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RNA helicase A interacts with divergent lymphotropic retroviruses and promotes translation of human T-cell leukemia virus type 1

Cheryl Bolinger[1,4], Alper Yilmaz[1,4], Tiffiney Roberts Hartman[1,2], Melinda Butsch Kovacic[1], Soledad Fernandez[5,6], Jianxin Ye[1,4], Mary Forget[1,4], Patrick L. Green[1,2,3,4,6] and Kathleen Boris-Lawrie[1,2,3,4,6,*]

[1]Center for Retrovirus Research, [2]Department of Veterinary Biosciences and [3]Department of Molecular Virology, Immunology & Medical Genetics, [4]Molecular, Cellular & Developmental Biology Graduate Program, [5]Center for Biostatistics and [6]Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210-1093, USA

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ABSTRACT

The 5′ untranslated region (UTR) of retroviruses contain structured replication motifs that impose barriers to efficient ribosome scanning. Two RNA structural motifs that facilitate efficient translation initiation despite a complex 5′ UTR are internal ribosome entry site (IRES) and 5′ proximal post-transcriptional control element (PCE). Here, stringent RNA and protein analyses determined the 5′ UTR of spleen necrosis virus (SNV), reticuloendotheliosis virus A (REV-A) and human T-cell leukemia virus type 1 (HTLV-1) exhibit PCE activity, but not IRES activity. Assessment of SNV translation initiation in the natural context of the provirus determined that SNV is reliant on a cap-dependent initiation mechanism. Experiments with siRNAs identified that REV-A and HTLV-1 PCE modulate post-transcriptional gene expression through interaction with host RNA helicase A (RHA). Analysis of hybrid SNV/HTLV-1 proviruses determined SNV PCE facilitates Rex/Rex responsive element-independent Gag production and interaction with RHA is necessary. Ribosomal profile analyses determined that RHA is necessary for polysome association of HTLV-1 gag and provide direct evidence that RHA is necessary for efficient HTLV-1 replication. We conclude that PCE/RHA is an important translation regulatory axis of multiple lymphotropic retroviruses. We speculate divergent retroviruses have evolved a convergent RNA–protein interaction to modulate translation of their highly structured mRNA.

INTRODUCTION

Retrovirus structural and enzymatic proteins are synthesized from mRNA transcript that contains a complex 5′ untranslated region (UTR) that inhibits ribosome scanning (1–4). The process used to circumvent this barrier to translation initiation is an issue that remains to be fully elucidated. Translation initiation is the rate-limiting step in protein synthesis and the primary target for translational control (5). The majority of eukaryotic mRNAs use a cap-dependent mode of translation initiation in which the multicomponent eIF4F complex binds the 5′ 7mpppG cap of mRNA and is joined by the small ribosomal subunit (5,6). Once assembled, the initiation complex scans the mRNA until an AUG or, in some cases CUG, in appropriate Kozak consensus is located. The large ribosomal subunit then joins the complex and the elongation phase begins. A 5′ UTR greater than 100 nt in length or with structural motifs with free energy of >-50 kcal/mol has been shown to block efficient ribosome scanning and significantly dampen the efficiency of translation initiation (1,7). The UTR of retroviruses is typically greater than 200 nt in length and contain a collection of *cis*-acting replication motifs with free energy greater than -50 kcal/mol (8). These motifs include the packaging signal and primer-binding site, which are stem loop structures that perform essential functions during particle assembly and reverse transcription, respectively. In order to ensure translation of the viral transcript, retroviruses require a specialized translational control mechanism to overcome structural barriers to cap-dependent ribosome scanning. Two viral RNA elements that facilitate translation despite a long and highly structured 5′ UTR are: (1) internal ribosome entry site (IRES); and (2) post-transcriptional control element (PCE). Both viral RNA elements operate by recruiting

*To whom correspondence should be addressed. Tel: +1-614-292-1392; Fax: +1-614-292-6473; Email: boris-lawrie.1@osu.edu

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host effector proteins and have been shown to have a homolog in cellular genes.

The IRES is an RNA structural motif originally identified in the picornaviruses encephalomyocarditis virus (EMCV) (9–11) and poliovirus (12), and more recently in selected cellular mRNAs including BIP (13), eIF4G (14), c-Myc (15,16), DAP5 (17), Apaf-1 (18), BAG-1 (19,20), c-IAP1 (21), FGF2 (22,23) and BCL-2 (24). Picornaviruses lack a 5′ cap and contain a 5′ UTR ~750 nt in length that is comprised of structural motifs, oligopyrimidine tracts and cryptic AUG and CUG codons upstream of the authentic initiation codon. Features of this complex 5′ UTR serve as a recognition site for a modified pre-initiation complex that delivers the small ribosomal subunit to an appropriate initiation codon independently of cap binding and ribosome scanning. Generally, picornaviruses encode proteins that modify the eIF4F complex and efficiently shutdown cap-dependent host translation initiation. Under such conditions, cap-independent translation initiation at the viral IRES is favored (25).

Unlike picornaviruses, retroviral mRNAs are capped, polyadenylated, and in some cases lack upstream AUGs. They also generally contain AUGs in appropriate Kozak consensus (8). These features are consistent with translation initiation by a cap-dependent mechanism. However, the structured replication motifs between the 5′ cap and distal translation start codon of retroviral RNAs have been directly demonstrated to inhibit efficient cap-dependent ribosome scanning and impede translation initiation at the downstream open reading frame (2–4). A growing collection of retroviruses have been identified to contain a 5′ terminal PCE that functions in conjunction with cellular protein to facilitate translation of retroviral genes. PCE activity has been identified in three lymphotropic retroviruses: avian SNV, simian Mason–Pfizer monkey virus (MPMV) and human foamy virus (26–28). Results derived from the retrovirus model system led to the first identification of PCE activity in a cellular gene, junD (29). Located at the 5′ RNA terminus, the PCE is a ~160 nt, orientation-dependent redundant stem-loop structure that was identified by its ability to facilitate Rev/RRE-independent expression of intron-containing HIV-1 *gag* reporter RNA (26,28,30). RNA co-immunoprecipitation and biochemical analysis identified that structural features of PCE are selectively recognized by the cellular DEaH box protein RNA helicase A (RHA) (29). PCE–RHA interaction is necessary for efficient translation initiation and functions by facilitating polyribosome association (26,29,31).

Results of bicistronic reporter assays determined that the 160-nt SNV PCE does not confer IRES activity (31). However, the potential for IRES activity in the distal 5′ UTR of SNV remained to be determined. SNV shares 90% sequence homology with reticuloendotheliosis virus A (REV-A) and IRES activity has been identified in distal 5′ UTR sequences of REV-A (32). This IRES activity was identified in the context of murine leukemia virus (MLV)-based bicistronic vectors containing various REV-A 5′ UTR segments. The presence of the complete REV-A 5′ UTR (sequences + 1-580) upstream of the neomycin phosphotransferase reporter gene correlated with expression of G418 resistance gene (32). The minimum sequence determinant for expression of G418 resistance was mapped to a 129-nt distal segment of the 5′ UTR (452–580).

Bicistronic reporter assays have also detected IRES activity in the 5′ UTR of human T-cell leukemia virus type 1 (HTLV-1) (33,34), Harvey murine sarcoma virus (35), MLV (36–38), Rous sarcoma virus (RSV) (39), simian immunodeficiency virus (SIV) (40), human immunodeficiency virus 1 (HIV-1) (41) and in the gag open reading frame of HIV-1 (42) and HIV-2 (43). Internal translation initiation in the natural context of the virus genome has yet-to-be characterized for REV-A or HTLV-1 and remains controversial in HIV-1 (2–4,8).

The goal of this study was comparative analysis of IRES and PCE activity within the 5′ UTR of SNV, REV-A and HTLV-1. Our results implicate PCE in association with RHA is a key translation regulatory axis of multiple retroviruses.

MATERIALS AND METHODS

Plasmid construction

Bicistronic reporter plasmids containing SNV sequences were constructed using polymerase chain reaction (PCR) of the designated sequences from SNV provirus clone pPB101 (44) and primers containing EcoRI, NcoI and XbaI sites and inserted into the EcoRI and NcoI sites of pTR250 (31). The SNV UTR-luc and polio IRES-luc cassettes were excised with XbaI and ligated at the XbaI site in pRL-CMV (Promega, Madison, WI) to create pSNV/1-591, pSNV/260-591, pSNV/393-591 and pPolio. pNoIRESfs is identical to previously described pRenLuc (45). The bicistronic reporter plasmids containing HTLV-1 5′ UTR sequences + 1-263 was created by PCR of HTLV-1 strain ACH with primer-containing SalI sites and inserted in SalI sites of intermediate bicistronic plasmid pCG201. pREV-A was created by PCR amplification of REV-A provirus pSW253 5′ UTR sequences + 1-580 using primers containing SalI and inserted into SalI sites of pCG201. To construct pSNVgagluc, pPB101 SNV pol was replaced with F-luc in three steps. pPB101 was digested with SmaI and religated to delete sequences 2964–5632. PCR-based site-directed mutagenesis was used to eliminate the pol stop codon and introduce a NcoI site. This plasmid, pMB109, was digested with NcoI and SmaI and ligated in-frame with the luc ORF from pGL3 (Promega, Madison, WI) to create pSNVgagluc. The previously described plasmids pYW100 and pYW205 (26) were used to generate REV-A derivatives. The REV-A LTR or U3 region in a PCR product of pSW253 containing terminal NdeI and BglII was inserted into NdeI and BamHI sites of pYW100 or pYW205 to generate pREV-A100 and pREV-A205. Plasmids containing HTLV-2 (46), BLV (47), EIAV (48) and FeLV (49) LTR regions were constructed similar to pREV-A100. pCG211 was constructed by digestion of pREV-A100 and pYW100 with NdeI and AvaI and subcloning of SNV U3 from pYW100 into pREV-A100. pMPMV100 is the

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previously described pMPMV (28). Generation of Rex-deficient HTLV-1 proviral clone HTLVRex- was described (50). To construct wt/PCE and Rex-/PCE, EcoRV and BamHI sites were generated in the R region of wild-type and the Rex-deficient HTLV-1^{ACH} (26) by site-directed mutagenesis (Quickchange^{TM}, Stratagene), and used for insertion of SNV PCE inserted between EcoRV and BamHI. The plasmid constructions were confirmed by DNA sequence analysis.

Transfections and protein analysis

Cultures of 293 and COS cells were grown to ~80% confluency in Dulbecco's modified eagle medium (Invitrogen) with 10% FBS (Invitrogen) and 1% antibiotic (Gibco) prior to transfection. DNA transfection was carried out with a 3:1 FuGene:DNA ratio following the manufacturers protocol (Roche). RNA transfections were performed using Transmessenger transfection reagent (Qiagen). Cells were pelleted by centrifugation at 3500 r.p.m., resuspended in NP40 lysis buffer, iced for 10 min, then centrifuged at 12000 r.p.m. at 4°C for 10 min to collect proteins as previously described (51). Luciferase activity was measured with Luciferase assay system (Promega) or Dual Luciferase Assay system (Promega) following the manufacturer's protocol quantified on a Lumicount luminometer (Packard). HIV-1 Gag levels were quantified by Gag enzyme-linked immunosorbent assay (ELISA) (Coulter Corp.) and HTLV Gag levels were quantified by ELISA (Zeptometrix).

For western blot analysis, proteins were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane (BioRad). Immunoblotting was performed with mouse monoclonal antibodies against histone H1, β-tubulin and β-actin (Abcam, Cambridge, MA), or rabbit polyclonal antibody against HIV-1 Gag (26). Secondary antibodies were from Amersham (mouse) or Santa Cruz (rabbit). Visualization was performed with Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

For siRNA transfection, COS cells were seeded at 5×10^4 cells per well in 35-mm plates. After 24 h, cells were incubated with a mixture of Oligofectamine (Invitrogen) and 10 ul of 20 μM siRNAs (Dharmacon) targeted to two regions of RHA (5 ul of each) or scrambled sequences (29) in OptiMEM media (Invitrogen) without serum. FBS (Invitrogen) was added 4 h post-treatment and cells were incubated at 37°C in 5% CO_2. After 72 h, siRNA treatment was repeated with either 5 μl of scrambled siRNAs or 5 μl of the RHA siRNAs. Cells were then transfected with PCE HIV-*gag* reporter plasmids or HTLV-1 proviral clones and pGL3 luciferase plasmid using FuGene6, as described above.

RNA isolation and analysis

For *in vitro* transcription, linearized bicistronic reporter plasmids that contain the T7 promoter upstream of the reporter gene were incubated with MAXIscript T7 polymerase and cap analog (Ambion), followed by treatment with poly(A) polymerase (Ambion), and subsequent DNase treated (52). After addition of 0.5 M EDTA and incubation at 70°C for 10 min, the RNA was isolated by acid phenol extraction and ethanol precipitation. Cellular RNAs were isolated in Trizol following the manufacturer's protocol (Invitrogen). Total cellular RNA and RNA extracts of nucleoplasm and cytoplasm were subjected to two DNase treatments, extraction with acid phenol, precipitation with ethanol and resuspension in diethyl pyrocarbonate-treated water. For northern blot analysis, 5 μg total RNA or 1 μg synthetic RNA was separated on 1.2% agarose gels containing 5% formaldehyde, transferred to Duralon-UV membrane (Stratagene, La Jolla, CA), and incubated with firefly luciferase (F-luc) DNA probe. The probe was an F-luc restriction product that had been gel purified and labeled with [α-^{32}P]dCTP by a random-primer DNA-labeling system (Invitrogen, CA). The hybridization products were scanned with PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For reverse transcription, polyA + mRNA was isolated using Oligotex (Qiagen) and the mRNA was reacted with Sensiscript reverse transcriptase (Qiagen) and random hexamer primer for 1 h at 37°C. Ten percent of the cDNA preparation was used for real-time PCR with primers complementary to HIV gag, β-actin, or gapdh and Quantitect SYBR Green PCR (Qiagen, Germany) in a Lightcycler (Roche, Germany). Standard curves to determine mRNA copy numbers were prepared with control reactions with pHIV^{NL4-3}, β-actin or gapdh plasmid in the range of 10^2–10^8 copies. RNA co-immunoprecipitation assays were performed as previously described (29). Ribosomal profiles were prepared as described previously (31). The samples were precipitated in warm ethanol and RNA was extracted in Trizol as described above. cDNA was created using Omniscript RT kit (Qiagen) and used as a template for real-time PCR as described above.

RESULTS

SNV 5′ UTR does not confer internal initiation

To assess IRES activity in selected retroviral 5′ UTRs, we evaluated the activity of bicistronic reporter plasmids in transiently transfected 293 cells. The bicistronic reporter transcript contains renilla luciferase (R-luc) in the first cistron, which serves as the cap-dependent translation reporter, and firefly luciferase (F-luc) in the second cistron, which is used to measure IRES activity (Figure 1A). The intercistronic region of pNoIRESfs introduces three stop codons into the R-luc open reading frame followed by a frameshift mutation (31). pNoIRESfs was used to measure the baseline level of read-through translation from R-luc into F-luc open reading frame. pPolio contains the poliovirus IRES and was used to measure efficient internal initiation of F-luc translation. The pSNV derivatives contain the indicated SNV 5′ UTR sequence to evaluate the possibility that they support internal initiation. Five independent transient transfection assays were performed and the results of dual luciferase assays were subjected to statistical analysis. A linear model using R-Luc as a covariate was used to determine whether or not F-Luc activity from pPolio was significantly different from the other groups. Box plot analysis, which summarized the mean and variance between the

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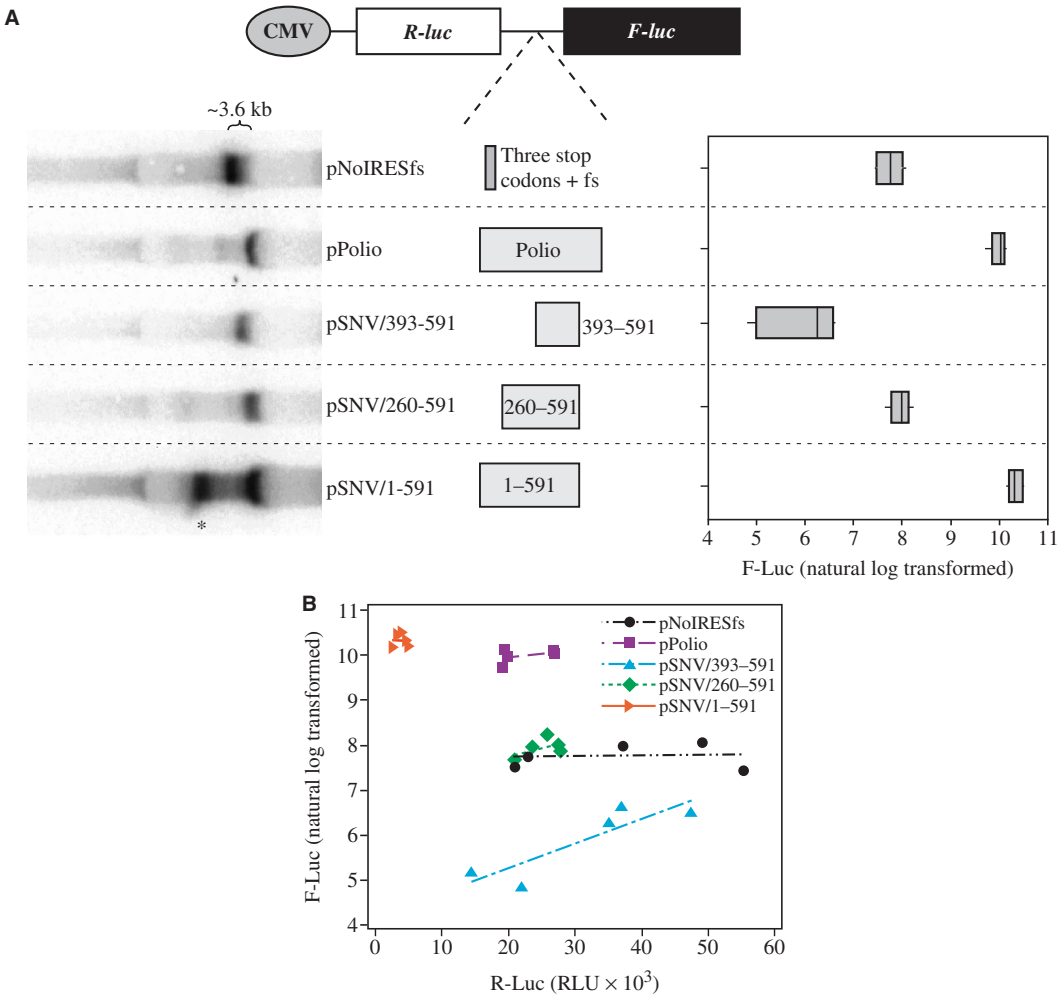


Figure 1. SNV 5′ UTR sequences do not confer IRES activity in bicistronic reporter plasmids. **(A)** Diagram of CMV IE-driven bicistronic reporter gene that encodes renilla luciferase (R-luc) in the first cistron, firefly luciferase (F-luc) in the second cistron and indicated intercistronic regions. The intergenic region of pNoIRESfs introduces three in-frame stop codons and a downstream frameshift mutation into the R-luc open reading frame for terminating cap-dependent translation of R-luc. pPolio contains the poliovirus internal ribosome entry site. The pSNV plasmids contain SNV 5′ UTR sequences with number relative to position in SNV RNA. Representative northern blot with F-luc DNA probe verifying expression of the expected ~3.6 kb transcript and an aberrant transcript marked with an asterisk. Five independent transfection assays were performed in 293 cells and total cellular proteins were harvested at 48 h post-transfection and subjected to dual Luciferase assay. Box plot analysis summarizes the range of F-Luc activity with internal line indicating mean and extending lines connecting extreme values. **(B)** Scatterplot analysis of the five replicate experiments demonstrated that pPolio and pSNV/1-591 segregated separately from the other plasmids. Natural log transformation was used to adjust non-normality and unequal variances.

samples, determined that F-Luc activity of pPolio and pSNV/1-591 segregated in one group, whereas pSNV/260-591 and pSNV/393-591 segregated with the negative control pNoIRESfs (Figure 1A). The results of the five replicate assays show that F-Luc activity from pPolio and pSNV/1-591 is at least two logs greater than pNoIRESfs, pSNV/260-591 or pSNV/393-591 (Figure 1A and B).

Northern blot analysis with ^{32}P-labeled DNA probe complementary to F-luc was used to evaluate the structure of the bicistronic transcripts. Analysis of total RNA from the transfected cells verified that pNoIRESfs, pPolio, pSNV/260-591 and pSNV/393-591 expressed the expected homogeneous ~3.6 kb transcript population (Figure 1A). The results indicated that IRES activity was not conveyed by the distal subsections of the SNV 5′ UTR. By contrast, pSNV/1-591 expressed a heterogeneous transcript population consisting of the expected 3.6 kb transcript plus another prominent transcript of ~2.5 kb. Therefore, the observation of significant F-Luc activity from pSNV/1-591 was associated with an aberrant F-luc transcript. We concluded that the observed F-Luc activity may be attributable to cap-dependent initiation of an internally initiated or spliced F-luc transcript. Therefore, we utilized an alternative approach to evaluate whether or not the SNV 5′ UTR supports cap-independent translation initiation.

As an alternative approach, we used infection with the picornavirus EMCV to downregulate cap-dependent translation and evaluated the effect on an SNV molecular clone. D17 cells, which are permissive for SNV replication, were permanently transfected with either SNV provirus, pSNVgagluc, cap-independent F-Luc bicistronic reporter

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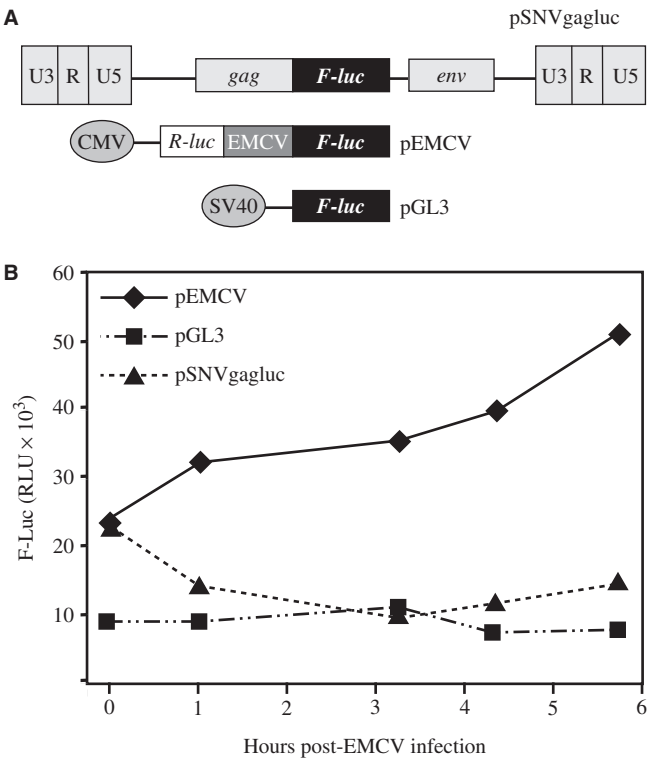


Figure 2. SNV initiates translation in a cap-dependent manner. (**A**) Diagram of SNV reporter provirus that contains F-luc in place of *pol*, and control cap-independent F-luc reporter plasmid that contains the encephalomyocarditis virus internal ribosome entry site (pEMCV), and cap-dependent F-luc reporter plasmid, pGL3. Transcription is driven by SNV U3, cytomegalovirus (CMV) or simian virus 40 (SV40) promoter, respectively. (**B**) Total cellular proteins were harvested at four times after infection with EMCV and equivalent protein samples were subjected to F-Luc assay. Duplicate experiments were performed and average Luciferase activity is presented. Relative light units, RLU.

plasmid pEMCV (31), or cap-dependent F-Luc expression plasmid pGL3 (Promega) (Figure 2A). pSNVgagluc is derived from pPB101 (44) and contains an in-frame fusion of gag-pol with F-luc (Figure 2A). The D17 cell lines were infected with EMCV (MOI > 0.5) and F-luc activity was quantified at sequential time points. By 4 h post-infection, cap-independent translation of F-luc from pEMCV had increased by ~200% (Figure 2B). In contrast, F-Luc activity from the pGL3 cap-dependent control failed to increase over time. The F-Luc production from pSNVgagluc segregated with pGL3 and failed to increase over time. These results indicate that SNV does not utilize cap-independent internal initiation as a major mode of translation initiation and is reliant on cap-dependent initiation.

5′ UTR of REV-A and HTLV-1 do not support internal translation initiation

The bicistronic luciferase reporter plasmids were used to evaluate the 5′ UTR sequences of the avian lymphotropic retrovirus, REV-A, which exhibits 90% sequence homology with SNV, and HTLV-1, a genetically more complex retrovirus. Four independent transient transfection assays were performed in HeLa cells with pREV-A, pHTLV-1 and the control plasmids pPolio and pNoIRESfs. Results of dual Luc assays showed that pREV-A and pHTLV-1 segregated with pPolio, similar to the results with pSNV/1-580 in Figure 1 (Figure 3A and B). The results of northern analysis of pREV-A and pHTLV-1 were also reminiscent of the results of pSNV/1-591; a heterogeneous transcript population was detected of the expected ~3.6 kb transcript, plus smaller prominent RNA species (Figure 3A). Therefore, the F-Luc activity of pREV-A and pHTLV-1 is associated with the presence of aberrant transcripts. Prominent aberrant transcripts were also identified when the experiment was conducted in D17 cells (Figure 3C). We concluded that plasmid transfection was not an appropriate assay for measuring IRES activity in these particular sequences.

A documented approach to avoid false-positive IRES activity associated with aberrant transcripts is direct transfection of *in vitro* transcribed bicistronic RNAs (53). To evaluate this method in our system, we generated capped and polyadenylated bicistronic *in vitro* transcripts and assessed internal initiation from pREV-A and pHTLV-1 RNA by RNA transfection into HeLa cells. Northern blot analysis verified expression of the expected ~3.6 kb homogeneous transcript population and aberrant transcripts were not detected (Figure 4). Five independent RNA transfection experiments were performed and results of dual Luc assays were used for statistical analysis. Tukey pairwise comparisons showed that the mean F-Luc activity of the pPolio positive control was significantly higher then all other groups (P-value < 0.0001) (Figure 4). In contrast, the mean F-Luc activity of pREV-A and pHTLV were not statistically different from the pNoIRESfs negative control (P-value 0.38 and 0.17, respectively). The results of the RNA transfection assays indicated that these 5′ UTR sequences do not support internal translation initiation from a bicistronic reporter in HeLa cells. The lack of internal initiation prompted us to examine the possibility that the 5′ UTR of REV-A and HTLV-1 contain a PCE to facilitate efficient translation.

5′ LTR of divergent lymphotropic retroviruses exhibit PCE activity

The hybrid SNV PCE-HIV gag reporter system was used previously to identify PCE activity within the 5′ proximal UTR sequences of SNV, MPMV and cellular junD (26,28,29). To test REV-A and HTLV-1 for PCE activity, we constructed derivatives of the hybrid SNV-HIV gag reporter plasmid (pYW100) that replaced the SNV LTR with the LTR of REV-A or HTLV-1 (Figure 5). Additional derivatives were constructed to evaluate the LTRs of feline leukemia virus (FeLV), HTLV-2, bovine leukemia virus (BLV) and equine infectious anemia virus (EIAV) for PCE activity. The plasmids were transiently transfected into 293 cells with Luciferase expression plasmid (pGL3), and F-Luc activity was used to standardize minor differences in transfection efficiency. In experiments to test the LTRs of the genetically more complex retroviruses, the gag reporter plasmids were co-transfected with expression plasmid of the

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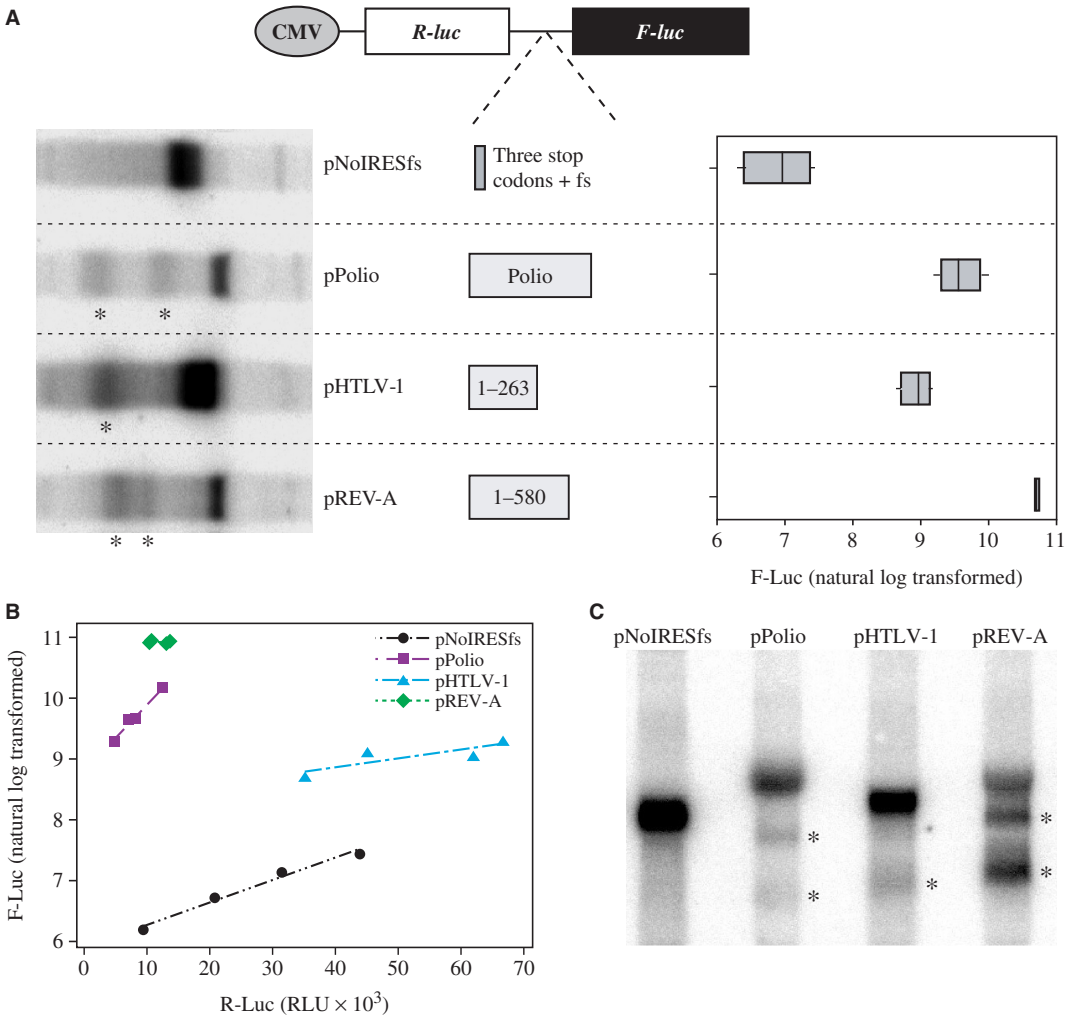


Figure 3. Transfection of REV-A and HTLV-1 bicistronic reporters yield heterogeneous transcript population. **(A)** Diagram of CMV IE-driven bicistronic reporter gene that encodes renilla luciferase (R-luc) in the first cistron, firefly luciferase (F-luc) in the second cistron and indicated intercistronic regions. Representative northern blot with F-luc DNA probe verifying expression of the expected ~3.6 kb transcript and aberrant transcripts marked with an asterisk. Four independent transfection assays were performed in HeLa cells and total cellular proteins were harvested at 48 h post-transfection and subjected to dual Luciferase assay. Box plot analysis summarizes the range of F-Luc activity with internal line indicating mean and extending lines connecting extreme values. **(B)** Scatterplot analysis of the four replicate experiments demonstrated that pPolio and pREV-A and pHTLV-1 segregated separately from the pNoIRESfs plasmid. Natural log transformation was used to adjust non-normality and unequal variances. **(C)** Northern blot with F-luc DNA probe indicates aberrant transcripts are also present in transfected D17 cells.

corresponding transcriptional transactivator (HTLV-1, HTLV-2 or BLV Tax and EIAV Tat), which is necessary to *trans*-activate the viral promoter. Total cellular protein was harvested 48 h post-transfection and Gag production was measured by HIV Gag ELISA. Representative results of three independent assays show that the positive control SNV LTR (pYW100) facilitated Rev/RRE-independent Gag production (Figure 5). PCE activity was also observed in response to the LTRs of REV-A (pREV-A100) and to a lesser extent, the gammaretrovirus FeLV, and the deltaretrovirus HTLV-1. In response to the BLV LTR, Gag production was near the detection limit of the assay, while the HTLV-2 and EIAV reporters did not produce detectable levels of Gag. To investigate PCE activity conferred by the REV-A LTR, the 5′ proximal 160-nt 5′ UTR of pREV-A100, which corresponds to RU5 region of the LTR, was deleted in pREV-A205 (see Figure 6A). Comparison of pREV-A100 and pREV-A205 determined that deletion of REV-A RU5 reduced Rev/RRE-independent Gag production (Table 1). Gag immunoblot verified the differences in Gag protein level (Figure 6B). These results indicate the REV-A RU5 is necessary for robust PCE activity.

To assess potential differences in steady-state gag RNA, total cellular RNA was subjected to RT-PCR analysis. After reverse transcription with random hexamer primer, the cDNAs were subjected to PCR with primers complementary to R and gag to amplify the region spanning the 5′ splice site. Electrophoresis of the RT-PCR products verified expression by pREV-A100 and pYW100 of the expected ~520-bp product without aberrant gag RNAs (Figure 6C). Control reactions without RT demonstrated a lack of DNA contamination and control reactions on the corresponding plasmid template demonstrated the

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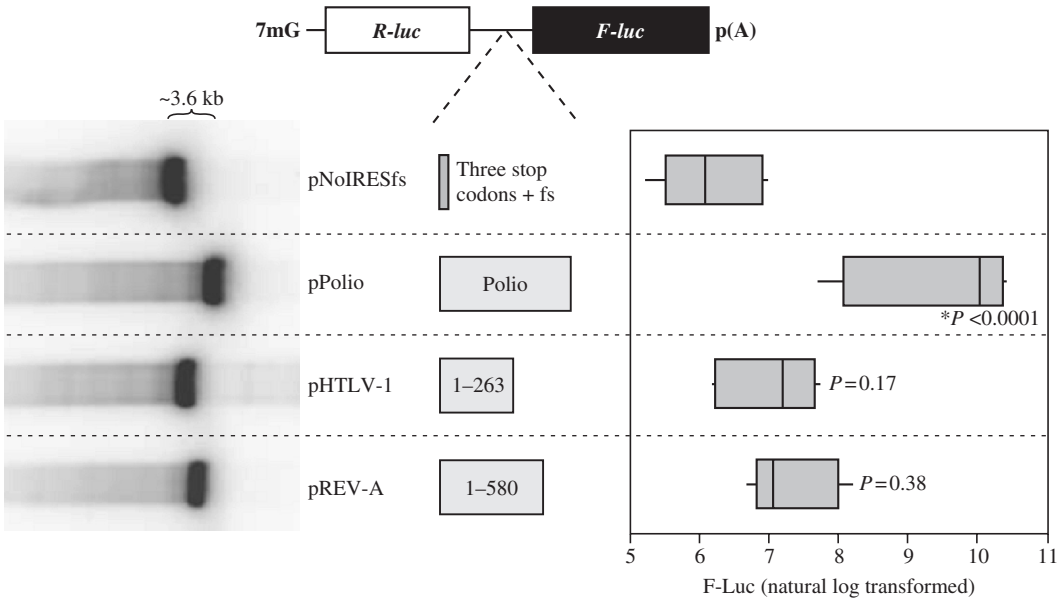
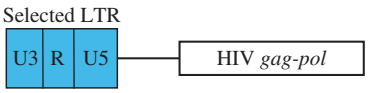


Figure 4. RNA transfection assays indicate that HTLV-1 and REV-A 5′ UTR sequences lack IRES activity. Diagram of the synthetic bicistronic transcripts that were transcribed *in vitro*, capped, polyadenylated and transfected into HeLa cells. Labels are as described in Figure 1. Northern blot with F-luc probe verified the homogeneous transcript populations. Dual Luciferase assays were performed on total cellular protein at 20 h post-transfection. Box plot analysis of the five independent experiments summarizes range of F-Luc activity with mean indicated by the internal line and extending lines connect box to extreme values. Tukey method was used to measure the significance level of each group relative to pNoIRESfs. Asterisk indicates *P*-value that is statistically different from pNoIRESfs group.



Measurement of Rev/RRE-independent Gag production

Selected LTR	Gag protein (pg/ml)
SNV	100037
REV-A	40157
FeLV	6332
HTLV-1	2851
BLV	734
HTLV-2	<MD
EIAV	<MD

Figure 5. Survey for PCE activity in the long terminal repeat (LTR) of seven divergent retroviruses. Diagram of PCE-HIV*gag* reporter plasmid used to assay the LTR of selected retroviruses for PCE activity. Observation of Rev/Rev responsive element (RRE) independent Gag protein production from unspliced HIV gag reporter RNA indicates PCE activity. The results are representative of three independent transfection assays in 293 cells. Gag in cellular protein was quantified by ELISA. <MD, below the detection limit of the assay (15 pg/ml).

amplicon size. Real-time PCR analysis of poly(A)+ mRNA was used to quantify steady-state gag RNA among the transfectants. Comparison of pREV-A100 and pREV-A205 in two independent experiments determined that the presence of RU5 did not correlate with increased steady-state gag RNA (Table 1). The results indicated that the greater Gag protein production from pREV-A100 compared to pREV-A205 is not attributable to greater steady-state RNA. Comparison of the ratio of Gag protein production relative to gag RNA from the same transfectant determined that gag mRNA translational efficiency was 3–7-fold greater for pREV-A100 compared to pREV-A205 (Table 1).

Comparison between pREV-A100 and the SNV control plasmid pYW100 indicated that pYW100 consistently yielded greater abundance of gag mRNA and Rev/RRE-independent Gag protein production (Table 1). To determine if the difference is attributable to the activity of the SNV U3 promoter region, we inserted SNV U3 in place of REV-A U3 in pREV-A100 and created pCG211. Results of three independent experiments determined that replacement of REV-A U3 with SNV U3 did not increase gag steady-state mRNA (compare pREV-A100 with pCG211) (Table 1). The copy number of steady-state gag RNA from pCG211 is less than the SNV U3-containing plasmids (pYW100 and pYW205). Comparison of Gag production from pCG211 and pYW205 verified that REV-A RU5 was sufficient to increase Gag protein production. The ratio of Gag protein level per gag mRNA was ~5-fold greater for pCG211 than pYW205. To address whether or not this activity is specific for REV-A RU5, REV-A RU5 was replaced

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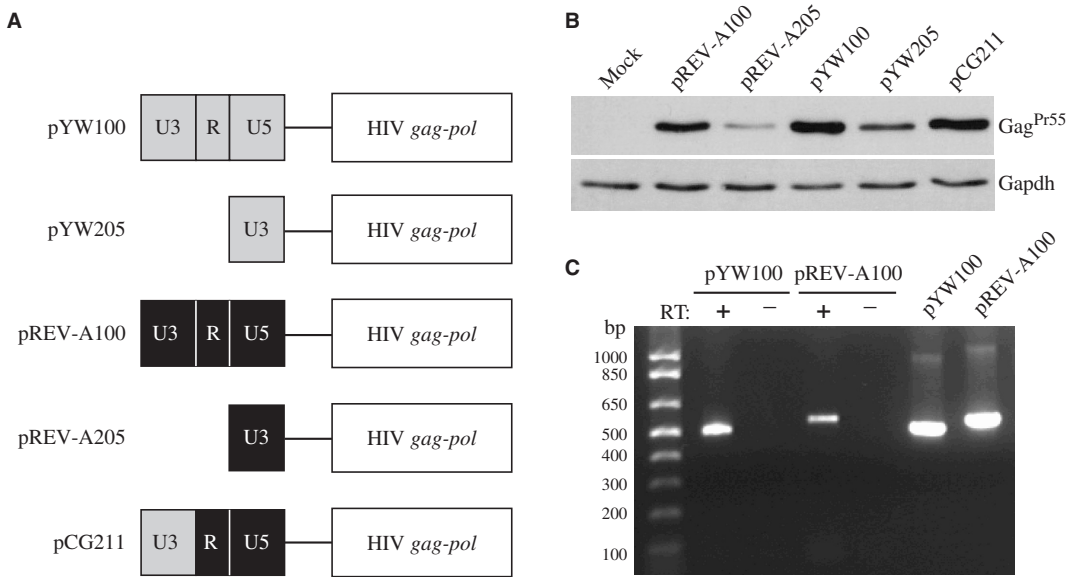


Figure 6. REV-A RU5 facilitates expression of HIV gag reporter RNA. (**A**) Diagram of PCE-gag reporter constructs. Gray rectangles, designated U3, R, U5 segments of SNV LTR; black rectangles, designated U3, R, U5 segments of REV-A LTR, white rectangle, HIV gag open reading frame within HIV intron. (**B**) Results of representative transfection assays in 293 cells with indicated PCE reporter plasmids. Immunoblot analysis of total cellular protein with antiserum to HIV-1 Gag or Gapdh. (**C**) RT-PCR analysis of RNA with PCR primers complementary to R (15–37) and gag (116 bp distal to gag ATG)-verified expression of the unspliced gag RNA. +/− indicates presence or absence, respectively of reverse transcriptase (RT). Indicated plasmid was used as a template in control reactions and indicates the expected size of each RNA amplicon. Results of Gag ELISA and RNA quantification are summarized in Table 1.

Table 1. gag RNA and Gag protein production from PCE reporter plasmids

		Gag[a]		
Replicate	PCE reporter plasmid	Protein (pg/ml)	RNA ($\times 10^3$)	Protein:RNA (relative to U3 only)
1	pREV-A100	53 877	4.879	2.9
	pREV-A205	26 425	6.890	1.0
	pYW100	1 14 040	10.239	2.8
	pYW205	29 093	7.193	1.0
	pCG211	54 903	2.888	4.8
2	pREV-A100	1 00 114	1.922	7.1
	pREV-A205	48 842	6.720	1.0
	pYW100	2 36 921	6.298	2.3
	pYW205	61 721	3.707	1.0
	pCG211	1 39 966	1.813	4.7

[a]Cultures of 293 cells were transfected with indicated reporter plasmids and total cellular protein and RNA were isolated. Gag protein production was determined by ELISA. The minimum detection limit of the assay was 15 pg/ml. RNA was subjected to reverse transcription and real-time PCR with gag and β-actin primers. Values represent gag copy number per nanogram standardized to β-actin copy number.

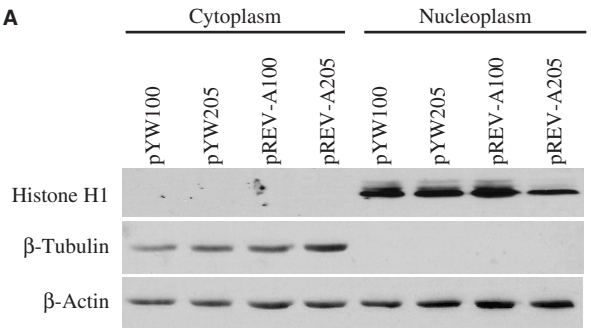
with MLV RU5 (pSH603). Gag protein production was below the minimum detection level of the assay, indicating that PCE activity is conferred by REV-A RU5, but not MLV RU5.

To investigate a possible effect of REV-A RU5 on the cytoplasmic accumulation of gag mRNA, the nucleoplasm and cytoplasm were fractionated, and polyadenylated nuclear and cytoplasmic mRNAs were isolated, reverse transcribed and quantified by real-time PCR. Western blot analysis of the fractionated proteins with antisera to nuclear protein histone H1 and cytoplasmic protein β-tubulin verified effective sub-cellular fractionation of the nucleoplasm and cytoplasm (Figure 7A). Comparison of gag mRNA copy numbers from pREV-A100 and pREV-A205 determined minor differences in gag mRNA level in the nucleus or cytoplasm (Figure 7B). The percentage of gag mRNA in the cytoplasm was not increased by REV-A RU5 (compare pREV-A100 and pREV-A205), similar to results with SNV PCE (compare pYW100 and pYW205) (26,28). The ratios of Gag protein level relative to gag mRNA copy number demonstrated that the presence of REV-A RU5 or SNV RU5 correlated with an increased translational efficiency of cytoplasmic gag mRNA (Figure 7B). We concluded that REV-A 5′ RU5 contains a PCE that facilitates the translational utilization of cytoplasmic gag RNA.

RHA is necessary for REV-A PCE activity and gag expression from HTLV-1 provirus

To determine if RHA is necessary for REV-A PCE activity, siRNAs were used to downregulate endogenous RHA. COS cells were transfected with siRNAs directed against RHA (DHX9) or the negative control scrambled sequence (Sc siRNA) (29). This siRNA treatment regiment was previously shown to specifically downregulate RHA without reducing cell viability, the rate of global cellular RNA and protein synthesis or gene-specific translation of gapdh and F-luc (29). Seventy-two hours after siRNA transfection, the cells were transfected with siRNAs and either pREV-A100 or pYW100

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B Cytoplasmic accumulation of gag RNA from PCE reporter plasmids

PCE reporter plasmid	gag RNA ($\times 10^2$)[a] Sub-cellular fraction: Nucleoplasm	gag RNA ($\times 10^2$)[a] Sub-cellular fraction: Cytoplasm	% Cytoplasmic accumulation	Ratio Gag protein: gag mRNA[b] (relative to U3 only)
pREV-A100	256.929	1.619	0.6	1.7
pREV-A205	135.865	1.265	0.9	1.0
pYW100	137.865	1.433	1.0	2.4
pYW205	139.354	2.404	1.7	1.0

Figure 7. Enhanced expression of reporter RNA by REV-A RU5 is not due to altered cytoplasmic accumulation. (**A**) Nucleoplasm and cytoplasm were effectively isolated for analysis of mRNA cytoplasmic accumulation. Here, 293 cells were transiently transfected with indicated REV-A or SNV PCE reporter plasmids for 48 h. Nucleoplasm and cytoplasm were isolated and aliquots were subjected to immunoblot with antiserum to nuclear protein histone H1 and cytoplasmic protein β-tubulin, and β-actin loading control. (**B**) Summary table of results of Gag ELISA and RNA quantification. [a]293 cells transfected with indicated reporter plasmids were seperated into nuculear and cytoplasmic fractions. p(A) + RNA was isolated from each fractions, subjected to reverse transcption and real-time PCR with gag and β-actin primers. Values represent gag copy number per nanogram standarized to β-actin copy number.
[b]Ratio of Gag protein production determined by ELISA relative to cytoplasmic gag RNA level.

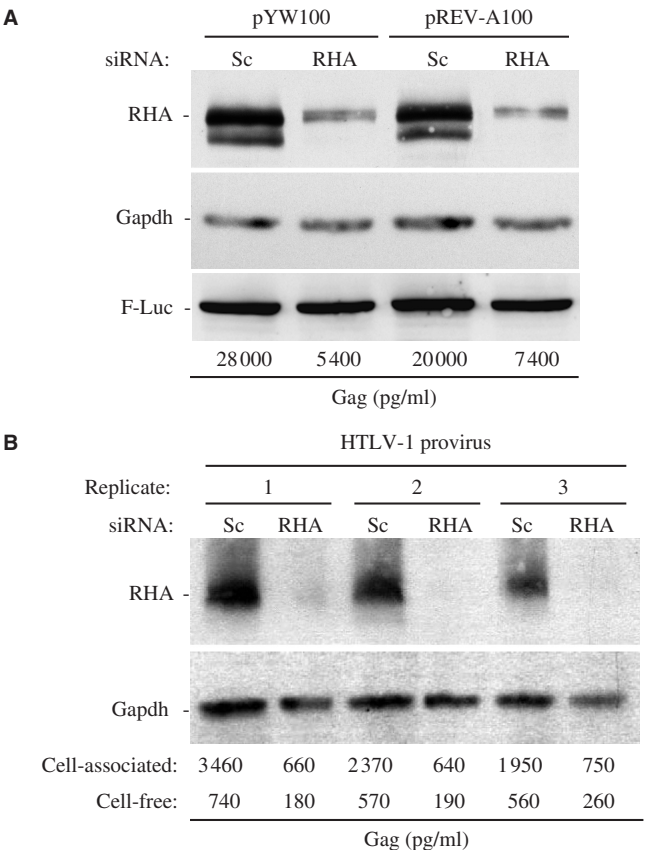


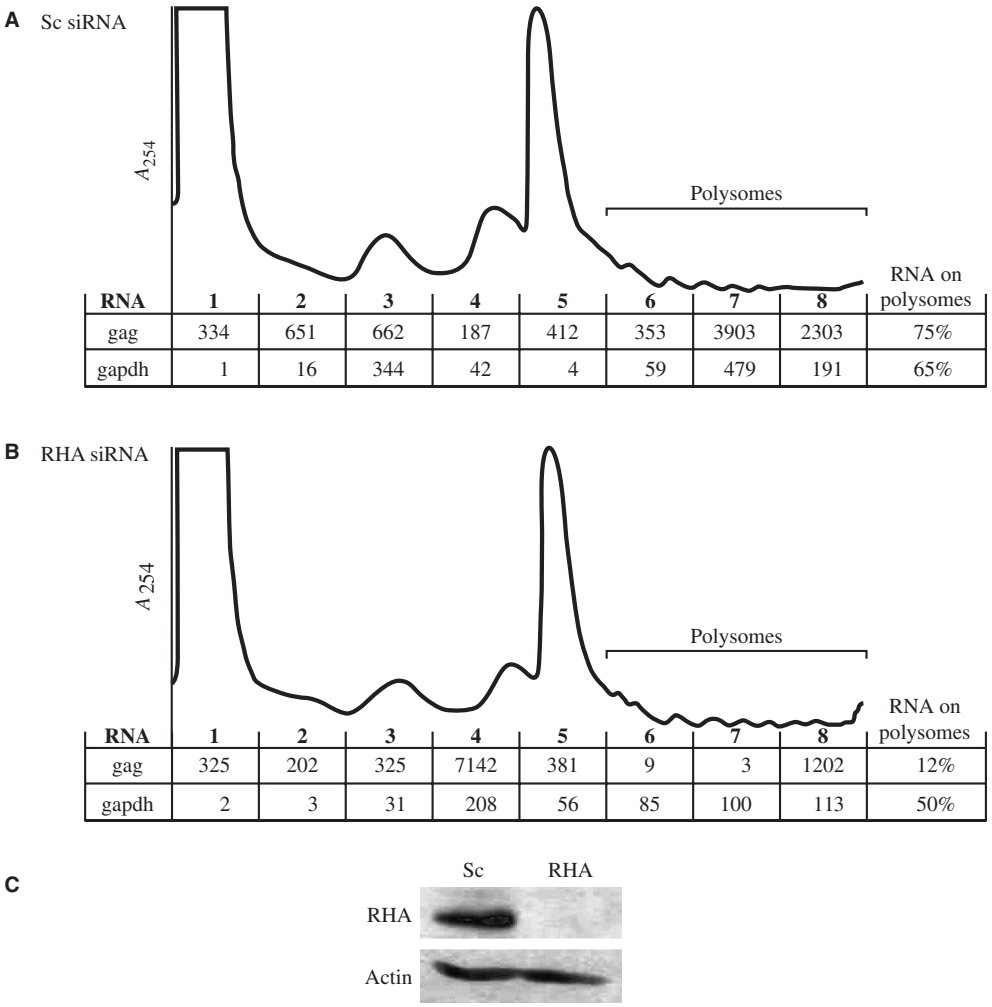
Figure 8. RNA helicase A is necessary for robust REV-A PCE activity and HTLV-1 Gag production. COS cells were transfected with siRNAs targeting RHA or with scrambled sequences (Sc), incubated for 72 h and co-transfected with the siRNAs and indicated PCE reporter plasmid or HTLV-1 provirus. After 48 h, total cellular protein was harvested and subjected to immunoblot with antiserum to RHA to assess downregulation, Gapdh antiserum to control for sample loading, and F-luc to control for non-specific effects of siRNAs. (**A**) Measurements of PCE activity by Gag ELISA on the total cellular protein are summarized below immunoblot. The detection limit of the assay was (25 pg/ml). Results are representative of three independent experiments. (**B**) Lysates from three independent transfection assays were used for immunoblot for RHA and Gapdh. Results of HTLV Gag ELISA on cell-associated or cell-free protein preparations are summarized below immunoblot.

reporter plasmid and pGL3 transfection efficiency control. Total cellular protein was harvested after 48 h, and downregulation of endogenous RHA was verified by RHA immunoblot (Figure 8A). Immunoblotting determined that the downregulation of RHA did not reduce Gapdh and F-luc levels, consistent with previously published results (29). Gag ELISA determined that RHA downregulation significantly reduced Gag protein production from pREV-A100 and pYW100 (factor of 3 to 5) (Figure 8A).

We also evaluated the effect of RHA downregulation on Gag production from HTLV-1 provirus. RHA immunoblots from three replicate experiments verified effective downregulation of RHA (Figure 8B). HTLV-1 Gag ELISA on cell-associated proteins revealed that RHA downregulation significantly reduced Gag protein production (factor of 2.5 to 5). The RHA downregulation produced a proportional reduction of Gag in cells and released into the supernatant medium, indicating that RHA does not affect HTLV-1 assembly and release.

To directly assess the effect of RHA on the translational efficiency of HTLV-1 gag mRNA, cells were transfected with RHA-specific siRNAs or Sc siRNAs, incubated for 48 h and then transfected with HTLV-1 provirus. Two days later, cytoplasmic lysates were harvested, subjected to sucrose gradient density centrifugation and ribosomal profiles were generated. RHA downregulation did not change the polysome profiles, indicating global cellular translation is not disrupted (compare Figure 9A with B) (29). These results are in agreement with results of metabolic labeling assays that determined global cellular translation is not affected by RHA downregulation (29). Each sample was subjected to reverse transcription and real-time PCR to monitor the distribution of gag and gapdh across the profiles. In Sc-siRNA-treated cells, the majority of gag RNA is associated with polyribosomes (75%). In RHA-siRNA-treated cells, the distribution of

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RNA	1	2	3	4	5	6	7	8	RNA on polysomes
gag	334	651	662	187	412	353	3903	2303	75%
gapdh	1	16	344	42	4	59	479	191	65%

RNA	1	2	3	4	5	6	7	8	RNA on polysomes
gag	325	202	325	7142	381	9	3	1202	12%
gapdh	2	3	31	208	56	85	100	113	50%

Figure 9. RHA promotes translation of HTLV-1 gag mRNA. COS cells were transfected with siRNAs that target two regions in RHA or scrambled sequence (Sc), incubated for 48 h, transfected with HTLV-1 provirus and incubated for 48 h. Cytoplasmic extracts were separated on 15–50% linear sucrose density gradients. Ribosomal RNA profiles (A_{254}) of cells treated with (**A**) Sc siRNAs or (**B**) RHA siRNAs. RNA was extracted from each gradient fraction, reverse transcribed and subjected to real-time PCR to quantify HTLV-1 gag RNA copies. Charts below each profiles indicate 10^2 copies of gag or gapdh present in equivalent fractions. (**C**) Immunoblotting with antisera against RHA and β-actin verifies efficient downregulation of RHA and indicates equivalent sample loading, respectively.

gag RNA was changed, and a minority of gag RNA is associated with polyribosomes (12%). By comparison, the majority of gapdh RNA remained associated with polyribosomes (65 and 50%, respectively). Effective downregulation of RHA was verified by immunoblot (Figure 9C). These results demonstrate that RHA is necessary for efficient HTLV-1 gag translation. We conclude that RHA is necessary for efficient Gag production from REV-A PCE reporter plasmid and from HTLV-1 provirus.

RHA is necessary for PCE enhancement of Gag production from HTLV-1 Rex-deficient provirus

The observations that SNV PCE requires interaction with RHA to facilitate HIV-1 Rev/RRE-independent Gag production (29) and that the SNV LTR facilitates Rex/RxRE-independent expression of BLV structural gene vectors (47,54) compelled us to examine whether or not SNV PCE can facilitate Rex/RxRE-independent expression of the related deltaretrovirus HTLV-1. To address this issue, we constructed chimeric HTLV-1 proviruses with an insertion of SNV PCE between the U3 and RU5 regions of the 5′ LTR (Figure 10A). The chimeric proviruses encode either wild-type Rex (wtHTLV, wt/PCE) or are Rex-deficient by introduction of a stop codon (HTLVRex-, Rex-/PCE) (Figure 10A) (50). The parental and chimeric proviruses were transfected into 293 cells, and total cellular protein and RNA were isolated. Gag protein production was quantified by ELISA, gag mRNA was reverse transcribed and quantified by real-time PCR. Results of two replicate experiments determined that insertion of SNV PCE upstream of HTLV-1 RU5 did not disrupt HTLV Gag production (compare wtHTLV-1 and wt/PCE) (Table 2). As expected, cells transfected with HTLVRex- yielded Gag levels near or below the limit of detection, and the steady-state gag RNA level was consistently lower from the proviruses

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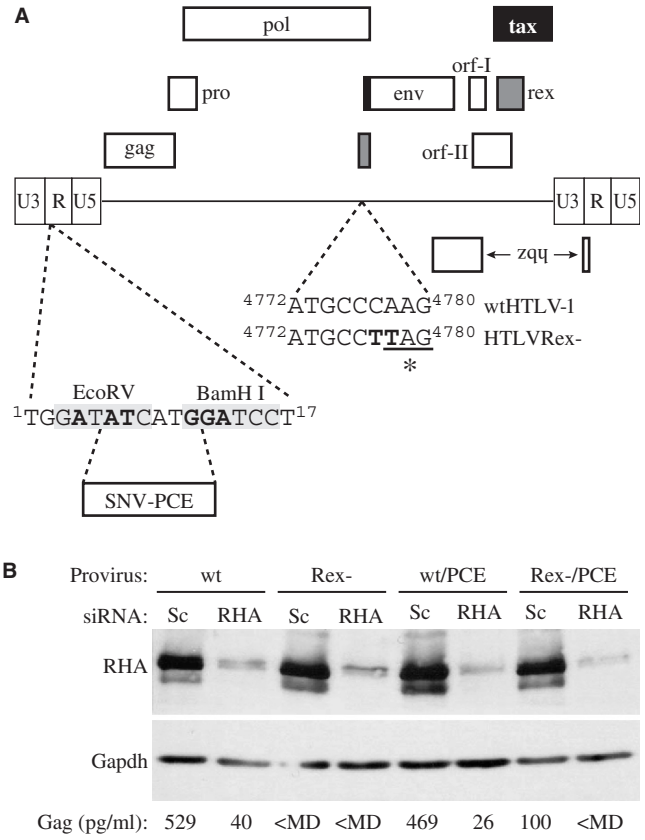


Figure 10. SNV PCE can enhance translation from HTLV-1 Rex-deficient provirus. (**A**) Diagram of the HTLV-1^{ACH} provirus. Terminal-labeled rectangles depict U3, R, U5 regions of the long terminal repeats. Labeled rectangles indicate gag, pro, pol, env, tax, rex, HBZ open reading frames, orf I and orf II. The wild-type DNA sequence near the Rex start codon (ATG) is labeled (wtHTLV-1). Bold letters indicate the site-directed mutation to introduce a stop codon in the Rex open reading frame (HTLVRex-). Location of SNV PCE insert is shown with bold letters indicating site-directed mutations that introduced the indicated restriction sites. Numbering refers to RNA sequence. (**B**) COS cells were treated with scrambled (Sc) and RHA siRNAs as described in Figure 8. Concurrent with second siRNA transfection, the cells were co-transfected with indicated HTLV-1^{ACH} provirus. Forty-eight hours later, total cellular protein was harvested and subjected to immunoblot with RHA antiserum to assess down-regulation and Gapdh antiserum to control for sample loading. Results of Gag ELISA are summarized below the immunoblot; <MD, less than the minimum detection limit of the assay (25 pg/ml).

that lack Rex (50). Insertion of SNV PCE into the Rex-deficient HTLV-1 provirus (Rex-/PCE) increased Gag production by >18-fold (compare HTLVRex- and Rex-/PCE). The results indicate that SNV PCE has the capacity to facilitate Rex/RxRE-independent Gag production from a Rex/RxRE-deficient HTLV-1 provirus. The ratios of Gag protein relative to gag mRNA indicated that insertion of SNV PCE into Rex-deficient provirus robustly increased translational utilization of the HTLV-1 gag transcripts (>20-fold).

To assess the effect of RHA on Gag protein production from the HTLV-1 proviruses, COS cells were transfected with the RHA siRNAs, incubated for 72 h and RHA downregulation was verified by RHA immunoblot on an aliquot of the cells (data not shown). The treated cells were transfected with siRNA, HTLV-1 provirus and pGL3 and incubated for 48 h. RHA immunoblot indicated sustained downregulation of RHA (Figure 10B). HTLV Gag ELISA indicated that RHA downregulation significantly decreased Gag production from each of the proviruses (Figure 10B). RHA downregulation reduced Gag production from wtHTLV-1 and wt/PCE to <10% and eliminated Gag production from Rex-/PCE provirus. The results are in agreement with the ribosomal profile analysis (Figure 9) and demonstrate that RHA is necessary for efficient expression of HTLV-1 Gag structural protein. Furthermore, the results indicate that SNV PCE facilitates Gag protein production from a Rex/RxRE-deficient HTLV-1 provirus in an RHA-dependent manner.

Table 2. Gag protein and RNA expression from HTLV-1 proviruses

Replicate	HTLV-1 provirus	Gag		
		Gag[a] pg/ml	gag RNA[b]	Ratio of Gag protein:RNA
1	wtHTLV-1	3125	417.5	7.5
	HTLVRex	31	228.4	0.1
	wt/PCE	4663	662.5	7.0
	Rex-/PCE	576	267.3	2.1
2	wtHTLV-1	2352	392.7	6.0
	HTLVRex	<MD	237.3	<MD
	wt/PCE	4118	729.7	5.6
	Rex-/PCE	796	285.4	2.8

[a]Cultures of 293 cells were co-transfected with indicated provirus and pGL3. Total cellular protein was isolated and Gag quantified by ELISA; <MD, below the minimum detection limit of the assay (25 pg/ml).
[b]gag copies per nanogram total RNA standardized to gapdh as determined by real-time PCR.

DISCUSSION

This study assessed the possibility that selected retroviral 5′ UTRs confer PCE activity or IRES activity. We surveyed seven retroviruses for PCE activity. Results of PCE reporter assays determined that the 5′ LTR of REV-A, HTLV-1 and FeLV contain a 5′ terminal PCE, which can substitute for SNV PCE to facilitate Rev/RRE-independent expression of HIV gag RNA. The analysis further determined that the RU5 region of the REV-A LTR is necessary for the Rev/RRE-independent Gag production, and REV-A PCE activity is attributable to increased translation efficiency of the REV-A-PCE gag RNA, rather than increased cytoplasmic accumulation of the gag RNA (29). This observation that PCE mutation disrupts translational utilization of the mRNA without detectable change in the cytoplasmic abundance supports the model that PCE–RHA interaction overcomes sequestration of the transcript in RNA storage granules (29). The experiments did not identify PCE activity in the 5′ LTR of HTLV-2, EIAV or MLV in 293 cells. Possible explanations are that these sequences do not contain a 5′ terminal PCE or that the level of LTR-driven transcription in 293 cells was insufficient for detection of Gag

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protein production. Further analysis is necessary to assess these possibilities.

Our findings show that the 5′ UTR of REV-A HTLV-1 and SNV do not support IRES activity in bicistronic RNA. Stringent bicistronic reporter assays by plasmid transfection, RNA transfection of synthetic RNAs, and RNA analysis identified a lack of IRES activity in the 5′ UTR of REV-A, HTLV-1 and SNV. Our findings contrast with published analysis of a bicistronic neomycin phosphotransferase reporter vector that identified IRES activity in REV-A after selection with G418 (32). IRES-like activity has also been identified from HTLV-1 R expressed adjacent to the SV40 early gene leader (33,34). Here, we identified that bicistronic reporter activity was detectable in the REV-A and HTLV-1 UTR, but is attributable to heterogeneous F-luc transcripts, rather than authentic IRES activity. A second, complementary approach assessed SNV translation initiation in the natural context of an SNV provirus. Results using EMCV to inhibit cap-dependent translation initiation determined that SNV is reliant on a cap-dependent initiation mechanism. Our results do not completely exclude the possibility of internal initiation, but indicate that cap-independent internal initiation is not a major mode of translation initiation for SNV in asynchronous cells. We speculate that retroviral 5′ UTR is a pliable template for mRNA translation initiation that is reorganized by changes in nucleoprotein architecture. The reorganization allows the virus to sustain translation during oscillations in the translation capacity of the cell, which alternatively favor cap-dependent initiation or cap-independent internal initiation (8). We speculate that the capacity to respond is orchestrated by alternative virus–host interactions: PCE–RHA and IRES–host factor, respectively.

The specific interaction between RHA and the SNV PCE and the PCE at the 5′ RNA terminus of the cellular junD gene has been shown to be necessary for efficient junD translation (29). Here, we verified that RHA is essential for PCE activity conferred by REV-A PCE reporter mRNA and for efficient translation of HTLV-1 gag mRNA from HTLV-1 provirus. Our results implicate RHA as an important translation regulatory effector of multiple lymphotropic retroviruses. Our bioinformatic searches have not identified a common primary sequence motif among SNV, MPMV, REV-A, HTLV-1, FeLV and junD PCE. However, genetic and co-immunoprecipitation experiments have identified that structural motifs in SNV PCE are necessary for the selective recruitment of RHA (29). We speculate that the growing collection of viral and cellular PCEs share related secondary or tertiary structural motifs that convey selective interaction with RHA.

HTLV-1 is dependent on the virally encoded Rex protein in association with the *cis*-acting RxRE for efficient export of viral transcripts. Here, we have shown that the PCE of SNV, a genetically simple retrovirus, is capable of facilitating Rex/RxRE-independent expression of HTLV-1 provirus. This finding is an example of the interchangeability of viral elements to facilitate robust gene expression in diverse gene expression systems. SNV PCE has also been shown to enhance translational efficiency of lentiviral vector transgene (55), modulate expression of BLV structural gene vectors (47,54) and to synergize with a heterologous transcriptional enhancer and constitutive transport element to increase protein yield (51,55).

Downregulation of endogenous RHA decreased Gag production from both wtHTLV-1 and PCE-containing Rex-deficient HTLV-1, implying that RHA is necessary for efficient translation of HTLV-1 transcripts that accumulate in the cytoplasm in either a Rex-dependent or Rex-independent manner. Not unexpectedly, the insertion of SNV PCE did not completely restore Gag production to the level of wtHTLV-1. This is attributable to impaired cytoplasmic accumulation of HTLV-1 transcripts because of the lack of Rex. We expect that SNV PCE facilitates Rex/RxRE-independent expression by upregulating the translational utilization of residual cytoplasmic gag mRNA that accumulates in the cytoplasm in the absence of Rex.

We propose that RHA associates with the 5′ proximal SNV PCE in PCE/Rex- and the PCE within HTLV-1 RU5 in wtPCE. This interaction facilitates RNP remodeling that promotes ribosome scanning and efficient translation initiation. A similar model is proposed for PCE/RHA translational stimulation of junD mRNA template as described in Hartman et. al, 2006 (29).

Recent studies with HIV-1 suggest that RHA is important for the process of reverse transcription and functions in an RNA-dependent manner to enhance infectivity of viral particles (56). If this phenomenon is also true for HTLV-1, then the role of RHA in HTLV-1 pathogenesis may be multifaceted. A potential mechanism is that RHA is packaged into viral particles, possibly in association with the HTLV-1 PCE and promotes RNA–protein and/or RNA–RNA remodeling that facilitates reverse transcription. After provirus formation and transcription of nascent viral RNA, RHA would interact with PCE to facilitate translation of the viral genome. The facilitation of efficient translation of viral structural and enzymatic proteins with the process of reverse transcription would function synergistically to promote infectivity and pathogenesis of HTLV-1. Compliant with this model, we did not observe PCE activity in HTLV-2, which correlates with reduced replication capacity and pathogenesis of HTLV-2 in relation to HTLV-1 (46).

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Conflict of interest statement. None declared.

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