Variable inhibition of cell-free translation by HIV-1 transcript leader sequences

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ABSTRACT

The 5' ends of all human immunodeficiency virus type I (HIV-1) transcripts have the potential to coordinately regulate translation of HIV-1 mRNAs. Conflicting observations of the translational impact of these sequences in various systems stimulated these analyses of translation in reticulocyte lysates. We report a sensitive, rapid, quantitative, and inexpensive cell-free translation assay in which translational efficiency is monitored by enzymatic assay of the translation products. Using this assay and conventional radiolabeling assays, we demonstrate that the HIV-1 transcript leader inhibits downstream translation and that the stem-loop structure is required. Under our assay conditions, this inhibition occurs predominantly in *cis* and is not mediated by the 68 kD, interferon-induced, double-stranded RNA-activated kinase (p68). However, under other assay conditions the HIV-1 leader may activate p68 and inhibit translation in *trans*. We show that variation between individual preparations of cell-free extracts can dramatically alter the magnitude of the translational inhibition by the HIV-1 leader. Further, we provide evidence that a heat-labile factor is required for efficient translation of transcripts containing the HIV-1 leader. These observations provide a foundation for identifying factors required for translation of HIV-1 transcripts.

INTRODUCTION

Investigations of the molecular biology of human immunodeficiency virus, type I (HIV-I) have revealed diverse mechanisms responsible for controlling the kinetics and abundance of viral gene expression (reviewed in 1). Among these, the interactions of the HIV-1 transactivator protein Tat with sequences at the 5' end of HIV RNA, known as TAR, have been the subject of numerous studies (reviewed in 2,3). Although some details are still not resolved, the interaction of Tat and TAR clearly results in a dramatic increase in the abundance of HIV-1 transcripts.

In addition to mediating transactivation by Tat, sequences at the 5' end of HIV-1 RNA, being present on all spliced and unspliced HIV-1 transcripts, have the potential to coordinately modulate the posttranscriptional expression of viral genes. In fact, in cell-free translation extracts and in *Xenopus* oocytes, the HIV-1

transcript leader profoundly inhibits translation (47). At least two distinct mechanisms may be involved in this effect. Some studies have revealed that RNA molecules containing the HIV-1 leader can inhibit translation by activating the 68 kD interferon-induced double-stranded-RNA-activated kinase (p68 kinase, also known as dsI or DAI; 5,7,8). However, others have reported that molecules containing TAR may block activation of the p68 kinase (9). The HIV-1 leader has also been reported to inhibit cell-free translation in *cis* by a mechanism independent of p68 kinase (7).

HIV-1 Tat activates translation of HIV-1 leader-containing mRNAs in some analyses. In *Xenopus* oocytes, the HIV-1 leader dramatically inhibits translation unless the RNA is modified in the nucleus in the presence of Tat (6,10,11). While Tat has also been reported to augment translation of HIV-1 leader mRNAs in RRL (7) it does not seem to alter translation of HIV-1 leader-containing RNAs in primate cells (12). Tat may also indirectly increase cellular translation by downregulating expression of p68 kinase (13).

While the magnitude of the inhibitory effect of the HIV-1 leader is quite variable, most reports have revealed a greater effect in cell-free translation systems (4,5,7) than in intact cells (12,14,15). To elucidate the basis for the variable translational impact of the HIV-1 leader in different systems, we investigated the influence of HIV-1 leader on translation in rabbit reticulocyte lysates (RRL) using a combination of a conventional ³⁵S-methionine labeling assay and a new enzymatic assay of translational efficiency. We report that under our assay conditions, the HIV-1 leader inhibits downstream expression predominantly in *cis*, independent of p68 kinase, and that this effect requires the stem-loop structure of the HIV-1 leader. Although we observe only a weak *trans* inhibitory effect of the HIV-1 leader, other assay conditions may elicit a stronger p68 kinase-mediated, *trans* effect. Notably, we find that the magnitude of the inhibitory effect leader varies dramatically among different lots of cell-free lysate. Further, we provide evidence that a heat-labile factor(s) is necessary for efficient translation of HIV-1 leader-containing RNA.

MATERIALS AND METHODS

Plasmids

Insertion of a HindIII/BamHI fragment from pEQ3 (16), containing the *lacZ* gene, into HindIII/BamHI digested pBS+ (Stratagene) resulted in pEQ4 in which β -gal transcripts without HIV-1 leader sequences were expressed from the T3 promoter.

pEQ292 resulted from insertion of the same HindIII to BamHI fragment into HindIII/BamHI sites of pSP64TARCAT (4; provided by Nahum Sonenberg), replacing the CAT reading frame with that of β -gal. A BglII collapse of pEQ292 resulted in pEQ293 in which HIV-1 sequences from +25 to +83 were deleted. The 3.6 kb HindIII/BamHI fragment from pEQ241 (15) containing HIV-1 sequences from +83 to +242 fused to β -gal was inserted into HindIII/BamHI sites of pSP64TARCAT (replacing the CAT gene) yielding pEQ306. Thus, transcripts expressed from SP6 promoter in pEQ293, pEQ292, and pEQ306 contained HIV-1 sequences from +1 to +25, +83, and +282, respectively, fused to β -gal (Fig. 1).

Plasmids pEQ390, pEQ389, and pEQ388 containing mutations in the HIV-1 TAR stem-loop structure (Fig. 4) were constructed by inserting β -gal as a HindIII/BamHI fragment from pEQ3 into HindIII/BamHI sites of TARI/III, TARI'/III' and TARI/III'/III'(8; provided by Nahum Sonenberg and Michael Katze), respectively.

The HindIII to BamHI fragment containing the CAT gene from pSP64TARCAT was inserted into the HindIII/BamHI sites of pBS+, resulting in pEQ319.

***In vitro* synthesis of RNA**

Plasmid DNA, prepared by alkaline lysis and banding twice in CsCl (17), was digested with either BamHI or ApaLI then purified using GeneClean (Bio 101, La Jolla, CA) following agarose gel electrophoresis. RNA was transcribed in a reaction containing 40 mM Tris-HCl pH7.9, 6 mM MgCl₂, 4 mM spermidine, 100 μ g/ml BSA, 10 mM DTT, 800 units/ml RNasin (Promega Corporation, Madison, WI), 1 mM ATP, CTP, and UTP, 0.1 mM GTP, 1 mM m⁷GpppG (New England Biolabs, Beverly, MA), and 2–5 μ g DNA template in the presence of 100 μ Ci/ml ³H-UTP and 800 units/ml T3 polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) or 400 units/ml SP6 polymerase (Bethesda Research Laboratories, Gaithersburg, MD). After 1 h at 37°C, another equal quantity of RNA polymerase was added, and the reaction continued for an additional 1 h. After the addition of RNase-free DNase I (Boehringer Mannheim Biochemicals) to 1400 units/ml for 15', 37°C, the RNA was purified by phenol:chloroform extraction and passed through a sephadex G-50 spin column. Following ethanol precipitation in potassium acetate, the RNA was resuspended in water and stored at -75°C. The RNA concentration was determined by measuring ³H incorporation, and the integrity of the RNA was verified by Northern blot analysis.

Cell-free translation assays

The enzymatic cell-free translation assay (IVT-MUG) used either lot A (#96101) or lot B (#07490) of micrococcal nuclease treated rabbit reticulocyte lysate (RRL, Promega Corporation). RNA was added to 12.5 μ l reactions containing 70% RRL, 20 μ M unlabeled amino acids (each, including methionine), and 800 units/ml RNasin. After translation at 30°C for 60', 8 reaction volumes of 60 mM Na₂HPO₄·7H₂O, 40 mM NaH₂PO₄·H₂O, 10 mM KCl, 1 mM MgSO₄·7H₂O, 50 mM 2-mercaptoethanol, and 1 mg/ml BSA containing 1% DMSO and 0.44 mM of the β -gal substrate methylumbelliferyl- β -D-galactoside (MUG, Sigma Chemical Co.) were added. After incubation for 60' at 30°C, a 10 μ l aliquot was dequenched by dilution into 190 μ l of 1.5% H₂O₂ and 1 N NaOH for 5' at room temperature, and fluorescence was measured using a Microfluor microfluorimeter

(Dynatech Laboratories, Alexandria, VA). In some experiments, the reaction volume and the volume of the aliquot dequenched were varied as indicated in the figure legends. Where indicated, RRL was heat treated by preincubation at 45°C 10' prior to addition of the amino acids, RNasin and RNA.

Cell-free translation with radiochemical labeling was carried out in a similar reaction with 1.2 μ M (1.2 mCi/ml) ³⁵S-methionine in place of unlabeled methionine. Labeled proteins were separated by SDS-PAGE, and autoradiographs were obtained following fluorography with Enhance (Dupont).

P68 kinase inhibitors

Poly I:C was kindly provided by Michael Katze, University of Washington. 2-aminopurine (Sigma Chemical Co.) was freshly prepared in 50 mM KOH.

RNA analyses

RNA was recovered from cell-free translation reactions by digestion in 10mM Tris pH7.8, 5mM EDTA containing 400 μ g/ml proteinase K, 0.5% SDS with 2 ng CAT RNA added to each sample to control for RNA recovery and Northern transfer efficiency. Following phenol:chloroform extraction and ethanol precipitation, RNA samples were separated on 1% formaldehyde agarose gels, transferred to nitrocellulose, and hybridized to a 2.1 kb HindIII/SacI fragment from pEQ3 specific for β -gal and the HindIII/BamHI fragment from pEQ319 specific for CAT.

RESULTS

Enzymatic Cell-free Translation (IVT-MUG) Assay

Published studies using cell-free translation systems demonstrated a strong inhibitory effect of HIV-1 leader sequences (4,5,7). In contrast, the same sequences had little or no apparent translational effect in intact cells (12,14,15). Comparisons of the polysomal distribution of RNAs in RRL and in intact human fibroblasts confirmed that the HIV-1 leader sequences only inhibited translation in RRL (C.R. Degrin and A.P. Geballe, unpublished data).

To facilitate investigation into the basis for the discordant translational effects of the HIV-1 leader in intact cells compared to cell-free lysates, we developed a quantitative cell-free translation assay in which translational efficiency was assessed by measurement of β -galactosidase (β -gal) enzymatic activity. As detailed in Materials and Methods, transcripts synthesized *in vitro* were translated in RRL (heated to 45°C for 10' prior to use where indicated [see below]) in the presence of unlabeled amino acids. The translation products were incubated with buffer containing the fluorogenic β -gal substrate MUG. Because of fluorescence-quenching activity in RRL (data not shown), aliquots of the reactions were dequenched with NaOH and H₂O₂ prior to measuring the fluorescence.

Using a β -gal transcript without HIV-1 leader sequences (pEQ4-RNA, Fig. 1), this assay detected the products of translation from as little as 8 ng/ml (in a 12.5 μ l reaction) of β -gal RNA (Fig. 2). Importantly, the enzymatic assay was linear with input levels of RNA up to approximately 800 ng/ml before a plateau was reached. For comparison, the same RNA samples were translated in RRL using a standard ³⁵S-methionine labeling assay (Fig. 2B). The approximately similar sensitivities of the two assays demonstrates that the enzymatic cell-free translation (IVT-MUG) assay is a suitable alternative to the conventional

Figure 1. HIV-1-*lacZ* transcripts. Transcripts derived from plasmids containing no HIV-1 leader (pEQ4) or the HIV-1 leader from +1 to either +25 (pEQ293), +83 (pEQ292), or +282 (pEQ306) fused to the β -gal reading frame are depicted. Digestion of plasmid templates with BamHI and ApaLI sites yielded full length (3.6 kb, excluding HIV-1 leader sequences) and truncated (2.4kb) transcripts, respectively.

Figure 2. Comparison of enzymatic (IVT-MUG) and ^{35}S -methionine labeling cell-free translation assays. (A) Following translation of RNA transcribed from BamHI-digested pEQ4 templates using heat-treated RRL lot B, β -gal activity was measured as described in Materials and Methods. The data represent the mean fluorescence from triplicate aliquots of reactions at each concentration less than from a control translation reaction containing no pEQ4 RNA. (B) The same RNAs were translated in the presence of ^{35}S -methionine as described in Materials and Methods, and proteins from one half of each reaction were electrophoretically separated on 10% SDS-polyacrylamide gels. The autoradiogram was exposed for 13 days. The faint band migrating at approximately 50 kD represents a background band present even in the no RNA control lane, while other bands correlate with the amount of input β -gal RNA and likely represent either partially synthesized or degraded β -gal protein. The positions of molecular weight (kD) markers are indicated on the left.

assay. Compared to the ^{35}S -methionine labeling assay, the IVT-MUG assay has the advantages of being rapid, inexpensive, and easily quantified.

Translational inhibition by HIV-1 leader sequences

Using the IVT-MUG assay, the effect of HIV-1 leader sequences on translation was examined. RNA was transcribed *in vitro* from the bacteriophage promoter in the plasmid pEQ4 which contains no HIV leader, or in pEQ293, pEQ292, and pEQ306 which express transcripts starting at the authentic HIV-1 cap site (+1) and containing 25, 83, or 292 nt, respectively, of HIV leader fused to *lacZ* (Fig. 1). Inclusion of the first 25 nt of the HIV-1 leader did not influence cell-free translation compared to RNA with no HIV-1 sequences (Fig. 3). In contrast inclusion of 83 or 282 nt of the HIV-1 leader at the 5' end of β -gal transcripts inhibited translation (Fig. 3). The magnitude of inhibition was similar over a range of RNA concentrations from 260 to 800 ng/ml and was more pronounced with +282 RNA (8–10 fold) compared to +83 RNA (3–4 fold). Because RNA transcribed from pEQ4 and pEQ293 (+25) consistently expressed nearly identical β -gal activities in multiple experiments (Fig. 3 and data not shown), only data using the +25 RNA is shown in subsequent experiments. Consistent with other reports (4,5,7), these data demonstrate an inhibitory effect of HIV-1 leader sequences on cell-free translation.

The HIV-1 leader stem-loop is required for translational inhibition

The stem-loop structure formed by the TAR region of the HIV-1 leader was shown to be important in mediating translational effects in previous studies (4,5,8). Therefore, we analyzed translation of RNAs transcribed from the stem-loop mutants pEQ390, pEQ389, and pEQ388. Destabilizing the TAR hairpin structure by mutation of 8 nt either on the 5' (pEQ390) or on the 3' (pEQ389) side of the stem inactivated the inhibitory signal (Fig. 4). Restoration of base pairing in the stem by complementary mutations (pEQ388) restored the signal. Thus, the hairpin structure is an essential component of the inhibitory signal.

Figure 3. HIV-1 leader sequences from +1 to +83 or to +282 inhibit translation. The indicated concentrations of RNA transcribed from pEQ4, pEQ293 (+25), pEQ292 (+83), or pEQ306 (+282) BamHI-digested templates were translated in the IVT-MUG assay using heat-treated RRL Lot B. Fluorescence in dequenched 5 μ l aliquots of the β -gal assay was measured as described in Materials and Methods.

Figure 4. The HIV-1 leader stem-loop structure is required for translational inhibition. RNA samples transcribed from the indicated plasmid templates digested with BamHI were translated in 6.25 μ l reactions containing 530ng/ml RNA and heat-treated RRL lot B for 1 h. Fluorescence measurements of each β -gal assay less than of a control translation containing no added RNA are shown. The stem-loop structure at the 5' end of each RNA is depicted at the top with thick lines representing sets of 4 base mutations (8). The mutations on the 5' and 3' sides of the stem which destabilize the stem in pEQ390 and pEQ389, respectively, are compensatory and restore the stem when present on both sides in pEQ388.

Translational inhibition acts predominantly in *cis*

To determine whether the translational inhibition found using our reaction conditions occurred in *cis* or *trans*, mixtures of two transcripts, one containing and the other lacking the inhibitory HIV-1 sequences, were translated together in RRL. The translation products of the two transcripts were distinguishable, since in each mixture one of the two transcripts was truncated at the 3' end (by transcribing the RNA from a plasmid template digested at an ApaLI site [Fig. 1]), resulting in a protein of expected molecular weight 92 kD. The other transcript was full length, yielding intact β -gal protein. For this experiment we used 35 S-methionine to label the proteins, since the truncated transcripts yielded enzymatically inactive proteins and hence could not be measured using the IVT-MUG assay.

Equimolar amounts of full-length (BamHI digested templates, 320 ng/ml) or 3' truncated (ApaLI digested templates, 220 ng/ml) RNAs derived from pEQ293, pEQ292, and pEQ306, when translated alone, showed the inhibitory effect of leader sequences to +83 or +282 compared to +25 (Fig. 5A, lanes 2–7), consistent with the results of the IVT-MUG assay (Fig. 3). Translation of a mixture of full length and truncated +25 RNA resulted in bands equal in intensity to either translated alone (compare lanes 2 and 3 with 8), indicating that the assay was linear with input RNA as expected from the IVT-MUG results which showed a linear response with input RNA up to 800 ng/ml (Fig. 2).

Translation of truncated +25 RNA mixed with either full-length +83 or +282 RNA (lanes 9 and 10) demonstrated that the inhibitory effect of the HIV-1 leader sequences acted predominantly in *cis*, since the protein levels corresponding to the +83 and +282 transcripts were markedly reduced compared

Figure 5. Translation of mixtures of RNA containing and lacking the inhibitory HIV-1 leader sequences. (A) Analysis of protein products. RNAs were translated for 30' in heat-treated RRL lot B in the presence of 35 S-methionine and the products separated by SDS-PAGE as described in Materials and Methods. The 37.5 μ l translation reactions contained RNA transcribed from plasmid templates (pEQ293 [+25], pEQ292 [+83], or pEQ306 [+282]) digested with either BamHI (lanes 3, 5, and 7) or ApaLI (lanes 2, 4, and 6) or a mixture of both (lanes 8–12), as indicated at the top. RNA from BamHI- and ApaLI-digested templates were translated in equimolar amounts (320ng/ml and 220ng/ml, respectively). The 119 kD and 92 kD products expected from translation of the RNA transcribed from BamHI and ApaLI digested templates, respectively, were separated on a 7.5% gel. The positions of molecular weight markers are indicated on the left. (B) RNA stability. RNA was recovered from 6.25 μ l aliquots of IVT-MUG reactions containing the indicated mixtures of RNAs like those in (A), before translation and after 30' of translation as described in Materials and Methods. Equal amounts of CAT RNA (2 ng, transcribed from BamHI digested pEQ319 template) were added to each sample along with SDS and proteinase K to control for variations in RNA recovery and Northern transfer. Autoradiograms of a blot probed first with β -gal specific probe then stripped and reprobed with a CAT-specific probe are shown.

to those of the +25 RNA. Corroborating data resulted from the complementary mixture of full-length +25 RNA translated with truncated +83 or +282 RNA (lanes 11 and 12). Although we did not extensively purify our RNA preparations to remove potential double-stranded RNA contaminants (9), the design of this experiment excludes the possibility that the inhibitory effect was caused by such RNA since the resulting inhibition would be expected to equally inhibit translation of both transcripts in the mixture. While the decrease in translation of the +25 RNA when translated with +83 or +282 RNA reveals a *trans* effect

Figure 6. Inhibitors of p68 kinase activation fail to relieve inhibition by the HIV-1 leader. RNA was translated in the IVT-MUG assay in 6.25 μ l reactions containing heat-treated RRL lot B and 530 ng/ml RNA transcribed from BamHI-digested pEQ293 (+25), pEQ292 (+83), or pEQ306 (+282). 2-aminopurine and poly I:C were added to a final concentration of 1, 5, or 10 mM and 25 μ g/ml, respectively, as indicated. Fluorescence was measured in 5 μ l aliquots after 1 h incubation in the presence of MUG for reactions containing no p68 kinase inhibitors (left set) or containing 2-aminopurine and in 10 μ l aliquots after 1.5 h incubation in the presence of MUG for samples containing poly I:C. Fluorescence in the samples less fluorescence in a control reaction containing no added RNA are shown.

of the HIV-1 leader (compare lanes 2 and 3 to lanes 9–12), these data demonstrate that using our assay conditions, the predominant inhibitory effect of HIV-1 leader sequences is *cis*-acting.

One potential explanation for an apparent decrease in the translational efficiency of +83 and +282 RNA is decreased stability of these transcripts compared to +25 RNA. To investigate this possibility, RNA was extracted from cell-free translation reactions after 0 and 30 minutes of translation. The decrease in β -gal RNA levels during the 30 minutes of translation was similar between both RNA samples in each mixture (Fig. 5B). CAT RNA, which was added to each sample at the beginning of the extraction procedure, was comparable in all lanes, demonstrating that recovery and blotting efficiency were consistent among samples. Thus differential translational efficiency rather than stability of RNAs explains the inhibitory effect of HIV leader sequences to +83 or +282.

p68 kinase activation does not account for translational inhibition

Our results demonstrate a *cis*-acting inhibitory effect of HIV-1 leader sequences to +83 or to +282 on cell-free translation. Other reports revealed that these sequences are capable of activating p68 kinase (5,8,18). While any *trans*-acting inhibitory effects of HIV-1 leader sequence cannot account for the data shown in Fig. 5, it is conceivable that HIV-1 RNAs act in *cis* by activating p68 locally (19,20). To determine whether the inhibitory effect is mediated by the p68 kinase, we blocked activation of p68 kinase by adding either 2-aminopurine or high levels of poly I:C to IVT-MUG assays (Fig. 6). Regardless of the level of 2-aminopurine used, we found that +83 and +282

Figure 7. Variation in magnitude of inhibition between RRL lots and in heat-treated RRL. Two different lots of RRL (A and B) were used in the standard IVT-MUG assay without (left) or with (middle) preincubation of the RRL at 45°C, 10' prior to addition of the amino acids, RNasin, and RNA. Translation using an equal-volume mixture of RRL lot A without heat treatment and RRL lot B with heat treatment (ht) is shown (right). Fluorescence measurements for reactions containing 530 ng/ml of +25 RNA (white bars), +83 RNA (striped bars), and +282 RNA (cross-hatched bars) less fluorescence in a control reaction containing no added RNA are shown. Numbers at the top of the bars indicate the fold inhibition of β -gal activity expressed from the +83 or +282 RNA compared to +25 RNA. Although the exact fold inhibition varied among experiments because of imprecision in measurements of the very low β -gal activity expressed by +83 and +282 transcripts in heat treated lysates, heat treatment consistently magnified the inhibitory effects of these leader sequences.

RNA expressed much less β -gal than +25 RNA. Similarly, sequences to +83 or +282 inhibited translation in the presence of 25 μ g/ml poly I:C which, contrary to the effects of low concentrations of dsRNA, blocks activation of p68 kinase (5,7,8,21). The failure of 2-aminopurine or high levels of dsRNA to alter the inhibitory effect of the +83 or +282 leaders suggests that the inhibition under these assay conditions is not due to p68 kinase activation by HIV-1 sequences. Further, these data support the conclusion from data presented above (Fig. 5) that the inhibition is not due to dsRNA contaminating our RNA preparations.

Influence of RRL lot variation and heat treatment on translational inhibition by HIV-1 leader sequences

During the course of these studies we noted the magnitude of the inhibitory effect of HIV-1 leader sequences varied substantially among lots of RRL obtained from the same manufacturer. While the HIV-1 leader in +83 RNA and +282 RNA inhibited downstream translation when translated in RRL lot A five- to ten-fold, the same leader inhibited translation only up to two-fold in lot B (Fig. 7, left). Repeated assays of these and additional lots (data not shown) demonstrated that the level of inhibition by HIV-1 leader sequences was reproducible for a particular lot but varied among lots from less than two-fold to approximately ten-fold. These data suggested that a factor (or factors) required for efficient translation through the HIV-1 leader was present in variable quantities in different lysates. Altering

the Mg^{++} and K^+ concentrations in the translation reaction did not change the relative translational efficiencies of the RNAs (data not shown).

We predicted that the activity that is variable among RRL lots might be quite labile, being inadvertently inactivated in some but not all preparations of RRL. To test this hypothesis we heated the RRL prior to the translation reaction. Heating RRL lot B to 45° for 10' disproportionately repressed translation of +83 or +292 RNA compared to +25 RNA (Fig. 7, middle), resulting in a markedly greater fold inhibition. Heating lot A also magnified the inhibitory effect of the HIV-1 leader (Fig. 7, middle). These data suggested that a heat-labile factor is required for efficient translation of +83 and +292 RNA in RRL.

To investigate whether the inefficient translation of +83 and +282 RNAs in RRL lot A (without heat treatment) could be complemented by heat-treated lysate B, RNAs were translated in an equal volume mixture of RRL lot A and heat-treated RRL lot B. The HIV-1 leader still inhibited translation in this mixture (Fig. 7, right). This result suggests that the factor deficient in RRL lot A is present in lot B but is heat-labile. Consistent with this hypothesis, equivalent results were obtained using either lot A without heat treatment (data not shown) or heat-treated lot B in each of the translation reactions reported (Figures 2–6).

DISCUSSION

Initiation of translation of most eukaryotic mRNAs involves loading of the 40s ribosomal subunit and associated factors onto the 5' end (cap site) of the mRNA and subsequent scanning in a 3' direction until an AUG codon is recognized. The 60s ribosomal subunit then joins the complex and polypeptide synthesis begins (reviewed in 22). Since approximately 290 nt at the 5' end of the HIV-1 genome are present on all spliced and unspliced viral transcripts, signals within this transcript leader have the potential to coordinately regulate the posttranscriptional expression of HIV-1 genes. The investigations reported here were designed to elucidate the basis for conflicting observations of the translational effects of the HIV-1 leader in different systems (4–9,12,14,15,18). In cell-free extracts, the HIV-1 transcript leader has been reported to inhibit translation (4,7). While the 5' proximal 25 nt of the HIV-1 leader do not inhibit translation (Fig. 3), our studies confirm that the 5' proximal 83 nt inhibit translation and that RNAs containing a leader including nearly the entire first exon common to all HIV-1 transcripts are translated even less efficiently (Figs. 3, 5–7).

Several possible mechanisms may account for the inhibitory effect of the HIV-1 leader. RNA secondary structure near the 5' end of a transcript can block the interactions of the RNA with translation initiation factors and thereby inhibit loading of 40s ribosomal subunits (reviewed in 22). The stem-loop structure of the HIV-1 transcript leader impairs interactions with eIF-4B as detected in cross-linking assays (4). Our results are consistent with this analysis in that mutations which destabilize the stem structure at the 5' end of the HIV-1 leader alleviate the inhibition, while compensatory mutations which restore the stem, restore the inhibition (Fig. 4). Furthermore, as we demonstrate (Fig. 5), the inhibition acts predominantly in *cis* as expected for such a mechanism.

However, other mechanisms may also require a stem-loop RNA structure and act in *cis*. Activation of p68 kinase results in phosphorylation of the α subunit of eIF-2 which in turn inhibits translational initiation (reviewed in 23). p68 may be activated

locally and therefore appear to act in *cis* (19,20). HIV-1 RNA, acting like low levels of dsRNA, can activate p68 kinase (5,8,18). Contrarily, highly purified HIV-1 leader RNA can prevent activation of p68 kinase and thereby preserve efficient initiation of translation (9). The basis for these contrary effects of the HIV-1 leader on p68 kinase activation is not clear but may be related to differences in quantity or purity of the RNA used in various studies. Under our conditions, translational inhibition occurs independent of p68 kinase activation. Standard inhibitors of p68 kinase activation, 2-aminopurine and high levels of dsRNA (7,21), failed to alleviate the inhibition (Fig. 6).

Despite these results, the HIV-1 leader may activate p68 kinase and inhibit translation in *trans*. In fact, we observe a decrease in translation of RNAs without the HIV-1 leader when mixed with HIV-1 leader-containing RNAs in Fig. 5 which may reflect p68-mediated *trans*-acting inhibition. Furthermore, we did not preincubate extracts in the presence of added RNA which may be necessary to elicit activation of p68 (5,7).

Another potential mechanism is suggested by studies in *Xenopus* oocytes, demonstrating that HIV-1 leader-containing RNAs must be modified in the nucleus in the presence of Tat in order to be efficiently translated (11). Recently, a double-stranded RNA-modifying activity was shown to change an A to an I in the HIV-1 leader in oocyte nuclei in the presence of Tat (24). Although the impact of this modification on translation is not yet known, it is possible that the inhibitory signal in the HIV-1 leader precludes translation until the signal is modified.

We found that the inhibitory effect of the HIV-1 leader varied from over 10-fold to less than 2-fold in different experiments. Data from another group revealed a similar wide range in inhibition mediated by the HIV-1 leader in RRL. The HIV-1 leader to +83 fused to CAT inhibited translation 200-fold in one report (4) but only 10-fold (5) in another. In our experiments, differences among lots of RRL, all obtained from the same commercial source, accounted for the variable effect (Fig. 7).

In addition to lot-to-lot variation in the magnitude of inhibition, we discovered that heating RRL prior to the translation reaction magnified the inhibition. The failure of heat-treated lot B RRL to complement lot A (Fig. 7) could be explained if the same factor that is deficient in lot A is also heat-labile. However, further studies will be required to identify and characterize factors required for efficient translation of HIV-1 mRNAs. Of interest, the HIV-1 leader does not inhibit translation at all in extracts derived from the mouse rhabdomyosarcoma cell line Sac⁻ (unpublished data). These extracts may therefore be useful in attempts to complement the translational defects of RRL lot A or heat-treated lot B. The convenience of the IVT-MUG assay should facilitate a biochemical approach to identifying the factor(s) required for efficient translation of HIV-1 mRNAs. Elucidating these factors necessary for translation of HIV-1 transcripts will undoubtedly enrich our understanding of eukaryotic translation in other viral and cellular systems.

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