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Efficiency of Reinitiation of Translation on Human Immunodeficiency Virus Type 1 mRNAs Is Determined by the Length of the Upstream Open Reading Frame and by Intercistronic Distance

B. G. MATTIAS LUUKKONEN, WEI TAN, AND STEFAN SCHWARTZ*

Microbiology and Tumorbiology Center, Karolinska Institute, S-171 77 Stockholm, Sweden

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In this study, we examined the mechanism of translation of the human immunodeficiency virus type 1 *tat* mRNA in eucaryotic cells. This mRNA contains the *tat* open reading frame (ORF), followed by *rev* and *nef* ORFs, but only the first ORF, encoding *tat*, is efficiently translated. Introduction of premature stop codons in the *tat* ORF resulted in efficient translation of the downstream *rev* ORF. We show that the degree of inhibition of translation of *rev* is proportional to the length of the upstream *tat* ORF. An upstream ORF spanning 84 nucleotides was predicted to inhibit 50% of the ribosomes from initiating translation at downstream AUGs. Interestingly, the distance between the upstream ORF and the start codon of the second ORF also played a role in efficiency of downstream translation initiation. It remains to be investigated if these conclusions relate to translation of mRNAs other than human immunodeficiency virus type 1 mRNAs. The strong inhibition of *rev* translation exerted by the presence of the *tat* ORF may reflect the different roles of Tat and Rev in the viral life cycle. Tat acts early to induce high production of all viral mRNAs. Rev induces a switch from the early to the late phase of the viral life cycle, resulting in production of viral structural proteins and virions. Premature Rev production may result in entrance into the late phase in the presence of suboptimal levels of viral mRNAs coding for structural proteins, resulting in inefficient virus production.

Three size classes of viral mRNAs are produced in human immunodeficiency virus type 1 (HIV-1)-infected cells: 9-kb mRNAs, 4- to 5-kb mRNAs, and 2-kb mRNAs. Together, more than 30 HIV-1 mRNA species are generated by differential splicing of the 9-kb precursor RNA. The small size class of mRNAs comprises more than 12 differently spliced mRNAs encoding the viral proteins Tat, Rev, and Nef (2, 19, 39, 45, 49, 53). The *tat* mRNAs contain three open reading frames (ORFs) located in the order *tat*, *rev*, and *nef*, while the *rev* mRNAs contain two ORFs, *rev* and *nef*, *rev* being the first ORF. Interestingly, the *tat* mRNAs on which all three ORFs, *tat*, *rev*, and *nef*, are present produce only Tat protein. We have shown that the presence of the upstream *tat* ORF efficiently inhibits translation of the *rev* and *nef* ORFs (49, 53). Removal of the *tat* ORF by deletion or inactivation of the *tat* AUG by site specific mutations resulted in high production of Rev and Nef (53). The *rev* mRNAs, encoding *rev* and *nef*, produced approximately equal levels of Rev and Nef (49, 53).

Comparison of the sequence surrounding the translational start codon of *tat* and *rev* revealed that the *tat* AUG conformed well to the consensus sequence for efficient translation initiation (33), while the *rev* AUG appeared as a weaker initiator of translation. Changing the sequence surrounding the *rev* AUG into the sequence that surrounds the *tat* AUG by site-directed mutagenesis resulted in elevated Rev production and a block in translation of the downstream *nef* ORF (53). These results were consistent with the scanning model for mRNA translation (33), which predicts that when the AUG of the first ORF lies in a favorable context, translation initiates primarily at the first AUG (30, 33). Following translation of this ORF, the ribosomes dissociate from the mRNA and downstream ORFs are not translated. If the AUG of the first ORF is not in a favorable context, only a fraction of the ribosomes initiate translation at the first AUG, while the remaining ribosomes continue scanning until they reach the second AUG, where translation initiation occurs.

Replacing the sequence surrounding the strong *tat* AUG with the sequence surrounding the weak *rev* initiator AUG, using site-specific mutagenesis, resulted in increased translation of the downstream *rev* ORF (53). However, while on the *rev* mRNA, the *rev* AUG allowed approximately 50% of the ribosomes to initiate translation at the downstream *nef* ORF, converting the strong *tat* AUG to a weaker *rev*-like AUG allowed only 5% of the ribosomes to initiate translation at the downstream *rev* ORF (53). These results showed that factors other than those determining the strength of the first AUG on the *tat* mRNA influence translation initiation at downstream ORFs, as has been described in other systems (1, 11, 15, 26, 55, 59).

The study presented here is a continuation of our previous work on the mechanism of translation of HIV-1 mRNAs in eucaryotic cells (49–51, 53). We have used the HIV-1 *tat* mRNA to investigate whether factors such as overlap of the first and second ORFs, length of the first ORF, and distance between the first and second ORFs influence downstream translation initiation. Our results show that in addition to the strength of the upstream AUG, the length of the first ORF is an important determinant of translation initiation at downstream AUGs. Proximity of the AUG of the downstream ORF to the translational terminator of the upstream ORF appeared to play a minor role, as did overlap between upstream and downstream ORFs. Interestingly, the degree of inhibition of translation of the downstream ORF was proportional to the length of the upstream ORF, if the AUG of the first ORF lies

* Corresponding author. Mailing address: Microbiology and Tumorbiology Center, Karolinska Institute, P.O. Box 280, S-171 77 Stockholm, Sweden. Phone: 468 728 6312. Fax: 468 331 399.

in a favorable context for translation initiation. In conclusion, the presence of a strong AUG at the *tat* ORF and the length of the *tat* ORF strongly influence the translation efficiency of *rev*.

MATERIALS AND METHODS

Plasmid constructs. pNLGAGRRE was constructed by first PCR amplifying the Gag coding sequence of pHXB2, using oligonucleotides BSS (5′-GGCTT GCTGAAGCGCGCACGGCAAGAGG-3′) and 7492 (5′-TTATTGTGACGA GGGGTCGTTGCC-3′). The PCR fragment was blunt-end ligated into an *Eco*RV-digested, calf intestine alkaline phosphatase-treated pBluescript plasmid, generating pT7-HIV-1GAG. A *Bss*HII-*Eco*RI fragment encoding p55^{gag} was excised and subcloned into *Bss*HII- and *Eco*RI-digested pNL17R (52), generating pNLGAGRRE. pHXB2fB (13) contains a frameshift mutation in the *rev* gene which inactivates Rev function. Plasmids prev, ptat, and ptatR were constructed by cloning of *rev*- or *tat*-encoding *Bss*HII-*Xho*I fragments from pNL14A7, pNL147, and pNL147T/R (49, 53), respectively, into *Bss*HII- and *Xho*I-digested pBS15EDSS (40), containing a pBluescript backbone.

Site-directed mutagenesis was performed by the method of Kunkel (35). Plasmid 147L, which contains a *Bss*HI-*Sac*I fragment spanning the *tat* ORF, was first transformed into *Escherichia coli* JM109 competent cells, which were grown on minimal medium agar plates containing ampicillin. Colonies were picked, grown to optical density at 600 nm of 1, infected with helper phage VCS-M13 at a multiplicity of infection of 20, and grown for 16 h in 2× YT in the presence of ampicillin and kanamycin. Cells were pelleted, and the extracellular medium containing packaged single-stranded DNA of 147L was used for infection of CJ236 cells, together with VCS-M13, in the presence of ampicillin, kanamycin, and chloramphenicol. After 6 h, uracil-containing single-stranded 147L DNA was prepared. Cells were pelleted, and the supernatants were treated with RNase A and subjected to polyethylene glycol (PEG) precipitation. The resulting pellets were resuspended in 10 mM Tris-HCl (pH 7.5) and then subjected to phenol-chloroform extraction and ethanol precipitation. The mutants were sequenced by dideoxy sequencing and transferred into ptat as *Bss*HII-*Xho*I fragments. ptatW, ptatM, p72tat, p87tat, p99tat, p111tat, and p120tat were created by using the following oligonucleotides for site-specific mutagenesis: 5′-GAGAGCAACAAA TGCATCCAGTA-3′, 5′-GAGCAAGAATTCGAGCCAGTAGATCC-3′, 5′-CCAATTGATATGGGCCCAAGTGTTGC-3′, 5′-TGTAAAAAGTGATATC TTCATTGC-3′, 5′-TGCTTTCATTGACGCGTTTGTTTC-3′, 5′-AAGTTTGT TGAATTCCAAAAGCCTTAG-3′, and 5′-CATTGCCACGCGTGTTTCATA ACATAGGGCCCAGGCATCTCCTATGGC-3′, respectively. The mutagenesis oligonucleotides used to create plasmids p72tat and p120tat also introduced an *Apa*I restriction site (underlined in the oligonucleotide sequences) immediately downstream of the terminated *tat* ORF in each plasmid. To create plasmids p72tat16 and p120tat64, the *Bss*HII-*Apa*I *tat* ORF-encoding fragments from plasmids p72tat and p120tat were swapped.

Plasmid pCS1X was constructed by cloning of a DNA fragment (nucleotides 7016 to 7525; numbers refer to the genomic human papillomavirus 1 [HPV-1] sequence [7]) containing the HPV-1 late poly(A) signals into pHCMVSEAP digested with *Hpa*I and *Eco*RI. These cloning steps replaced the insulin poly(A)-containing sequence with the HPV-1 sequence.

Cells and transfections. HLtat cells (49) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Twenty-four hours prior to transfection, cells were seeded at 3×10^5/60-mm-diameter culture dish and 10^6/100-mm-diameter dish. Transfections were carried out by the calcium phosphate coprecipitation technique (17). The various amounts of plasmids used for transfection were adjusted with pBluescript DNA to 15 μg in 0.5 ml of precipitate for 60-mm-diameter dishes and 30 μg in 1 ml of precipitate for 100-mm-diameter dishes. Transfected cells were washed twice with phosphate-buffered saline (PBS) 6 h posttransfection, refed with fresh Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, and cultured overnight. Cells were harvested 24 h posttransfection. pCMVCAT or pCS1X was included in transfections to control for transfection efficiency. All plasmid DNAs used for transfection were purified on Qiagen columns (Diagen).

Western immunoblotting and HIV-1 p24^{gag} antigen capture enzyme-linked immunosorbent assay (ELISA). HLtat cells were harvested in 500 μl of lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl [pH 7.5]) and freeze-thawed three times. Cell lysates were cleared of insoluble material by centrifugation at 12,000 × *g* for 15 min. Proteins were electrophoretically separated on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels (37.5:1 acrylamide/bisacrylamide) at 150 V for 1 h and transferred onto nitrocellulose filters (Schleicher & Schuell) by electroblotting in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) at constant current (300 mA) for 1 h. Membranes were blocked for 1 h at 37°C with 10% nonfat milk in PBS containing 0.3% Tween 20 (PBS-T). After being washed three times in PBS-T, the blots were incubated for 1 h at 37°C with a mouse monoclonal antibody against HIV-1 p24^{gag} (EF7) (23) diluted 1:5,000, washed in PBS-T, and incubated with a horseradish peroxidase-conjugated anti-mouse antibody (Amersham) diluted 1:20,000 in PBS-T. Specific proteins were visualized by using an enhanced chemiluminescence detection system (Amersham).

Samples (100 μl) of cell extract in lysis buffer were analyzed in an in-house HIV-1 p24^{gag} antigen capture ELISA as described before (56). Briefly, plates coated with mouse monoclonal antibodies against HIV-1 p24^{gag} were washed six times in washing buffer (0.16 M NaCl containing 0.1% Tween 20), a 100-μl sample was added to each well, and the plates were incubated at room temperature overnight. The following day, plates were washed six times in washing buffer, and a horseradish peroxidase-labeled mouse monoclonal antibody against HIV-1 p24^{gag} was added. Plates were incubated at 37°C for 2 h, washed six times in washing buffer, and incubated in *o*-phenylenediamine dihydrochloride substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding 100 μl of 2.5 M H_2SO_4 per well and the A_{490} was determined. In transfections with pHXB2fB (13), extracellular medium was harvested and adjusted to 12% PEG and 0.2 M NaCl, and then samples were incubated overnight at 4°C. The samples were subjected to centrifugation at 12,000 × *g* for 15 min, and pellets were resuspended in 0.5% Triton X-100. Particle-associated HIV-1 Gag was quantitated in HIV-1 p24^{gag} antigen capture ELISA.

RNA extractions. Cytoplasmic poly(A)$^+$ mRNA was isolated by using Dynabeads Oligo $(dT)_{25}$ (Dynal AS). At 24 h posttransfection, cells from 100-mm-diameter dishes were washed twice in ice-cold PBS and then subjected to lysis in 500 μl of lysis buffer D (10 mM Tris-HCl [pH 7.5], 0.14 M NaCl, 5 mM KCl, and 1% Nonidet P-40) and low-speed centrifugation at 8,000 × *g* for 2 min. Supernatants were mixed with an equal volume of 2× binding buffer (20 mM Tris-HCl [pH 7.5], 1.0 M LiCl, 2 mM EDTA, 0.5% SDS) containing 400 μg of Dynabeads Oligo $(dT)_{25}$. Cell extracts and Dynabeads were incubated for 5 min at room temperature, and then the Dynabeads were washed with washing buffer (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 2 mM EDTA). The poly(A)$^+$ mRNAs were eluted in 150 μl of 2 mM EDTA (pH 7.5) at 65°C and stored at −70°C until use.

RT-PCR. Aliquots (20 μl) of poly(A)$^+$ mRNA purified using by Dynabeads Oligo $(dT)_{25}$ were reverse transcribed at 42°C for 1 h in a total volume of 30 μl containing 4 U of avian myeloblastosis virus reverse transcriptase (RT; Promega), 19 U of RNA guard (Pharmacia), 0.2 mM each dATP, dCTP, dGTP, and dTTP, 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 50 μg of random hexamer oligonucleotides (Pharmacia) per ml. A reaction without RT was performed in parallel. Five-microliter aliquots of cDNA product were PCR amplified in a 50-μl reaction volume, using HIV-1 *tat* mRNA-specific oligonucleotides NARS (49) and 2316A (5′-GATGAGCTCTTCGTCGCTGTCTCCG-3′). Oligonucleotide 2316A was end-labeled with [γ-^{32}P]ATP prior to use. PCR was performed in a total volume of 50 μl with 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) for 20 to 25 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. Ten-microliter samples from each RT-PCR were analyzed by electrophoresis on 5% polyacrylamide gels. The gels were dried and exposed to X-ray film at −70°C.

RESULTS

A quantitative, virus-free complementation assay for Rev. To investigate the effects of various mutations in the *tat* ORF on translation efficiency of the downstream *rev* ORF, we first developed a quantitative, virus-free functional assay for Rev. Rev acts through an RNA sequence termed the Rev-responsive element (8, 10, 21, 22, 37, 48) to induce production of HIV-1 Gag protein in eucaryotic cells. A plasmid named pNLGAGRRE (Fig. 1A), containing the HIV-1 *gag* gene and Rev-responsive element under control of the HIV-1 long terminal repeat promoter, was constructed. This plasmid was cotransfected with the serially twofold-diluted Rev cDNA prev (Fig. 1A). Analysis of Gag production by Western immunoblotting using a mouse monoclonal antibody against HIV-1 p24^{gag} (23) showed that HIV-1 Gag could not be detected in the absence of Rev, as expected, while in the presence of Rev, high levels of Gag were produced (Fig. 1B). The amount of Gag protein produced increased with increasing levels of Rev-producing plasmid used in the transfection (Fig. 1B).

To investigate if the amount of Gag protein reflected the levels of Rev protein produced, extracts of cells transfected with pNLGAGRRE and serially twofold-diluted prev were analyzed by using an in-house HIV-1 p24^{gag} antigen capture ELISA (56). The results showed that HIV-1 Gag is produced in a Rev dose-dependent manner (Fig. 1C). Transient transfection of Rev-producing plasmid with pNLGAGRRE, followed by quantitation of HIV-1 Gag protein in the antigen capture ELISA, was therefore used to determine the amount of Rev produced from various mutant *tat* mRNAs.

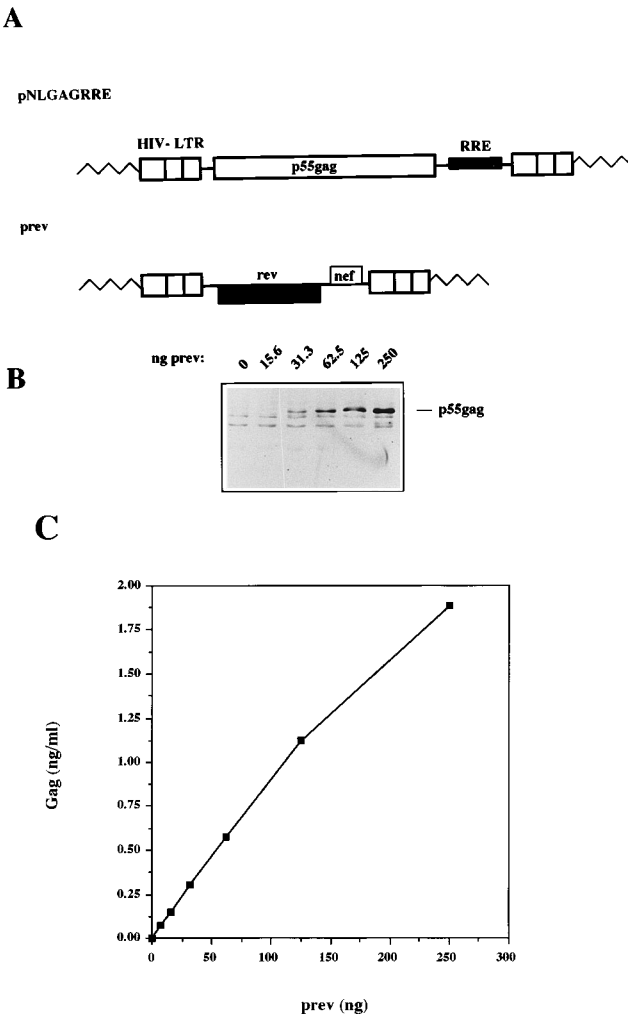

FIG. 1. (A) Schematic structure of the HIV-1 p55^{gag}-producing plasmid pNLGAGRRE and the *rev* cDNA prev. LTR, long terminal repeat; RRE, Rev-responsive element. (B) Western immunoblot of cell extracts from HLtat cells transfected with pNLGAGRRE and a serially diluted prev plasmid. The amount of Rev-producing plasmid (prev) used in each transfection is indicated. (C) HLtat cells were cotransfected with pNLGAGRRE and various amounts of prev. The concentration of intracellular HIV-1 Gag protein in each transfection was quantitated in an HIV-1 p24^{gag} antigen capture ELISA. The concentration of Gag in each sample was plotted against the amount of *rev* plasmid used for transfection.

Translation of the *rev* ORF in response to alterations of the sequence surrounding the *tat* AUG on the HIV-1 *tat* mRNA. We have previously shown that the HIV-1 *tat* cDNA does not produce detectable levels of Rev (49, 53). Transfection experiments revealed that the *tat* cDNA ptat (Fig. 2A) failed to activate HIV-1 Gag production from pNLGAGRRE (Fig. 2B), as expected. The sequence surrounding the *tat* AUG conforms with the consensus sequence for efficient initiation of translation at positions −3 and +4 (Fig. 2A) (30, 31). A purine (A or G) at position −3 and a G nucleotide at position +4 are required for efficient initiation of translation at a given AUG (30, 31). Alteration of these positions results in less efficient translation initiation and allows scanning ribosomes to bypass the AUG and initiate translation at downstream AUGs. Changing the sequence surrounding the *tat* AUG to a sequence homologous to that surrounding the *rev* AUG (Fig. 2A), which has a C at position −3 and a G at position +4, resulted in increased Rev production (Fig. 2B) (53). Comparison of the levels of Rev produced in cells transfected with 125 ng of the mutant *tat* cDNA ptatR with Rev levels produced in cells transfected with 125 ng prev revealed that only ~12% of the ribosomes initiated translation at the *rev* AUG (Fig. 2B), which is in agreement with previous results (53). To investigate whether changing both position −3 and position +4 to nonconsensus nucleotides would result in higher Rev production, positions −3 and +4 were both changed to C (30, 31), resulting in plasmid ptatW (Fig. 2A). This plasmid was cotransfected with plasmid pNLGAGRRE, using amounts of ptatW plasmid that resulted in a Gag response in the linear range of the assay. Interestingly, transfection with ptatW gave rise to a Gag response similar to that observed for ptatR (Fig. 2B), in which

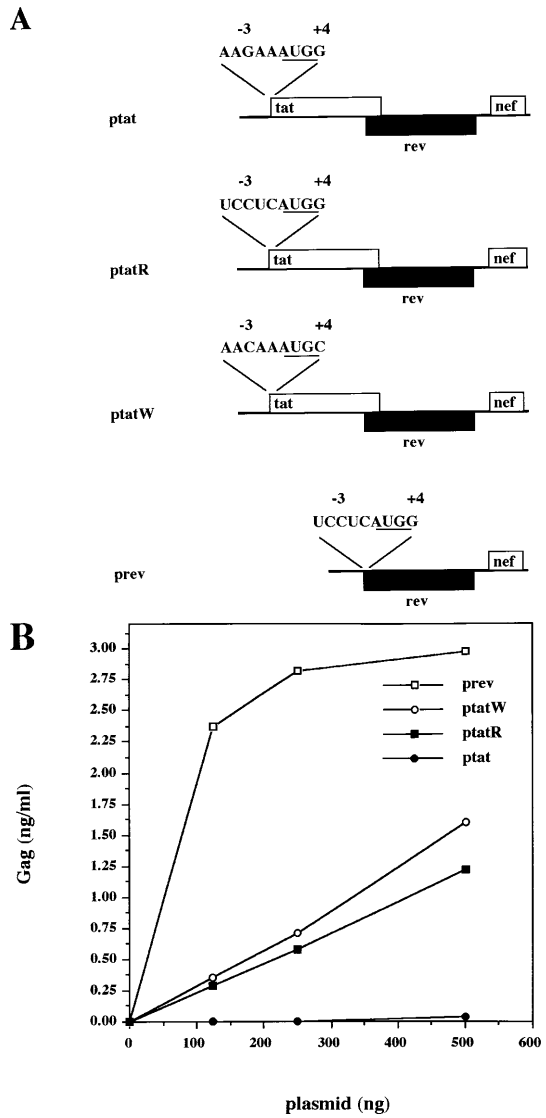

FIG. 2. (A) Schematic structures of mRNAs produced by *rev*, *tat*, and mutated *tat* cDNAs. Plasmid names are indicated on the left. The sequence encompassing the *tat* initiator of translation is displayed. Nucleotide positions −3 and +4 are indicated. (B) Intracellular concentration of HIV-1 Gag in transfected cells versus amount of plasmid used for transfection. The plasmids indicated were serially diluted and cotransfected with pNLGAGRRE into HLtat cells. At 24 h posttransfection, cells were lysed and the amount of intracellular Gag was determined in each transfection by using an HIV-1 p24^{gag} antigen capture ELISA.

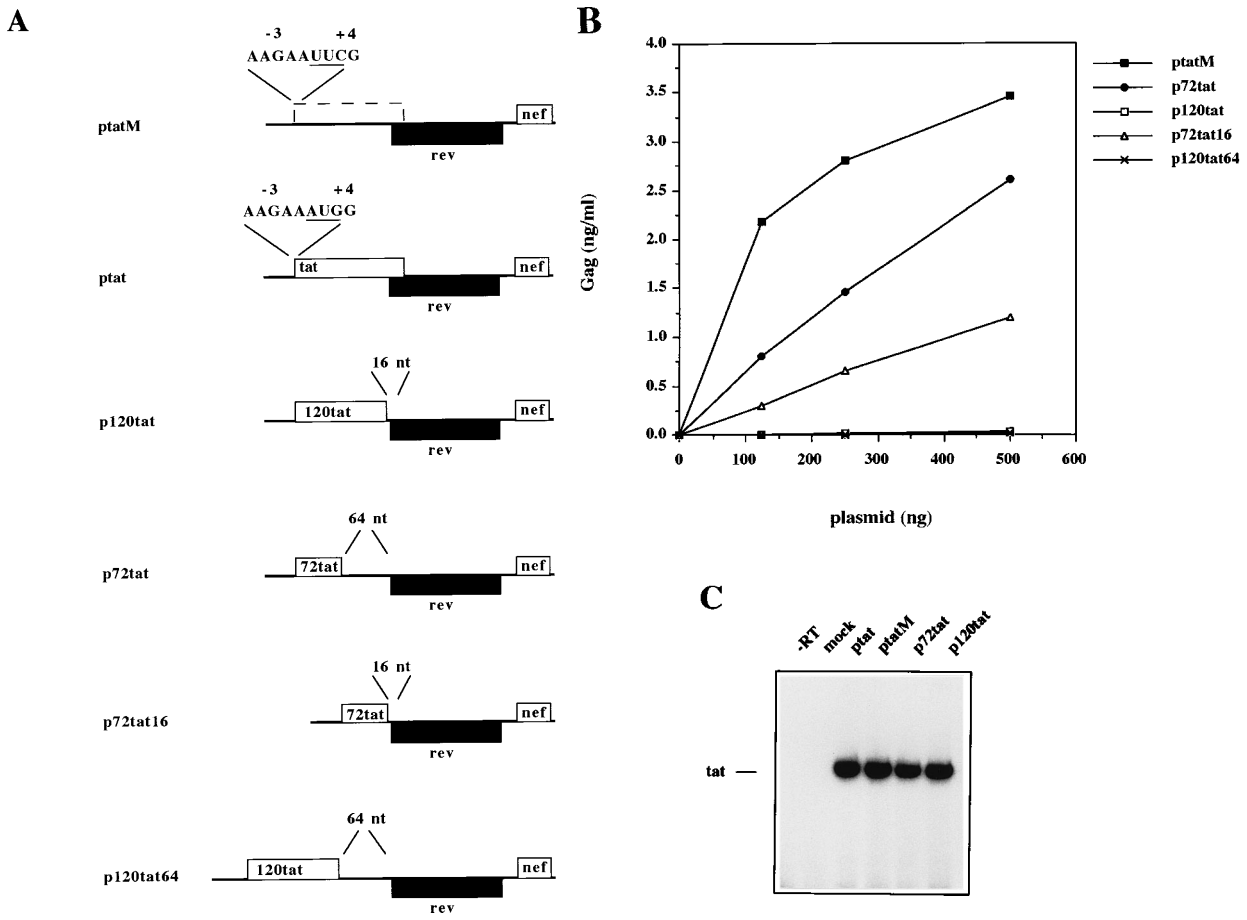

FIG. 3. (A) Schematic structures of mRNAs produced by *rev*, *tat*, and mutated *tat* cDNAs. Plasmid names are indicated on the left. The numbers in the plasmid names indicate the lengths of the various mutant *tat* ORFs in nucleotides. The sequence encompassing the *tat* initiator of translation is displayed, and nucleotide positions −3 and +4 are indicated. The distance between the *tat* and *rev* ORFs is indicated on some mRNAs. (B) Intracellular concentration of HIV-1 Gag in transfected cells versus amount of plasmid used for transfection. The plasmids indicated were serially diluted and cotransfected with pNLGAGRRE into HLtat cells. At 24 h posttransfection, cells were lysed and the amount of intracellular Gag was determined in each transfection by using an HIV-1 $p24^{gag}$ antigen capture ELISA. (C) RT-PCR specifically detecting mRNAs produced from plasmids ptat, ptatM, p72tat, and p120tat. Cytoplasmic poly(A)$^+$ mRNA was extracted from cells transfected with the plasmids indicated. The mRNAs were subjected to random hexamer oligonucleotide-directed cDNA synthesis followed by PCR using oligonucleotides specific for the *tat* mRNAs. The number of PCR cycles was adjusted so as to detect cDNAs in the linear range of the assay.

position −3 but not position +4 was mutated. We concluded that changing the *tat* AUG to a weak start codon resulted in increased *rev* translation. However, the Rev levels were only ~12% of those produced from the wild-type *rev* mRNA, suggesting that factors other than those determining the efficiency of translation initiation at the *tat* AUG inhibit translation of *rev*.

Premature termination of *tat* translation results in high Rev production. We have previously shown that a *tat* cDNA with mutations that inactivated the *tat* AUG produced high levels of Rev, approximately 50% of those produced from the *rev* cDNA (53). However, this mutant cDNA contained an out-of-frame AUG upstream of *rev*. We therefore created a mutant *tat* cDNA in which the *tat* AUG was replaced with UUC, named ptatM (Fig. 3A). This plasmid does not contain any AUGs upstream of *rev* and produced Rev levels similar to those produced by the *rev* cDNA (data not shown), indicating that translation of *tat* is required for inhibition of *rev* translation and that the presence of the sequences upstream of the *rev* splice acceptors did not affect *rev* translation substantially.

To investigate whether the overlap between the *tat* and *rev* ORFs negatively affects translation initiation at the *rev* AUG, a translational stop codon was introduced in the *tat* ORF, 16 nucleotides upstream of the *rev* AUG. The *tat* ORF on the resulting mutant plasmid p120tat (Fig. 3A) is 120 nucleotides long and is predicted to produce a polypeptide of 40 amino acids. pNLGAGRRE was cotransfected with serially diluted p120tat. However, p120tat did not produce detectable levels of Rev (Fig. 3B), demonstrating that inhibition of *rev* translation is not caused by the overlap of the *tat* and *rev* ORFs. Since the p120tat mutant displayed a negative phenotype, the *tat* ORF was deleted and the levels of Rev produced were determined. This plasmid produced similar levels of Rev as did the *rev* cDNA prev (data not shown), verifying that the inability of p120tat to produce Rev was not a result of secondary mutations introduced during the cloning steps.

Inhibition of *rev* translation may be caused by the length of the upstream *tat* ORF. Therefore, we constructed a plasmid in which the *tat* ORF was terminated after 72 nucleotides (Fig. 3A). Interestingly, the resulting plasmid, named p72tat, produced high levels of Rev (Fig. 3B). The levels constituted approximately 38% of those produced by plasmid ptatM, in which the *tat* AUG had been replaced with UUC. These results did not discriminate between inhibition of *rev* translation by *tat*

ORF length or *tat* ORF proximity to the *rev* AUG. To resolve this question, the 72-nucleotide *tat* ORF present in p72tat was moved closer to the *rev* AUG. This resulted in p72tat16 (Fig. 3A), in which the *tat* ORF from p72tat is located 16 nucleotides upstream of the *rev* AUG (the same distance that separates *tat* and *rev* ORFs in p120tat). Figure 3B shows that p72tat16 produced approximately twofold-lower levels of Rev compared with p72tat. Therefore, the major reason for the inability of p120tat to produce Rev is the length of the upstream *tat* ORF, while the intercistronic distance between *tat* and *rev* played a minor role. If this conclusion is correct, increasing the intercistonic distance between the *tat* and *rev* ORFs in p120tat from 16 nucleotides to 64 nucleotides (the same intercistonic distance that separates *tat* and *rev* ORFs in p72tat) should not result in increased Rev production. Figure 3B shows that p120tat64 (Fig. 3A), in which the 120-nucleotide *tat* ORF terminates 64 nucleotides upstream of the *rev* AUG, does not produce increased levels of Rev compared with p120tat. We concluded that the major reason for the inability of p120tat and p120tat64 to produce Rev is the length of the *tat* ORF.

It could be argued that the sequence alterations introduced in p72tat activated cryptic splice signals, which generated spliced mRNAs on which the *tat* AUG had been omitted. Such mRNAs could potentially produce high levels of Rev. To investigate if spliced mRNAs were produced from p72tat, poly(A)$^+$ mRNA was prepared from cells transfected with ptat, ptatM, p120tat, and p72tat, respectively, and subjected to random hexamer-primed cDNA synthesis followed by PCR using one oligonucleotide hybridizing to the 5′ untranslated leader sequence and one oligonucleotide hybridizing downstream of the *rev* AUG. This oligonucleotide pair would detect mRNA species from which the *tat* AUG had been deleted by splicing. RT-PCR analysis of poly(A)$^+$ mRNA produced from cells transfected with the various *tat* cDNAs resulted in only one band, corresponding in size to the wild-type *tat* mRNA (Fig. 3C). The number of PCR cycles was adjusted so as to amplify cDNAs in the linear range of the assay. Figure 3C shows that similar levels of mRNAs were produced from the various plasmids. These results demonstrated that the high level of Rev produced from p72tat was not a result of aberrant splicing. We concluded that premature termination of *tat* translation resulted in high Rev production.

Complementation of a *rev*-defective provirus with a mutant *tat* cDNA: production of functional levels of Rev as a result of premature termination of *tat* translation on the *tat* mRNA. To further investigate the importance of the length of the upstream *tat* ORF in inhibition of *rev* translation, we examined the ability of p72tat to complement in *trans* the *rev*-minus mutant HIV-1 provirus HXB2fB (13). As a result of the mutation in the *rev* gene, plasmid pHXB2fB does not produce virus upon transfection into eucaryotic cells (13). To investigate if the mutant *tat* cDNAs could complement in *trans* the *rev* defect, pHXB2fB was cotransfected separately with p72tat, p120tat, ptatM, ptatW, and ptat. Cell culture medium was collected at different time points posttransfection and subjected to PEG precipitation. The amount of extracellular particle associated HIV-1 p24^{gag} antigen was determined in a p24^{gag} antigen capture ELISA. The results showed that p72tat efficiently activated production of HIV-1 virions (Fig. 4). As expected, ptatM also activated production of virus, while ptat and p120tat failed to complement the *rev*-minus mutant provirus (Fig. 4). The amount of virus produced in the presence of p72tat was approximately 42% of that produced in the presence of ptatM. This result is similar to those in Fig. 3B, which show that when 125 ng of p72tat or ptatM was used in transfections with pNLGAGRRE, the levels of Rev produced from

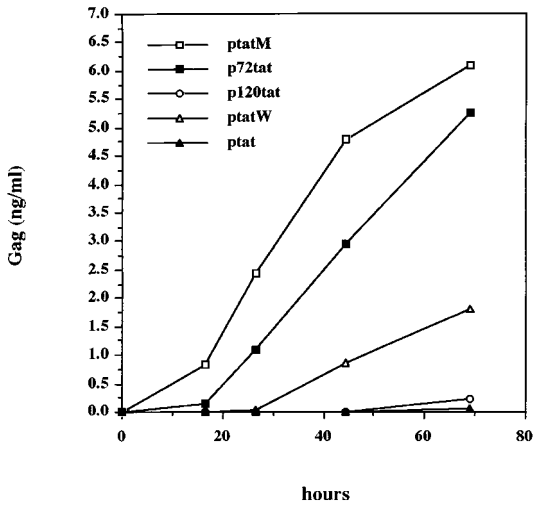

FIG. 4. The *rev*-defective HIV-1 proviral molecular clone pHXB2fB (13) was cotransfected with each of the five cDNA expression plasmids ptatM, p72tat, p120tat, ptatW, and ptat. Cell culture supernatants were harvested at different time points after transfection and were subjected to PEG precipitation. The amount of particle associated HIV-1 Gag was determined in a Gag antigen capture ELISA assay.

p72tat were ~38% of those produced from ptatM. Therefore, pHXB2fB- and pNLGAGRRE-based complementation assays gave similar results. The levels of Rev produced from p72tat were sufficient to restore virus production from a *rev*-defective HIV-1 proviral clone, while the mutant tat cDNA p120tat, which contains a longer *tat* ORF, failed to complement the Rev defect (Fig. 4). Virus production from cells transfected with p72tat or with ptatW, which contains a weak *tat* AUG, revealed that approximately fourfold-lower levels of virus were produced from cells transfected with ptatW compared with cells transfected with p72tat (Fig. 4). These results verified that mutations that decreased the length of the *tat* ORF had a greater effect on Rev translation than mutations that decreased the strength of the translational initiator of *tat*, thus underscoring the importance of the length of the *tat* ORF for inhibition of *rev* translation.

Correlation between length of the *tat* ORF and inhibition of translation initiation at downstream AUGs. Since the 72-nucleotide *tat* ORF present on p72tat allowed high production of Rev, while the 120-nucleotide *tat* ORF present on p120tat efficiently blocked Rev production, we wished to determine the minimal length of the *tat* ORF that inhibited translation of *rev*. We therefore generated additional mutants in which translational terminators were introduced at various positions in the *tat* coding region to create *tat* ORFs consisting of 87, 99, or 111 nucleotides (Fig. 5A). The amount of Rev protein produced from the resulting mutant cDNA expression plasmids p87tat, p99tat, and p111tat was assessed by analyzing their abilities to complement in *trans* the *rev*-defective provirus pHXB2fB. Production of extracellular virus was monitored by the HIV-1 p24^{gag} antigen capture ELISA (56) at various time points posttransfection. The results from one representative experiment are shown in Fig. 5B. These results demonstrated that all mutants except p120tat produced Rev and induced production of virus (Fig. 5B). Comparison of virus levels induced by the various mutants revealed that there was a gradual decrease in Rev production with increasing length of the *tat* ORF (Fig. 5B).

To examine in detail the correlation between length of the

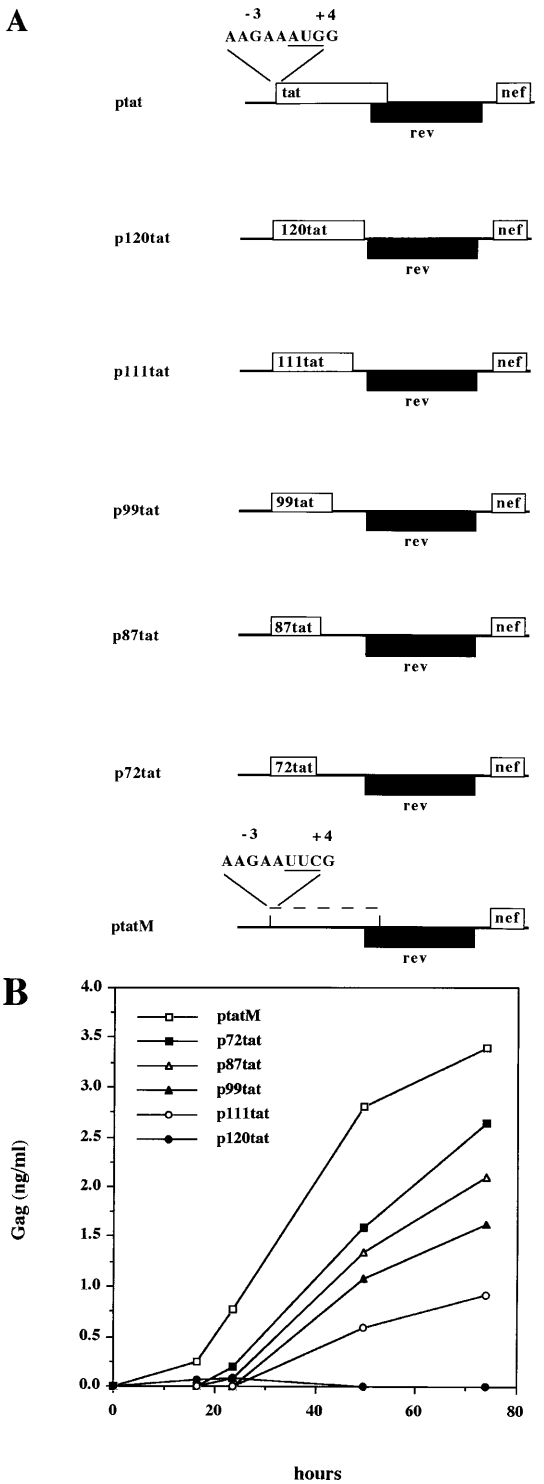

FIG. 5. (A) Schematic structures of mRNAs produced by *rev*, *tat*, and mutated *tat* cDNAs. On two mRNAs, the sequence encompassing the *tat* initiator of translation is displayed, and nucleotide positions −3 and +4 are indicated. Plasmid names are indicated on the left. The numbers in the plasmid names indicate the lengths of the various mutant *tat* ORFs in nucleotides. (B) The *rev*-defective HIV-1 proviral molecular clone pHXB2fB was cotransfected with 0.5 μg of each cDNA expression plasmid indicated. Cell culture medium was harvested at different time points after transfection and was subjected to PEG precipitation. The amount of particle associated HIV-1 Gag was determined in a Gag antigen capture ELISA assay.

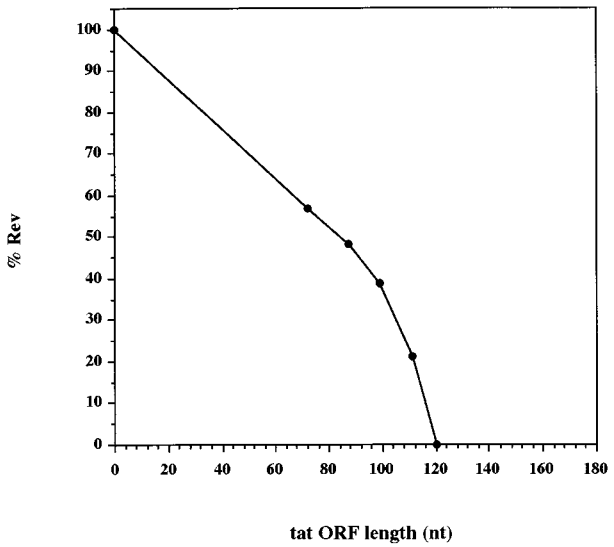

FIG. 6. Graph showing percent Rev translation versus length of the *tat* ORF on the transfected *tat* cDNAs. The levels of extracellular HIV-1 Gag produced in cells cotransfected with pHXB2fB and the various *tat* cDNAs at 48 h posttransfection (shown in Fig. 5) were used to calculate percentage of *rev* translation. nt, nucleotides.

tat ORF and downstream translation initiation, we plotted the percentage of virus produced after cotransfection of pHXB2fB with the mutant cDNAs (compared with cotransfection with ptatM) against *tat* ORF length. Figure 6 shows that there is a linear response between virus production and length of the *tat* ORF for *tat* ORFs shorter than 99 nucleotides. The nonlinear inhibition seen with *tat* ORFs longer than 99 nucleotides may be caused by the close proximity of the *tat* terminator and the *rev* AUG on these mRNAs. This may cause inhibition of *rev* translation, as shown by comparison of Rev production from p72tat and p72tat16 (Fig. 3B). An upstream ORF of 84 nucleotides would inhibit translation at downstream AUGs by 50%. We concluded that the length of the upstream ORF is of critical importance for the ability of ribosomes to reinitiate translation at downstream AUGs.

DISCUSSION

Here we have shown that the ability of ribosomes to reinitiate translation at downstream AUGs is critically dependent on the length of the upstream ORF. The results obtained here allowed us to predict that downstream translation initiation is inhibited 50% by an upstream ORF of 84 nucleotides and should be entirely abrogated by an ORF longer than 165 nucleotides (predicted by extrapolating the linear range of the curve in Fig. 6). Furthermore, an intercistronic distance shorter than 37 nucleotides, as in p111tat, appeared to negatively affect initiation frequency at downstream AUGs. By introducing mutations into an HIV-1 *tat* cDNA that produces an mRNA exactly identical to the mRNA produced by the virus (49), we could examine the mechanism of translation of mRNAs that contain a minimal number of nucleotide alterations compared with the wild-type mRNA. Furthermore, using a cDNA corresponding to the terminally spliced mRNA ensures that splicing of the mRNA does not occur. This is an advantage since hybrid plasmid constructs may produce mRNAs on which cryptic splice sites are activated (24), which may affect the interpretation of data.

We show that when the upstream *tat* ORF, which normally

efficiently suppresses translation of the downstream *rev* ORF, is shortened and terminates upstream of the *rev* AUG, *rev* is efficiently translated. This occurs despite the fact that the *tat* initiator AUG is located in a favorable context for translation initiation. Several virus species produce mRNAs containing short ORFs located upstream of the coding sequence (5, 25, 41, 54, 57). However, although the short ORFs may contain initiator AUGs situated in a favorable context for translation initiation, efficient translation of the downstream ORFs occurs. Such results have been explained by reinitiation of translation; after translation of the first ORF on the mRNA, ribosomes may remain associated with the mRNA, continue scanning, and initiate translation at downstream AUGs (20, 25, 32, 36, 42–44). Interestingly, it has been reported that the coding sequence of the 66-nucleotide upstream ORF2 present on the human cytomegalovirus gp48 mRNA leader was important for inhibition of downstream translation initiation (9). In yeast cells, expression of the *GCN4* gene is regulated at the level of translation by a complex mechanism involving translation termination and reinitiation of translation at upstream short ORFs (18, 38). In contrast to reinitiation, on picornavirus mRNAs, which lack an mRNA cap structure, ribosomes initiate translation directly at internal AUGs by binding to large highly structured RNA sequences (26, 55). It could be argued that a signal for internal initiation of translation is located at the *rev* AUG and that efficient usage of this internal ribosomal binding site is prevented as a result of steric hindrance of ribosomes translating the overlapping *tat* ORF. It has been proposed that ribosomes engaged in translation of one ORF interfere with ribosomes on an overlapping ORF (11). Translating ribosomes may also not be uniformly distributed on the mRNA (59), which may lead to translational interference when two ORFs overlap. However, steric hindrance appears unlikely since p120tat, in which *tat* terminates before *rev*, does not produce detectable levels of Rev. Furthermore, a linear correlation between *tat* ORF length and *rev* translation is not consistent with internal ribosomal binding. We concluded that a combination of *tat* ORF length, overlap of *tat* and *rev* ORFs, and strength of the *tat* AUG efficiently inhibit translation of Rev. It should be noted that although we altered the sequence surrounding the *tat* AUG (both position −3 and position +4 were changed to nonconsensus nucleotides in ptatW), thereby creating a weak initiator of translation, the Rev levels were still only ~12% of those produced from the *rev* cDNA prev or from ptatM. The fraction of reinitiating ribosomes may be different in different expression systems. Our results suggest that sequences other than those located in the immediate vicinity of the *tat* AUG contributed to the efficiency of translation initiation at the *tat* AUG. Such sequences may form secondary structures which slow down scanning of the 40S subunit (29). This may increase the initiation efficiency at the *tat* AUG and would result in relatively low Rev production even from ptatR and ptatW.

The reason for the strong inhibitory effect of the *tat* ORF on *rev* translation may reflect the fact that in the viral life cycle, Rev has a negative effect on Tat, Rev, and Nef production through a feedback mechanism (12). The *tat* initiator of translation conforms well with the consensus for efficient translation initiation whereas the *rev* AUG does not (53), indicating that production of Tat is favored over production of Rev at an early stage of the viral life cycle. When high levels of all viral mRNAs have been induced by Tat, Rev induces a switch from the early to the late phase in the viral life cycle (6, 14, 28, 46). One may speculate that high production of Rev at an early stage would prematurely downregulate Tat production and promote entrance into the late phase of the viral life cycle in the presence of suboptimal mRNA levels. This would result in inefficient virus production and may affect the ability of the virus to escape the immune surveillance. Another example of temporally regulated gene expression is found in the herpesvirus family. It has been shown for human cytomegalovirus that some viral mRNAs appear long before their gene products (16), suggesting that translation is under negative control. Delay of translation may be mediated by short upstream ORFs present on these mRNAs. Therefore, the presence of upstream ORFs may be a general mechanism to delay expression of temporally regulated viral genes.

Both the presence of a strong *tat* AUG and the location of the major part of the *tat* coding sequence upstream of the *rev* AUG are conserved properties of all primate lentiviruses. On the other hand, on the equine lentivirus equine infectious anemia virus (EIAV) bicistronic *tat-rev* mRNA, the initiator of the *tat* ORF is CUG, which allows for leaky scanning translation and expression of the downstream *rev* ORF (4). In addition, the *tat* ORF terminates upstream of *rev*. Interestingly, as a result of mutating the EIAV *tat* initiator into a strong AUG initiator, translation of the downstream *rev* ORF was dramatically reduced (4), indicating that Rev is produced as a result of leaky scanning and not as a result of termination and reinitiation. The EIAV *tat* ORF is longer than 165 nucleotides. According to the results obtained here, the EIAV *tat* ORF therefore should not allow efficient reinitiation of translation at downstream ORFs as a result of its length. Similarly, on the HIV-1 *rev* mRNA, *rev* terminates upstream of the *nef* ORF, and as a result of leaky scanning at the *rev* AUG, high levels of Nef, in addition to Rev, are produced from this mRNA (53). Alteration of the *rev* initiator to generate a *rev* AUG in a good context for translation initiation resulted in dramatically reduced Nef production from the biscistronic *rev-nef* mRNA (53). This occurred despite the fact that the upstream *rev* ORF terminates upstream of the *nef* AUG and is efficiently translated, demonstrating that reinitiation of translation did not occur. Again, the upstream *rev* ORF is longer than 165 nucleotides and therefore is predicted by the results obtained here not to allow for reinitiation of translation. A weak *rev* AUG allows for high *nef* expression on the *rev* mRNA by leaky scanning, while a mutated, weak *tat* AUG on the *tat* mRNA does not result in efficient initiation at the downstream *rev* AUG. This may be due to the fact that *tat* and *rev* ORFs overlap. Ribosomes translating *tat* may negatively affect translation initiation of scanning ribosomes at the *rev* AUG. Alternatively, the *tat* mRNA may contain sequences that enhance the initiation frequency at the *tat* AUG.

Determination of mRNA sequences from various viruses has revealed that several viral mRNAs are structurally multicistronic. In many cases the upstream ORFs encode only short peptides, while in other cases more than one functional protein is produced from each mRNA. It is interesting that when upstream ORFs present in the leader sequences on many viral mRNAs contain initiators which are in a good context for translation initiation, the ORFs are shorter than 165 nucleotides (20, 25, 41, 44). Therefore, these ORFs allow for efficient reinitiation of translation. On hybrid mRNAs containing two nonoverlapping ORFs encoding functional products, 100- to 1,500-fold inhibition of translation of the downstream ORF has been reported (1, 27), while hybrid mRNAs containing short upstream ORFs show efficient reinitiation of translation (32, 36). This indicates that termination of translation of the 5′-proximal ORF upstream of the second ORF is not sufficient for reinitiation to occur. This is in agreement with the results obtained here, demonstrating that termination-reinitiation of translation is dependent on the length of the upstream ORF.

When more than one functional protein is produced from one mRNA species, it is in most cases a result of leaky scanning at the first AUG (4, 50, 58). However, the cauliflower mosaic virus 35S mRNA contains several ORFs that are translated in infected cells (3). In this case, upstream ORFs longer than 165 nucleotides allow downstream translation initiation. However, this occurs only in the presence of a virally encoded transactivator protein that selectively enhances translation of downstream ORFs (15). The arrangements of ORFs on an mRNA may therefore allow for regulation at the level of translation.

It has been reported that efficiency of reinitiation of translation on mRNAs expressed in eucaryotic cells increased by inserting multiple copies of the AGCTTACTTACTTACTT sequence between the upstream minicistron and the downstream preproinsulin ORF (32). It was suggested that increasing the intercistronic distance would provide more time for the small ribosomal subunit to acquire Met-tRNA. In yeast cells, translation of *GCN4* was reduced as the upstream short ORF1 was moved progressively closer to the *GCN4* AUG (18). Binding to ribosomes of factors required for reinitiation was proposed to be a rate-limiting step, thereby explaining the requirement for a certain intercistronic distance. Here we show that the length of the upstream *tat* ORF is the major determinant of expression levels of the downstream *rev* ORF. However, comparison of the amounts of Rev produced from p72tat and p72tat16 revealed that p72tat16 produced approximately twofold-lower levels of Rev. The p72tat and p72tat16 plasmids encode a Tat protein of the same length, but the *tat* ORF is located 64 and 16 nucleotides, respectively, upstream of the *rev* AUG. The intercistronic distance therefore plays a minor role in determining efficiency of reinitiation on the mRNAs used here. Decreasing the distance between the *tat* and *rev* ORFs further may have a more pronounced effect on *rev* translation. The results displayed in Fig. 6 show that when *tat* ORFs longer than 99 nucleotides were present on the mRNAs, inhibition of *rev* translation increased dramatically. Our interpretation is that factors in addition to the length of the *tat* ORF affect *rev* translation. It is reasonable to assume that as the *tat* terminator is moved closer to the *rev* AUG, efficiency of reinitiation of translation decreases, perhaps as a result of lower efficiency in association of ribosomes with essential translational initiation factors as previously suggested (18, 32). Although the presence of the 120-nucleotide *tat* ORF on p120tat inhibits Rev production entirely, a more accurate estimation of the length of an upstream ORF that abrogates downstream initiation of translation is probably obtained by extrapolating the linear range of the curve. This led to the prediction that a *tat* ORF longer than 165 nucleotides would fully inhibit initiation of translation at downstream AUGs.

To explain the correlation between the length of the upstream ORF and efficiency of reinitiation of translation, we propose that ribosomes that translate short upstream ORFs may still be associated with factors required for translation initiation when they reach the termination codon of the upstream ORF, as has been suggested previously (32, 34). Association with such factors may constitute a prerequisite for efficient reinitiation of translation. These factors may be transiently associated with translating ribosomes and may be gradually lost as translation proceeds. Therefore, to prevent reinitiation of translation at the *rev* AUG and premature Rev production, the portion of the *tat* ORF that is located upstream of the *rev* AUG is longer than 165 nucleotides, and *tat* and *rev* ORFs overlap. It is interesting that a large portion of the Tat protein encoded by the sequence that overlaps the *rev* gene is not required for Tat function (47). The extension at the 3′ end of the *tat* gene may therefore be important to inhibit translation of the *rev* ORF on the *tat* mRNAs.

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