Mechanism of Translation of Monocistronic and Multicistronic Human Immunodeficiency Virus Type 1 mRNAs

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We have used a panel of cDNA clones expressing wild-type and mutant human immunodeficiency virus type 1 (HIV-1) mRNAs to study translation of these mRNAs in eucaryotic cells. The *tat* open reading frame (ORF) has a strong signal for translation initiation, while *rev* and *vpu* ORFs have weaker signals. The expression of downstream ORFs is inhibited in mRNAs that contain the *tat* ORF as the first ORF. In contrast, downstream ORFs are expressed efficiently from mRNAs that have *rev* or *vpu* as the first ORF. All *env* mRNAs contain the upstream *vpu* ORF. Expression of HIV-1 Env protein requires a weak *vpu* AUG, which allows leaky scanning to occur, thereby allowing ribosomes access to the downstream *env* ORF. We concluded that HIV-1 mRNAs are translated by the scanning mechanism and that expression of more than one protein from each mRNA was caused by leaky scanning at the first AUG of the mRNA.

Human immunodeficiency virus type 1 (HIV-1) produces three size classes of RNAs: full-length (9-kb) mRNA, intermediate (4- to 5-kb) mRNAs, and small multiply spliced (2-kb) mRNAs. The size class of small mRNAs consists of more than 12 differently spliced mRNA species that produce the regulatory proteins Tat, Rev, and Nef (4, 23, 49, 59, 67). The *tat* mRNAs contain all three open reading frames (ORFs) for *tat*, *rev*, and *nef* but efficiently express only the *tat* ORF, while the *rev* mRNAs that contain the *rev* and *nef* ORFs express both proteins (67). The intermediate size class of HIV-1 mRNAs is also heterogeneous, consisting of more than 12 differently spliced mRNA species that are either bicistronic, producing Vpu and Env, or monocistronic, producing the functional one-exon Tat protein (68, 69). All intermediate-size HIV-1 mRNAs contain both *vpu* and *env* ORFs, but the mechanism of Env production has not been determined. It was shown that when the *tat* ORF was present upstream of the *vpu* and *env* ORFs on some of the intermediate-size mRNAs, Env and Vpu expression was efficiently inhibited from these mRNAs (68).

In addition to these mRNAs, some strains of HIV-1 generate a fourth class of mRNAs, which contain an additional 116-bp exon (6D in Fig. 1) in the *env* region and express alternative forms of Tat and Rev (5, 65, 67). We have recently characterized two proteins, Tev and 6D-Rev (5), produced from these mRNAs. The *tev* ORF contains the first exon of *tat*, 116 bp of *env*, and the last exon of *rev*. The 6D-*rev* ORF encodes an alternative, inactive form of Rev initiated at an AUG in exon 6D. The large number of mRNAs generated by alternative splicing, many of which express more than one protein, prompted the study of the mechanism of their translation in human cells.

Most cellular mRNAs express only one protein, while several viral mRNAs have been found to contain and express more than one ORF (7, 27, 34, 36). The mechanism by which most eucaryotic mRNAs are translated appears to require binding of the 40S subunit of the ribosome at the 5' end of a capped mRNA (71, 72). The ribosome then scans

the mRNA in the 3' direction until a suitable AUG codon is encountered and initiation of translation occurs (34, 39). In this model, the efficiency of translation initiation depends on the strength of the AUG, which is determined by the surrounding sequence (35). A consensus sequence for efficient initiation of translation, CCPuCCAUGG, where positions -3 and +4 relative to the A in the AUG have been shown to be the most important nucleotides for efficient translation (35, 37), has been derived. If the first AUG of the mRNA lies in a favorable context, it is recognized as a start codon by the majority of the ribosomes and only the first ORF of the mRNA is expressed efficiently. If the first start codon lies in a suboptimal context, leaky scanning takes place: some ribosomes bypass this AUG and continue scanning until another initiator is found. This model explains expression of more than one protein from one mRNA species (34, 39).

A second model for expression of downstream ORFs concerns mRNAs in which the first ORF terminates before the downstream ORF starts (nonoverlapping ORFs). In this case, ribosomes may not always dissociate from and fall off the mRNA when translation of the first ORF is completed but instead may stay on the mRNA and resume scanning (7, 34, 39). These ribosomes may therefore be able to reinitiate at other AUGs and express downstream ORFs. Efficient expression of various genes located after short ORFs with initiator AUGs in optimal contexts for translation initiation have been reported. Some viral mRNAs (13, 19, 22, 28, 33, 51) and yeast mRNAs (47, 81) have long leader sequences containing small ORFs but still express the major downstream ORF efficiently. Regulation of translation by upstream small ORFs has been demonstrated in yeast cells (46–48, 81, 82). Taken together, these findings imply that a short ORF that terminates upstream of the major ORF does not inhibit expression of the major ORF as severely as does an ORF that overlaps the AUG of downstream ORFs, suggesting that reinitiation occurs. As a result of reinitiation of translation, initiation at a downstream AUG should be less dependent on the strength of the AUG of the preceding ORF.

As an alternative to the scanning model, it has been shown

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FIG. 1. (A) Genomic organization of HIV-1. ORFs are indicated as open or shaded boxes. (B) HIV-1 exons (black bars) present in the small multiply spliced mRNAs. The exons are numbered according to Muesing et al. (49) and Schwartz et al. (67), and the positions of the splice sites in the HIV-1 genome are indicated. The numbering system of the HIV-1 genome follows that of the revised HXB2 sequence, where the first nucleotide of the R region is designated +1. (C) Structures of the *tat*, *rev*, and *nef* mRNAs produced by HIV-1 (4, 23, 49, 59, 67). The mRNAs are named for the exons that they contain, as indicated on the left. The proteins expressed by each mRNA are indicated at the far left (49, 67). (D) Structures of the HIV-1 mRNAs containing exon 6D. The mRNAs are named for the exons that they contain. The proteins expressed by each mRNA are indicated. The ORFs are represented by boxes, and the two AUGs at the beginning of the 6D-*rev* ORF (AUG-1 and AUG-2) are indicated.

that ribosomes can initiate translation at internal AUGs of some mRNAs without scanning from the 5' end (for a review, see reference 27). These mRNAs, found in a number of different members of the picornavirus family (29–32, 53–55, 80), contain sequences other than the cap structure that direct the ribosomes to initiate translation directly at the internal AUG of the major ORF (41, 73, 79). As a result, several ORFs in the leader sequence of these mRNAs are bypassed. On the basis of *in vitro* and *in vivo* studies, it has been proposed that internal initiation of translation may also occur in mRNAs that contain the cap structure (8, 11, 26, 66).

We took advantage of the many alternative spliced mRNAs produced by HIV-1 to study the mechanism of translation of the different ORFs. In addition, we mutated the region surrounding the initiator AUGs in some of these mRNAs by mutating cDNA clones corresponding to different mRNAs. All cDNAs and their derivatives were cloned into eucaryotic expression plasmids and analyzed in human cells, since it has been shown that *in vitro* translation systems may not accurately reflect translation of mRNAs in eucaryotic cells (39, 68). The results presented here revealed that HIV-1 mRNAs are translated by the scanning mechanism. The efficient production of more than one protein from some mRNAs was attributed to leaky scanning. The *tat* AUG constitutes a strong signal for initiation of translation, while *rev* and *vpu* AUGs are weak, thereby explaining why some mRNAs efficiently express more than one protein. Furthermore, it was shown that leaky scanning at the *vpu* AUG is necessary for production of HIV-1 Env protein.

MATERIALS AND METHODS

Recombinant plasmids. pNL1.5.7, pNL1.4A.7, pNL1.4.7, pNL1.5.6D.7, pNL1.4.6D.7, and pNL1.5E have been described previously (5, 67, 68). The identification of cDNAs pNL1.4A.6D.7 and pNL1.4B.6D.7 has been reported elsewhere (67); cloning into eucaryotic expression vectors was performed as previously described for other HIV-1 cDNAs (67). pNL1.4A.7fB was generated by digestion of pNL1.4A.7 (67) with *Bam*HI, filling in of the 5' overhang by the large fragment (Klenow) of DNA polymerase I, and subsequent religation with T4 DNA ligase. The resulting plasmid has a 4-bp insertion in the *rev* ORF that inactivates the Rev protein by frameshifting into a reading frame that terminates 111 bp upstream of the authentic *rev* terminator (15, 25, 45, 74). pNL2rev was constructed by subcloning a *Stu*I-*Xho*I fragment of pNL1.3.4A.7 (67) into the *Hpa*I and *Xho*I sites of pNL1.4A.7fB. pNL1.4.7T⁻ was generated by replacing the *Sall*-*Sac*I fragment in exon 4 of pNL1.4.7 with the *Sall*-*Sac*I fragment from the *tat*-mutated HIV-1 proviral clone M80 (61, 62), resulting in an mRNA having the *tat* AUG replaced by a UGA terminator. pNL1.4A.7R/T, pNL1.4.7RL, pNL1.4.7T/R, and pNL1.5EU/T were generated by site-directed mutagenesis (Mutator kit; Stratagene) after subcloning into an M13 vector. The clones were screened by sequencing, and mutated clones were transferred back to the eucaryotic expression plasmids as *Sall*-*Bam*HI fragments for pNL1.4A.7R/T, pNL1.4.7RL, and pNL1.4.7T/R or as *Bss*HII-*Bam*HI fragments for pNL1.5EU/T. Plasmid gp120Δss is a derivative of pNL1.5E that has been cloned into the Bluescript KS⁻ vector and will be described in detail elsewhere (50a). A termination codon has been introduced in the *env* ORF at the processing site, generating gp120 and gp41. Thus, gp120Δss produces only gp120 protein, which is secreted from the transfected cells.

The two plasmids gp120U/T and gp120U⁻ were generated by site-directed mutagenesis (Muta-Gene kit; Bio-Rad). For mutagenesis, single-stranded DNA was prepared from gp120Δss. DH5αF' bacteria were transformed and grown on minimal medium agar plates supplemented with ampicillin. Single colonies were picked, grown to an optical density at 600 nm of 1, and subsequently infected with VCS-M13 (Stratagene) helper phage at a multiplicity of 20:1. The colonies were grown for 16 h in 2× YT supplemented with ampicillin and kanamycin. Single-stranded DNA was prepared according to a standard protocol.

Cells, transfections, and immunoprecipitations. Plasmids were transfected into HLtat cells (67), a HeLa-derived cell line constitutively producing the truncated but functionally active Tat-1 protein, or into HL3T1 cells, a HeLa-derived cell line containing silent copies of an HIV-1 long terminal repeat (LTR)-chloramphenicol acetyltransferase (CAT) construct. Alternatively, plasmids were transfected into the HLfB cells (45) containing a *rev*-minus mutant HIV-1 proviral clone, fB (25), integrated into their genome. Twenty-four hours before transfection, 0.6×10^6 cells were seeded in 60-mm plates. The various amounts of plasmids used for transfection were adjusted to 17 μg/0.5 ml per plate with pUC19 carrier DNA and were transfected by the calcium phosphate coprecipitation technique (21) as described previously (16, 18). Cotransfection with a plasmid producing secreted human placental alkaline phosphatase (HCMV-SEAP) (6) or a plasmid producing CAT (pHCMV-CAT) (a gift from B. Fleckenstein) was used to control for transfection efficiency. Plasmids pNL1.5E, pNL1.5EU/T, gp120Δss, gp120U/T, and gp120U⁻ were cotransfected with the Rev-expressing plasmid pL3crev (17), since Rev protein is required for expression of Env and Vpu proteins from these plasmids (68). The transfected HLtat cells were metabolically labeled with [³⁵S]methionine or [³⁵S]cysteine for 3 h at day 2 after transfection. The cells were lysed in 0.5× RIPA buffer, immunoprecipitated, and analyzed by electrophoresis in 10 or 15% denaturing polyacrylamide gels as described previously (16). The cells transfected with gp120Δss or its derivatives secreted gp120 protein into the medium, which was harvested directly for immunoprecipitation. Immunoprecipitations were performed with rabbit anti-Tat, anti-Rev, anti-Nef, or rabbit anti-gp120 antiserum.

Western immunoblots, p24^{agg} antigen assays, and CAT assays. Transfected HLtat or HLfB cells (67) were harvested in 0.5× RIPA buffer 1 to 2 days after transfection. Proteins were separated on 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose filters. The filters were probed with anti-gp120 antiserum, anti-Nef antiserum, or HIV-1 patient serum followed by ¹²⁵I-labeled protein A as described previously (25). Transfected HLfB cells (45) were harvested in 400 μl of 100 mM Tris-HCl (pH 7.4)–0.5% Triton X-100 and lysed by freezing and thawing. Cell lysates were cleared by centrifugation and analyzed by the p24^{agg} antigen capture assay (Dupont, NEN) according to the manufacturer's recommendations. All Rev-expressing plasmids were titrated in the Rev-complementation assay to determine the amount of plasmid that resulted in p24^{agg} response within the linear range. The p24^{agg} values were then normalized to the amount of plasmid used for transfection. Tat-producing plasmids were transfected into HL3T1 cells, which contain silent copies of an HIV-1 LTR-CAT construct. CAT expression is activated by Tat in a dose-dependent manner. All plasmids were titrated in this functional assay for Tat, and the amount of plasmid that resulted in a CAT response within the linear range of the assay was

determined. The CAT values were then normalized to the amount of plasmid used for transfection. CAT assays were performed as described previously (20).

RNA extraction and Northern (RNA) blot analysis. Total RNA was extracted from transfected cells by the heparin-DNase procedure (40) and subjected to Northern blot analysis. The filters were hybridized to a nick-translated DNA fragment (nucleotides 8304 to 9008) from the 3' end of the HIV-1 genome. The fragment was produced by polymerase chain reaction (63, 64) amplification. The polymerase chain reaction was carried out for 20 cycles under standard conditions with 200 ng of pNL1.4.7 (67) as a DNA template, using the following two primers: 5'-GAAGAATAAGACAGGGCTTGGAAGG-3' (HIV positions 8304 to 8329) and 5'-CCCTGGAAAGTCCCCAGCGGAAAGTCCC-3' (HIV positions 9008 to 8981). The numbering system of the HIV-1 genome follows that of the revised HXB2 sequence (56–58, 75), where the first nucleotide of the R region is designated +1. The revised sequence (HIVHXB2R [50]) is included in the data base under accession number K03455. The amount of radioactivity on the blots was quantitated using the AMBIS radioanalytic imaging system.

RESULTS

The *tat* AUG is a strong signal for initiation of translation and inhibits expression of downstream ORFs. The small multiply spliced HIV-1 mRNAs 1.4.7, 1.4A.7, 1.4B.7, and 1.5.7 contain one to three ORFs (*tat*, *rev*, and *nef*; Fig. 1) (4, 23, 49, 59, 67). Expression of these mRNAs in eucaryotic cells demonstrated that mRNA 1.4.7, which contains three ORFs (*tat*, *rev*, and *nef*), expressed primarily the first ORF (*tat*). These results suggested that internal initiation of translation did not occur at the *rev* or *nef* AUG, since if that was the case, mRNA 1.4.7 would have also produced high levels of Rev and Nef. The two *rev* mRNAs, 1.4A.7 and 1.4B.7, which contain both *rev* and *nef* ORFs, produced high levels of both proteins (67). These results were consistent with the hypothesis that the multiply spliced mRNAs were translated by the scanning mechanism and that the presence of the *tat* ORF prevented expression of downstream ORFs, while the presence of the *rev* ORF did not (67).

To investigate whether the inability of mRNAs containing the *tat* ORF to express downstream ORFs was due to the presence of the *tat* AUG on these mRNAs, *tat* cDNAs with different mutations in the sequence surrounding *tat* AUG were generated and analyzed for expression of Tat, Rev, and Nef. pNL1.4.7T/R has five nucleotides upstream of the *tat* AUG replaced by the five nucleotides located upstream of the *rev* AUG (Fig. 2A and 3), while pNL1.4.7T⁻ has the *tat* AUG converted to the stop codon UGA (Fig. 2A). The production of Tat, Rev, and Nef from these mutants was determined by immunoprecipitation after transfections into HLtat cells (67), which constitutively produce the 14-kDa one-exon Tat (Tat-1) protein, and was compared with the expression of Rev and Nef from the *rev* mRNA 1.4A.7 and from the *tat* mRNA 1.4.7. Figure 2B shows that, as expected, pNL1.4.7T⁻ did not produce Tat but expressed high levels of Rev and Nef. The levels of Rev and Nef were comparable to those produced by the *rev* mRNA 1.4A.7 (Fig. 2B). The second mutant, pNL1.4.7T/R, expressed high levels of Tat (indicated as Tat-2 in Fig. 2B), similar to those produced by the wild-type *tat* mRNA 1.4.7 (67). Rev could not be detected by immunoprecipitation from 1.4.7T/R or from 1.4.7. Nef was expressed at similar but very low levels from both mRNA 1.4.7T/R and mRNA 1.4.7. These results

FIG. 2. (A) Structures of the wild-type *rev* mRNA 1.4A.7, wild-type *tat* mRNA 1.4.7, and mutated *tat* mRNAs 1.4.7T/R, 1.4.7RL, and 1.4.7T-. In 1.4.7RL, the portion that is derived from the *rev* cDNA is in black, while the portion that is derived from the *tat* cDNA is indicated in gray. The sequences surrounding the *tat* and *rev* AUGs are shown on top for each mRNA. Sequence changes introduced by site-directed mutagenesis are indicated in smaller letters. Protein expression levels are summarized at the right. Tat

	-3	+4
CONSENSUS	CC A/G CC	AUG G
gag	GA G AG	AUG G
env	UG G CA	AUG A
vif	GG A UU	AUG G
vpr	GA U AG	AUG G
vpu *	AU G UA	AUG C
tat	AA G AA	AUG G
rev	CU C CU	AUG G
6D-rev-1	GG A GA	AUG A
6D-rev-2	UG A UA	AUG G
nef	AU A AG	AUG G

FIG. 3. Translation initiation regions for HIV-1 ORFs compared with the consensus. AUG start codons are underlined. Nucleotides in agreement with the consensus are in boldface. *The sequence surrounding the *vpu* AUG was obtained from the proviral clone pNL43 (1), while the other sequences were from clone HXB2 (56–58).

indicated that the *tat* AUG inhibits expression of downstream ORFs.

To examine the mRNA levels produced by the various constructs, we analyzed RNA from cells transfected with the various cDNAs (Fig. 2C). These experiments demonstrated that all of the plasmids examined expressed similar levels of mRNA and that only one mRNA species was produced by each plasmid. These results indicated that the differential protein expression was determined at the level of translation.

To detect low levels of Rev produced by the various *tat* cDNAs, a very sensitive and quantitative complementation assay for Rev function was used. A HeLa-derived cell line (HLfB) (45) containing a *rev*-minus mutant HIV-1 provirus (fB [25]) stably integrated in the genome of the cells was transfected with the various cDNA expression plasmids. HLfB cells produce low levels of p55^{gag}; complementation by Rev results in high expression of p55^{gag} and the appearance of p24^{gag} and p17^{gag}. Complementation was quantitated by a p24^{gag} antigen capture assay using cell extracts from transfected HLfB cells. To demonstrate that increasing amounts of Rev protein resulted in proportionally increased levels of p24^{gag}, we transfected HLfB cells with serial dilutions of the *rev* cDNA expression plasmid pNL1.4A.7 and measured p24^{gag} production (Fig. 4A). The same samples were also analyzed by Western immunoblot (Fig. 4B), which detects both p24^{gag} and p17^{gag}. The results demonstrated that for *rev* plasmid levels of less than 100 ng, there

expression from the wild-type *tat* cDNA pNL1.4.7 and Rev expression from the *rev* cDNA pNL1.4A.7 were set as 100%. NT, not tested. (B) Immunoprecipitations of Tat, Rev, and Nef produced by the wild-type and mutated *tat* mRNAs. Expression levels of pNL1.4A.7, pNL1.5.6D.7, and pNL1.4.6D.7 are also shown for comparison. Tat-2 is the complete, two-exon Tat protein produced by the *tat* cDNA. Three immunoprecipitations with preimmune sera (P) are also shown. M, molecular weight markers; T, anti-Tat serum; R, anti-Rev serum; N, anti-Nef serum. (C) Northern blot analysis of mRNAs produced from the various cDNA expression plasmids after transfection into HLtat cells. Total RNA was prepared as described in Materials and Methods and was subjected to blotting and hybridization. The blots were probed with a nick-translated fragment spanning the *nef* ORF as described in Materials and Methods.

FIG. 4. Titration of the *rev* cDNA expression plasmid pNL1.4A.7 in the *trans*-complementation assay for Rev. HLfB cells, which contain a *rev*-minus mutant provirus stably integrated in their genome, were transfected with serially diluted *rev* cDNA. As a result of Rev expression, p24^{gag} is produced by the virus. The cells were lysed, and the levels of p24^{gag} were determined by p24^{gag} antigen capture ELISA (A) or by Western immunoblot (B). The Western blot was probed with HIV-1 patient serum that also detected p17^{gag}.

is a linear response in p24^{gag} expression. This assay was used to quantitate Rev expression from the various *tat* cDNAs. We transfected HLfB cells with serial dilutions of the different cDNA expression plasmids to ensure a p24^{gag} response within the linear range of the assay. The absorbance value obtained in the p24^{gag} enzyme-linked immunosorbent assay (ELISA) was plotted versus the amount of plasmid DNA used in each transfection (Fig. 5). The results are also summarized in Fig. 2A as percentage of p24^{gag} produced by each plasmid compared with the amount produced by *rev* cDNA pNL1.4A.7, which produced the highest amount of Rev. Transfection with *tat* cDNA pNL1.4.7

FIG. 5. Quantitation of Rev production from the mutant *tat* and *rev* mRNAs. The mutant *tat* and *rev* cDNAs were serially diluted and transfected into HLfB cells. p24^{gag} production was determined and plotted versus the amount of transfected DNA. The amount of plasmid DNA that resulted in a p24^{gag} response in the linear range of the assay was determined. p24^{gag} values were then normalized to the amount of plasmid that was used for transfection and are summarized in Fig. 2A. OD₄₉₀, Optical density (absorbance) at 490 nm.

resulted in production of 500-fold less p24^{gag} (0.2%) compared with the level produced by *rev* cDNA pNL1.4A.7, verifying that very small amounts of Rev are produced from the wild-type *tat* mRNA, as reported previously (67). Production of Rev by pNL1.4.7T⁻ was almost as efficient as that by pNL1.4A.7 (~50% of that obtained by the *rev* cDNA). Mutation of *tat* AUG to UGA in pNL1.4.7T⁻ resulted an out-of-frame AUG which generated a short ORF (Fig. 2A). This may explain the twofold-lower expression of Rev from pNL1.4.7T⁻ than from pNL1.4A.7. Interestingly, complementation by pNL1.4.7T/R showed that this mRNA produced higher levels of Rev than did *tat* cDNA pNL1.4.7 (~5% versus ~0.2% of pNL1.4A.7 levels). To verify that the replacement of the five nucleotides upstream of the *tat* AUG included the complete signal for initiation of translation, we replaced the complete 5' untranslated leader sequence of *tat* mRNA 1.4.7 with the leader sequence of the *rev* mRNA. This resulted in plasmid pNL1.4.7RL (Fig. 2B). Expression of Rev from this plasmid was similar to Rev expression from pNL1.4.7T/R (Fig. 5), demonstrating that translation initiation properties were determined at least primarily by the region surrounding the initiator AUG.

We also quantitated the amounts of Tat produced from the various derivatives of pNL1.4.7. The *tat* cDNA plasmids were transfected into HL3T1 cells, which contain silent copies of an HIV-1 LTR-CAT construct. CAT expression is activated in a Tat dose-dependent manner and therefore reflects the levels of Tat protein (70). The CAT protein

FIG. 6. (A) Structures of *nef* mRNA 1.5.7 and *rev* mRNA 1.4A.7. (B) Transfection assay. *rev* cDNA pNL1.4A.7 and *nef* cDNA pNL1.5.7 were serially diluted and transfected into HLtat cells. The amount of DNA used in the transfections is indicated. Nef expression was determined by Western immunoblot probed with rabbit anti-Nef antiserum. (C) RNA levels produced by pNL1.4A.7 and pNL1.5.7, determined by Northern blot analysis of total RNA prepared from HLtat cells transfected with pNL1.4A.7 or pNL1.5.7.

expressed by each mutant is shown as percentage of CAT activity produced after transfection with the *tat* cDNA pNL1.4.7 (Fig. 2A). pNL1.4.7T⁻ did not produce Tat, as expected. The mutant *tat* cDNAs pNL1.4.7T/R and pNL1.4.7RL produced lower levels of Tat than did the *tat* cDNA 1.4.7. This was predicted by the scanning model, since Rev production from both pNL1.4.7T/R and pNL1.4.7RL increased from ~0.2% to 5% compared with the level from *tat* cDNA pNL1.4.7. We concluded that the *tat* AUG region provides a strong signal for initiation of translation, which severely inhibits expression of downstream ORFs. Replacement of the sequence surrounding the *tat* AUG with the sequence surrounding the *rev* AUG allowed higher expression of the downstream *rev* ORF, indicating that the *rev* AUG is a weaker signal for translation initiation than is the *tat* AUG.

The *rev* ORF allows high expression of downstream ORFs. To investigate the inhibitory effect of the *rev* ORF on expression of the downstream *nef* ORF, we compared Nef expression from the bicistronic *rev* mRNA 1.4A.7 with Nef expression from *nef* mRNA 1.5.7 (Fig. 6A). Plasmids pNL1.4A.7 and pNL1.5.7 were serially diluted and transfected into HLtat cells. Quantitative assessment of nef expression from mRNA 1.4A.7 and 1.5.7 by Western immunoblot revealed that the *rev* mRNA 1.4A.7 produced approximately twofold less Nef protein than did the *nef* mRNA 1.5.7 (Fig. 6B). Northern blot analysis of RNA from HLtat cells transfected with pNL1.4A.7 or pNL1.5.7 showed that the two plasmids produced similar mRNA levels (Fig. 6C). These results demonstrated that the *rev* ORF exerted a

FIG. 7. (A) Structures of the wild-type and mutant *rev* mRNAs 1.4A.7, 1.4A.7fB, and 2rev. fB denotes an insertion of 4 bp in the *rev* ORF, resulting in a frameshift that inactivates the Rev protein. The Rev production from each mRNA was determined in the Rev-complementation assay and normalized to the amount of mRNA produced by each plasmid (see panel C). (B) Immunoprecipitation analysis of Rev and Nef expression. The mutant Rev protein produced from revfB could not be detected by the anti-Rev antiserum used. M, molecular weight markers; P, preimmune serum; R, anti-Rev serum; N, anti-Nef serum. (C) mRNA production by each plasmid, determined by Northern blot analysis in parallel experiments. The amount of radioactivity on the blots was quantitated by using the AMBIS radioanalytic imaging system and is indicated in 10^3 cpm (kcpm) at the bottom.

twofold inhibitory effect on expression of downstream ORFs.

In an attempt to better quantitate the inhibitory effect of the *rev* ORF on the expression of a downstream ORF, we inserted a *rev* ORF expressing an inactive Rev protein upstream of the *rev* ORF on pNL1.4A.7. The resulting plasmid, pNL2rev (Fig. 7A), carries a frameshift mutation within the *rev* ORF that generates a Rev protein displaying a recessive negative phenotype. When this mutation was introduced into plasmid pNL1.4A.7 to generate pNL1.4A.7fB (Fig. 7A), no complementation could be detected after transfection into the HLfB cells, as expected (Fig. 7A). Therefore, analysis of Rev expression from plasmid pNL2rev measured the amount of Rev expressed from the second, nonmutated *rev* ORF. By comparing Rev expression from plasmid pNL2rev with Rev expression from pNL1.4A.7, we were able to measure the effect of the first *rev* ORF expressing the mutant Rev on the expression of the downstream *rev* ORF. Rev and Nef expression from the three plasmids was first analyzed by immunoprecipitation (Fig. 7B). The results revealed that pNL1.4A.7fB produced levels of Nef similar to those produced by the wild-type *rev* mRNA 1.4A.7, verifying that the frameshift mutation in the *rev* ORF

did not affect Nef expression. The mutant Rev protein produced by the *rev*FB ORF could not be detected by the Rev antiserum used here. The pNL2rev plasmid produced low levels of both Rev and Nef, indicating that the *rev*FB ORF formed a strong barrier for expression of downstream ORFs. The levels of Rev produced by pNL2rev were quantitated in the complementation assay for Rev and shown to be 7% of those produced by pNL1.4A.7. However, Northern blot analysis of total RNA from HLTat cells transfected with pNL2rev or pNL1.4A.7 showed that pNL2rev produced approximately 10-fold less mRNA than did pNL1.4A.7 (2,300 cpm versus 24,000 cpm) (Fig. 7C). To evaluate the difference in Rev production between the pNL2rev mRNA and the 1.4A.7 mRNA, the Rev protein levels were normalized to the mRNA levels. This analysis revealed that the Rev production from the pNL2rev mRNA was 70% of the Rev production from *rev* mRNA 1.4A.7, indicating that the *rev* ORF inhibits downstream expression by approximately 30%. These results were in good agreement with the results shown in Fig. 6, which indicated that the *rev* ORF exerts a twofold inhibitory effect on expression of the downstream *nef* ORF. In contrast, the wild-type *tat* ORF allowed only ~0.2% of Rev expression from mRNA 1.4.7 compared with *rev* mRNA 1.4A.7 (Fig. 5). This analysis indicated that the *rev* AUG is weak and allows higher expression of downstream ORFs than does the strong *tat* AUG.

High expression of the downstream *nef* ORF on the bicistronic *rev* mRNA is a result of leaky scanning at the *rev* AUG. The results described above suggested that the ability of *rev* mRNA 1.4A.7 to express efficiently both the *rev* ORF and the downstream *nef* ORF was due to leaky scanning at the *rev* AUG (34, 39). Ribosomes that do not translate the *rev* ORF could therefore initiate translation at the *nef* ORF. Alternatively, these results could be explained by different properties of the terminators for *tat* and *rev*. Ribosomes that translate *rev* could reinitiate at the downstream *nef* ORF, since *rev* terminates upstream of *nef*. This could be achieved by a signal at the *rev* terminator that would prevent ribosomes from dissociating, thus allowing scanning for new AUGs (7, 27, 34, 39). To distinguish between these two mechanisms, we generated a *rev* cDNA in which the five nucleotides upstream of the *rev* AUG were replaced by the five nucleotides upstream of the *tat* AUG. This resulted in pNL1.4A.7R/T (Fig. 8A). A comparison between pNL1.4A.7 and pNL1.4A.7R/T revealed that pNL1.4A.7R/T produced approximately twofold-higher levels of Rev than did pNL1.4A.7 (Fig. 8B). This finding is in agreement with the results shown above demonstrating that initiation of translation at the *tat* AUG occurs with higher efficiency than initiation of translation at the *rev* AUG. More importantly, immunoprecipitation of Nef showed that pNL1.4A.7R/T produced drastically lower levels of Nef than did pNL1.4A.7 (Fig. 8B), while the two plasmids produced equal amounts of RNA (Fig. 8C). These results verified that initiation of translation occurs with higher efficiency at the *tat* AUG than at the *rev* AUG and that a strong AUG in the first ORF on the mRNA efficiently inhibits expression of downstream ORFs. This verified that the major mechanism of expression of both Rev and Nef from the same mRNA is leaky scanning at the weak *rev* AUG. 1.4A.7R/T still produced low levels of Nef protein. This may be the result of leaky scanning at the first AUG of pNL1.4A.7R/T or, alternatively, of reinitiation of translation or internal initiation of translation at a low frequency.

To verify these results, we also analyzed protein expres-

FIG. 8. (A) Structures of *rev* mRNA 1.4A.7 and mutant *rev* mRNA 1.4A.7R/T. The sequence upstream of the *rev* AUG is shown. Sequence changes introduced by site-directed mutagenesis are indicated in smaller letters. (B) Immunoprecipitation of Rev and Nef produced by pNL1.4A.7 and pNL1.4A.7R/T. M, molecular weight markers; R, anti-Rev serum; N, anti-Nef serum. (C) Northern blot analysis of RNA from HLTat cells transfected with pNL1.4A.7 or pNL1.4A.7R/T.

sion from the alternatively spliced HIV-1 mRNA 1.4.6D.7 (5, 67) (Fig. 1D). The first ORF in this mRNA is the *tev* ORF, consisting of the first exon of *tat* linked via the small exon 6D in *env* to the second exon of *rev* (Fig. 1D), followed by the *nef* ORF (5, 67). Therefore, *tev* has the *tat* initiator and the *rev* terminator of translation. We reasoned that mRNA 1.4.6D.7 should not express Nef, since the *tev* ORF contains the *tat* AUG, which inhibits expression of downstream ORFs. However, if the *rev* terminator, used in the *tev* ORF, allowed reinitiation of translation to occur at the *nef* AUG, high levels of Nef should be produced. Expression of plasmid pNL1.4.6D.7 in HLTat cells was analyzed by immunoprecipitation with anti-Tat, anti-Rev, and anti-Nef antisera. The results showed that high levels of Tev were produced, as predicted. Since Tev is a hybrid protein containing Tat and Rev domains, it was detected by both the anti-Tat and anti-Rev antisera (Fig. 9). A direct comparison of Nef expression from 1.4.6D.7 and the bicistronic *rev* mRNA 1.4A.7 revealed that 1.4A.7 produced high levels of Nef, while Nef expression from the *tev* mRNA was undetectable (Fig. 2B). These results verified that the *rev* terminator does not allow ribosomes to reinitiate efficiently at the downstream *nef* AUG, which is in agreement with the results shown in Fig. 8B. These results are also in agreement with data presented above, indicating that a frameshift mutation in the *rev* ORF that changed the *rev* terminator did not affect *nef* expression from the *rev* mRNA 1.4A.7fB (Fig. 7). The

FIG. 9. Immunoprecipitation of Tev, 6D-Rev, and Nef after expression of mRNAs containing exon 6D in HLTat cells. The expressed mRNAs are indicated at the top. The 14-kDa protein precipitated with anti-Tat antiserum is the truncated one-exon Tat protein constitutively produced by HLTat cells (67). A representative parallel immunoprecipitation with preimmune serum (P) is shown. M, molecular weight markers; R, anti-Rev serum; N, anti-Nef serum; T, anti-Tat serum.

rev mRNA 1.4.6D.7 produced less Nef than did the mutant *rev* mRNA 1.4A.7R/T (Fig. 8 and 9). The reason for this is probably that *rev-1* (i.e., the first exon of *rev*; Fig. 1D) and the 6D-*rev* AUGs act as additional barriers for Nef expression from the *tev* mRNA. These results also support the conclusion that internal initiation of translation does not occur on these mRNAs, since that would have resulted in high expression of Nef both by the *tev* and *tat* mRNAs.

The 6D-*rev* initiator AUG is weak and does not support internal initiation of translation. HIV-1 has the potential to express a protein named 6D-Rev. 6D-Rev protein initiates at one of two methionines in exon 6D within the *tev* ORF (5, 67). Several cloned HIV-1 mRNAs contain the 6D-*rev* ORF, as shown in Fig. 1D. The results described above predicted that the 6D-*rev* ORF should not be expressed from 1.4.6D.7 mRNA. To verify that internal initiation of translation did not occur at the 6D-*rev* initiator, expression of 6D-Rev from mRNAs 1.5.6D.7, 1.4A.6D.7, 1.4B.6D.7, and 1.4.6D.7 (Fig. 1D) (5, 67), was analyzed. The results showed that all mRNAs containing exon 6D except for 1.4.6D.7 expressed the 18-kDa 6D-Rev protein (Fig. 9). This finding indicated that internal initiation of translation did not occur at the 6D-*rev* AUG and further verified that the *tat* AUG prevented expression of all downstream ORFs, while the *rev* AUG did not.

To investigate the strength of the 6D-*rev* AUG, we analyzed the expression of the downstream *nef* ORF from 1.5.6D.7, 1.4A.6D.7, and 1.4B.6D.7 by immunoprecipitation. The results showed that mRNA 1.5.6D.7, on which 6D-*rev* is the first ORF, produced high levels of Nef, comparable to those produced by *rev* mRNA 1.4A.7 (Fig. 2B and 9). This finding demonstrated that the 6D-*rev* ORF exerted an approximately twofold inhibitory effect on Nef expression; therefore, the 6D-*rev* AUG constitutes a weak signal for translation initiation. Interestingly, both 1.4A.6D.7 and 1.4B.6D.7 expressed approximately twofold-lower levels of 6D-Rev and Nef protein than did 1.5.6D.7, in which the first exon of *rev* (*rev-1*) is the first ORF. This finding indicated that the *rev-1* ORF inhibits expression of downstream ORFs to the same extent as does the complete *rev*

FIG. 10. (A) Structures of wild-type *env* mRNA 1.5E and mutant *env* mRNA 1.5EU/T. The sequence surrounding the *vpu* AUG is shown on top of each mRNA, and the sequence changes introduced by site-directed mutagenesis are indicated in smaller letters. (B) Immunoprecipitations of Vpu after metabolic labeling of HLTat cells transfected with pNL1.5E or pNL1.5EU/T. M, molecular weight markers; P, preimmune serum; U, anti-Vpu antiserum. (C) Western immunoblot analysis of Env expression from pNL1.5E and pNL1.5EU/T in HLTat cells, as described in Materials and Methods. (D) Northern blot analysis of RNA prepared from HLTat cells transfected with pNL1.5E or pNL1.5EU/T.

ORF. The polypeptide expressed from the *rev-1* ORF would consist of 30 amino acids including the amino terminus of Rev and was not detected by the anti-Rev antiserum used here, which was raised against the carboxy terminus of Rev. In conclusion, translation of HIV-1 mRNAs containing exon 6D is also consistent with the scanning model.

Env expression requires a weak AUG in the preceding *vpu* ORF. The results presented above indicated that expression of more than one ORF per mRNA was dependent on the efficiency of translation initiation at the first initiator AUG. We next analyzed the mechanism of *env* expression by the bicistronic *env* mRNAs of HIV-1. We have previously shown that *env* is the only HIV-1 ORF that does not have a splice acceptor immediately upstream of its initiator AUG. Env is expressed as the second ORF from multiple mRNAs such as 1.5E (Fig. 10A), which encode both *env* and *vpu* (68). We proposed that expression of *env* was due to leaky scanning at the *vpu* AUG. We reasoned that replacement of the sequence surrounding the *vpu* AUG with the sequence surrounding the *tat* AUG (which does not allow leaky scanning) should result in inhibition of expression of the *env* ORF. To test this, the cDNA expression plasmid pNL1.5E (Fig. 10A), which produces both Vpu and Env, was subjected to site-directed mutagenesis at the *vpu* AUG. In the resulting plasmid, pNL1.5EU/T (Fig. 10A), the sequence surrounding the *vpu* AUG has been replaced by the sequence surrounding the *tat* AUG. pNL1.5E and pNL1.5EU/T were transfected into HLTat cells in the presence of Rev and analyzed for Vpu and Env expression by immunoprecipitation and immunoblotting, respectively. The results showed that both plasmids expressed Vpu (Fig. 10B) and that Vpu expression was slightly increased in the mutant pNL1.5EU/T. Interestingly, only pNL1.5E produced high

FIG. 11. (A) Structures of mRNAs gp120 Δ ss, gp120U/T, and gp120U⁻. The sequence surrounding the *vpu* AUG is shown on top of each mRNA, and the sequence changes introduced by site-directed mutagenesis are indicated in smaller letters. (B) Immunoprecipitations of gp120 protein from gp120 Δ ss, gp120U/T, and gp120U⁻. Three independent clones of gp120U/T were analyzed. M, molecular weight markers; P, preimmune serum; E, anti-gp120 antiserum. (C) Northern blot analysis of RNA from HLTat cells transfected with gp120 Δ ss, gp120U/T, or gp120U⁻.

levels of Env, while Env was undetectable in cells transfected with pNL1.5EU/T (Fig. 10C). RNA analysis of HLTat cells transfected with pNL1.5E or 1.5EU/T revealed that the two plasmids produced similar levels of mRNA (Fig. 10D). These data verified that the *tat* AUG constitutes a strong signal for translation initiation, which inhibits expression of all of the downstream ORFs. An exchange of three nucleotides surrounding the *vpu* AUG, which resulted in a sequence similar to the *tat* AUG, led to a more efficient signal for translation initiation and severely inhibited *env* expression. The mutations also altered an AUG that is located at position -5 upstream of the *vpu* AUG (Fig. 10A). Although this AUG is out of frame and immediately followed by a terminator, its location may have contributed to the poor recognition of the *vpu* AUG. We concluded that production of HIV-1 Env protein requires a weak *vpu* initiator AUG that allows leaky scanning to occur.

To verify these findings, we analyzed Env expression from another HIV-1 *env* expression plasmid, gp120 Δ ss (Fig. 11) (50a). This plasmid is a derivative of pNL1.5E (68) and produces only the surface glycoprotein gp120 due to a terminator codon introduced in the *env* ORF (Fig. 11A). This plasmid also has a point mutation that inactivates the *vpu* initiator AUG (Fig. 11A) and does not produce Vpu protein

(data not shown). The mutated *vpu* AUG region in plasmid gp120 Δ ss was replaced by the *tat* AUG by site-directed mutagenesis, resulting in plasmid gp120U/T (Fig. 11A). Expression of gp120 from plasmid gp120U/T was analyzed by radioimmunoprecipitation and revealed that gp120 was undetectable, as expected (Fig. 11B). We analyzed three independent clones of this plasmid, since it could be argued that the *env* ORF might have been inactivated by mutations that occurred during the cloning steps. To further exclude this possibility, we also inactivated the initiator AUG of the *vpu* ORF in gp120U/T by mutating it back into an ACG by site-directed mutagenesis. The resulting plasmid, gp120U⁻ (Fig. 11A), produced gp120 (Fig. 11B), demonstrating that the *env* ORF is intact both in plasmid gp120U⁻ and gp120U/T. The expression of gp120 from gp120U⁻ was somewhat lower than that from gp120 Δ ss. This may be the result of competition of translation initiation at the ACG at the beginning of the *vpu* ORF, since this ACG is located in a very good context for initiation of translation, and the *env* AUG. Northern blot analysis of HLTat cells transfected with plasmid gp120 Δ ss, gp120U/T, or gp120U⁻ revealed that the RNA and protein data were in agreement (Fig. 11C), indicating that the differential protein expression was regulated at the level of translation. We concluded that Env expression is dependent on a weak AUG in the preceding *vpu* ORF.

DISCUSSION

We have used a panel of alternatively spliced mRNAs generated by HIV-1 to study the mechanism of mRNA expression in eucaryotic cells and the regulation of HIV-1 mRNA translation. We have shown that expression of several proteins from one mRNA is regulated mainly by the strength of the AUG of the first ORF of the mRNA and that translation of the HIV-1 mRNAs follows the scanning mechanism. These conclusions are based on the finding that none of the mRNAs having the *tat* AUG as the first ORF was able to express downstream ORFs efficiently. This was true for naturally occurring mRNAs with a *tat* AUG in the first ORF of the mRNA (1.4.7 and 1.4.6D.7) as well as for mRNAs that were engineered to have a *tat*-like AUG in the first ORF such as 1.4A.7R/T, pNL1.5EU/T, and gp120U/T. Different mutations in the sequence immediately upstream of the *tat* AUG resulted in increased production of the downstream ORFs. The inhibitory effect of the *tat* AUG on the translation of other ORFs was seen only when it was the first AUG of the mRNA. Other ORFs upstream of *tat* were translated efficiently in the presence of the *tat* AUG, such as the *vpr* ORF from *vpr* mRNA (69). This finding further indicates that the HIV-1 mRNAs are translated by the scanning mechanism (34, 39) and that the AUGs of the ORFs are the major determinants of expression of downstream ORFs from bicistronic mRNAs.

The *rev* AUG inhibits expression of downstream ORFs by approximately 30 to 50% (Fig. 6 and 7), while the *tat* AUG inhibits downstream expression by >99.8%. However, when the *tat* AUG is replaced by the *rev* AUG as in pNL1.4.7T/R or pNL1.4.7RL, we still measured substantial inhibition of *rev* expression (95%). Compared with the 30% inhibition on downstream expression exerted by the *rev*FB ORF (Fig. 7) or the 50% inhibition exerted by the *rev* ORF (Fig. 6), it appears that the *rev* AUG is a stronger inhibitor of downstream expression when it is located in the *tat* ORF. This may be due to the fact that the *tat* ORF overlaps the downstream *rev* ORF. Since mRNA 1.4.7T⁻, on which the *tat* ORF is present but not translated, produces almost as high levels of

Rev as does *rev* mRNA 1.4A.7, the results argue that a combination of the strong *tat* AUG and translation per se inhibit expression of the downstream overlapping *rev* ORF. Ribosomes that translate the *tat* ORF may negatively affect initiation of translation at the *rev* AUG. This may be caused by steric hindrance exerted by the translating ribosomes on the scanning ribosomes. This would also explain why mRNA 1.4.7 and 1.4.7T/R produced different amounts of Rev but similar levels of Nef (Fig. 2B). According to this model, the increase in scanning ribosomes would result primarily in an increase in Rev expression, while translation of the downstream *nef* ORF would be less affected. The small increase in Nef expression that would be predicted may not be detected by immunoprecipitation. Alternatively, scanning ribosomes that reach the *rev* AUG on mRNA 1.4.7T/R may stall as a result of translational interference and dissociate from the mRNA. On mRNA 1.4.7, the *rev* AUG may not be recognized efficiently as a result of translational hindrance and the majority of the ribosomes may continue scanning and express Nef. This would result in similar Nef production from mRNA 1.4.7 and mRNA 1.4.7T/R. The efficient inhibition of Rev expression from the *tat* mRNA 1.4.7 may therefore be due partly to the fact that *tat* and *rev* are overlapping. Alternatively, sequences downstream of the *tat* AUG may contribute to the efficient initiation of translation at the *tat* AUG. Further experiments are in progress to determine in detail the mechanism of inhibition of translation of *rev* from the *tat* mRNA.

The fact that the *rev* AUG inhibited expression of downstream ORFs more when located in the *tat* ORF (1.4.7T/R; Fig. 2A) than when located in the *rev* ORF (1.4A.7) could suggest that reinitiation occurred after translation of *rev*. Reinitiation of translation would have resulted in equal levels of Nef from mRNAs 1.4A.7R/T (Fig. 8) and 1.4.6D.7 (Fig. 9). Since these plasmids produced different amounts of Nef, these experiments suggest that leaky scanning is the major mechanism determining the levels of translation. Expression of lower levels of Nef by 1.4.6D.7 is most likely due to the presence of the *rev* and the 6D-*rev* AUGs on 1.4.6D.7. These AUGs overlap the *tev* ORF and constitute an additional barrier for those ribosomes that do not initiate translation at the *tev* AUG.

Replacement of the *vpu* AUG with the *tat* AUG on the Env mRNA 1.5E resulted in a small increase in Vpu production, while Env expression was undetectable. This finding suggests that almost all of the ribosomes initiated translation at the *vpu* AUG on mRNA 1.5EU/T, which would predict a dramatic decrease in Env expression. This is in agreement with the results shown in Fig. 8C. Therefore, a small increase in the efficiency of initiation of translation at the AUG of the first ORF greatly affected the expression of the second ORF. This further supports the conclusion that the HIV-1 mRNAs are translated by the scanning mechanism.

Some mRNAs containing exon 6D (1.4A.6D.7, 1.4B.6D.7, and 1.5.6D.7) have the ability to express Nef even though the second of the two AUGs of 6D-*rev* is predicted to be strong when compared with the consensus (Fig. 3). Since the amount of Nef produced is much higher than that produced from the 1.4.6D.7 mRNA, reinitiation or internal initiation of translation (7, 27, 34, 36) at the *nef* AUG is not the likely mechanism. An alternative explanation is that the close proximity of the two 6D-*rev* AUGs (5) affects the recognition of the strong AUG-2 and that only the weak AUG-1 is recognized. The distance between AUG-1 and AUG-2 is three nucleotides. Two of the mRNAs containing exon 6D, 1.4A.6D.7 and 1.4B.6D.7, have the first exon of *rev* (*rev-1*)

(68) as the first ORF, in contrast to mRNA 1.5.6D.7, which has 6D-*rev* as the first ORF. Comparison of 6D-Rev expression from these mRNAs showed that similar levels of 6D-Rev were produced from 1.4A.6D.7 and 1.4B.6D.7 but that only twofold-higher levels were produced from mRNA 1.5.6D.7. This finding is consistent with results of previous studies, which showed that intermediate-size HIV-1 mRNAs containing the *rev-1* ORF upstream of *env* and *vpu* expressed high levels of Env and Vpu (68). It has been reported that short ORFs that terminate before the AUG of a major downstream ORF appear to inhibit expression of the major ORF to a lesser extent than does an ORF that overlaps the downstream ORF. This has in some cases been attributed to reinitiation of translation (28, 38, 43, 46, 52). We could not assay for expression of Rev-1, since the available antisera recognize the carboxy terminus of Rev. The low inhibition by *rev-1* may be due to reinitiation of translation after translation of *rev-1* or the inability of ribosomes to recognize the short *rev-1* ORF. However, taking into account the results obtained with mRNA 1.4A.7, it is reasonable to assume that expression of 6D-Rev and Nef from mRNA 1.4A.6D.7 and 1.4B.6D.7 is a result of leaky scanning at the *rev-1* AUG.

Our data show that the *tat* AUG is strong and does not allow expression of downstream ORFs. On the contrary, the *rev* AUG is weaker, resulting in high Nef expression from the Rev mRNAs. mRNAs carrying the *nef* ORF as the first ORF are also generated by alternate splicing (mRNA 1.5.7); therefore, the virus does not depend on leaky scanning for Nef expression. A comparison of the AUGs of HIV-1 with the consensus sequence for efficient initiation of translation (Fig. 3) revealed that the *tat* AUG agreed with the consensus at positions -3 and +4, which have been shown to be the major determinants of efficient initiation of translation (35, 37). In contrast, the *rev* AUG did not conform to the consensus, which is in agreement with our results. We next examined whether the strength of these AUGs were conserved among other closely related lentiviruses that are similar in genomic organization to HIV-1 and produce proteins that are similar in function to those of HIV-1 Tat and Rev (3, 10, 12, 14, 24, 42, 44, 83, 84). The sequences surrounding the *tat* and *rev* AUGs in some clones of HIV-2 and simian immunodeficiency virus (SIV) (50) were compared with the consensus for efficient initiation of translation (35, 37). The comparison showed that both HIV-2 and SIV have *tat* AUGs that should be considered strong compared with the consensus, while the *rev* AUGs of both HIV-2 and SIV are predicted to be weaker. This arrangement appears to be conserved among the primate lentiviruses. This may reflect a requirement for higher production of Tat than of Rev protein or, alternatively, a requirement for expression of Tat in the absence of Rev. Since the Rev protein has a negative effect on the expression of Tat, Rev, and Nef through a feedback mechanism (2, 16, 60), it may be beneficial for the virus to inhibit the expression of Rev at an early stage of the viral life cycle.

All HIV-1 *env* mRNAs contain the *vpu* AUG upstream of the *env* ORF (68). Env expression is therefore dependent on leaky scanning at the *vpu* AUG. The *vpu* AUG conformed poorly to the consensus sequence (Fig. 12) (35, 37). Comparison of the *vpu* AUGs of other HIV-1 isolates (50) with the consensus sequence (Fig. 12) revealed that all known HIV-1 isolates have *vpu* initiator AUGs that are predicted to be weak. Interestingly, 3 of 17 viral clones carried mutations that destroyed the *vpu* AUG, while none of them had mutations that were predicted to convert the weak *vpu* AUG

	-3		+4	
CONSENSUS	CC	A/G	CC	AUG G
HIVBRU	AU	G	UA	AUG C
HIVHXB2	AU	G	UA	ACG C
HIVMN	AU	G	UA	AUG C
HIVSC	AU	G	UA	AUG C
HIVJH3	AU	G	UA	AUG C
HIVBRVA	AU	G	UA	AUG C
HIVSF2	AU	G	UA	AUG C
HIVHAN	AU	G	UG	AUG U
HIV0Y1	AA	U	AC	AUG U
HIVCDC451	AU	G	UC	AUG C
HIVSWB882	AU	G	UA	AUG C
HIVRF	AU	G	UA	AUG C
HIVMAL	AU	G	UA	AUA C
HIVELI	AU	G	UA	AUG C
HIVZZ6	AU	G	UA	AUG C
HIVZ6	AU	G	UA	AGC C
HIVNDK	AU	G	UA	AUG C

FIG. 12. Initiator AUGs of the *vpu* ORFs in different HIV-1 clones.

into a strong initiator AUG. This supports the conclusion that Env expression is dependent on leaky scanning at the weak *vpu* AUG. The presence of the *vpu* ORF on the *env* mRNAs may decrease the levels of Env produced by the virus. Some HIV-1 isolates that are adapted to rapid and continuous growth in vitro have mutations eliminating the *vpu* initiator AUG (50, 56–58), in agreement with a role of *vpu* in modulating *env* expression. A comparison of the *env* AUG to the consensus sequence revealed that the *env* AUG should be regarded as suboptimal (Fig. 3), further suggesting a requirement for low Env production. It has been reported that *vpu*-minus mutants of proviral HIV-1 clones induce a higher number of syncytia in transfected or infected cells than does the wild-type viral clone, which has an intact *vpu* ORF (9, 76–78). This observation may be explained by higher production of Env protein from the *env* mRNAs after removal of the *vpu* AUG, which may cause an increase in syncytium formation. *vpu*-minus mutant viral clones also show slower release of viral particles from transfected or infected cells compared with wild-type virus (9, 76–78). This effect was attributed to the function of the Vpu protein, since it was shown that a *vpu* defect could be overcome by *trans* complementation with Vpu. Therefore, an alternative explanation for the bicistronic *env* mRNAs may be that this arrangement reflects a requirement for coordinate expression of Env and Vpu, since both proteins may act at a late stage of the viral life cycle.

In summary, the HIV-1 mRNAs described here are translated by the scanning mechanism. This allows regulation of expression of the different proteins by modulation of the strength of the initiator AUGs. Env expression is completely dependent on a weak *vpu* AUG. This may reflect the need to decrease the production of Env, while the strong *tat* AUG may be critical for inhibition of Rev expression at an early stage of the viral life cycle. Other primate lentiviruses have similar genome organization and complex splicing patterns. It therefore appears that the mechanisms used for HIV-1 expression may also be used in other lentiviruses.

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