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Both Linear and Discontinuous Ribosome Scanning Are Used for Translation Initiation from Bicistronic Human Immunodeficiency Virus Type 1 *env* mRNAs[▽][†]

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Human immunodeficiency virus type 1 (HIV-1) generates 16 alternatively spliced isoforms of *env* mRNA that contain the same overlapping open reading frames for Vpu and Env proteins but differ in their 5′ untranslated regions (UTR). A subset of *env* mRNAs carry the extra upstream Rev initiation codon in the 5′ UTR. We explored the effect of the alternative UTR on the translation of Vpu and Env proteins from authentic *env* mRNAs expressed from cDNA constructs. Vpu expression from the subset of *env* mRNA isoforms with exons containing an upstream Rev AUG codon was minimal. However, every *env* mRNA isoform expressed similar levels of Env protein. Mutations that removed, altered the strength of, or introduced upstream AUG codons dramatically altered Vpu expression but had little impact on the consistent expression of Env. These data show that the different isoforms of *env* mRNA are not redundant but instead regulate Vpu production in HIV-1-infected cells. Furthermore, while the initiation of Vpu translation conforms to the leaky ribosome-scanning model, the consistent Env synthesis infers a novel, discontinuous ribosome-scanning mechanism to translate Env.

Like most cellular pre-mRNAs, the human immunodeficiency virus type 1 (HIV-1) 9.2-kb genome-length RNA transcribed from the provirus acquires a 5′ m^7GpppN cap and a 3′ poly(A) tail. Multiple splice donor (SD) and splice acceptor (SA) sites in the genome-length HIV-1 mRNA support alternative splicing to a pool of 4-kb and 2-kb mRNAs that differ in their 5′ untranslated regions (UTR) (46, 52). These mRNAs contain partly overlapping open reading frames (ORFs) and collectively encode the nine HIV-1 proteins and polyproteins. In the early phase, multiple splicing produces more than 23 different 2-kb HIV-1 mRNAs that are constitutively exported to the cytoplasm for the translation of Rev, Tat, Nef, and Vpr regulatory and accessory proteins (46, 52, 54). Later, after sufficient levels of Rev accumulate in the nucleus, Rev multimers bind to the highly structured RNA of the Rev-responsive element (RRE) that underlies the Env reading frame. This binding promotes the engagement of proteins required for the nuclear export (22, 42) and cytoplasmic accumulation of unspliced genome-length RNA and 23 different spliced 4-kb mRNAs containing the RRE (reviewed in references 15 and 26). The viral Gag and Gag-Pol structural polyproteins are translated from the 9.2-kb RNA, with a ribosome frameshift producing Gag-Pol on 5% of occasions during translational scanning (24, 36). The structural envelope (Env) glycoprotein and accessory and regulatory Vpu, Vif, and Vpr proteins and

amino acids 1 through 72 of Tat (Tat_{72}) are translated from the 4-kb spliced mRNAs (53, 54).

The high level of diversity among the spliced HIV-1 mRNAs results in part from the incorporation of the upstream noncoding exons 2 and 3 (46, 52). These exons may be included separately or together or excluded from the 5′ UTR of alternative 2-kb *nef*, *tat*, and *rev* and 4-kb *env* and *tat* mRNAs. Furthermore, the use of alternative SA sites can expand the diversity of spliced mRNAs (46, 52). For instance, the use of three competing alternative SA sites immediately 5′ to the Rev AUG codon, SA4b, SA4a, and SA4c, results in 12 isoforms of 2-kb *rev* mRNA. These alternative SA sites, together with SA5, are also used for 4-kb *env* mRNA, resulting in 16 *env* isoforms that differ in their 300- to 900-nucleotide-long 5′ UTR (see Fig. 1) (46, 53). For simplicity, the *env* isoforms are referred to herein as *env1* to *env16*, as described previously (46). Selection among the various weak splice sites is controlled by interspersed exonic and intronic splice enhancer and silencer elements (2) whose activity is dictated by RNA binding proteins, including 9G8, SC35, ASF/SF2, and SRp40 (13, 49, 66).

All 16 *env* mRNAs contain ORFs for Vpu, Env, and Nef (46, 53). The Vpu ORF lies upstream of but overlapping that of Env, and the translation of both proteins is reported to proceed by leaky scanning, in which some ribosomes bypass the weak upstream Vpu initiation codon to initiate translation at the strong Env start signal (53). HIV-1 Env is synthesized as a 160-kDa precursor (gp160) that is cleaved to a 120-kDa surface protein (gp120) and a 41-kDa transmembrane protein (gp41) that assemble into mature trimers that protrude from the virion surface (64). Interestingly, Vpu controls the level of mature Env protein available for assembling virions during viral replication (reviewed in reference 10). The Env gp160 precursor and its cellular receptor, CD4, are both translated in the endoplasmic reticulum, where they interact and potentially

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become entrapped (9). Vpu is also translated in the endoplasmic reticulum, where it binds and targets CD4 for degradation (63) through the ubiquitin/proteasome pathway (51). This mechanism frees the Env precursor for correct processing and expression at the cell surface, where it is incorporated into virions (reviewed in reference 10). The coordinated expression of Vpu and Env through leaky scanning translation from the same *env* mRNA may optimize the inclusion of mature Env into virions.

Ultimately, the purpose of the alternatively spliced HIV-1 mRNA isoforms that contain the same ORFs but differ in the 5′ UTR is largely unknown. However, changing the proportions of the alternative mRNAs by mutating redundant SA sites or by modulating the expression of splice control proteins alters the expression of HIV-1 proteins and impairs viral replication and infectivity (13, 46, 47, 49, 61, 66). Mutating specific splice sites in the HIV-1 molecular clone, NL4.3, to redistribute the mRNA isoforms dramatically alters the expression of the viral Env and regulatory Rev and Nef proteins (46). Changes in Env expression for some splice mutants were inversely related to the levels of Rev expression and instead correlated with changes in the isoforms of *env* mRNA (46), suggesting that individual *env* isoforms differing only in their 5′ UTR may express Env protein with different degrees of efficiency.

Here we tested whether the alternative *env* mRNAs provide a means to regulate translation. HIV-1 mRNAs are produced in the nucleus and acquire a 5′ m⁷GpppN cap that binds the eukaryotic initiation factor 4E (eIF4E) complex, which initiates the recruitment of 40S ribosomes that scan for a suitable AUG initiation site (reviewed in references 18 and 30). The different 5′ UTR of HIV-1 *env* mRNAs contain several features predicted to have an impact on ribosome-scanning efficiency. These include (i) highly structured 5′ RNA elements of various lengths (5); (ii) upstream AUG codons for Rev and/or Vpu (28, 29, 41); and (iii) binding sites for various RNA binding proteins implicated either directly in translation, like eIF2 (4), or in translation modulation, like the protein kinase regulated by RNA (PKR), transactivation-responsive RNA binding protein (TRBP), the La autoantigen, and the viral Tat protein (6, 14, 19, 38, 60). In previous work, the noncoding exon 2 of the HIV-1 5′ UTR was also reported to stimulate protein expression while noncoding exon 3 reduces the expression of a heterologous reporter, suggesting that exon 2 and/or 3 may perturb mRNA stability (31). Similar effects may occur with the *env* mRNA containing these noncoding exons, possibly altering Env protein expression.

The use of SA4b, SA4a, and SA4c splice acceptors during *env* mRNA splicing incorporates a weak translation start codon for Rev into the 5′ UTR (see Fig. 1) that may influence the progression of 40S ribosome subunits to translate the downstream Env ORF. Here we tested the effects of the different 5′ UTR of *env* mRNA isoforms on Vpu and Env expression. Unexpectedly, we showed that the different 5′ UTR affect Vpu synthesis, but not Env synthesis, from the *env* mRNA. The differences in Vpu synthesis patterns are explained by the upstream Rev translation initiation codon encountered in some 5′ UTR during ribosome scanning. However, the uniform expression of Env protein despite upstream initiation codons indicates that a discontinuous scanning mechanism akin to an internal ribosome entry site (IRES) or a ribosome shunt is used for Env translation initiation from the HIV-1 *env* mRNA.

MATERIALS AND METHODS

Plasmid construction. The HIV-1 molecular clone pNL4.3 was provided by Malcolm Martin (National Institutes of Health, Bethesda, MD) (1). The derivative clone pDRNL4.3, with a deletion of 1.5-kb of cellular DNA flanking the proviral sequence, was constructed by joining the 5′ and 3′ half clones provided by Ron Desrosiers (New England Primate Research Center, Southborough, MA) (20). Klaus Strebel (National Institutes of Health, Bethesda, MD) generously provided the constructs pNLA1U− (containing a Vpu ATG-to-GTG mutation) and pNLΔU35 (containing an 8-bp insertion at Vpu amino acid 32, inducing the premature termination of Vpu at amino acid 35) (57).

pEGFP-NI that expresses green fluorescent protein (GFP) was obtained from Clontech (BD Biosciences). The Rev reporter plasmid pDM128 expresses chloramphenicol acetyltransferase (CAT) in response to Rev activity in the cell (23). pHIVlacZ containing the *Escherichia coli* β-galactosidase gene cloned downstream of the HIV-1 long terminal repeat promoter was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, from Joseph Maio (35). The human β-actin plasmid was provided by Johnson Mak (Burnet Institute, Prahran, Australia).

Cloning and mutagenesis. To generate a library of NL4.3 cDNA clones representing 1.8-kb spliced mRNAs, HeLa cells were transfected with 20 μg of pNL4.3, poly(A)⁺ RNA was harvested, and the viral cDNA was reverse transcribed as described previously (46). cDNA representing spliced, HIV-1 1.8-kb mRNA was PCR amplified using primers Odp.001 and Odp.032 (sequences are given in Table 1) with the Expand high-fidelity PCR system per the instructions of the manufacturer (Roche). The DNA was purified (Progen PCR Spinclean kit), digested with BssHII plus BamHI, and cloned into the analogous region of pNL4.3 to produce a library of 1.8-kb cDNA clones.

cDNA clones representing specific HIV-1 1.8-kb spliced mRNAs were identified from the library by using restriction enzyme analysis, sequencing (ABI Prism kit; Perkin Elmer), and colony lifts (colony/plaque screen hybridization transfer membranes; DU PONT) probed with [γ-³²P]dATP-labeled oligonucleotides spanning different HIV-1 exon-exon junctions. This allowed the following 1.8-kb cDNA clones to be isolated: pNL*nef2* (exons 1, 5, and 7 [1/5/7]), pNL*nef3* (1/2/5/7), pNL*nef4* (1/3/5/7), pNL*nef5* (1/2/3/5/7), pNL*rev1* (1/4b/7), pNL*rev2* (1/4a/7), pNL*rev3* (1/4c/7), pNL*rev4* (1/2/4b/7), pNL*rev5* (1/2/4a/7), pNL*rev7* (1/3/4b/7), pNL*rev8* (1/2/3/4b/7), pNL*rev10* (1/2/3/4b/7), and pNL*tat1* (1/4/7).

HIV-1 *env* cDNA clones were then generated using a three-fragment ligation strategy. The BssHII-SacI 5′ UTR from the appropriate 1.8-kb cDNA was cloned with the SacI-BamHI and BssHII-BamHI fragments from pDRNL4.3. The following *env* cDNA constructs were thus generated, and their exon contents (shown in parentheses) were verified by restriction enzyme analysis and sequencing: pDR*env1* (1/5E), pDR*env2* (1/4bE), pDR*env3* (1/4aE), pDR*env4* (1/4cE), pDR*env5* (1/2/5E), pDR*env6* (1/2/4bE), pDR*env7* (1/2/4aE), pDR*env8* (1/3/5E), pDR*env10* (1/3/4bE), pDR*env11* (1/3/4aE), pDR*env13* (1/2/3/5E), and pDR*env14* (1/2/3/4bE).

Mutations that prematurely terminated Rev translation at an arginine residue at amino acid 38 (R38), removed the Rev AUG codon (Rev−) and/or the Vpu AUG codon (Vpu−), increased the Kozak strength of the Rev AUG codon, added a strong Kozak AUG codon to exon 2, or mutated this AUG codon to AGG were all synthesized by PCR mutagenesis by using the Expand high-fidelity PCR system (Roche) and the following protocol with the template and primer sets outlined in Table 2. Briefly, 5′ and 3′ mutagenic fragments were PCR amplified using the plasmid template plus wild-type forward and mutagenic reverse primers or mutagenic forward and wild-type reverse primers, respectively. Both fragments were purified, pooled together, and PCR amplified with wild-type forward and wild-type reverse primers, producing a larger fragment containing the mutation used in subsequent cloning. The pNL-M2A2 construct that introduces a translation stop codon corresponding to Vpu amino acid 2 was the result of a random PCR mutation arising during the preparation of an SA4b proviral splice site mutant (46). All oligonucleotides used throughout this study were synthesized by Invitrogen and are outlined in Table 1.

The R38 PCR mutation was cloned into the analogous regions of pDR*env1* to pDR*env4* by using MfeI and BamHI to generate the pDR*envR38* cDNA constructs. The Rev− mutation was cloned into the analogous region of pDR*env2* by using BssHII and MfeI to generate pDR*env2Rev−* cDNA. The same strategy was used to clone the mutation increasing the Kozak strength of the Rev AUG codon into pDR*env2* and pDR*env2R38*, producing pDR*env2RevK* and

TABLE 1. Primer sequences

Primer	5′→3′ sequence[a]
Odp.001	CTCTAGCAGTGGCGCCCGAAC
Odp.002	CTCTGGTAACTAGAGATCCCTCAG
Odp.031	AGTAGAGCAAAATGGAATGCCAC
Odp.032	CCGCAGATCGTCCCAGATAAG
Odp.067	GCTCATCAGAACAGTCAGACTCA
Odp.080	GCTGCCTTGTAAGTCATTGGTCTTAA
Odp.081	TCAGCTCGTCTCATTCTTTCCCTTAC
Odp.084	TCATTGCCACTGTCTTCTGCTCT
Odp.087	TACCCCATAATAGACTGTGACCCAC
Odp.092	CCAGACTGTGAGTTGCAACAG
Odp.151	GGGACCCGACAGGCCCGAAGGAA
Odp.263	GCGGCGACTGGCATCTCCT**ACG**GCAGGAAGAAGCGGAGACAG
Odp.264	CTGTCTCCGCTTCTTCCTGCC**CGT**AGGAGATGCCAGTCGCCGC
Odp.267	GACCCGACAGGCC**TGA**AGGAATAGAAG
Odp.268	CTTCTATTCCT**TCA**GGCCTGTCGGGTC
Odp.281	GCTGTAAGGGAAAGA**TGA**AGACGAGCTGAGCCAGC
Odp.282	GCTGGCTCAGCTCGTCT**TCA**TCTTTCCCTTACAGC
Odp.330	GCGAGGGGCGGCGACTGGCA**GCC**ACCATGGCAGGAAGAAGCGGAGAC
Odp.331	GTCTCCGCTTCTTCCTGCCATG**GTG**GCTGCCAGTCGCCGCCCCTCGC
Odp.513	GACAGCAGAGATCCAGT**GGCACC**ATGGCCAGCT**A**AGCTCCTCTGGAAAG
Odp.514	CTTTCCAGAGGAGCTT**A**GCTGGCC**ATGGTGCC**ACTGGATCTCTGCTGTC
Odp.515	GACAGCAGAGATCCAGT**GGCACC**AGGGCCAGCT**A**AGCTCCTCTGGAAAG
Odp.516	CTTTCCAGAGGAGCTT**A**GCTGGCCCT**GGTGCC**ACTGGATCTCTGCTGTC
5′ human β-actin gene fragment	CCCAAGCTTGCGGCCGCCCATGTGCAAGGCCGGCTTCG
3′ human β-actin gene fragment	GCGGGCCCGAAGGTGTGGTGCCAGATC

[a] Mutations are shown in bold.

pDR*env2R38RevK*, respectively. The pDR*env1R38* to pDR*env3R38* constructs containing the Vpu⁻ mutation and the mutations terminating Vpu translation at amino acid 2 and amino acid 35 were cloned by ligating three fragments: the BssHII-SacI 5′ UTR from pDR*env1*, pDR*env2*, or pDR*env3*; the SacI-MfeI fragment of pNLA1U−, pNLM2A2, or pNLΔU35; and the BssHII-MfeI plasmid backbone from pDR*env1R38*. The same cloning strategy was used to generate pDR*env2Rev⁻Vpu⁻* (except that the 5′ UTR was derived from pDR*env2Rev⁻*, the SacI-MfeI fragment from pNLA1U−, and the plasmid backbone from pDR*env1*). The exon 2 mutations adding a strong Kozak AUG codon or mutating the codon to AGG were cloned into pDR*env5* and pDR*env6* by using BssHII and MfeI to generate pDR*env5KAUG* and pDR*env6KAUG* and pDR*env5KAGG* and pDR*env6KAGG*. Finally, the pDR*env1ΔS* clone, containing a deletion of the splice sites SD4, SD5, SA6, SA7, SA7a, and SA7b, was generated by a multistep assembly of fragments mutated using PCR templates and primers listed in Tables 1 and 2. All clones were verified by restriction enzyme analysis and sequencing. A derivative clone of pEGFP-N1, pCMVempty, carried a deletion of the GFP gene between the AgeI and NotI sites; overhangs were end-filled with Klenow DNA polymerase before blunt-end ligation. The pCMVTat2X construct was generated by inserting the SalI-BamHI fragment from pNL*tat1* (1/4/7) into pCMVempty.

Cells and transfections. HeLa cells (CCL-2; American Type Culture Collection) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Plasmids for transfection were generated with QIAGEN Mega prep kits. HeLa cells were transfected using calcium phosphate coprecipitation (62). Cells (1×10^6) were typically transfected in 25-cm² flasks with 7.5 μg of *env* cDNA, 5 μg of pCMVTat2X, 2.5 μg of pEGFP-N1, and either 8 or 4 μg of pNL*rev1* for wild-type or R38 mutant *env* cDNA, respectively. To quantify Rev, 5 μg of the pDM128 reporter was also included in the DNA mix. pHIVlacZ and pCM-Vempty were used to normalize for differences in DNA amounts and promoter activities among samples.

Antibodies. The HIV-1-positive patient serum used to detect Env (gp160) was kindly provided by Johnson Mak (Burnet Institute, Prahran, Australia). The Vpu antibody raised in rabbits by using *E. coli*-derived Vpu amino acids 31 to 81 (37) was generously donated by Klaus Strebel (National Institutes of Health, Bethesda, MD). The rabbit anti-human, donkey anti-rabbit, and rabbit anti-sheep sera conjugated to horseradish peroxidase (HRP) were obtained from Dako (Denmark), Amersham Pharmacia Biotech (England), and Zymed (United States), respectively.

Immunoblots. Forty hours after transfection, 10^6 cells were washed twice in phosphate-buffered saline (PBS) and lysed on ice for 2 h in a solution containing 150 μl of 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% (vol/vol) Triton X-100 supplemented with 1 μg/ml aprotinin and 2 mM phenylmethylsulfonyl fluoride. Cell debris and nuclei were removed by centrifugation at $12,000 \times g$ for 2 min. GFP in the cell extracts was quantified to measure transfection efficiency by using opaque 96-well plates (Corning) and a FLA-2000 fluorescent-image analyzer (Fujifilm, Japan). Extracts with equivalent levels of GFP were resolved on 10% (Env) or 12.5% (Vpu) sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (DU PONT) by using a semidry electroblotting apparatus (Integrated Separation

TABLE 2. Primers and initial plasmid template for PCR mutagenesis

Mutation	Initial PCR template	Primer[a]			
		WT F	WT R	MUT F	MUT R
R38	pNL4.3	Odp.031	Odp.081	Odp.267	Odp.268
N20	pDR*env2*	Odp.151	Odp.080	Odp.281	Odp.282
Rev⁻	pDR*env2*	Odp.002	Odp.092	Odp.263	Odp.264
Strengthening of Rev AUG codon	pDR*env2*	Odp.002	Odp.092	Odp.330	Odp.331
Addition of Kozak AUG codon	pDR*env5* or pDR*env6*	Odp.001	Odp.092	Odp.513	Odp.514
Change from Kozak AUG to AGG	pDR*env5* or pDR*env6*	Odp.001	Odp.092	Odp.515	Odp.516

[a] WT, wild-type; F, forward; R, reverse; MUT, mutagenic.

Systems, Enprotech). Membranes were blocked in PBS containing either 5% (wt/vol) skim milk powder (for Env detection) or 3% (wt/vol) bovine serum albumin (for Vpu detection). To detect Env, blots were probed with HIV-1-positive patient sera (1:5,000) followed by HRP-conjugated anti-human sera (1:4,000). Vpu sera (1:500) and HRP-conjugated anti-rabbit sera (1:10,000) were used to detect Vpu. Probed bands were visualized using enhanced chemiluminescence plus autoradiography and the LAS-1000 luminescent-image analyzer (Fujifilm, Japan). The densities of protein bands were quantified using accompanying MacBAS version 3.3 software.

CAT assays. Rev activity was indirectly measured by transfecting cells with the pDM128 Rev reporter plasmid and analyzing cell extracts for CAT activity 40 h posttransfection. Cells (10^6) were washed in PBS, resuspended in 120 μl of 0.25 M Tris-HCl (pH 7.8)–0.5% (vol/vol) Nonidet P-40, and lysed by three consecutive rounds of rapid freeze-thawing with a 10-s vortexing step after each thaw. Cell debris was removed by centrifugation at 15,300 × g for 10 min at 4°C, and extracts were heat inactivated for 10 min at 65°C and centrifuged as before. Final lysates were normalized for transfection efficiency by quantifying GFP, and the samples were diluted with 0.25 M Tris (pH 7.8)–0.5% (vol/vol) Nonidet P-40 to contain equivalent levels of GFP. Normalized extract (12.5 μl) was mixed with 12.5 μl of 0.5 M Tris-HCl (pH 7.8), 5 μl of acetyl coenzyme A (3.5 mg/ml; Roche), and 2.5 μl of D-threo-dichloroacetyl-[1,2-^{14}C]chloramphenicol (52.8 mCi/mmol; NEN Life Science Products). Samples were incubated for 2 to 4 h at 37°C, and the extracts were typically diluted 1:10 for analysis to keep the assay samples within the linear range (below 30% conversion). The chloramphenicol forms were extracted with ethyl acetate, resolved using thin-layer chromatography in 5% (vol/vol) methanol and 95% (vol/vol) chloroform, and visualized using the FLA-2000 fluorescent-image analyzer (Fujifilm, Japan). The acetylated and unacetylated forms were quantified using MacBAS version 3.3 software.

RPA. A T7 promoter was added to DNA templates required for RNA probe synthesis by using the Lig'nScribe RNA polymerase promoter addition kit (Ambion). The *env* probe template was PCR amplified from pDR*env1* by using primers Odp.067 and Odp.087 (Table 1). The β-actin probe template was PCR amplified by using a human β-actin plasmid and PCR primers (Table 1) provided by Johnson Mak (Burnet Institute, Prahran, Australia). RNA probes were synthesized using the MAXIscript™ in vitro transcription kit (Ambion) with the supplied T7 polymerase and [α-^{32}P]UTP (NEN Life Science Products). The RNase protection assay (RPA) was performed using the RPA II TM kit per the instructions of the manufacturer (Ambion). Total RNA was isolated from transfected cells by using TRIzol (Invitrogen). In the RPA, the probe and total RNA were both hybridized at 42°C for 14 h, followed by digestion with either RNase A-RNase T1 or RNase T1 at a 1:50 dilution. Samples were resolved on a prewarmed, 8 M urea–6% polyacrylamide gel (Shelton Scientific-IBI). The gel was dried on 3MM chromatography paper (Whatman International Ltd., England), and bands were visualized using autoradiography and the FLA-2000 fluorescent-image analyzer (Fujifilm, Japan).

RNA stability analysis. Twenty-four hours after transfection, medium containing 5 μg/ml actinomycin D was added to cells. Total RNA was harvested 0, 4, 8, and 12 h later using TRIzol (Invitrogen). The amount of *env* mRNA present in 5 μg of total RNA was analyzed via the RNA protection assay and quantitated using the FLA-2000 (Fujifilm, Japan) and MacBAS version 3.3 software.

RESULTS

Alternative isoforms of *env* mRNA express similar levels of Env but different levels of Vpu. To determine the effects on protein synthesis of the different 5′ UTR of the 16 *env* mRNA isoforms, we generated a panel of cDNA constructs that each expressed one isoform of *env* mRNA. These subgenomic cDNA constructs expressed native *env* isoforms with the 5′ UTR exons already spliced together. Therefore, these *env* mRNA isoforms had the same structures as those arising naturally after alternative splicing of the 9.2-kb genomic RNA (Fig. 1). These *env* mRNAs all contained exon 1 and included either, neither, or both exon 2 and/or exon 3 and interchange exon 5E, 4bE, 4aE, or 4cE (Fig. 1C) from the HIV-1 NL4.3 molecular clone. These mRNAs all carried the same Vpu, overlapping Env, and downstream Nef ORFs (Fig. 1D) but differed in the 5′ UTR upstream of the region corresponding to Vpu depending on their exon contents (Fig. 1C).
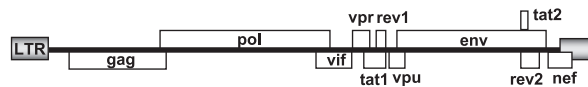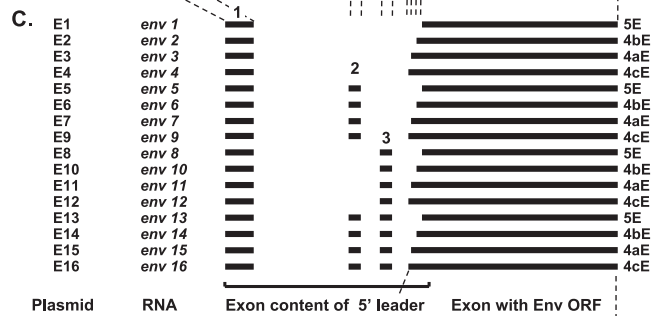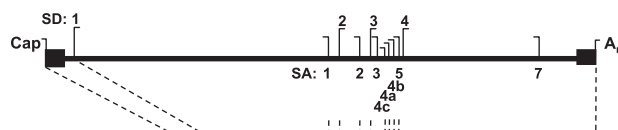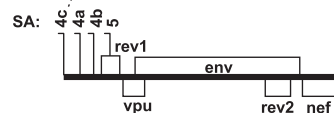

FIG. 1. The 16 isoforms of HIV-1 *env* mRNA. (A) Schematic diagram of the HIV-1 provirus genome. LTR, long terminal repeat. (B) HIV-1 provirus transcribes a genome-length 9.2-kb RNA with an m^{7}Gppp cap and a poly(A) tail (A$_n$) containing multiple SD and SA sites. (C) Sixteen different spliced forms (isoforms) of *env* mRNA are present in the mRNA pool following infection with HIV-1$_{NL4.3}$, and these are assembled from four alternative Env-encoding exons, designated 5E, 4bE, 4aE, and 4cE, together with 5′ noncoding exon 1 and combinations of two alternatively spliced exons, exons 2 and 3 (46). E1 to E16, cDNA plasmids pDR*env1* to pDR*env16* expressing the mRNA isoforms described in this study. (D) The *env* mRNAs all contain ORFs for Vpu, Env, and Nef but differ in the sequence of the 5′ UTR due to the different exons spliced together to make the mRNA.

To express the individual *env* mRNAs from these cDNA plasmids in HeLa cells, cells were cotransfected with an HIV-1 Tat expression plasmid to transactivate the viral long terminal repeat promoter together with the pEGFP-N1 transfection efficiency reporter expressing GFP. Protein expression from *env* mRNA that contains the RRE is known to be acutely reliant on the level of Rev protein for nucleocytoplasmic export (22, 42). Because the subset of *env* mRNA transcripts containing exon 4bE, 4aE, or 4cE, such as those produced by the pDR*env2*, pDR*env3*, and pDR*env4* cDNA plasmids, can actively splice to SA7, leading to Rev expression (31a; Fig. S1 in the supplemental material), it was important to develop a system to equalize the levels of Rev activity among the samples. Two approaches were used to equalize the levels of Rev activity in experiments with the *env* isoforms, allowing us to focus on the effects of the 5′ UTR on protein synthesis.

Firstly, cells were cotransfected with individual *env* isoform cDNA plasmids and high levels of the Rev expression plasmid
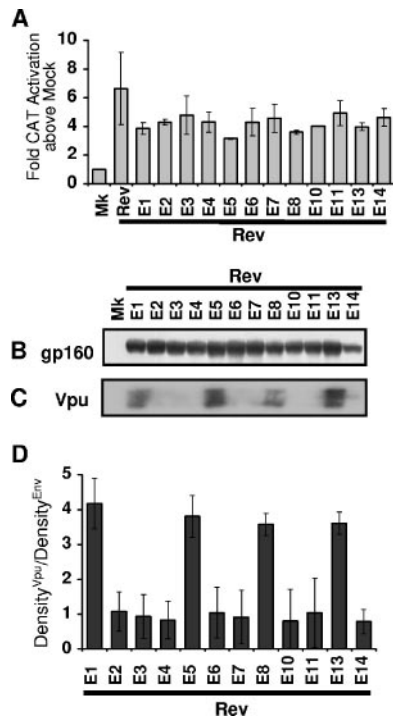
FIG. 2. Similar levels of Env but different levels of Vpu coexpressed by the *env* isoforms with saturating Rev in *trans*. (A) Rev activity 40 h after triplicate transfections of HeLa cells with pLTR*rev1* (8 μg) along with the *env* cDNA construct, pCMVTat2x, pEGFP, and pDM128 was determined by CAT assays. (B and C) Samples normalized for transfection efficiency by using GFP were immunoblotted for gp160 Env (B) and Vpu (C), with representative blots shown. (D) The density of the Vpu band was divided by the density of the Env band for each *env* isoform, and the average ratio from three independent experiments was graphed. Mk, mock (no *env* or pLTR*rev1*) transfection control; Rev, pLTR*rev1* (no *env*) transfection control; E1 to E14, plasmids expressing isoforms *env1* to *env14*.
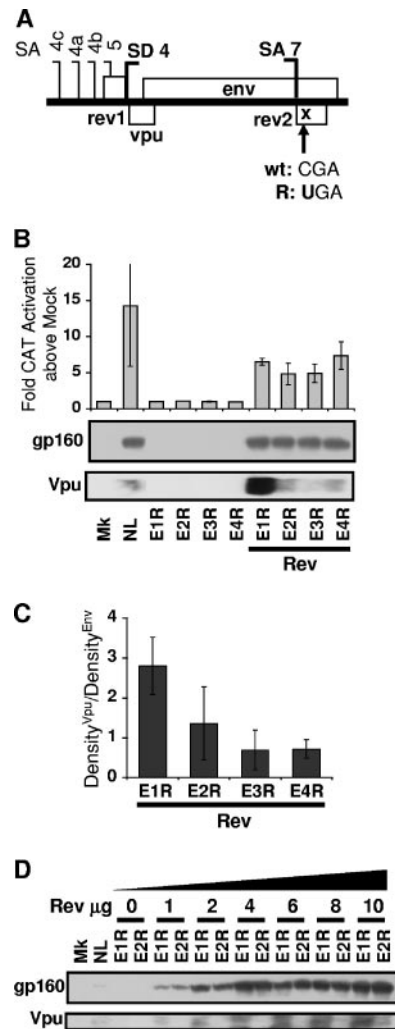

FIG. 3. Rev R38 mutant *env* isoforms express different levels of Vpu but similar levels of Env irrespective of the Rev titer. (A) The R38 mutation introduces a premature translation termination codon corresponding to amino acid 38 in the second Rev exon of the *env* cDNA constructs. (B) The activity of Rev R38 *env* isoform mutants was measured by CAT assays 40 h after the cotransfection of HeLa cells with pCMVTat2X, pEGFP, and pDM128 with or without pLTR*rev1*. (C) Densitometry analysis of Env and Vpu expression from the *env* cDNA constructs. (D) Immunoblot assay of gp160 Env and Vpu synthesized in HeLa cells cotransfected with *env* cDNA, pCMVTat2X, pEGFP, pDM128, and increasing amounts of pLTR*rev1*. wt, wild-type sequence; R, R38 mutant sequence; Mk, mock (no *env* or pLTR*rev1*) transfection control; NL, HIV$_{NL4.3}$ proviral plasmid control; E1R to E4R, *env1* to *env4* R38 mutant plasmids.

pNL*rev1* to produce similar levels of saturating Rev among the samples (Fig. 2A). When similar levels of Rev activity among the samples with the native *env* isoforms were induced using this method (Fig. 2A), the different isoforms expressed similar levels of gp160 Env protein but dramatically different levels of Vpu (Fig. 2B and C). A densitometry analysis of the protein synthesized from each Env cDNA plasmid revealed that constructs producing *env* mRNA isoforms containing exon 5E, such as pDR*env1*, pDR*env5*, pDR*env8*, and pDR*env13*, consistently expressed approximately fourfold more Vpu than Env relative to their exon 4bE-, 4aE-, and 4cE-containing counterparts (Fig. 2D). This protein expression profile was conserved irrespective of whether noncoding exon 2 and/or exon 3 was included in the *env* mRNA. The differences in levels of Vpu expression measured by immunoblotting were significant because titrating lysate from cells transfected with pDR*env2* cDNA revealed that this quantification method detected these protein levels in the linear range, supporting its use here for Env and Vpu quantification (Fig. S2 in the supplemental material). Hence, this analysis revealed that the *env* isoforms expressed similar levels of Env but different levels of Vpu, coincident with the presence in the mRNAs of exon 5E, which

has no upstream Rev AUG codon, or that of exon 4bE, 4aE, or 4cE, which has an upstream AUG codon.

To overcome any discrepancy in levels of endogenous Rev expression among cDNA constructs (Fig. S1 in the supplemental material), we adopted a second method of equalizing levels of Rev activity obtained with the Env constructs by introducing a premature translation termination codon in the Rev ORF in the *env* mRNAs to terminate Rev translation at the arginine residue at amino acid position 38 (Fig. 3A). This mutation led to the expression of a nonfunctional truncated Rev protein after splicing (Fig. 3B). The cotransfection of cells with these

R38 mutant *env* cDNA plasmids and equal amounts of the Rev expression plasmid equalized the levels of Rev activity between the different *env* isoforms (Fig. 3B). Neither Vpu nor Env protein was expressed from the *env* R38 mutant mRNAs unless wild-type Rev was supplied in *trans* (Fig. 3B). When Rev was supplied in *trans*, the *env1* to *env4* R38 mutant cDNA plasmids expressed similar levels of Env protein but the *env1* R38 mutant cDNA plasmid that expressed an exon 5E-containing *env1* mRNA isoform produced two- to fourfold more Vpu than *env2*, *env3*, and *env4* R38 mutant cDNA plasmids that expressed 4bE-, 4aE-, and 4cE-containing *env2*, *env3*, and *env4* isoforms, respectively (Fig. 3B and C). Titrating the *env1* and *env2* R38 cDNA mutants with increasing amounts of Rev further revealed that at each Rev concentration tested, both *env1* and *env2* mRNA isoforms expressed similar amounts of Env but that the *env1* R38 mutant isoform that used exon 5E always synthesized more Vpu than the *env2* R38 mutant isoform that used exon 4bE (Fig. 3D). Using the two methods for equalizing levels of Rev activity, we consistently demonstrated that the *env* isoforms expressed similar levels of Env protein but different levels of Vpu, depending upon the structure of the 5′ UTR resulting from the use of exon 5E, 4bE, 4aE, or 4cE. Furthermore, equivalent levels of Env were also expressed from the *env1* to *env4* mRNA isoforms following the transfection of the Env cDNA clones into Jurkat T cells, which express CD4 viral receptor (33) (Fig. S3 in the supplemental material). Nef expression did not have an impact on the amounts of Env and Vpu expressed from these Env cDNA clones (Fig. S4 in the supplemental material). While Vpu translation conformed to the leaky ribosome-scanning model, Env expression was not influenced by upstream AUG codons.

Consistent Env expression from the *env* isoforms is not due to cryptic splicing or increased RNA stability. The leaky scanning model of translation predicts that ribosomes engaging the m⁷Gppp cap of mRNA at a constant rate proportionally initiate protein synthesis at the weak upstream Vpu AUG codon (53). While the inclusion of the weak upstream Rev AUG codon in the 5′ UTR of some isoforms of *env* mRNA altered Vpu synthesis, consistent with a leaky ribosome-scanning model, constant levels of Env synthesis from the alternative isoforms of *env* mRNA were not easily explained. We anticipated that 5′ UTR-induced changes in Vpu synthesis would alter the number of ribosomes available for Env translation and also cause changes in Env production. But the level of Env expression by the different *env* isoforms remained consistent (Fig. 2 and 3). It was possible that the *env* isoforms transcribed from our cDNA constructs underwent cryptic splicing to an mRNA monocistronic for the Env ORF, leading to similar levels of Env expression by the different isoforms despite the changes in Vpu production. To explore if such cryptic splicing occurred, RNA harvested from cells transfected with Env cDNA constructs expressing different *env* isoforms was analyzed by RPA (Fig. 4). For the RPA, a probe binding the NL4.3 *env* mRNA after SA5 and along the first 136 nucleotides of the Env ORF was employed (Fig. 4A). This probe protects a 356-nucleotide fragment corresponding to the native *env* isoforms. Importantly, this probe can also detect any cryptic mRNA species that remove the Vpu ORF and are thus monocistronic for the Env ORF. Treating the hybridized RNA-probe complexes with either a mix of RNaseA-RNase T1 (Fig. 4B) or
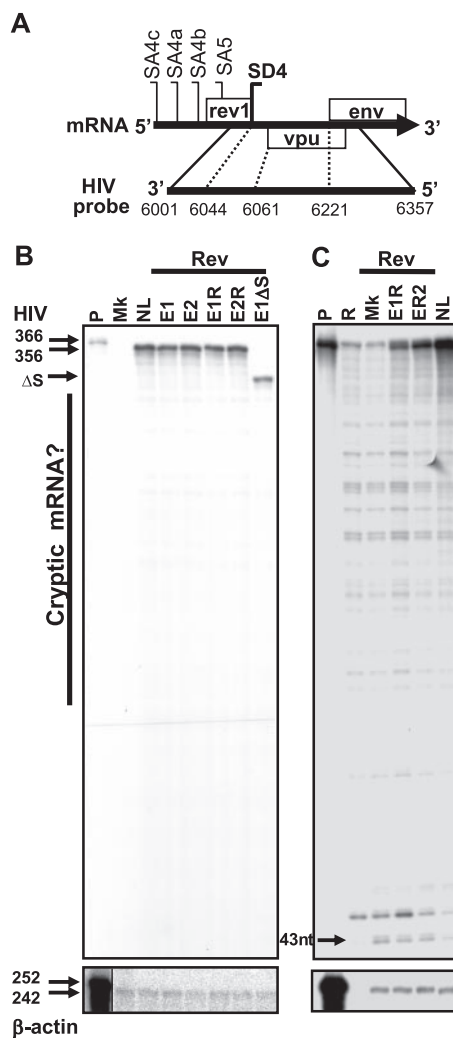

FIG. 4. Consistent Env expression among *env* isoforms is not due to cryptic splicing to monocistronic *env* mRNA. (A) Location of the probe in *env* mRNA used for RPA to explore RNA species generated from our *env* cDNA constructs. Numbers at the bottom indicate nucleotide positions. (B and C) Five micrograms of total RNA harvested from HeLa cells transfected with either NL4.3 or *env* cDNA plasmids plus pLTR*rev1* was hybridized with the probe and digested with either an RNase A-RNase T1 mix (B) or RNase T1 alone (C) during the RPA. Protected fragments detected by autoradiography are shown. A β-actin-specific probe was used as a loading control. Molecular sizes in nucleotides are shown on the left. P, undigested probe; R, probe plus RNase; Mk, mock (no *env* or pLTR*rev1*); NL, HIV_{NL4.3} proviral plasmid control; E1 and E2, plasmids expressing isoforms *env1* and *env2*; E1R and E2R, plasmids expressing *env1* and *env2* R38 mutant isoforms; E1ΔS, *env1*Δ splice site control; ΔS, deletion of SD4, SD5, SA7, SA7a, and SA7b; nt, nucleotides.

RNase T1 alone (Fig. 4C) allowed the 356-nucleotide fragments of native *env* isoforms to be seen. However, no extra cryptic mRNA species indicative of an mRNA monocistronic for the Env ORF could be observed above the background radiolytic decay of the probe. A 313-nucleotide protected band from a mutant *env* construct which cannot actively splice was also included as a control to demonstrate that this method will detect aberrant *env* mRNA species (Fig. 4B). A 43-nucleotide band was also seen, representing a protected fragment from
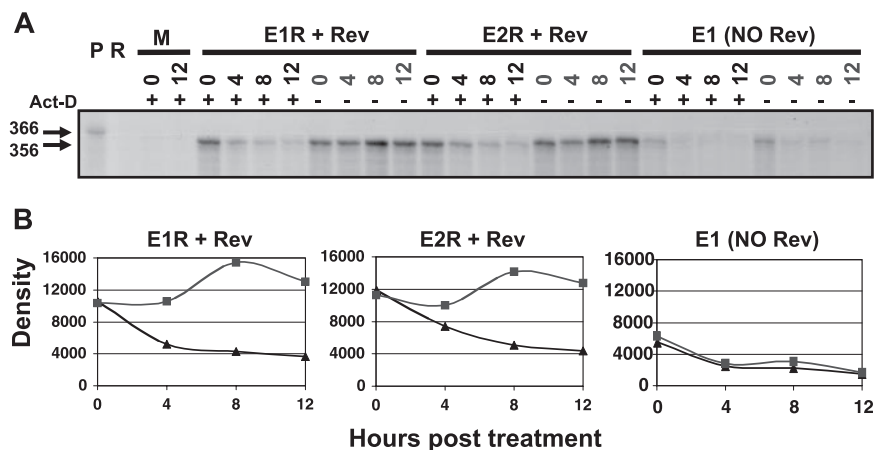
FIG. 5. Exon 5E isoforms do not have greater stability than exon 4bE isoforms. (A) HeLa cells were transfected with *env* cDNA plasmids with or without pLTR*rev1*, and 24 h later, cells were either treated (+) or untreated (−) with actinomycin D (Act-D) for a further 0, 4, 8, and 12 h prior to RNA harvesting. Five micrograms of RNA per sample was analyzed for native *env* mRNA via RPA using RNase A-RNase T1 digestion and autoradiography. Molecular sizes in nucleotides are shown on the left. (B) Densitometry analysis of protected bands (untreated cells, ■; treated cells, ▲). P, undigested probe; R, probe plus RNase; M, mock (no *env* or pLTR*rev1*); Rev, pLTR*rev1*; E1R and E2R, plasmids expressing *env1* and *env2* R38 mutant isoforms; E1, pDR*env1*. Density equals photostimulated luminescence minus background.

the SD4 splicing event in the 1.8-kb mRNA of pCMVTat2X (Fig. 4C). Therefore, the Env cDNA constructs did not appear to produce a cryptic mRNA monocistronic for the Env ORF that could explain the consistent Env production by the various *env* mRNA isoforms despite changes in Vpu levels.

To test whether the consistent Env expression was instead due to an increased stability of the exon 5E-containing isoforms relative to that of the exon 4bE-, 4aE-, and 4cE-containing isoforms, we compared the degrees of stability of the *env1* (1/5E) and *env2* (1/4bE) mRNAs with or without actinomycin D treatment when normalized for Rev activity. Adding actinomycin D prevents transcription by RNA polymerase II so that the fate of the existing pool of mRNA over time can be examined. The amount of *env* mRNA present in cells expressing the *env1* or *env2* isoform was measured 0, 4, 8, and 12 h after treatment by using RPA (Fig. 5A) and densitometry for quantification (Fig. 5B). The similar accumulation and degradation profiles of both *env1* and *env2* isoforms normalized for Rev activity clearly showed that *env1* mRNA containing exon 5E expressed by the *env1* R38 mutant plasmid was no more stable than the *env2* mRNA isoform containing exon 4bE expressed by the *env2* R38 mutant plasmid. The similar profiles of these isoforms in the presence of Rev also contrasted with that of the no-Rev control, which displayed limited accumulation and rapid degradation of the mRNA with and without actinomycin D treatment. These data clearly show that the similar levels of Env synthesis from the *env* isoforms cannot be attributed to any increased stability of *env* isoforms containing exon 5E. If anything, the greater slope of the decay curve over the first 4 h for the *env1* mRNA produced from the *env1* R38 mutant plasmid than for the *env2* mRNA produced from the *env2* R38 mutant plasmid would indicate that the exon 5E-containing *env1* mRNA may have a shorter half-life.

The effects of the Rev AUG codon upon Vpu expression are consistent with leaky ribosome scanning. The *env* isoforms containing exons 4bE, 4aE, and 4cE from pDR*env2*, pDR*env3*, and pDR*env4* cDNA constructs, respectively, that expressed

diminished levels of Vpu also incorporated the weak, upstream Rev AUG codon in the 5′ UTR. This Rev AUG codon was excluded from the exon 5E *env* isoforms produced by pDR*env1* cDNA due to the position of SA5. As Vpu is reported to be translated by ribosome scanning (53), we reasoned that including this weak, upstream Rev AUG codon in the 5′ UTR of *env* mRNA may impede scanning 40S ribosomes from reaching the Vpu AUG codon and diminish Vpu expression. To explore the role of the upstream Rev AUG codon in downstream Vpu expression, we constructed the pDR*env2RevK* cDNA mutant, which carried a strengthened Rev AUG codon, and the pDR*env2Rev⁻* mutant, which had the Rev AUG codon removed from the *env* mRNA (Fig. 6A). Strengthening the Kozak consensus of the Rev AUG codon increased Rev activation in the pDM128 Rev-dependent CAT reporter assay by almost twofold above that from the wild-type *env2* isoform (Fig. 6B), suggesting that Rev expression was increased by improving the AUG start context. In contrast, deleting the Rev AUG codon extinguished the production of functional Rev, similar to the result for *env* R38 mutant constructs containing the Rev premature termination codon corresponding to amino acid 38 (Fig. 6B). Combining the enhanced Rev Kozak AUG consensus and the Rev R38 premature termination codon mutations in the same construct also blocked Rev function (Fig. 6B), allowing levels of Rev activity to be equalized in subsequent experiments (Fig. 6C). The Rev AUG Kozak translation initiation mutation that increased Rev expression from *env2* cDNA decreased Vpu expression from this cDNA (Fig. 6C). Conversely, removing the Rev AUG codon increased Vpu synthesis from *env2* to match the high levels of Vpu from *env1* (Fig. 6C). These data indicate that Vpu synthesis from the native mRNA is affected by the upstream Rev AUG codon in a leaky ribosome-scanning manner because (i) increasing initiation at the Rev AUG codon decreased Vpu output, indicating that fewer ribosomes escape the Rev AUG codon to translate downstream Vpu, and (ii) removing the Rev AUG codon increased Vpu output, as scanning ribosomes were no longer
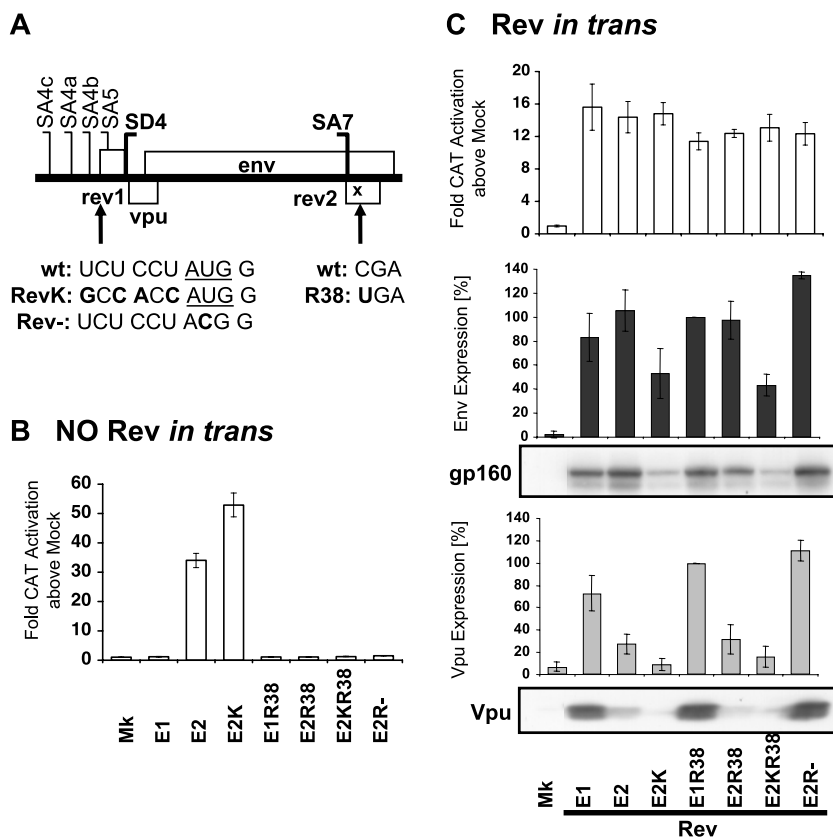
FIG. 6. Increasing the strength of or removing the Rev AUG modulates Vpu expression. (A) Mutations in the *env2* cDNA constructs that either strengthened the Rev AUG codon (underlined; RevK) or removed it (Rev⁻). (B and C) HeLa cells were transfected with the *env* cDNA constructs, pCMVTat2X, pEGFP, and pDM128 without (B) or with (C) pLTR*rev1*. Rev activity was measured by CAT assay. Env and Vpu were detected by immunoblotting followed by densitometry analysis, and the percentage of expression relative to the expression of the *env1* R38 mutant (E1R38; set at 100%) is graphed. wt, wild type; Mk, mock (no *env* or pLTR*rev1*); E1 and E2, pDR*env1* and pDR*env2*; E2K, *env2* cDNA construct expressing RevK mutation; E2R38, *env2* cDNA construct expressing R38 mutation; E2KR38, *env2* cDNA construct expressing RevK and R38 mutations; E2R−, *env2* cDNA construct expressing Rev⁻ mutation.

lost to the upstream Rev AUG codon. Furthermore, this result indicates that the upstream Rev AUG codon in the 5′ UTR of the exon 4bE, 4aE, and 4cE *env* isoforms produced from pDR*env2*, pDR*env3*, and pDR*env4* cDNA constructs was responsible for their diminished Vpu synthesis relative to that of the exon 5E isoform from pDR*env1* cDNA clones that lacked the upstream Rev AUG codon.

Given that the native weak Rev AUG codon had no impact upon Env expression from *env* isoforms (Fig. 2), it was surprising that Env expression was also affected by the Rev AUG codon mutations in a pattern consistent with effects on leaky scanning (Fig. 6C). The pDR*env2Rev⁻* cDNA construct that removed the Rev AUG codon in the *env2* isoform led to a 1.3-fold increase in Env expression, and the pDR*env2R38RevK* cDNA construct that increased the strength of the Rev AUG codon led to a twofold reduction in Env expression (Fig. 6C). However, *env1*, *env5*, *env8*, and *env13* cDNA constructs that produced *env* mRNA isoforms containing exon 5E, which lacks the Rev AUG codon, expressed levels of Env protein similar to those expressed by the other isoforms with the Rev AUG codon (Fig. 2), not fitting the leaky scanning mechanism model. These data suggest that the translation mechanism for

the Env ORF is more complex than the standard leaky ribosome-scanning mechanism.

Upstream AUG codons and the length of the Vpu ORF do not alter Env expression. To further explore potential elements in the 5′ UTR, we reasoned that removing both the upstream Rev and Vpu AUG codons should allow more scanning ribosomes to reach the Env AUG codon and thus induce greater synthesis of Env than if either AUG codon was removed in isolation. *env2* R38 cDNA mutants which had the Vpu and/or Rev AUG codon removed were generated for this purpose (Fig. 7A). Removing the Rev or Vpu AUG codon in isolation caused minor increases in Env protein synthesis (Fig. 7B), but no greater increase in Env synthesis from a construct with the double AUG codon mutation was observed (Fig. 7B). Again, Env synthesis from these *env2* mRNAs was clearly not consistent with leaky ribosome-scanning translation.

To explore the importance for Env synthesis of the overlap of the Vpu ORF with the 5′ portion of the Env ORF, the Vpu ORF was prematurely terminated to produce Vpu mutants of two different lengths, one ending at amino acid 2 and the other terminating at amino acid 35 following an 8-nucleotide insertion at Vpu amino acid 32 (57) (Fig. 7A). As anticipated, these
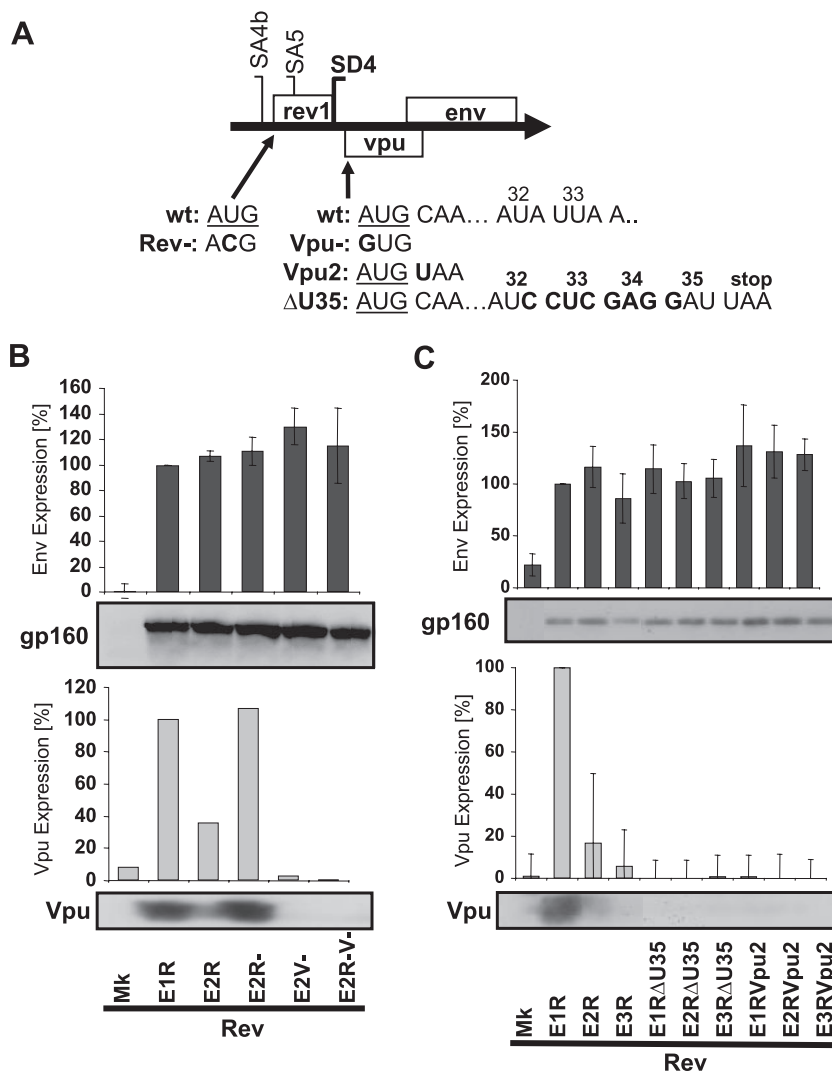
FIG. 7. Removing upstream AUG codons and changing Vpu ORF length does not alter consistent Env expression. (A) Mutations in the *env* cDNA constructs that removed the upstream Rev and/or Vpu AUG codon (underlined; Rev− and/or Vpu−) or shortened the Vpu ORF at the position corresponding to amino acid 2 (Vpu2) or 35 (ΔU35). Numbers indicate amino acid positions corresponding to the codons. (B and C) HeLa cells were transfected with the *env* cDNA constructs, pCMVTat2X, pLTR*rev1*, and pEGFP. Env and Vpu expression was analyzed by immunoblotting and then densitometry. The percentage of expression relative to that of the *env1* R38 mutant (E1R; set at 100%) is graphed for the *env* isoforms lacking upstream AUG codons (B) or containing altered Vpu ORF lengths (C). wt, wild type; Mk, mock (no *env*); E1R to E3R, pDR*env1* to pDR*env3* cDNA constructs containing the Rev R38 premature termination codon mutation; E2−, *env2* cDNA construct expressing the Rev− mutation; E2V−, *env2* cDNA construct expressing the Vpu− mutation; E2R−V−, *env2* cDNA construct expressing the Rev− and Vpu− mutations; E1RΔU35 to E3RΔU35, *env1* to *env3* R38 mutant cDNA constructs with the Vpu ORF shortened at the position corresponding to amino acid 35; E1RVpu2 to E3RVpu2, *env1* to *env3* R38 mutant cDNA constructs with the Vpu ORF shortened at the position corresponding to amino acid 2.

premature termination mutations in the Vpu ORF eliminated Vpu expression (Fig. 7C). However, minimal impact on the consistent expression of Env was observed. The constructs with the mutation at amino acid 35 expressed wild-type levels of Env protein (Fig. 7C), while Env synthesis by the Vpu mutants with the termination mutation at amino acid 2 increased merely 1.3-fold (Fig. 7C). Therefore, Env expression was not perturbed by changes to the length of the Vpu ORF, ruling out this parameter as an influence on consistent Env expression.

Env translation uses discontinuous ribosome scanning. To delineate whether the translation mechanism for Env protein involved a continuous or discontinuous ribosome-scanning mechanism, we developed Env cDNA constructs producing

mRNAs containing a strong Kozak AUG codon (Fig. 8A) in the upstream noncoding exon 2 that was shown not to affect Env expression from the *env5* and *env6* isoforms from pDR*env5* and pDR*env6* cDNA constructs (Fig. 2B). If HIV-1 Env protein is translated via a continuous ribosome-scanning mechanism, then adding a strong, upstream Kozak AUG codon should usurp the scanning 40S ribosomes and block the downstream translation of Env (and Vpu). Conversely, if Env uses a discontinuous scanning mechanism for translation initiation, this upstream AUG codon could be bypassed and no decline in downstream Env translation would occur. Mutants of cDNA plasmids pDR*env5* and pDR*env6* with the addition of an upstream, strong Kozak AUG codon and a translation termina-
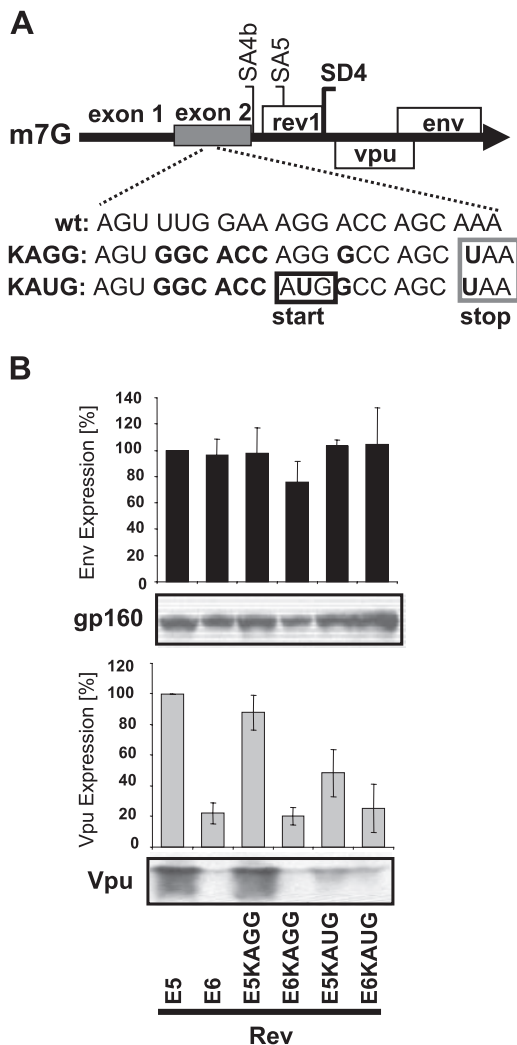
FIG. 8. Adding an upstream, strong Kozak AUG codon in exon 2 of the *env* isoforms does not diminish Env expression. (A) Mutations in exon 2 of the *env* cDNA constructs that introduced either a strong Kozak AUG codon (KAUG) or the same mutations minus the AUG codon (KAGG). A UAA translation termination motif was included at codon 4 to avoid aberrant protein synthesis. (B) HeLa cells transfected with the *env* cDNA constructs, pCMVTat2X, pLTR*rev1*, and pEGFP were analyzed for Env and Vpu by immunoblotting and densitometry. The percentage of expression relative to that of the E5 (E5; set at 100%) is graphed. wt, wild type; E6, pDR*env6*; E5KAGG and E6KAGG, *env5* and *env6* constructs without the Kozak AUG codon; E5KAUG and E6KAUG, *env5* and *env6* constructs with the Kozak AUG codon.

tion signal in exon 2 of the *env5* (1/2/5E) and *env6* (1/2/4bE) mRNAs (Fig. 8A) showed reduced Vpu expression but no effect on Env production, supporting a discontinuous initiation mechanism for Env (Fig. 8B). Importantly, the control constructs lacking the exon 2 AUG start signal (Fig. 8A) did not show effects on Vpu or Env expression relative to the wild-type *env5* and *env6* isoforms (Fig. 8B). This finding indicates that changes to protein translation in the constructs with the strong Kozak AUG codon were specifically the result of the Kozak AUG motif and not of other nucleotide changes added to exon 2. Collectively, our data indicate that the Env ORF is trans-

lated mostly via a discontinuous ribosome-scanning mechanism, as introducing an upstream strong Kozak codon (Fig. 8) did not diminish the synthesis of Env.

DISCUSSION

We examined the significance of the different 5′ UTR for Env and Vpu synthesis from the multiple *env* isoforms produced by RNA splicing during HIV-1 replication. We found that the different 5′ UTR that arise from alternative splicing regulate Vpu synthesis while curiously maintaining a uniform level of Env production. Our analysis used cDNA plasmids expressing a single *env* mRNA isoform with the unique 5′ UTR exon combination already spliced together to reproduce native *env* mRNA isoforms made during viral replication. This method is likely to avoid changes to 5′ UTR mRNA elements and protein binding sites potentially important for translation control (4). This issue may have complicated the interpretation from earlier cDNA constructs and reporters (53). We found that only pDR*env1*, pDR*env5*, pDR*env8*, and pDR*env13* cDNA constructs that produced *env* mRNA isoforms containing exon 5E, and not exons 4bE, 4aE, and 4cE, efficiently expressed Vpu.

This differential expression of Vpu by the *env* isoforms indicates the purpose of the alternately spliced isoforms of *env* mRNA. While factors influencing the preferential selection among the various weak splice sites by interspersed exonic and intronic splice enhancer and silencer elements (2), such as the levels of RNA binding proteins including 9G8, SC35, ASF/SF2, and SRp40 (13, 49, 66), have been identified, the effects of this regulation on HIV-1 replication and pathogenesis have been unclear. Our results show that alternative splice control mechanisms may modulate Vpu production during HIV-1 infection. Vpu plays two important roles in the natural replication cycle of HIV-1, reviewed in references 3 and 10. Firstly, Vpu promotes CD4 degradation in infected cells, allowing Env to efficiently accumulate at the cell surface for incorporation into new virions, thereby enhancing virus infectivity (10, 51, 63). Secondly, Vpu enhances virion release (27, 56), overcoming a restriction on virion release in human producer cells (58). Hence, changing the ratio of the *env* mRNA isoforms during viral infection may allow the virus to control Vpu-directed CD4 degradation, the accumulation of Env at the cell surface, and/or particle release, adjusting viral replication to suit the cellular environment. The alternative *env* isoforms are not redundant and appear, at the very least, to regulate Vpu production to perhaps aid HIV-1 replication in specific cellular environments.

Vpu expression by the native *env* isoforms clearly depended on the translation initiation signals in the 5′ UTR. The *env* mRNA isoforms containing exons 4bE, 4aE, and 4cE, such as those expressed by the pDR*env2*, pDR*env3*, and pDR*env4* cDNA constructs, respectively, contained the weak upstream AUG codon for Rev that would allow a proportion of scanning 40S ribosomes to initiate at the Rev AUG codon, thus depleting the number of ribosomes available to initiate at the downstream Vpu codon. The mutation of the Rev AUG codon confirmed its involvement in controlling Vpu expression from the *env* isoforms in a leaky ribosome-scanning manner (Fig. 6 and 7). Previous studies that described efficient Vpu transla-

tion from in vitro-transcribed *env* RNA isoforms containing exon 4bE and 4aE used the rabbit reticulocyte lysate translation system that ignores known control elements such as the 5′ m⁷Gppp cap structure and the 3′ poly(A) tail (17, 53). Rabbit reticulocyte lysate translation also ignores powerful upstream ORFs such as the Tat AUG codon in 4-kb *tat* mRNA, where the upstream Tat ORF otherwise blocks Env translation in vivo (53).

The most perplexing finding of this study was that even though the Rev AUG codon in the 5′ UTR controlled Vpu synthesis, the levels of Env synthesis by the *env* isoforms remained uniform. Therefore, contrary to that of Vpu, this consistent expression of Env protein does not lend itself to the leaky scanning translation mechanism previously reported by others (53). The native *env* isoforms expressed similar levels of Env irrespective of the 5′ UTR and Rev AUG codon. Furthermore, removing the upstream Rev and/or Vpu AUG codon or adding a strong Kozak consensus AUG codon in the 5′ UTR had little impact on the uniform synthesis of Env protein (Fig. 7 and 8). Explanations involving cryptic splicing to a monocistronic Env mRNA or highly stable RNA were also ruled out experimentally (Fig. 4 and 5). Instead, we propose that the consistent expression of Env from these isoforms arises from a discontinuous translation initiation mechanism that can bypass the effects of upstream AUG codons on downstream ORFs, enabling consistent Env production irrespective of the upstream Rev, Vpu, or strong AUG codon inserted into exon 2.

Examples of translation initiation by discontinuous initiation methods include the use of an IRES and ribosome shunting. Both mechanisms allow ribosomes to bypass upstream AUG codons and ORFs, targeting translation initiation to the correct, downstream start codon. For initiation by an IRES, ribosome components are recruited directly to the initiation codon mRNA without the 40S subunits engaging the 5′ cap structure, hence the description of this process as cap independent translation. In contrast, ribosome shunting is cap dependent, but the scanning 40S ribosome subunit can jump over or bypass large regions of RNA between shunt "donor" and "acceptor" motifs and does not require continuous scanning to locate downstream initiation codons (reviewed in references 18 and 40). Common to both of these translation initiation mechanisms are long 5′ UTR rich in secondary structures, a feature of HIV-1 5′ UTR (5, 7).

The translation of Env via the discontinuous IRES or ribosome shunt initiation would fit with the timing of Env translation late in HIV-1 replication, when viral Gag and Gag-Pol structural polyproteins are also synthesized. The Pol polyprotein contains the viral protease that was shown to cleave eIF4G and inhibit cap-dependent translation (59). Furthermore, IRES elements for the translation of both HIV-1 and simian immunodeficiency virus Gag have been defined, as well as elements for the translation of Env protein from other retroviruses (8, 11, 12, 16, 34, 39). Therefore, HIV-1 Env may use a discontinuous translation mechanism at a time late in replication if cap-dependent translation is diminished by the cleavage of eIF4G by the viral protease (59). The HIV Vpr accessory protein is also translated from mRNA containing RRE late in HIV-1 replication (53, 54). Vpr arrests the cell cycle in the G₂/M phase (25, 48), which coincides with limiting cellular levels of the eIF4E, a critical 5′ cap binding translation factor

that restricts cellular translation during cell division (50). Therefore, HIV-1 Env may also use an IRES or ribosome shunt translation mechanism to maximize Env production during G₂/M cell cycle arrest. In a cellular environment in which viral protease and/or Vpr G₂/M arrest represses the cap-dependent translation of Vpu and CD4, efficient Env synthesis may potentially overwhelm entrapment by CD4, ensuring efficient Env incorporation into virions.

Env translation may use cap-dependent initiation, but thereafter short ORFs in the 5′ UTR of the *env* isoforms may direct discontinuous ribosome shunting, similar to that in the cauliflower mosaic virus (43–45). Short ORFs are present in the *env* isoforms just upstream of but overlapping the Vpu ORF. Mutation studies of the highly conserved short ORF (32) suggest that it is a promising candidate for a donor site for potential Env ribosome shunt translation (31a).

The introduction of a strong upstream AUG codon can usurp all the scanning 40S ribosome subunits and block the translation of downstream ORFs (21). For example, in HIV-1 *tat* mRNAs, the high level of Kozak strength of the Tat ORF blocks downstream Rev and Nef translation in 1.8-kb mRNA (55). We found that adding the strong Kozak AUG codon to upstream exon 2 in *env5* and *env6* mRNAs produced from pDR*env5* and pDR*env6* cDNA plasmids did not block consistent Env production, despite significant declines in the levels of Vpu, which is translated by leaky scanning (Fig. 8). This result supports a discontinuous ribosome-scanning mechanism for Env translation to avoid the effect of the strong, upstream, artificial AUG codon in exon 2. In contrast, when the Kozak strength of the Rev AUG codon in the *env2* isoform was increased nearly twofold (Fig. 6B), Env synthesis from the same isoform declined twofold (Fig. 6C), as would be expected if Env translation conformed to leaky ribosome-scanning initiation. But this mutation may also perturb the RNA structure involved in a discontinuous IRES or shunt initiation mechanism, for which RNA structure is often critical, and thus cause Env production to decline. The deletion of the Rev or Vpu AUG codon in isolation also led to minor, 1.3-fold increases in Env production (Fig. 6 and 7), consistent with leaky-scanning initiation, although the significance of such minor changes is questionable. Perhaps instead, these data indicate that HIV-1 Env is translated via dual mechanisms of discontinuous scanning (like the use of an IRES or shunt) and continuous leaky scanning after the translation of Vpu, analogous to the translation of adenovirus late mRNAs. With adenovirus late mRNAs, proportions of 40S ribosome subunits either shunt or continuously scan to translate downstream ORFs (65). A similar fate for ribosomes on HIV-1 *env* mRNAs would allow Env synthesis by a discontinuous IRES or shunt mechanism and Vpu (and, to a lesser extent, Env) synthesis by continuous leaky scanning. Therefore, the decline in Env levels corresponding to the Kozak Rev AUG codon mutation (Fig. 6C) may reflect a decline in Env production from leaky scanning while Env production by the discontinuous IRES or shunt mechanism proceeds.

HIV-1 *env* mRNAs are a compelling example in which separate translation mechanisms seem likely to translate the viral Vpu and Env genes. Perhaps lessons from the adenovirus late mRNAs, where 40S subunits scan continuously or shunt (65), will provide a useful model for understanding Vpu and Env

production from HIV-1 *env* mRNAs. We have identified a mode of HIV-1 translational control in which two different translation initiation mechanisms enable the virus to regulate Vpu expression while maintaining a constant supply of Env protein. Delineating the translation initiation mechanism for Env may reveal key features that may benefit future DNA vaccines that seek efficient expression of diverse HIV-1 Env proteins in vivo without synthetically optimizing the codons in the mRNA.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Amendt, B. A., D. Hesslein, L. J. Chang, and C. M. Stoltzfus.** 1994. Presence of negative and positive *cis*-acting RNA splicing elements within and flanking the first *tat* coding exon of human immunodeficiency virus type 1. Mol. Cell. Biol. **14:**3960–3970.
3. **Anderson, J. L., and T. J. Hope.** 2004. HIV accessory proteins and surviving the host cell. Curr. HIV/AIDS Rep. **1:**47–53.
4. **Ben-Asouli, Y., Y. Banai, H. Hauser, and R. Kaempfer.** 2000. Recognition of 5′-terminal TAR structure in human immunodeficiency virus-1 mRNA by eukaryotic translation initiation factor 2. Nucleic Acids Res. **28:**1011–1018.
5. **Berkhout, B.** 2000. Multiple biological roles associated with the repeat (R) region of the HIV-1 RNA genome. Adv. Pharmacol. **48:**29–73.
6. **Berkhout, B., R. H. Silverman, and K. T. Jeang.** 1989. Tat trans-activates the human immunodeficiency virus through a nascent RNA target. Cell **59:**273–282.
7. **Berkhout, B., and J. L. B. Van Wamel.** 2000. The leader of the HIV-1 RNA genome forms a compactly folded tertiary structure. RNA **6:**282–295.
8. **Berlioz, C., and J.-L. Darlix.** 1995. An internal ribosomal entry mechanism promotes translation of murine leukemia virus *gag* polyprotein precursors. J. Virol. **69:**2214–2222.
9. **Bour, S., F. Boulerice, and M. A. Wainberg.** 1991. Inhibition of gp160 and CD4 maturation in U937 cells after both defective and productive infections by human immunodeficiency virus type 1. J. Virol. **65:**6387–6396.
10. **Bour, S., and K. Strebel.** 2000. HIV accessory proteins: multifunctional components of a complex system. Adv. Pharmacol. **48:**75–119.
11. **Brasey, A., M. Lopez-Lastra, T. Ohlmann, N. Beerens, B. Berkhout, J. L. Darlix, and N. Sonenberg.** 2003. The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G$_2$/M phase of the cell cycle. J. Virol. **77:**3939–3949.
12. **Buck, C. B., X. Shen, M. A. Egan, T. C. Pierson, C. M. Walker, and R. F. Siliciano.** 2001. The human immunodeficiency virus type 1 *gag* gene encodes an internal ribosome entry site. J. Virol. **75:**181–191.
13. **Caputi, M., M. Freund, S. Kammler, C. Asang, and H. Schaal.** 2004. A bidirectional SF2/ASF- and SRp40-dependent splicing enhancer regulates human immunodeficiency virus type 1 *rev*, *env*, *vpu*, and *nef* gene expression. J. Virol. **78:**6517–6526.
14. **Chang, Y.-N., D. J. Kenan, J. D. Keene, A. Gatignol, and K.-T. Jeang.** 1994. Direct interactions between autoantigen La and human immunodeficiency virus leader RNA. J. Virol. **68:**7008–7020.
15. **Cullen, B. R.** 2003. Nuclear mRNA export: insights from virology. Trends Biochem. Sci. **28:**419–424.
16. **Deffaud, C., and J.-L. Darlix.** 2000. Characterization of an internal ribosome entry segment in the 5′ leader of murine leukemia virus *env* RNA. J. Virol. **74:**846–850.
17. **Furtado, M. R., R. Balachandran, P. Gupta, and S. M. Wolinsky.** 1991. Analysis of alternatively spliced human immunodeficiency virus type-1 mRNA species, one of which encodes a novel TAT-ENV protein. Virology **185:**258–270.
18. **Gale, M., S.-L. Tan, and M. G. Katze.** 2000. Translational control of viral gene expression in eukaryotes. Microbiol. Mol. Biol. Rev. **64:**239–280.
19. **Gatignol, A., A. Buckler-White, B. Berkhout, and K. T. Jeang.** 1991. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. Science **251:**1597–1600.

20. **Gibbs, J. S., D. A. Regier, and R. C. Desrosiers.** 1994. Construction and in vitro properties of HIV-1 mutants with deletions in nonessential genes. AIDS Res. Hum. Retrovir. **10:**343–350.
21. **Gray, N. K., and M. Wickens.** 1998. Control of translation initiation in animals. Annu. Rev. Cell Dev. Biol. **14:**399–458.
22. **Hope, T. J.** 1999. The ins and outs of HIV Rev. Arch. Biochem. Biophys. **365:**186–191.
23. **Hope, T. J., X. Huang, D. McDonald, and T. G. Parslow.** 1990. Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif. Proc. Natl. Acad. Sci. USA **87:**7787–7791.
24. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus.** 1988. Characterization of ribosomal frameshifting in HIV-1 *gag-pol* expression. Nature **331:**280–283.
25. **Jowett, J. B., V. Planelles, B. Poon, N. P. Shah, M. L. Chen, and I. S. Y. Chen.** 1995. The human immunodeficiency virus type 1 *vpr* gene arrests infected T cells in the G$_2$ + M phase of the cell cycle. J. Virol. **69:**6304–6313.
26. **Kjems, J., and P. Askjaer.** 2000. Rev protein and its cellular partners. Adv. Pharmacol. **48:**251–298.
27. **Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein.** 1990. The human immunodeficiency virus type 1-specific protein Vpu is required for efficient virus maturation and release. J. Virol. **64:**621–629.
28. **Kozak, M.** 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. **196:**947–950.
29. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:**283–292.
30. **Kozak, M.** 1992. Regulation of translation in eukaryotic systems. Annu. Rev. Cell Biol. **8:**197–225.
31. **Krummheuer, J., C. Lenz, S. Kammler, A. Scheid, and H. Schaal.** 2001. Influence of the small leader exons 2 and 3 on human immunodeficiency virus type 1 gene expression. Virology **286:**276–289.
31a.**Krummheuer, J., A. T. Johnson, I. Hauber, S. Kammler, J. L. Anderson, J. Hauber, D. F. Purcell, and H. Schaal.** 27 February 2007, posting date. A minimal uORF within the HIV-1 *vpu* leader allows efficient translation initiation at the downstream *env* AUG. Virology. doi:10.1016/j.virol.2007.01.022.
32. **Kuiken, C. L., B. Foley, E. O. Freed, B. Hahn, B. Korber, P. A. Marx, F. McCutchan, J. W. Mellors, and S. M. Wolinsky.** 2002. HIV Sequence Compendium 2002. Los Alamos National Laboratory, Los Alamos, NM.
33. **Levy, J. A.** 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. **57:**183–289.
34. **Lopez-Lastra, M., C. Gabus, and J.-L. Darlix.** 1997. Characterization of an internal ribosomal entry segment within the 5′ leader of avian reticuloendotheliosis virus type A RNA and development of novel MLV-REV-based retroviral vectors. Hum. Gene Ther. **8:**1855–1865.
35. **Maio, J. J., and F. L. Brown.** 1988. Regulation of expression driven by human immunodeficiency virus type 1 and human T-cell leukemia virus type 1 long terminal repeats in pluripotential human embryonic cells. J. Virol. **62:**1398–1407.
36. **Mak, J., M. Jiang, M. A. Wainberg, M. L. Hammarskjold, D. Rekosh, and L. Kleiman.** 1994. Role of Pr160$^{gag-pol}$ in mediating the selective incorporation of tRNA^{Lys} into human immunodeficiency virus type 1 particles. J. Virol. **68:**2065–2072.
37. **Maldarelli, F., M.-Y. Chen, R. L. Willey, and K. Strebel.** 1993. Human immunodeficiency virus type 1 Vpu protein is an oligomeric type 1 integral membrane protein. J. Virol. **67:**5056–5061.
38. **McMillan, N. A. J., R. F. Chun, D. P. Siderovski, J. Galabru, W. M. Toone, C. E. Samuel, T. W. Mak, A. G. Hovanessian, K. T. Jeang, and B. R. G. Williams.** 1995. HIV-1 Tat directly interacts with the interferon-induced, double-stranded RNA-dependent kinase, PKR. Virology **213:**413–424.
39. **Ohlmann, T., M. Lopez-Lastra, and J.-L. Darlix.** 2000. An internal ribosome entry segment promotes translation of the simian immunodeficiency virus genomic RNA. J. Biol. Chem. **275:**11899–11906.
40. **Pe'ery, T., and M. B. Mathews.** 2000. Viral translational strategies and host defense mechanisms, p. 371–424. *In* N. Sonenberg, J. W. B. Hershey, and M. B. Mathews (ed.), Translational control of gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
41. **Pestova, T. V., V. G. Kolupaeva, I. B. Lomakin, E. V. Pilipenko, I. N. Shatsky, V. I. Agol, and C. U. T. Hellen.** 2001. Molecular mechanisms of translation initiation in eukaryotes. Proc. Natl. Acad. Sci. USA **98:**7029–7036.
42. **Pollard, V. W., and M. H. Malim.** 1998. The HIV-1 Rev protein. Annu. Rev. Microbiol. **52:**491–532.
43. **Pooggin, M. M., J. Futterer, K. G. Skryabin, and T. Hohn.** 2001. Ribosome shunt is essential for infectivity of cauliflower mosaic virus. Proc. Natl. Acad. Sci. USA **98:**886–891.
44. **Pooggin, M. M., J. Futterer, K. G. Skryabin, and T. Hohn.** 1999. A short open reading frame terminating in front of a stable hairpin is the conserved feature in pregenomic RNA leaders of plant pararetroviruses. J. Gen. Virol. **80:**2217–2228.
45. **Pooggin, M. M., T. Hohn, and J. Fütterer.** 2000. Role of a short upstream open reading frame in ribosome shunt on the cauliflower mosaic virus RNA leader. J. Biol. Chem. **275:**17288–17296.

46. **Purcell, D. F. J., and M. A. Martin.** 1993. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. J. Virol. **67:**6365–6378.

47. **Riggs, N. L., S. J. Little, D. D. Richman, and J. C. Guatelli.** 1994. Biological importance and cooperativity of HIV-1 regulatory gene splice acceptors. Virology **202:**264–271.

48. **Rogel, M. E., L. I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. J. Virol. **69:**882–888.

49. **Ropers, D., L. Ayadi, R. Gattoni, S. Jacquenet, L. Damier, C. Branlant, and J. Stevenin.** 2004. Differential effects of the SR proteins 9G8, SC35, ASF/SF2, and SRp40 on the utilization of the A1 to A5 splicing sites of HIV-1 RNA. J. Biol. Chem. **279:**29963–29973.

50. **Sachs, A. B.** 2000. Cell cycle-dependent translation initiation: IRES elements prevail. Cell **101:**243–245.

51. **Schubert, U., L. C. Antón, I. Bacík, J. H. Cox, S. Bour, M. BenniOrlowski, K. Strebel, and J. W. Yewdell.** 1998. CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. J. Virol. **72:**2280–2288.

52. **Schwartz, S., B. K. Felber, D. M. Benko, E.-M. Fenyö, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64:**2519–2529.

53. **Schwartz, S., B. K. Felber, E. M. Fenyo, and G. N. Pavlakis.** 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J. Virol. **64:**5448–5456.

54. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1991. Expression of human immunodeficiency virus type 1 vif and vpr mRNAs is rev-dependent and regulated by splicing. Virology **183:**677–686.

55. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1992. Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs. Mol. Cell. Biol. **12:**207–219.

56. **Strebel, K., T. Klimkait, F. Maldarelli, and M. A. Martin.** 1989. Molecular and biochemical analyses of human immunodeficiency virus type 1 Vpu protein. J. Virol. **63:**3784–3791.

57. **Strebel, K., T. Klimkait, and M. Martin.** 1988. A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science **241:**1221–1223.

58. **Varthakavi, V., R. M. Smith, S. P. Bour, K. Strebel, and P. Spearman.** 2003. Viral protein U counteracts a human host cell restriction that inhibits HIV-1 particle production. Proc. Natl. Acad. Sci. USA **100:**15154–15159.

59. **Ventoso, I., R. Blanco, C. Perales, and L. Carrasco.** 2001. HIV-1 protease cleaves eukaryotic initiation factor 4G and inhibits cap-dependent translation. Proc. Natl. Acad. Sci. USA **98:**12966–12971.

60. **Waysbort, A., S. Bonnal, S. Audigier, J.-P. Estève, and A.-C. Prats.** 2001. Pyrimidine tract binding protein and La autoantigen interact differently with the 5′ untranslated regions of lentiviruses and oncoretrovirus mRNAs. FEBS Lett. **490:**54–58.

61. **Wentz, M. P., B. E. Moore, M. W. Cloyd, S. M. Berget, and L. A. Donehower.** 1997. A naturally arising mutation of a potential silencer of exon splicing in human immunodeficiency virus type 1 induces dominant aberrant splicing and arrests virus production. J. Virol. **71:**8542–8551.

62. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as a donor. Cell **14:**725–731.

63. **Willey, R., F. Maldarelli, M. A. Martin, and K. Strebel.** 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J. Virol. **66:**7193–7200.

64. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884–1888.

65. **Yueh, A., and R. J. Schneider.** 1996. Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells. Genes Dev. **10:**1557–1567.

66. **Zahler, A. M., C. K. Damgaard, J. Kjems, and M. Caputi.** 2004. SC35 and heterogeneous nuclear ribonucleoprotein A/B proteins bind to a juxtaposed exonic splicing enhancer/exonic splicing silencer element to regulate HIV-1 tat exon 2 splicing. J. Biol. Chem. **279:**10077–10084.