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Translation of Equine Infectious Anemia Virus Bicistronic *tat-rev* mRNA Requires Leaky Ribosome Scanning of the *tat* CTG Initiation Codon

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We have examined the translational regulation of the equine infectious anemia virus (EIAV) bicistronic *tat-rev* mRNA. Site-directed mutagenesis of the *tat* leader region followed by expression of the *tat-rev* cDNA both in vitro and in transiently transfected cells established that *tat* translation is initiated exclusively at a CTG codon. Increasing the efficiency of *tat* translation by altering the CTG initiator to ATG resulted in a dramatic decrease in translation of the downstream (*rev*) cistron, indicating that leaky scanning of the *tat* CTG initiation codon permitted translation of the downstream *rev* cistron. Since the *tat* leader sequences precede the major EIAV splice donor and are therefore present at the 5′ termini of both spliced and unspliced viral mRNAs, the expression of all EIAV structural and regulatory proteins is dependent on leaky scanning of the *tat* initiator.

Equine infectious anemia virus (EIAV), a macrophage-tropic lentivirus, is the etiologic agent of a disease characterized by cyclic episodes of fever, anemia, and viremia (8). Like other lentiviruses, EIAV encodes both structural and regulatory gene products (20, 45, 53). One of these regulatory gene products, Tat, stimulates EIAV long terminal repeat (LTR)-directed gene expression in *trans* (11, 33). EIAV Tat is structurally and functionally similar to human immunodeficiency virus type 1 Tat (5, 10), and like human immunodeficiency virus type 1 Tat, EIAV Tat is translated from a multiply spliced mRNA (11, 33, 35, 50, 54).

The EIAV *tat* coding sequences possess several features of biological interest, foremost of which is the absence of an ATG initiation codon (11, 33, 54). Additionally, the genomic location of the EIAV *tat* coding exons is unique among lentiviruses in that the first coding exon is located 5′ to the major splice donor site (11, 33, 34). This exon is therefore present in all classes of EIAV mRNA, including unspliced, singly spliced, and multiply spliced transcripts. An important consequence of this unusual genomic organization is that EIAV mRNAs are, by necessity, functionally bicistronic. A number of mechanisms can be envisioned by which the translation of upstream and downstream open reading frames is regulated, including leaky scanning, internal initiation, and termination-reinitiation of translation (17, 25). Identification of the mechanism by which this coordinate synthesis is accomplished first requires an unambiguous identification of the codon at which translation of EIAV Tat is initiated. Deletion analysis of the EIAV *tat* open reading frame has generated conflicting results, with two different codons postulated to serve as the initiator (11, 35). By mutagenesis of individual codons within the EIAV *tat* open reading frame, we have definitively identified the *tat* initiation codon. Furthermore, the effects of altering the sequence of the *tat* initiation codon on the levels of Rev expression were consistent with a leaky scanning mechanism of translational control.

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MATERIALS AND METHODS

Construction of *tat-rev* bicistronic cDNAs. Full-length, multiply spliced *tat-rev* coding sequences were cloned in three steps. In the first step, D17 (canine osteosarcoma) cells were transfected with the EIAV proviral clone FL85 (gift of Nancy Rice). RNA was isolated 48 h later by the hot acid-phenol method, and random-primed cDNA was prepared (47). The cDNAs were amplified by polymerase chain reaction (PCR) with primer 1 (5′-CGCAGACCCTACCTGT TGAAC-3′, nucleotides [nt] 352 to 372) and primer 2 (5′-ACTGGTCACTTTCCAGAGGG-3′, nt 7263 to 7244). All coordinates are with respect to the published EIAV genomic sequence (20). The amplified DNA was digested at internal *Bam*HI (nt 386 to 391) and *Apa*I (nt 7241 to 7246) sites (Fig. 1). The resulting 323-nt fragment, encompassing all the splice junctions present in the mature, four-exon mRNA (54), was ligated into *Bam*HI- and *Apa*I-digested Bluescript (Stratagene), generating pKS-Ed1234d. Sequences missing from both the 5′ and 3′ termini of the random-primed cDNAs were restored by PCR amplification of EIAV clones. Exon 1 *tat* leader sequences were PCR amplified from the EIAV genomic clone FL85 by using primer 3 (5′-ATGACCGCG GCACTCAGATTCTGCGG-3′, nt 211 to 225) and primer 4 (5′-CTGGCCAGGAACACCTCC-3′, nt 444 to 426). Primer 3 contains an *Sst*II site (underlined). The amplified DNA, cleaved at *Sst*II and internal *Bam*HI sites, was ligated into *Sst*II- and *Bam*HI-digested pKS-Ed1234d, generating pKS-E1234d. The remaining exon 4 (*rev*) coding sequences were obtained by PCR amplification of the *rev* expression plasmid pK-Rev (courtesy of N. Rice) with primers 5 (5′-CATGGC AGAATCGAAGGAA-3′, nt 5445 to 5459) and 6 (5′-GTAC TCTAGAGCTTTAATGCAACAGTCATAA-3′, nt 7655 to 7635). Amplified DNA was digested at the internal *Apa*I site and the *Xba*I site introduced in primer 6 (underlined). The gel-purified fragment and the *Sst*II-*Apa*I restriction fragment from pKS-E1234d were ligated into *Sst*II- and *Xba*I-digested Bluescript, generating pKS-ET/R, which contained the entire four-exon *tat* and *rev* coding sequences.

Construction of *tat-rev* expression vectors. The in vitro transcription vector pUCT7 was constructed by inserting oligonucleotides containing bacteriophage T7 promoter se-

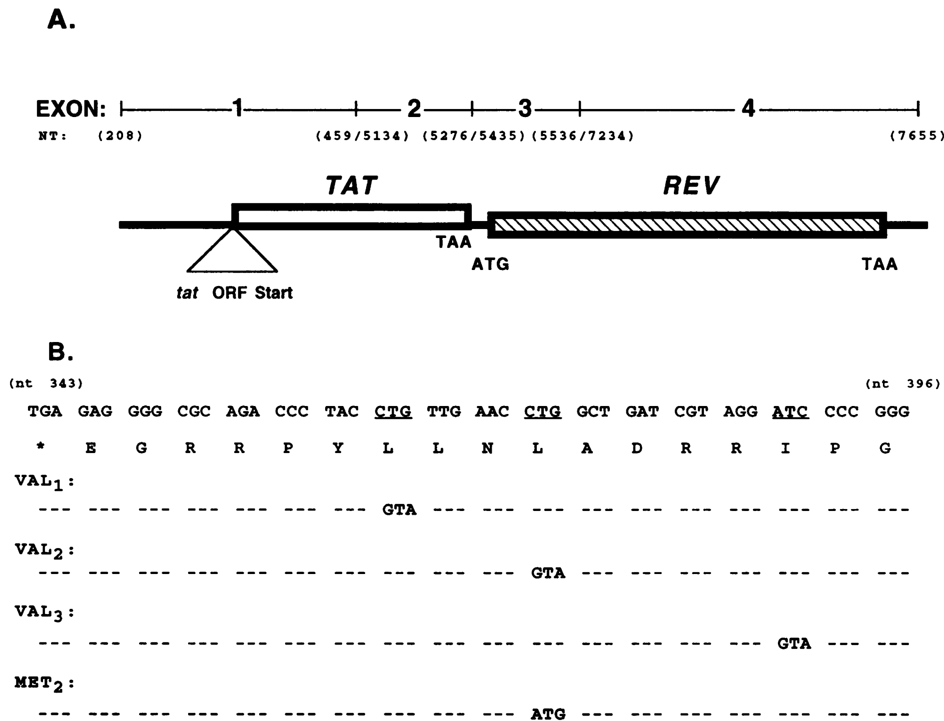

FIG. 1. Structure of bicistronic EIAV *tat-rev* mRNA. (A) Diagram of *tat* (open rectangle) and *rev* (hatched rectangle) open reading frames. The exons containing the open reading frames are indicated. Their nucleotide coordinates are given with respect to the previously published EIAV genomic sequence (20). The *rev* start codon and the *rev* and *tat* stop codons are indicated. *tat* ORF start refers to the beginning of the *tat* open reading frame. (B) Nucleotide and predicted amino acid sequence of the beginning of the EIAV *tat* open reading frame. The potential *tat* initiation codons examined in the text are underlined. The sequences of mutants containing alterations within this region are shown. Only altered nucleotides are indicated.

quences (5′-TAATACGACTCACTATA-3′ and 5′-TATAGT GAGTCGTATTA-3′) into *Hin*dIII- and *Pst*I-digested, blunt-ended pUC19. The *tat-rev* coding sequences from pKS-ET/R were digested with *Sst*II, blunt ended, digested with *Xba*I, and ligated into *Apa*I-digested, blunt-ended, and *Xba*I-digested pUCT7. The blunt-end fusion of *Apa*I-digested vector and *Sst*II-digested insert generated the native EIAV cap site (7). For in vivo expression of *tat-rev* DNA, the expression vector pRSPA was modified to permit insertion of cDNA coding sequences at the Rous sarcoma virus transcription start site. pRSPA contains the Rous sarcoma virus promoter and the simian virus 40 polyadenylation signals cloned into Bluescript (11). An *Sst*I site was introduced at the Rous sarcoma virus cap site by site-directed mutagenesis with the PCR overlap technique (16). The oligonucleotides used were 5′-GGTGAATGGTCAGAGCTCGTTTATTGTATCGAG-3′ and 5′-GATACAATAAACGAGCTCTGACCATTCACCA CAT-3′; the *Sst*I sites are underlined. Additionally, the Bluescript polylinker sequences and *Eco*RI sites in the simian virus 40 polyadenylation signal were removed to facilitate subsequent cloning and mutagenesis procedures. The resulting modified expression vector was termed pRSSPA. The *tat-rev* coding sequences were removed from pKS-ET/R by *Sst*II digestion, blunt ending, and *Xba*I digestion. The purified fragment was inserted into *Sst*I-digested, blunt-ended, *Xba*I-digested pRSSPA. The fusion of blunt-ended *Sst*I vector and *Sst*II insert fragments reconstituted the native EIAV cap site.

Mutagenesis of *tat* coding sequences. The PCR overlap procedure was used to mutate individual codons within the *tat* open reading frame. The following oligonucleotides were used to alter the CTG at nt 364 to 366 (CTG-364) to GTA: 5′-CGCAGACCCTACGTATTGAACCTGGCTGATC-3′ and 5′-AGCCAGGTTCAATACGTAGGGTCTGCGCCCC-3′. The following oligonucleotides were used to alter CTG-373 to GTA: 5′-TACCTGTTGAACGTAGCTGATCGTAG GATCC-3′ and 5′-CCTACGATCAGCTACGTTCAACAGG TAGGGT-3′. The following oligonucleotides were used to alter CTG-373 to ATG: 5′-TACCTGTTGAACATGGCTGA TCGTAGGATC-3′ and 5′-CCTACGATCAGCCATGTTCA ACAGGTAGGGT-3′. The following oligonucleotides were used to alter ATC-388 to GTA: 5′-GCTGATCGTAGGGTA CCCGGGACAGCAGAGG-3′ and 5′-TGCTGTCCCGGGT ACCCTACGATCAGCCAGG-3′. The mutagenized sites are underlined. Both pUCT7-ET/R and pRSS-ET/R were subjected to the mutagenesis procedures, and conveniently sized restriction fragments containing the mutations were cut out and inserted in place of their wild-type counterparts. The nucleotide sequence of all constructs, both wild-type and mutant, was determined by dideoxynucleotide sequencing with Sequenase (U.S. Biochemicals) or by automated sequence analysis with an Applied Biosystems model 373A DNA sequencer.

In vitro transcription and translation. pUCT7 expression constructs were linearized and capped mRNAs were synthesized by using T7 RNA polymerase (New England Biochemicals) and m^{7}GpppG (Boehringer Mannheim). mRNA integrity and yield were evaluated on formaldehyde-agarose gels (47). mRNAs were translated in wheat germ extracts (Promega) in the presence of [^{35}S]cysteine (New England

Nuclear). Translation products were analyzed by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (49) on 15% acrylamide gels and visualized by fluorography with Enlightening (New England Nuclear). Alternatively, translation products were immunoprecipitated from the wheat germ extract prior to gel analysis (see below).

Transfections and CAT assays. Transfections and chloramphenicol acetyltransferase (CAT) assays were performed essentially as described before (5). D17 cells (3×10^5) were transfected with *tat-rev* expression plasmid and 3 μg of pEI-CAT, a reporter construct containing the EIAV LTR and the bacterial CAT gene (12), by the calcium phosphate procedure (14). At 48 h after transfection, cell extracts were prepared by suspending the cell pellet in 0.1 M Tris-HCl (pH 7.8)–0.5% Nonidet P-40. The suspension was incubated at room temperature for 5 min and then centrifuged for 5 min in a microcentrifuge. CAT activity in the supernatant was determined by the solvent partition method (31).

Antisera. The preparation of the peptide antisera used in this study has been described previously (6, 54). Peptide Etat-30 (NYHCQLCFLRSLGIDYLDASLRKKNKQRLK) comprises residues 34 to 63 of the product of the EIAV *tat* expression vector pRS-ETat-M (11). Rev peptides include Rev-N (AESKEARDQEMNLKE, amino acids 2 to 16 of EIAV Rev), Rev-1 (LIDPQGPLESDQ, amino acids 29 to 40), Rev-2 (LRQSLPEEKIPSQT, amino acids 45 to 58), Rev-3 (GPGPTQHPSR, amino acids 66 to 76), and Rev-C (SPRVLRPGDSK, amino acids 149 to 158).

Radioimmunoprecipitation analysis. Radioimmunoprecipitations were performed essentially as described before (47). D17 cells (3×10^5) were transfected with 5 μg of *tat-rev* expression plasmid. After 48 h, the cells were starved in cysteine-free, methionine-free medium containing 5% dialyzed fetal calf serum and then labeled in medium containing 250 μCi each of [^{35}S]cysteine and [^{35}S]methionine per ml. The cells were lysed in triple detergent lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 100 μg of phenylmethylsulfonyl fluoride per ml, 1% Nonidet P-40, 0.5% sodium deoxycholate) (47) and sheared by passage through a 26-gauge needle, and insoluble debris was pelleted by centrifugation for 20 min in a microcentrifuge. The supernatant was divided into two parts. Tat peptide antiserum was added to one portion, while Rev peptide antiserum was added to the other. Immune complexes were collected by addition of protein A–Sepharose CL-4B (Pharmacia). The immune complexes were washed twice in RIPA buffer (47) and once in 10 mM Tris-HCl (pH 7.5)–0.5% Nonidet P-40. The precipitated proteins were released from the protein A-Sepharose by boiling in 1× Tricine-SDS-PAGE sample buffer (49), resolved by Tricine-SDS-PAGE on 15% acrylamide gels, and analyzed by fluorography. Radioimmunoprecipitation analysis of [^{35}S]cysteine-labeled in vitro translation products was accomplished by adding 10 volumes of IVL buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 1.0% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) directly to the wheat germ extract. Samples were precipitated and collected as described above, washed three times in IVL buffer, and analyzed by Tricine-SDS-PAGE and fluorography.

RESULTS

The bicistronic *tat-rev* cDNA shown in Fig. 1 results from multiple splicing events (54). *tat-rev* cDNAs obtained from EIAV-infected D17 cells were reconstructed so that their 5′ termini were fully intact, as described in Materials and Methods. The indicated nucleotide coordinates are with respect to the previously published EIAV genome sequence (20). The *tat* coding sequences are contained in exon 1, the leader exon (nt 208 to 459, where nt 208 is the cap site), and exon 2 (nt 5134 to 5276), while the *rev* coding sequences are contained in exon 3 (nt 5435 to 5536) and exon 4 (which starts at nt 7234 and is truncated immediately downstream of the *rev* termination codon; see Materials and Methods). In order to examine how translation of this bicistronic mRNA is regulated, we examined Tat and Rev synthesis both in vitro in a wheat germ cell-free translation system and in vivo in transiently transfected cells.

In vitro translation of *tat-rev* bicistronic mRNA. The *tat-rev* cDNA was inserted into the in vitro expression plasmid pUCT7, generating plasmid pUCT7-ET/R. Linearized plasmid DNA was transcribed in vitro, and the resulting mRNA was translated in a wheat germ extract. Analysis of the [^{35}S]cysteine-labeled translation products by tricine-SDS-PAGE revealed three labeled proteins (Fig. 2A). The fastest-migrating band had an apparent size of approximately 8 kDa, consistent with the predicted size of EIAV Tat, and it was precipitated by EIAV Tat-specific antiserum (Fig. 2A, lane 3). The two more slowly migrating bands, with apparent sizes of 20 and 15 kDa, were both recognized by EIAV Rev-specific antiserum (Fig. 2A, lane 5). The 20-kDa protein corresponded to the predicted size of EIAV Rev (46, 54). The anomalous 15-kDa protein was characterized by radioimmunoprecipitation analysis, in which both Rev-related proteins were mapped with a panel of Rev-specific antisera (Fig. 2B and C). The 20-kDa protein was precipitated by the entire panel of antisera. The 15-kDa protein was precipitated by antisera directed against Rev amino-terminal peptides but not by antisera directed against Rev carboxy-terminal peptides, indicating that this protein was a carboxy-terminal truncated version of Rev. Pulse-chase experiments failed to detect a precursor-product relationship between the 20-kDa and 15-kDa proteins, indicating that the 15-kDa protein did not arise from proteolytic degradation of full-length Rev (data not shown). Therefore, these experiments suggest that the 15-kDa Rev-related protein arose from a premature termination of translation.

Identification of the EIAV *tat* initiation codon. Three in-frame codons at the 5′ terminus of the EIAV *tat* open reading frame were selected as potential initiation codons (Fig. 1). The CTG codon at nt 364 to 366 (CTG-364) was selected for examination because of earlier reports of translation initiation at CTG codons in both cellular and viral mRNAs (13, 15, 42). (Since all manipulations were performed at the DNA level, nucleotides are referred to in their DNA form.) The CTG codon at nt 373 to 375 (CTG-373) and the ATC codon at nt 388 to 390 (ATC-388) were selected for examination because of the results of prior deletion analyses of EIAV cDNAs (11, 35, 54).

Codons CTG-364, CTG-373, and ATC-388 were individually altered to GTA (valine) codons by site-directed mutagenesis, and the resulting mutations, termed Val_1, Val_2, and Val_3, respectively, were inserted into pUCT7. Translation of in vitro-transcribed Val_1 and Val_3 mRNA in wheat germ extracts resulted in protein patterns identical to that obtained from wild type *tat-rev* mRNA, with high levels of both Tat and Rev synthesis observed (Fig. 3). Translation of Val_2 mRNA yielded a different result. Rev levels remained high, but Tat was completely absent. This experiment demonstrated that translation of the EIAV *tat* open reading frame

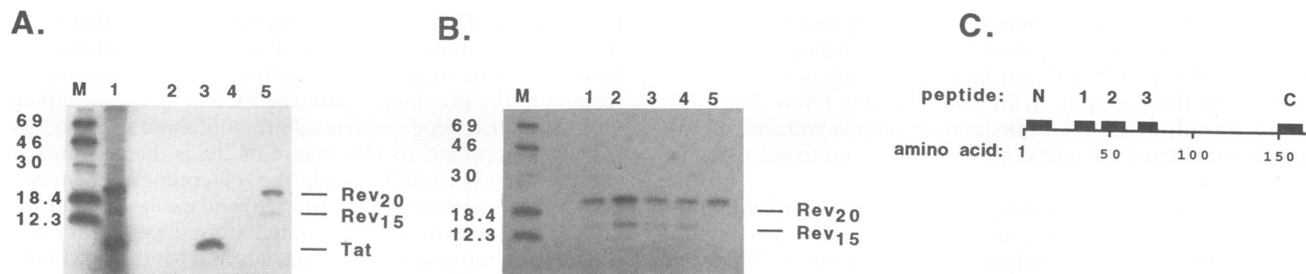

FIG. 2. (A) In vitro translation of *tat-rev* bicistronic mRNA. Linearized, in vitro-transcribed pUCT7-ET/R mRNA was translated in a wheat germ extract cell-free translation system. Where indicated, the [^{35}S]cysteine-labeled proteins were immunoprecipitated prior to Tricine-SDS-PAGE analysis as described in Materials and Methods. Lane M contains ^{14}C-labeled molecular size markers (albumin [69 kDa], ovalbumin [46 kDa], carbonic anhydrase [30 kDa], lactoglobulin [18.4 kDa], and cytochrome *c* [12.3 kDa]). Lane 1 contains the unprecipitated wheat germ translation products. Translation products were precipitated with Tat preimmune serum (lane 2), Tat peptide antiserum Etat-30 (lane 3), Rev preimmune serum (lane 4), and Rev peptide antiserum Rev-3 (lane 5). Rev_{20} and Rev_{15} indicate the bands of 20 and 15 kDa, respectively, that reacted with the Rev-specific antiserum. (B) In vitro translation and immunoprecipitation of Rev-related proteins. In vitro translation products of the bicistronic *tat-rev* mRNA were precipitated with a battery of Rev peptide antisera, and the precipitates were analyzed by Tricine-SDS-PAGE. Lane M contains ^{14}C-labeled molecular size markers. Proteins precipitated by Rev antisera Rev-N (lane 1), Rev-1 (lane 2), Rev-2 (lane 3), Rev-3 (lane 4), and Rev-C (lane 5) are shown. (C) Schematic diagram of the *rev* open reading frame, depicting the approximate locations of peptides from which Rev-specific antisera were derived. The exact peptide locations and sequences are given in Materials and Methods.

initiates exclusively at CTG-373. If, as has been suggested (50), translation of the EIAV *tat* open reading frame could be initiated at several codons, then mutation of CTG-373 would have merely diminished rather than abolished *tat* translation.

In several other instances when translation is initiated at non-ATG codons, methionine, rather than the amino acid specified by the initiator codon (in the case of EIAV Tat, leucine), serves as the initiator amino acid (2, 36). In order to determine whether methionine is inserted in response to the *tat* CTG initiation codon, we took advantage of the fact that the EIAV *tat* coding sequence contains no methionine codons. Therefore, unless methionine is the initiator amino acid, in vitro translations performed in the presence of [^{35}S]methionine as the sole radioisotope should not generate labeled Tat protein. Translation of wild type *tat-rev* mRNA in a wheat germ extract generated an 8-kDa, [^{35}S]methionine-labeled protein that was specifically precipitated by EIAV Tat peptide antiserum (Fig. 3B). The lower intensity of [^{35}S]methionine-labeled Tat relative to [^{35}S]cysteine-labeled Tat (Fig. 3B) can be attributed, at least in part, to the presence of two cysteine codons within the *tat* coding sequence. This experiment suggests that methionine functions as the initiator amino acid in EIAV Tat.

Translation of downstream cistrons in response to changes in the efficiency of the *tat* initiation codon. Since the EIAV *tat* initiation codon is present in all EIAV mRNAs, translation of EIAV messages must be regulated so that adequate levels of expression of both upstream and downstream cistrons are achieved. In order to determine how this coordinate translational regulation is effected, we examined Rev protein synthesis under conditions in which translation of the upstream (*tat*) open reading frame was normal, absent, or substantially higher than normal. We used the following *tat-rev* constructs: pUCT7-ET/R, which contains the wild-type CTG-373 initiation codon; pUCT7-Val_2, in which the CTG codon was altered to a GTA codon; and pUCT7-Met_2, in which CTG-373 was altered to an ATG codon (Fig. 1). In vitro-transcribed mRNA was translated in a wheat germ extract, and the [^{35}S]cysteine-labeled products were analyzed by SDS-PAGE. As described above, translation of

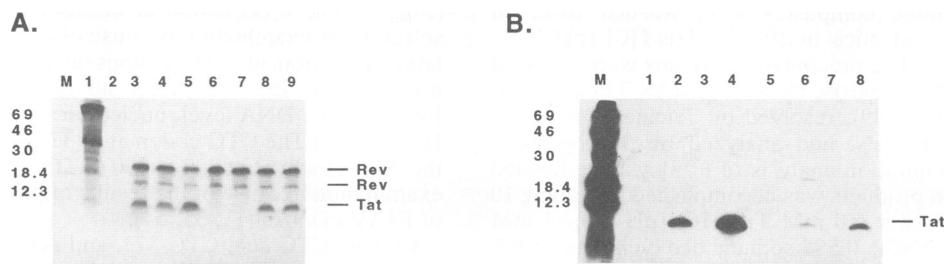

FIG. 3. (A) Identification of the *tat* initiation codon. mRNA obtained from wild-type and mutant *tat-rev* constructs (see Fig. 1) was translated in wheat germ extracts, and the [^{35}S]cysteine-labeled proteins were analyzed by Tricine-SDS-PAGE. Two independent clones of all *tat* open reading frame mutants were analyzed. The autoradiogram depicts the translation products obtained when brome mosaic virus RNA (lane 1), no exogenous RNA (lane 2), wild-type *tat-rev* mRNA (lane 3), Val_1 RNA (lanes 4 and 5), Val_2 RNA (lanes 6 and 7), or Val_3 RNA (lanes 8 and 9) was added to the wheat germ extract. Molecular size markers are shown in lane M (sizes in kilodaltons). The positions of Tat and Rev are indicated. (B) Identification of the amino acid inserted in response to the *tat* CTG initiation codon. Wild-type *tat-rev* RNA (lanes 1, 2, 5, and 6) and Met_2 RNA (see Fig. 1) (lanes 3, 4, 7, and 8) were translated in the wheat germ cell-free system in the presence of [^{35}S]cysteine (lanes 1 to 4) or [^{35}S]methionine (lanes 5 to 8). Translation products were precipitated with Tat preimmune serum (lanes 1, 3, 5, and 7) or the Tat peptide antiserum Etat-30 (lanes 2, 4, 6, and 8) prior to analysis by Tricine-SDS-PAGE and autoradiography. Lane M contains molecular size markers.

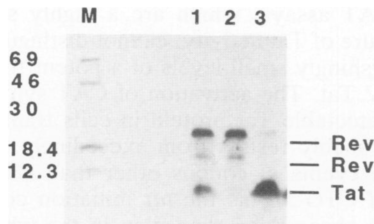

FIG. 4. Alteration in Rev synthesis in response to changes in the *tat* initiation codon. In vitro-transcribed *tat-rev* mRNA was translated in wheat germ extracts, and the [^{35}S]cysteine-labeled proteins were analyzed by Tricine-SDS-PAGE and autoradiography. The translation products of wild-type *tat-rev* mRNA (lane 1), Val_2 mRNA (lane 2), and Met_2 mRNA (lane 3) are shown. The *tat* initiation codons in these constructs are CTG, GTA, and ATG, respectively. Lane M contains ^{14}C-labeled molecular size markers (shown in kilodaltons).

wild-type (pUCT7-ET/R) mRNA resulted in high levels of both Tat and Rev synthesis, while translation of pUCT7-Val_2 mRNA resulted in high levels of Rev synthesis but no Tat synthesis (Fig. 3 and 4). In contrast, translation of pUCT7-Met_2 mRNA yielded a marked increase in Tat synthesis and a corresponding abrupt decrease in Rev synthesis (Fig. 4). While Rev was still detectable, it was evident that translation of the *rev* open reading frame had been inhibited dramatically. The virtual abolition of translation of the downstream reading frame concomitant with increased initiation capacity of the upstream reading frame strongly suggests that translation of EIAV mRNA is regulated by a leaky scanning mechanism (25).

In order to put the leaky-scanning hypothesis to a more rigorous test, the *tat-rev* sequences from pUCT7-ET/R (containing a CTG *tat* initiation codon), pUCT7-Val_2 (containing a nonfunctional GTA *tat* initiation codon), and pUCT7-Met_2 (containing an ATG *tat* initiation codon) were inserted into the eukaryotic expression plasmid pRSSPA. The resulting expression vectors, termed pRSS-ET/R, pRSS-Val_2, and pRSS-Met_2, respectively, were transfected into D17 cells, a canine osteosarcoma cell line in which EIAV Tat is active (11). Tat and Rev synthesis in transfected cells was examined by metabolic labeling with both [^{35}S]cysteine and [^{35}S]methionine, followed by radioimmunoprecipitation and Tricine-SDS-PAGE (Fig. 5). Cells transfected with pRSS-ET/R synthesized high levels of Rev and low but significant levels of Tat. The ratio of Tat to Rev in transfected cells was much lower than that observed in wheat germ extracts, in concurrence with the observation that recognition of non-ATG initiation codons is more relaxed in vitro than in vivo (24, 37). While the inclusion of [^{35}S]methionine in the labeling medium would enhance the apparent ratio of Rev to Tat (due to the presence of two methionine residues in Rev versus only one apparent methionine residue in Tat), similar low levels of Tat synthesis were observed in cells labeled with [^{35}S]cysteine only (data not shown). In cells transfected with pRSS-Val_2, Rev was produced in abundance, but as seen in wheat germ extracts, Tat was completely absent, confirming that CTG-373 is the *tat* initiation codon. In cells transfected with pRSS-Met_2, Tat synthesis increased to high levels, while Rev synthesis declined to barely detectable levels. The analysis of Tat and Rev synthesis in transiently transfected cells confirms the results obtained in in vitro translation assays and further supports the suggestion that translation of the downstream Rev cistron is accomplished by leaky scanning.

***trans* activation by *tat-rev* constructs with altered *tat* initiation codons.** The ability of pRSS-ET/R, pRSS-Val_2, and pRSS-Met_2 to activate the EIAV promoter was assayed by cotransfecting D17 cells with the *tat-rev* expression plasmids and pEI-CAT, a reporter construct containing the EIAV LTR fused to the bacterial CAT gene (12). When small amounts (10 ng) of *tat* plasmids were used both pRSS-ET/R and pRSS-Met_2 activated CAT expression, while pRSS-Val_2 failed to do so (Fig. 6). However, the level of pRSS-Met_2-stimulated CAT activity was less than twofold higher than the level obtained with pRSS-ET/R, despite the substantially greater quantity of Tat protein produced by pRSS-Met_2 (Fig. 5). The discrepancy between the levels of Tat synthesis and the resulting CAT activity became even more apparent when cells were transfected with larger amounts (100 ng) of *tat-rev* expression plasmids. Under these conditions, pRSS-Val_2 also activated CAT expression (Fig. 6); the pRSS-Val_2-stimulated increase in CAT activity occurred in the absence of detectable Tat protein (Fig. 5). These observations indicate that even at the lowest level of transactivator assayed (10 ng), a nonlinear relationship exists between the amount of Tat protein present and the resulting CAT activity. This suggests that translation initiation at either CTG-373 or ATG yielded saturating quantities of Tat, while aberrant initiation at other non-ATG codons produced minute levels of the potent transactivator that could only be detected by the highly sensitive but indirect CAT assay.

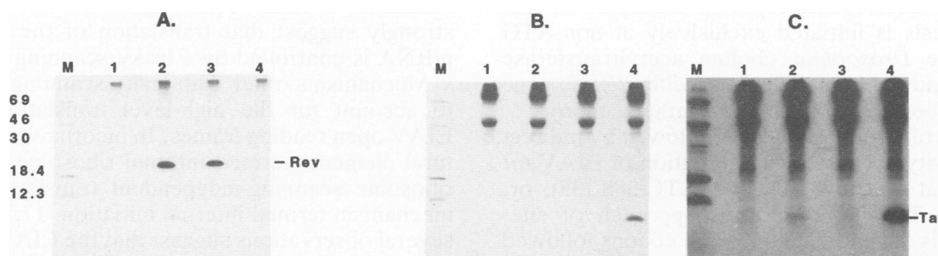

FIG. 5. Tat and Rev synthesis in transiently transfected D17 cells. D17 cells were transfected with pRSSPA, containing no *tat* or *rev* coding sequences (lane 1); pRSS-ET/R, containing the wild-type *tat-rev* expression construct (lane 2); pRSS-Val_2, in which the *tat* initiation codon is altered to GTA (lane 3); or pRSS-Met_2, in which the *tat* initiation codon is altered to ATG (lane 4). [^{35}S]cysteine- and [^{35}S]methionine-labeled cells were lysed, and the lysate was split into two portions. One portion was precipitated with the Rev peptide antiserum Rev-3 (A), while the other was reacted with the Tat peptide antiserum Etat-30 (B). Autoradiograms A and B were exposed for identical periods of time, while autoradiogram C represents a longer exposure of autoradiogram B. The positions of Tat and Rev are indicated. Sizes are shown in kilodaltons.

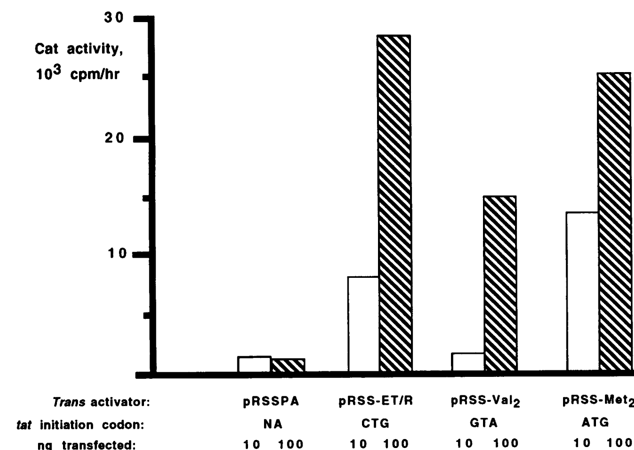

FIG. 6. *trans* activation of the EIAV LTR by bicistronic constructs with altered *tat* initiation codons. D17 cells were transfected with the EIAV LTR-CAT construct pEI-CAT and with either 10 ng (open bars) or 100 ng (hatched bars) of the indicated *tat-rev* construct. The resulting CAT activity is represented. pRSSPA contains no *tat* or *rev* coding sequences and was used to determine basal pEI-CAT activity. NA, not applicable.

DISCUSSION

Through site-directed mutagenesis of the EIAV *tat* open reading frame, we have demonstrated that initiation of *tat* translation occurs at a CTG codon, and we have examined the impact of non-ATG initiation on the coordinate translational control of upstream and downstream open reading frames in a bicistronic *tat-rev* mRNA.

Translation initiation at non-ATG codons has been observed for the human c-*myc* (15), murine *int-2* (1), and murine *pim-1* genes (48), the adeno-associated virus capsid protein B (2), and the C′ proteins of both Sendai virus (9) and parainfluenza virus (3), while translation initiation at multiple non-ATG codons has been reported for the human basic fibroblast growth factor gene (4, 13, 43). However, in these instances, translation is also initiated from a downstream ATG codon, and non-ATG initiation serves to produce amino-terminally extended proteins. The ACG-initiated adeno-associated virus capsid protein B serves a unique biological function (2), while CTG-initiated basic fibroblast growth factor and Int-2 have different subcellular locations than their ATG-initiated counterparts (1, 4). Proteins, such as EIAV Tat, whose expression relies solely on non-ATG initiation are much rarer. Other reported examples of proteins whose synthesis is initiated exclusively at non-ATG codons include the *Drosophila* choline acetyltransferase gene product (55) and the human testis-specific *PRPS3* gene product, phosphoribosylpyrophosphate synthetase (56).

Deletion analysis of EIAV *tat* cDNAs followed by indirect assays for Tat activity suggested that initiation of EIAV *tat* translation occurs at CTG-373 (11, 54), ATC-388 (35), or, conceivably, both. The more rigorous approach of site-directed mutagenesis of candidate initiation codons followed by direct measurement of Tat protein levels by radioimmunoprecipitation has demonstrated that translation of *tat* mRNA initiates at CTG-373. The transactivation data presented in Fig. 6 indicated that even when cells were transfected with small quantities of *tat* expression plasmids, there was a nonlinear relationship between Tat synthesis and resulting CAT activity, as reported previously (11). It is likely that CAT assays, which are a highly sensitive but indirect measure of Tat activity, cannot distinguish between high and vanishingly small levels of a potent transactivator such as EIAV Tat. The activation of CAT synthesis in the absence of detectable Tat protein in cells transfected with pRSS-Val_2 probably results from exceedingly rare translation initiation events at codons other than CTG-373. The assignment of CTG-373 as the *tat* initiation codon is supported by the observation that even in the wheat germ in vitro translation system, in which non-ATG initiation is artificially enhanced (24), alteration of CTG-373 to GTA completely abolished Tat synthesis.

The CTG-373 codon lies in an optimal context for translation initiation (22), and the potential for formation of secondary structure downstream from the *tat* initiation codon has been reported (28). RNA stem-loop structures downstream from initiation codons have been shown to facilitate initiation of translation from suboptimal initiation codons (24, 26). Despite these contextual and structural features, which should enhance translation of the *tat* open reading frame, translation initiation at CTG-373 was remarkably inefficient, as judged from the relative levels of Tat and Rev synthesis in transfected cells (Fig. 5). The obviously inefficient synthesis of EIAV Tat suggests that, at least in permissive cell lines (6), Tat is a powerful transactivator and that only small amounts of Tat are required to stimulate EIAV LTR-directed gene expression.

In eukaryotes, the vast majority of mRNAs are monocistronic (21, 27). While a number of mRNAs, mainly of viral origin, are structurally bicistronic, analysis of their translation products indicates that most are functionally monocistronic; the downstream open reading frame is not translated. There are exceptions to this general rule, including the *vpu-env* and *nef-rev* bicistronic mRNAs of human immunodeficiency virus type 1 (52), the *tax-rex* mRNA of human T-cell leukemia virus type I (30), and the P/C mRNA of Sendai virus (9).

The unusual genomic location of the first EIAV *tat* coding exon places the *tat* CTG initiation codon at the 5′ end of all EIAV mRNAs (11, 33, 34) and thus renders all EIAV mRNAs bicistronic. In the *tat-rev* bicistronic mRNA under investigation in this study, as well as all other EIAV mRNAs, the translation of upstream (*tat*) and downstream reading frames must be modulated to ensure the coordinated expression of regulatory and structural gene products. Increasing the strength of the *tat* initiation codon (by converting CTG-373 to ATG) virtually abolished Rev synthesis (Fig. 4 and 5). The strong 5′ translational polarity observed when the EIAV *tat* CTG initiation codon was altered to ATG is the hallmark of leaky scanning (25), and these experiments strongly suggest that translation of the bicistronic *tat-rev* mRNA is controlled by a leaky scanning mechanism.

Mechanisms other than leaky scanning can be postulated to account for the high-level translation of downstream EIAV open reading frames. In picornaviruses, defined structural elements termed internal ribosome entry sites permit ribosome scanning-independent translation initiation by a mechanism termed internal initiation (17, 18, 41). However, several observations suggest that the EIAV *rev* open reading frame is not translated by this mechanism. The picornavirus internal ribosome entry site is a large, highly structured RNA element (19, 29, 32); no similarly structured regions can be detected in the *tat-rev* mRNA. Furthermore, picornavirus RNAs contain numerous ATG codons upstream of the translation initiation site that do not affect translation (40), whereas in the EIAV *tat-rev* mRNA, introduction of

one in-frame ATG codon upstream of the *rev* open reading frame resulted in a strong inhibition of Rev synthesis. Finally, the internal-initiation model predicts that increasing the translation of an upstream, nonoverlapping open reading frame, such as *tat*, would have only a relatively minor effect on the translation of the downstream (*rev*) open reading frame (17). This hypothesis is clearly contrary to the data presented above. Taken together, these observations rule out the possibility that translation of the downstream (*rev*) open reading frame is accomplished via an internal-initiation mechanism.

While the variation in levels of Tat and Rev synthesis in response to changes in the efficiency of the *tat* initiation codon is consistent with a leaky scanning mechanism, leaky scanning may not entirely explain the coordinate regulation of Tat and Rev synthesis. This suggestion is based on the observation that when an ATG initiation codon is inserted into the *tat* open reading frame, in optimal context and with potential secondary-structure determinants downstream, translation of Rev is greatly diminished but not entirely abrogated. Under these favorable circumstances for translation initiation, it is unlikely that the *tat* initiation codon would be bypassed by many scanning ribosome subunits. It is conceivable that the residual Rev synthesis observed under these conditions results not from leaky scanning but from a termination-reinitiation of translation. The conditions which promote termination-reinitiation of translation in eukaryotes are not clearly defined. Both short (38, 39) and long (23) intercistronic distances have been reported to enhance translation reinitiation. Similarly, the size of the upstream cistron may play a role in the efficiency of reinitiation (23). It is therefore unclear whether the relatively short length of the *tat* open reading frame and the proximity of the *tat* termination codon to the *rev* initiation codon favor reinitiation, and the experiments presented herein cannot rule out such a possible mechanism. However, the dramatic drop in Rev synthesis in response to an increase in Tat translation indicates that a termination-reinitiation mechanism of Rev translation plays at best a minor role in the coordinate regulation of Tat and Rev synthesis.

As is the case in primate lentiviruses (51), EIAV utilizes differential splicing to generate various mRNA classes from which viral proteins are translated (11, 34, 44). The evidence presented in this report suggests that the synthesis of all EIAV structural and regulatory proteins is dependent on leaky scanning of the *tat* CTG initiation codon present at the 5′ terminus of each mRNA class. Furthermore, it is conceivable that the efficiency of Tat translation (and therefore the efficiency of downstream open reading frame translation) is modulated by cellular or viral *trans*-acting factors, suggesting yet another potential level of translational control of retroviral gene expression.

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