a C-terminal domain (two-thirds of the molecule) (28). Previous studies have shown that this proteolytic cleavage results in an inhibition of cap-dependent protein synthesis, whereas translation driven by an IRES is unaffected or even stimulated (14, 23, 29, 30). Therefore, we used the recombinant L protease from FMDV (a kind gift from Drs. S. J. Morley and V. M. Pain) to study translation of the bicistronic SIV construct under conditions in which cap-dependent protein synthesis would be inhibited.

As shown in Fig. 4A, the addition of increasing concentrations of the recombinant L protease to the RRL programmed with capped bicistronic SIV RNA resulted in the inhibition of translation of the upstream cistron (neo) in a dose-dependent manner (compare lane 1 with lanes 2–4), with the maximum inhibition (lane 4) being obtained with the highest amount of the L protease used. The translation of uncapped bicistronic RNA revealed quite a different pattern. Expression of the first cistron was stimulated at a low concentration of the L protease (lane 6) or was unchanged (lanes 7 and 8). This stimulatory effect on translation of uncapped RNAs has been previously described (23). Expression of the downstream cistron (β-galactosidase) was stimulated upon treatment with the L protease, and this occurred with both the capped (compare lane 1 with...
Fig. 2. The SIV leader region can drive gene expression in a bicistronic RNA context. A, the leader of SIV was inserted upstream of the lacZ gene, creating pSIV-mono. Different concentrations of the capped (lanes 1–3) or uncapped (lanes 4–6) SIVmono RNAs were incubated for 60 min in the RRL (10 μl) as indicated. 1-μl samples of each assay were analyzed on SDS-15% polyacrylamide gel, and the dried gel was subjected to autoradiography. The position of the β-galactosidase product (Δβ-Gal) is indicated. B, the complete leader of SIV was inserted into the intercistronic spacer of a bicistronic construct coding for neomycin (first gene) and β-galactosidase (second gene), creating pSIVBi. Capped (lanes 1–4) and uncapped (lanes 5–8) RNAs were translated at different concentrations (as indicated) in the RRL (10 μl). A control incubation with 10 μg/ml capped bicistronic EMCV-D260–837 was set in parallel. The samples were processed as described above, but were run on SDS-10% polyacrylamide gel. The positions of neomycin and β-galactosidase (Δβ-Gal) translation products are indicated.

lanes 2–4) and uncapped (compare lane 5 with lanes 6–8) RNA templates. However, the stimulatory effect of the protease on β-galactosidase expression was greater with the capped transcripts, a possible explanation being that cleavage of eIF4G has a dual effect: it reduces the competition with the first capped cistron, and it specifically stimulates gene expression mediated by the SIV leader. The lack of translation inhibition upon eIF4G cleavage strongly suggests that the SIV leader is capable of driving internal initiation in the rabbit reticulocyte system.

A 72-Nucleotide 3′-Deletion Is Sufficient to Inhibit IRES Activity—In view of the above results, we decided to look for the sequences involved in IRES activity. Thus, a 3′-deletion of 72 nucleotides was engineered to give a truncated leader inserted into the bicistronic vector, generating pSIV3′ (Fig. 5A). The AUG initiation codon was reconstructed at position 465 with the following surrounding context: GTTAUGG. Capped and uncapped SIV3′ RNAs were translated in the RRL, and a control incubation translating the uncapped bicistronic SIV RNA was set in parallel.

As shown in Fig. 5B, β-galactosidase expression was impaired by this 72-nucleotide deletion whether the RNA transcript was capped or uncapped. Increasing the RNA concentration of SIV3′ did not change the pattern of β-galactosidase expression, nor did addition of the L protease to the system (compare lanes 4–6 with lane 8). Thus, these data suggest that the ability of the SIV leader to promote translation of the downstream cistron requires sequences between nucleotide 465 and 537.

Hybridization of 2′-O-Methyloligoribonucleotides on the 5′-Leader of the Bicistronic SIV RNA Does Not Inhibit Translation—Previous work has shown that antisense 2′-O-alkyloligoribonucleotides bound to the mRNA 5′-untranslated region inhibit translation of capped mRNAs in the RRL (31) without affecting internal initiation (32). The chemical modification of the 2′-OH group stabilizes these oligonucleotides against degradation, and the oligonucleotide/mRNA hybrids do not appear to be substrates for double-stranded RNA helicase/deaminases (32). Thus, we have exploited this approach and designed three distinct antisense oligonucleotides complementary to different regions of the SIV leader. Oligo 1 spans nucleotides 127 to 112; oligo 2 covers nucleotides 320 to 303 and is therefore complementary to the primer-binding site; and oligo 3 is complementary to the region from nucleotides 535 to 519 and thus covers the AUG initiation codon (Fig. 6A). These antisense oligonucleotides were annealed to the SIV leader under the conditions described under “Experimental Procedures,” and the resulting oligonucleotide/mRNA hybrids were translated in the RRL (Fig. 6B).

In agreement with the results of Johansson et al. (32), oligo 3, which spans the AUG initiation site, severely impaired translation of the lacZ gene without affecting neo production (first gene). This inhibition of translation is most probably due to impairment of scanning of the 43 S ribosomal subunit. It is also interesting to note that the β-galactosidase protein appeared to be slightly larger than the normal size protein when oligo 3 was annealed around the AUG codon (Fig. 6B, compare
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Fig. 4. Protein synthesis driven by the SIV leader is not inhibited by the addition of the FMDV L protease. A, capped or uncapped SIV RNAs (30 μg/ml) were translated in the RRL (10 μl) in the absence (lanes 1 and 5) or presence of 7.5 × 10⁻⁴ μg (lanes 2 and 6), 1.5 × 10⁻⁴ μg (lanes 3 and 7), or 2 × 10⁻⁴ μg (lanes 4 and 8) of FMDV recombinant L protease. A 1-μl sample of each assay was analyzed on SDS-15% polyacrylamide gel, and the dried gel was subject to autoradiography. The positions of the β-galactosidase (Δ β-Gal) and neomycin products are indicated. B, the relative intensities of the bands were quantified using a STORM 850 phosphoimager, and the results are expressed as percentage of the control (no L protease added) for both capped and uncapped RNAs. Beta-Gal, β-galactosidase.

Fig. 5. A 72-nucleotide 3'-deletion severely impairs translation driven by the SIV leader. A, shown is a schematic diagram of the constructions used. The wild-type (SIV) or the 3'-deleted (SIVΔ3') leader was inserted between the neo and lacZ genes. B, uncapped SIVΔ3' RNAs were translated in the RRL (10 μl) at different concentrations in the absence (lanes 1–3) or presence (lanes 4–6) of 1.5 μg of FMDV recombinant L protease. A control incubation containing 30 μg/ml SIV RNAs was translated with or without the L protease (as indicated). A 1-μl sample of each assay was analyzed on SDS-15% polyacrylamide gel, and the dried gel was subject to autoradiography. The positions of the β-galactosidase (Δ β-Gal) and neomycin translation products are indicated.

lane 4 with lanes 1–3). This may be the result of initiation events at in-frame non-AUG codons, as has been described previously (32). However, hybridization of oligos 1 and 2 to the RNA did not affect translation of lacZ (compare lane 1 with lanes 2 and 3), suggesting that the segment of the SIV leader covered by these oligonucleotides is not scanned by a 43S preinitiation complex. It should also be noted that hybridization of the oligonucleotides is likely to disrupt some secondary (and tertiary) structures of the RNA molecule. However, the lack of inhibition with oligos 1 and 2 suggests that disruption of the 5'-proximal RNA structures of the SIV leader does not impair IRES activity. These data provide additional evidence that the 43S preinitiation complex can gain access to the RNA molecule internally.

Transfection of NIH-3T3 Cells with a Bicistronic Vector Containing the SIV Leader—To substantiate the data obtained in the reticulocyte lysate system, we studied the ability of the SIV leader to direct cap-independent initiation in cells. Thus, we designed a new bicistronic vector in which the SIV leader was inserted between the gene encoding the human placental alkaline phosphatase (PLAP) and the second gene coding for neomycin phosphotransferase (neo), creating pMLV-SIV T1 (Fig. 7A).

The pMLV-SIV T1 vector was transfected in NIH-3T3 cells as described under “Experimental Procedures.” After 1 month of G418 selection (neo resistance), all the clones obtained were also found to be positive for PLAP histochemical staining (data not shown). Cell clones were then mixed and expanded. Although the obtaining of neo-resistant clones strongly suggests that the SIV leader enables cap-independent translation, we measured neomycin expression in the presence of rapamycin. The macrolide antibiotic rapamycin has been shown to block phosphorylation of the 4E-BP1 protein, also known as PHAS-1. In its dephosphorylated form, PHAS-1 acts as a natural repressor of the cap-binding protein eIF4E (27, 33–35), whose nonsequestered levels are probably rate-limiting during cap-dependent translation initiation (36). Phosphorylation of PHAS-1 results in the release of eIF4E and increased translational activity (37). Beretta et al. (27) have shown that in NIH-3T3 cells, rapamycin blocks PHAS-1 phosphorylation, inhibiting cap-dependent (but not cap-independent) translation.

Thus, the effect of rapamycin was measured on the expression of PLAP and neo in stably transfected pMLV-SIV T1 NIH-3T3 cell populations. The enzymatic activities of both PLAP and Neo are shown in Fig. 7B and represent a summary of three separate experiments. As expected, rapamycin reduced PLAP expression (first cistron), whereas neo expression (second cistron) was either unaffected or stimulated (Fig. 7B). A Western blot showing the accumulation of the Neo protein in the absence or presence of rapamycin is shown in Fig. 7C. In conclusion, the above data show that the SIV 5'-leader enables cap-independent translation initiation in a bicistronic context and...
that expression of the two cistrons takes place independently.

The SIV IRES Can Be Used for the Design of New Bicistronic Retroviral Vectors for Gene Transfer—The final aim of this work was to utilize the SIV IRES activity for the design and use of new bicistronic retroviral vectors. Thus, we constructed the retroviral vector pMLV-SIV T1 E+, in which the MMLV E+ packaging sequence (positions 210–1035) (38) was inserted upstream of the PLAP gene (Fig. 7A). Previous work has shown that the MMLV E+ sequence promotes packaging of the recombinant RNA and exhibits IRES activity (18). Therefore, in the pMLV-SIV T1 E+ construct, expression of the first cistron (PLAP) is under the control of the MLV IRES, whereas expression of the second cistron (neo) is driven by the SIV IRES. We used both pMLV-SIV T1 E+ and pMLV-SIV T1 to transfect ecotropic GP+E-86 helper cells. neo-resistant clones were obtained, indicating that the SIV IRES is functional in these cells. All neo-resistant GP+E-86 cells were also found to stably express PLAP (data not shown). Upon selection, recombinant viral titers were determined by transducing NIH-3T3 cells with virus-containing medium. The control vector pSIV T1 was unable to transduce NIH-3T3 cells in the absence of the MMLV packaging sequence. However, pMLV-SIV T1 E+ containing the MMLV E+ IRES sequence could be efficiently encapsidated. Histochemical staining of transduced NIH-3T3 cells revealed a titer of \((2.6 \pm 1) \times 10^6\) transducing units/ml.

The results presented above were confirmed by using another helper cell line and a different target cell line. For this end, pMLV-SIV T1 E+ was transfected in PG-13 helper cells; and after selection and verification of double gene expression (PLAP and neo), supernatants were used to transduce COS-7 cells. After 30 days of selection with G418, stable double gene-expressing COS-7 cells (renamed COS-PN) were obtained. In both target cell lines (NIH-3T3 and COS-7), the level of Neo and PLAP expression was monitored after treatment with rapamycin as described under “Experimental Procedures.” As expected, the production of PLAP (first cistron) driven by the MMLV IRES was not impaired in NIH-3T3 cells and was even stimulated in COS-7 cells by the addition of rapamycin (Fig. 7B). The addition of rapamycin did not alter the level of expression of Neo (second cistron) in NIH-3T3 or COS-7 cells as shown by determining the enzymatic activity (Fig. 7B) or protein accumulation (Fig. 7C). These results confirm that the SIV leader promotes translation in a cap-independent manner; and interestingly, the relative level of expression of PLAP and neo varied among the cell types.

DISCUSSION

Internal ribosome entry segments have been characterized in all picornaviruses identified to date (8), other RNA viruses (39–41), and some cellular mRNAs (42–45). In retroviruses, internal initiation has been shown to take place for gag precursor proteins of MLV type C retrovirus (17–20). Here, we report for the first time in lentiviruses that the SIV leader contains an internal ribosome entry segment.

The complete SIV leader was found capable of driving gene expression in a bicistronic context (Fig. 1). At the same RNA concentration, β-galactosidase expression was lower than that obtained with the EMCV IRES. Nonetheless, it should be noted that EMCV is one of the most efficient IRESs in the RRL (46). The SIV leader in the reverse orientation failed to promote internal initiation, suggesting that SIV-directed translation results from specific features of the leader. By using the recombinant L protein from the foot-and-mouth-disease virus, we have shown that translation driven by the SIV leader is not impaired and rather is stimulated by the cleavage of the initiation factor eIF4G (Fig. 3), in agreement with a previous report (20). To characterize the cis-acting RNA sequences of the SIV leader involved in internal initiation, we truncated 72 nucleotides of the IRES. Translation of this construct showed a significant decrease in the expression of β-galactosidase. Interestingly this 72-nucleotide RNA segment is located between the major splice donor and the AUG initiation codon of gag and is therefore eliminated upon splicing of the genomic RNA.

Finally, we have adapted the results of Johansson et al. (32) and Le Tinevez et al. (31) to design a novel experimental approach to characterize internal ribosome entry segments. Antisense 2'-O-methyloligoribonucleotides were hybridized to different regions of the SIV 5'-leader in a bicistronic context. The chemical modification of the 2'-OH group stabilizes these oligonucleotides against degradation, and they display a strong affinity for complementary RNA sequences (47). As previously reported, binding of 2'-O-methyloligoribonucleotides anywhere...
within the 5'-untranslated region of a reporter RNA blocked cap-dependent translation initiation with high specificity, and this was due to impairment of the progression of a scanning 43S preinitiation complex (31, 32). The resulting oligonucleotide/mRNA hybrids cannot be displaced, as they do not seem to be recognized by RNA helicases involved in translation (32). In our experiments, translation of these oligonucleotide/mRNA hybrids revealed that oligos 1 and 2 did not affect the pattern of gene expression driven by the SIV leader, whereas oligo 3 (which encompasses the initiating AUG codon) was inhibitory. These data suggest that the 5'-proximal region of the SIV leader is not scanned by a 43S preinitiation complex.

To further assess the data presented above, we studied translation driven by the SIV leader in cell culture. Using stably transfected cells, we showed that SIV-driven expression was not inhibited, but instead was stimulated upon treatment with rapamycin. The increase in cap-independent protein expression in the presence of the L protease (Fig. 4) or rapamycin (Fig. 7) probably reflects competition between the 5'-cap structure and the IRES for the recruitment of initiation factors. In agreement with previous studies by Beretta et al. (27), it should be noted that rapamycin inhibited cap-dependent protein synthesis by only 30–40%. However, this is sufficient to show that expression of the second cistron (driven by the SIV leader) occurs independently from that of the first one and is not the result of a termination-reinitiation mechanism.

Viral transduction experiments showed that the SIV leader could be used in a retroviral vector, suggesting that the SIV leader does not interfere with the upstream packaging sequences of MLV. Surprisingly, a competition effect was also observed when the two IRESs (MLV and SIV) cohabited in the pMLV-SIV T1 E+ construct. In this particular case, the SIV
IRES was more efficiently expressed in NIH-3T3 cells than in COS-7 cells, whereas PLAP activity was higher in COS-7 than in NIH-3T3 cells (Fig. 7). These variations are likely to depend on the relative strength of the IRES (46) and the cell type (48). These relative levels of expression should be taken into account for the design of bicistronic retroviral vectors to ensure stable expression and optimum IRES activity in different cell types.

To date, IRES elements have been identified in the genomic RNA of several retroviruses and retro-elements, suggesting that they may be present in most, if not all, retroviruses. The full-length genomic RNA acts both as mRNA and as genomic RNA. However, based on the current models of genomic RNA packaging, both functions do not seem compatible. One possibility is that two pools of RNA coexist in the cytoplasm of the infected cell: one for translation and one for packaging (49, 50). According to this model, the RNA that will be selected for packaging must be inhibited at the translational level. It is tempting to speculate that this inhibition may be mediated by the binding (or the absence of binding) of a factor to the IRES.

Another possibility is that there is only one pool of RNA; this RNA would first be used as a messenger to synthesize the viral proteins necessary for dimerization, packaging, and release of the virions. In this case, translation of this unique genomic RNA must be tightly controlled to stop producing proteins and to allow packaging. Once again, this switch between translation and replication could be controlled at the level of the IRES.

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Theophile Ohlmann, Marcelo Lopez-Lastra and Jean-Luc Darlix

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