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The Tat protein of equine infectious anemia virus (EIAV) activates cellular gene expression by read-through transcription

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Abstract

The Tat protein of equine infectious anemia virus, EIAV, was shown to augment viral gene expression, presumably through interaction with the Tat responsive element, TAR. Recently, cell-free polyadenylation assays suggested that perturbation of the EIAV TAR secondary structure diminished polyadenylation efficiency. The present study indicates that the EIAV TAR regulates the efficiency of the 3′-end processing of viral RNA also in transfected cells. Moreover, our data suggest that the provision of the EIAV Tat protein in *trans* potentiates read-through transcription through the 3′ viral long terminal repeat (3′ LTR), thus suggesting activation of downstream-located cellular genes.

Keywords: Lentiviruses; Polyadenylation; TAR

1. Introduction

The inefficient polyadenylation signal of the retroviral downstream long terminal repeat (3′LTR) has been documented [reviewed by Guntaka (1993)]. In avian retroviral infections, it was shown that 1–15% of transcripts are read-through mRNAs extending beyond the 3′ end of the downstream R domain of the viral LTR, into the flanking cellular sequences (Herman and Coffin, 1987; Iwasaki and Temin, 1990; Cleavinger et al., 1991; Swain and Coffin, 1993). Moreover, it was suggested that accurate polyadenylation within the genomic RNA is not essential for efficient virus replication (Swain and Coffin, 1989). In fact, read-through transcripts represent intermediates in the process of transduction of cellular genes by retroviruses (Swain and Coffin, 1992).

The endonucleolytic cleavage and polyadenylation of a pre-mRNA in mammalian cells require a core poly(A) site containing two *cis*-acting elements: a highly conserved polyadenylation signal, AAUAAA, residing 10–30 nucleotides upstream of the cleavage site and a downstream non-conserved GU/U-rich region located within 50 nucleotides downstream of the cleavage site [reviewed by Wahle (1995) and Keller (1995)]. Upstream of the core poly(A) site, additional sequences regulating 3′ processing were identified in various viral genes. In retroviruses, the terminal redundancy of viral pre-mRNA requires that the processing machinery bypasses the core poly(A) site at the 5′ end of the transcript and efficiently utilizes it at the 3′ end. Thus, in retroviruses, including the human immunodeficiency virus type 1 (HIV-1), it was shown that the polyadenylation signal might be activated also by distal sequences located in the U3 that are absent from the 5′ end of the viral transcripts [reviewed by Guntaka (1993)]. Recently, 3′ processing sequences were shown to reside also in the U3 domain of equine infectious anemia virus (EIAV) LTR (Graveley and Gilmartin, 1996). It has also been suggested that promoter proximity plays a role in reducing poly(A) site efficiency at the 5′ end of the transcripts (DeZazzo et al., 1991; Weichs an der Glon et al., 1991; Cherrington and Ganem, 1992; Weichs an der Glon et al., 1993). Additionally, it was suggested that the HIV-1 poly(A) site is inhibited or stimulated by downstream or upstream splicing events, respectively (Ashe et al., 1995; Scott and Imperiale, 1996). In HIV-1, the Tat responsive element (TAR) located within the R of the

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Abbreviations: bp, base pair; EIAV, equine infectious anemia virus; LTR, long terminal repeat; nt, nucleotide; TAR, Tat responsive element.

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LTR was shown to play a role in regulating the correct 3′ processing of viral transcripts. It was suggested that enhancement of both processing efficiency and complex stability requires spatial juxtaposition of the U3 regulatory element and the core poly(A) site by the RNA stem–loop structure of TAR (Gilmartin et al., 1992). Moreover, data showed that when transcription from the HIV promoter is activated by Tat, the promoter proximal core poly(A) site is specifically occluded (Weichs an der Glon et al., 1993). The HIV Tat was also shown to stimulate transcriptional read-through of artificial terminator sequences placed downstream of the LTR (Graeble et al., 1993). Recent cell-free studies showed that perturbation of the TAR secondary structure diminished polyadenylation efficiency also in EIAV (Graveley and Gilmartin, 1996).

The present study suggests that the EIAV TAR located at the 3′ LTR is involved in the regulation of correct 3′ processing of viral transcripts in vivo. Moreover, our data show that the EIAV Tat is responsible for bypassing the viral 3′ core poly(A) site, thus enabling transcriptional read-through, resulting in the expression of cellular genes located downstream of the integrated proviral EIAV genome.

2. Materials and methods

2.1. Plasmid construction and site-directed mutagenesis

The various reporter constructs used in the present study are schematically presented in Fig. 1. To construct pTat, the *Bam*HI–*Sal*I fragment [*Bam*HI is located at nt 389 of EIAV genome (Kawakami et al., 1987) and *Sal*I is located within the plasmid sequences, downstream of the Tat coding region] of EIAV *tat* cDNA, p180 (Noiman et al., 1991), that spans the *tat* open reading frame (ORF), was inserted into pCEV21, downstream of cytomegalovirus (CMV) early transcriptional unit, as described previously (Rosin-Arbesfeld et al., 1993). To construct pEIAV/neo, the *Hin*dIII–*Eco*RI (nt 2688–5790) fragment from pMAMneo (Clontech, Palo Alto, CA) which spans the *neo*-coding region together with SV40 polyadenylation signal was inserted 14 bp downstream of the 3′ LTR of the cloned EIAV genome, designated pEIAV, described previously (Kawakami et al., 1987). Next, to construct pTat-LTR-neo, the *Spe*I–*Hpa*I fragment of pTat (the *Spe*I site is located at nt 7715 of pEIAV, and *Hpa*I is located within the polyadenylation signal downstream of the *neo*-coding region in the pCEV21 sequence) was replaced by the *Spe*I–*Eco*RI fragment from pEIAV/neo, which contains the 3′LTR, the *neo*-encoding region and the SV40 polyadenylation signal. To construct pΔTat-LTR-neo, the strategy of site-directed mutagenesis by overlap extension using PCR was employed as described elsewhere (Vallejo et al., 1994), using Vent DNA Polymerase (New England BioLabs, Beverly, MA). The oligonucleotides used as primers in the PCR reactions are detailed below. A fused antisense E5179/459(as) and a fused sense E457/5161(s) complementary primer were used in two independent PCR reactions together with an upstream viral E389(s) primer and downstream E7727(as), respectively, using pTat-LTR-neo as a template. The two resulting PCR products were gel-purified and used as mixed templates in a third PCR reaction performed in the presence of E389(s)/E7727(as) primer pair, thus yielding a fused PCR fragment in which four amino acids of the Tat core domain (nt 5150–5161 of EIAV genome; Kawakami et al., 1987) were deleted. The fused PCR product was digested with *Sal*I and *Spe*I, residing in the upstream and downstream primers, respectively, and inserted back into pTat-LTR-neo, thus resulting in pΔTat-LTR-neo. To construct pCell-LTR-neo, the 1.2-kb *Bam*HI–*Spe*I fragment of pTat-LTR-neo that spans the *tat* ORF was replaced by a heterologous 1.3-kb DNA fragment. To construct pLTR-neo, the 1.2-kb *Bam*HI–*Spe*I fragment of pTat-LTR-neo that spans the *tat* ORF was omitted. pLTR(TAR*)-neo was constructed by site-directed mutagenesis using overlapping PCR as described above. The mutagenizing primers, an antisense E8144 and a sense E8122, in which the C at nt 8131 of EIAV genome (Kawakami et al., 1987) was omitted, were used in two independent PCR reactions together with an upstream viral E7704(s) primer and a downstream N325(as) *neo*-specific primer, respectively, using pEIAV as a template. The two resulting PCR products that contained the TAR mutation within the overlapping region were used as mixed templates in a third PCR reaction performed in the presence of the upstream E7704(s) viral and the downstream N325(as) *neo* primers, thus yielding a fused TAR mutated PCR fragment. The mutated fused PCR product was digested with *Spe*I and *Bgl*II, residing in the upstream viral and the downstream *neo* primers, respectively, and then inserted back into pLTR-neo. The PCR-synthesized inserts of both pΔTat-LTR-neo and pLTR(TAR*)-neo were subjected to nucleotide sequence analysis to confirm the existence of the desired mutations, as well as to definitely exclude any additional changes that might have been introduced during the PCR. To construct pTat-LTR-LacZ, the *Sma*I–*Hin*dIII 3750-bp fragment of pCMVβ vector (Clontech, Palo Alto, CA) spanning the *LacZ* coding region and the SV40 polyadenylation signal was inserted 14 bp downstream of the 3′ LTR in pTat-LTR-neo, substituting the *neo* gene. Subsequently, the *neo* gene controlled by SV40 promoter was inserted. To construct pΔTat-LTR-LacZ, the *Bam*HI–*Spe*I fragment spanning the *tat*-coding region of pTat-LTR-LacZ was replaced by the homologous fragment from pΔTat-LTR-neo. To construct pCell-LTR-LacZ, the *Bam*HI–*Spe*I fragment was replaced by a heterologous

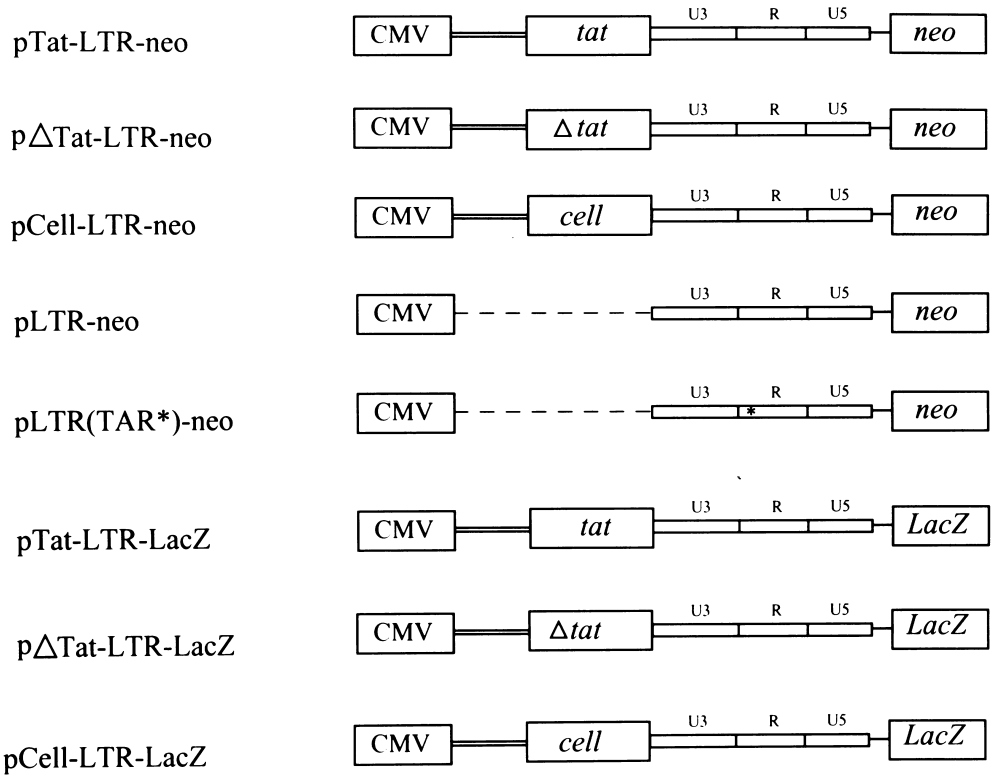

Fig. 1. Structure of constructs. The *tat* ORF (designated Δ*tat*), a four-amino-acid deleted *tat* (designated Δ*tat*) of the p180*tat* cDNA fragment (Noiman et al., 1991) or a heterologous cellular fragment (designated *cell*) was inserted downstream of CMV early promoter-enhancer unit (designated CMV) and upstream of EIAV 3′ LTR (designated U3RU5), whereas the *neo* or *lacZ* coding regions, deprived of enhancer-promoter elements and bordered downstream by the SV40 polyadenylation site, were inserted downstream of the EIAV 3′ LTR. Flanking cellular sequences (14-nt) are marked by solid lines, plasmid sequences are marked by parallel lines, and deleted regions are marked by dashed lines. Point-mutated TAR within the 3′R of pLTR(TAR*)-neo is marked by an asterisk.

1.3-kb DNA fragment. The plasmid pBSpac conferring puromycin resistance was described elsewhere (de la Luna et al., 1988).

2.2. Recombinant Tat production

A biologically functional recombinant Tat protein was produced in bacteria and purified as described previously (Rosin-Arbesfeld et al., 1994).

2.3. Cell culture and transfections

Canine thymus cells (Cf2Th) (ATCC CRL 1430), permissive for EIAV replication, were grown as previously described (Noiman et al., 1990). Transfection of equimolar amounts of the various plasmids into semiconfluent Cf2Th cells was performed by the calcium phosphate coprecipitation technique, as previously described (Rosin-Arbesfeld et al., 1993). To generate aminoglycoside G418-resistant transfectants, cells were transfected with *neo*-expressing plasmids, and 3 days later, cells were grown in the presence of the G418 (400 μg/ml; GIBCO BRL, Life Technologies, UK) for 3 weeks, and G418-resistant clones were isolated or Giemsa-stained. To obtain puromycin-resistant transfectants, reporter plasmids were cotransfected with one-tenth of the amount of pBSpac plasmid (de la Luna et al., 1988), and 2 days later, cells were grown for 14 days in the presence of 2.5 μg/ml of puromycin. Puromycin-resistant clones were first assayed by PCR for the presence of reporter plasmids, and then isolated. The bacterially synthesized Tat protein was inserted into Cf2Th cells by cell scraping as described by Rosin-Arbesfeld et al. (1994).

2.4. RNA isolation

Total RNA was isolated by the cesium chloride–guanidinium isothiocyanate method, as previously described (Noiman et al., 1990). To eliminate DNA contamination in RT-PCR, total RNA was subsequently treated with RQ1 DNaseI (Promega, Madison, WI) at 10 u/ml for 15 min at 37°C, followed by phenol extractions.

2.5. PCR and RT-PCR

RT-PCR was performed as follows: total RNA (2 μg) was reverse-transcribed using recombinant AMV reverse

transcriptase, in the presence of an antisense oligonucleotide primer. After 60 min at 42°C, one-tenth of the cDNA product was PCR-amplified using AmpliTaq polymerase in the presence of specific antisense and sense primers. Amplification was performed in a Perkin-Elmer/Cetus DNA thermal cycler for 35 cycles (40 s of denaturation at 94°C, 1 min of annealing at 60°C and 1 min of polymerization at 72°C). For efficient amplification of transcripts longer than 1 kb, the StrataScript cDNA Synthesis Kit (Stratagene Ltd, Cambridge) was used as follows: total RNA (2 μg) was reverse-transcribed at 37°C for 60 min using MoMLV RNase H-minus reverse transcriptase, in the presence of a specific antisense primer. One-tenth of the reaction mixture was then PCR-amplified for 30 cycles (40 s of denaturation at 94°C, 2 min of annealing at 60°C and 10 min of polymerization at 72°C) using 1 u of KlenTaq1 (Ab Peptides) and 0.1 u of Pfu DNA polymerase (Stratagene). For each RT-PCR assay, a parallel control reaction was performed in the absence of reverse transcriptase, to ensure the absence of DNA contamination. The semi-quantitative RT-PCR was performed as previously described (Vider et al., 1997). Serially twofold-diluted total RNA was reverse-transcribed in the presence of an antisense primer, and 5 μl of each cDNA were PCR-amplified in the presence of specific sense and antisense primers in a 50-μl reaction mixture. Amplification was performed for 35–40 cycles to ensure a linear range of amplification. The same serially diluted RNA samples were reverse-transcribed using an oligo dT primer, and cDNAs were used as templates for PCR reactions performed for 20 cycles in the presence of β-actin primers, 5′-GTTTGAGACCTTCAACACCCC-3′ and 5′-GTGGCCATCTCTTGCTCGAAGTC-3′. The RT-PCR products were analyzed by electrophoresis on NuSieve agarose (FMC Bioproducts) gels and photographed. Quantitation of ethidium-bromide-stained RT-PCR products was performed using the Imagine System of EL-MUL Technologies (Israel). The EIAV-specific oligonucleotides that served as sense (s) or antisense (as) primers were as follows (Kawakami et al., 1987):

E1(s)-5′-ggcctctgagTGTGGGGTTTTTATGAGG-3′ (lower-case letters denote a restriction enzyme recognition site bordered by 4 nt);
E235(as)-5′-gatccGGGACTCAGACCGCAGAATC-TGAGTGCCC-3′;
E319(as)-5′-ggccaagcttTAGGATCTCGAACAGAC-AAAC-3′;
E385(as)-5′-ggccaagcttTACGATCAGCCAGGTTC-AACAGGTAGG-3′;
E389(s)-5′-gaggtcgacTCCCCGGGACAGCAGAG-G-3′;
E457/5161(s)-5′-CAGGAAGCAAGACCCAAC/CT-GTGTTTCCTGAGGTCT-3′;
E5179/5150(as)-5′-AGACCTCAGGAAACACAG-/GTTGGGTCTTGCTTCCTG-3′;
E7727(as)-5′-CTATAATTACTAGTCCCCAAAA-TA-3′;
E7704(s)-5′-TATTTTGGGGACTAGTAATTATAG-3′;
E8122(s)-5′-CAGATTCTGGGTCTGAGTCCCTT-C-3′;
E8144(as)-5′-GAAGGGACTCAGACCCAGAATC-TG′-3.

Neo-specific primers were as follows (Pridmore, 1987):
N368(as)-5′-ggccaagcttGCGTGCAATCCATCTTG-TTCAATCATGCGA-3′
N325(as)-5′-CTCTTGATCAGATCTTGATCCCCT-3′.

The CMV-specific primer was (Boshart et al., 1985):
C3(s)-5′-ccgggtcgacGATCGCCTGGAGACGCCAT-CCACGCTG-3′.

2.6. RNase protection assay

A riboprobe, 321 bases in length, was synthesized by T7 RNA polymerase using the pBluescript KS vector (Stratagene), containing the EIAV LTR fragment as a template. Hybridization was carried out with 0.1 pmol of ^{32}P-labeled riboprobe and 20 μg of total RNA, for 18 h at 49°C, in a 30-μl solution containing 80% formamide, 40 mM PIPES pH 6.4, 400 mM NaCl and 1 mM EDTA. Following hybridization, 0.2 μg RNase T1 (GIBCO BRL, Life Technologies, UK) and 0.035 μg RNase A (Sigma, St. Louis, MO) were added for 15 min at 30°C in a 350-μl reaction mixture containing 10 mM Tris–HCl, pH 7.5, 300 mM NaCl and 5 mM EDTA. Following treatment with proteinase K (50 μg/ml) and 0.5% SDS for 15 min at 37°C and phenol extraction, RNase-resistant fragments were resolved on 6% polyacrylamide–7.8 M urea gels. The size standards were obtained by ^{32}P-end-labeling of *Hae*III-digested ΦX174. The intensity of the ^{32}P-labelled RNase protected bands was quantitated using the NIH Image 1.55. For PCR amplification of the RNase-protected bands following gel electrophoresis, the ^{32}P-labelled RNA bands were eluted from gels during overnight incubation at 37°C in a solution containing 0.75 M ammonium acetate, 0.01 M magnesium acetate, 0.1 mM EDTA and 0.1% SDS, followed by phenol extraction. RT was performed in the presence of a specific sense primer, and then PCR was performed in the presence of sense and antisense specific primers as described above.

2.7. Nucleotide sequence analysis

For sequence analysis, subclones were prepared by using pBluescript KS vectors. Nucleotide sequence was determined employing the Sequenase DNA Sequencing Kit (USB, Cleveland, Ohio).

2.8. Determination of the level of β-Gal activity

The level of β-Gal activity was determined as described elsewhere (MacGregor et al., 1989). Briefly, the level of β-Gal activity in cell extracts was assessed spectrophotometrically using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. Cell extracts containing equal amounts of cell protein (ranging from 15 to 150 μg) were incubated with ONPG at 37°C for 20 min. Absorbance was determined at 420 nm with the aid of a spectrophotometer. Units of β-Gal activity were calculated [units = $380 \times A_{420}$/time (min)], so that 1 unit is equivalent to the conversion of 1 nmol of ONPG per minute (Norton and Coffin, 1985). The histochemical assay of β-Gal activity was performed using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as a substrate.

3. Results

3.1. The level of neo expression due to read-through transcription in the presence of the EIAV Tat

To facilitate detection of read-through transcription, a strategy was employed in which cells expressing read-through transcripts could be selected due to their resistance to the antibiotic G418. A plasmid, designated pTat-LTR-neo, was constructed, in which the *tat* ORF of cDNA p180 (Noiman et al., 1991) was inserted downstream of the CMV early promoter-enhancer unit and upstream of the EIAV 3′ LTR. To enable expression of the *neo* gene from the CMV promoter by a read-through transcription, the *neo* coding region, deprived of the promoter-enhancer element and bordered downstream by the SV40 polyadenylation site, was inserted downstream of the EIAV 3′ LTR (Fig. 1). By using this construct, the G-418-resistant cells would emerge from translating the *neo* gene using the CMV promoter. The use of the CMV promoter-enhancer unit, rather than the EIAV 5′LTR, was preferred in order to exclude the possibility of augmented transcription due to Tat-TAR binding. As a control, a pΔTat-LTR-neo, lacking Tat activity (data not shown; Dorn et al., 1990), was constructed by deleting four amino acids of the Tat core domain (Leu, Cys, Phe, Leu). Alternatively, pCell-LTR-neo, in which a heterologous DNA sequence replaced the *tat* ORF, served as a control. Cf2Th cells, highly permissive for EIAV replication (Noiman et al., 1990), were transfected, and *neo*-expressing colonies resistant to G418 were selected. Data showed that transfection of pTat-LTR-neo DNA generated a higher number of G418-resistant colonies than cells transfected with pΔTat-LTR-neo (Fig. 2A) or with pCell-LTR-neo (data not shown). In addition to the difference in the number of G418-resistant colonies, a marked difference was observed also with respect to their size. Thus, pTat-LTR-neo-transfectants yielded larger colonies than those obtained by pΔTat-LTR-neo- (Fig. 2B) or pCell-LTR-neo- (data not shown) transfectants.

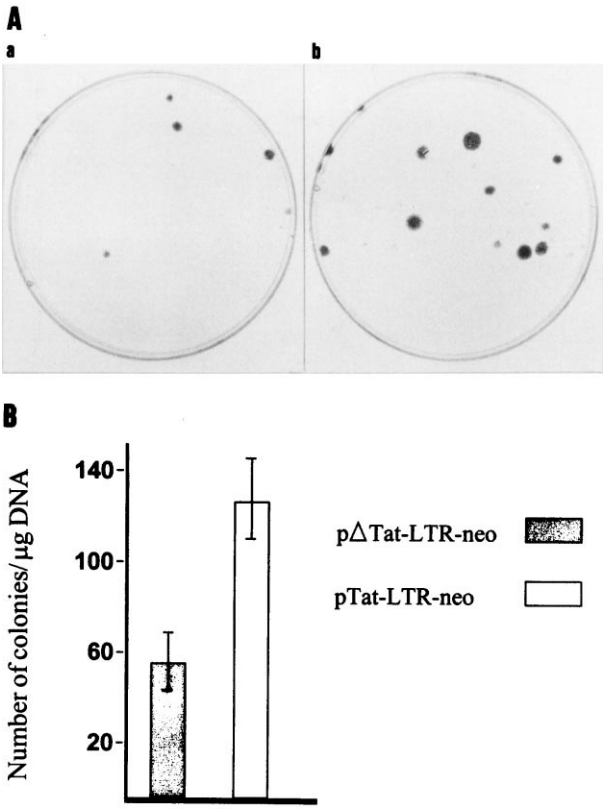

Fig. 2. Development of G418-resistant colonies by *neo* gene expression from the viral promoter. 5×10^5 Cf2Th cells were transfected with 0.15 μg DNA of the various reporter *neo*-expressing constructs. Following 3 weeks of growth in the presence of G418, resistant colonies were Giemsa-stained and enumerated. (**A**) Photographs of representative cell cultures of (a) pΔTat-LTR-neo or (b) pTat-LTR-neo. (**B**) The results are the average of five experiments

In order to confirm that *neo* gene expression was switched on via read-through transcription, RT-PCR was performed on total RNA of several G418-resistant colonies. First, RT was performed in the presence of an antisense primer from the *neo*-coding region [(N368(as)], and PCR was then performed using KlenTaq and Pfu polymerases, in the presence of sense and antisense primers derived from the CMV promoter [(C3(s)] and *neo* coding region [(N325(as)], respectively. The use of a CMV/neo primer pair yielded full-length cDNAs of 1.8 kb in length both in pTat-LTR-neo- and in pΔTat-LTR-neo-transfectants, although there was a marked difference in their levels of expression (Fig. 3). Sequence analysis of these RT-PCR products (data not shown) confirmed that they were full-length read-through *tat-neo* transcripts, which initiated at the CMV promoter, proceeded along the entire *tat* ORF, spanned the U3RU5 region and ended at the *neo* coding region. To

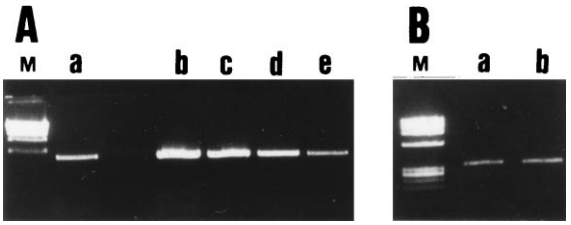

Fig. 3. Determination of the level of read-through transcription by semi-quantitative RT-PCR. (**A**) Two micrograms of total RNA of (a) pΔTat-LTR-neo- or (b–e) pTat-LTR-neo- stable transfectants were serially diluted (a,b, undiluted; c, 1:2; d, 1:4; e, 1:8) and reverse-transcribed using an antisense primer N3(as) in a 50-μl reaction mixture. Five microliters of the reaction were subjected to PCR in the presence of C3(s)/N325(as) primer pair. (**B**) Undiluted total RNA of (a) pΔTat-LTR-neo- or (b) pTat-LTR-neo- stable transfectants was reverse-transcribed using an antisense oligo(dT) primer, and then PCR was performed using actin-specific primers. (**A**) *Hin*dIII-digested λDNA or (B) *Hae*III-digested ΦX DNA served as size markers. Following gel electrophoresis, the ethidium-bromide-stained bands were quantitated. The results show that the intensity of the RT-PCR product obtained from the 1:8 dilution of pTat-LTR-neo RNA (lane e) was comparable to that obtained from the undiluted pΔTat-LTR-neo RNA (lane a).

evaluate the level of expression of the read-through transcripts, a semi-quantitative assay was performed as previously described (Vider et al., 1997). Serially diluted total RNA was reverse-transcribed in the presence of an antisense primer N368(as), and then PCR was performed in the presence of C3(s)/N325(as) primer pair. Data showed that the intensity of the PCR product obtained from the undiluted RNA of pΔTat-LTR-neo transfectants (Fig. 3A lane a) was comparable to that obtained from a 1:8 dilution of the RNA obtained from pTat-LTR-neo transfectants (Fig. 3A, lane e).

Next, an RNase protection assay was performed to confirm that the *neo* gene expression was switched on via read-through transcription, rather than initiating at the viral 3′LTR. Total RNA of several G418-resistant colonies was obtained from cells transfected with pCell-LTR-neo or with pTat-LTR-neo, and RNase protection was performed using U3RU5 sequence as a riboprobe (Fig. 4A). Following hybridization, hybrids were treated with RNase and fractionated on denaturing polyacrylamide gels (Fig. 4B). In order to conclusively determine the genetic structure of the various RNase-protected bands, the labeled RNA bands were eluted from the gel, amplified by RT-PCR in the presence of sense and antisense primers complementary to various regions of U3, R and U5 domains [E1(s), E235(as) and E319(as), respectively], and the PCR products were nucleotide-sequenced. Data showed that the 321-base fragment contained the U3RU5 sequence and thus represented the read-through transcripts, whereas the 285 fragment contained the U3R sequence and accordingly represented the correctly processed transcripts ending at the 3′ end of R (Fig. 4A). The level of transcripts initiating at the 3′LTR promoter would be evaluated by the 103-base fragment corresponding to the RU5 domain. Data showed the presence of higher levels of U3RU5-spanning transcripts in pTat-LTR-neo transfectants as compared to pCell-LTR-neo transfectants (Fig. 4B), thus suggesting the augmentation of read-through transcription in the presence of Tat expression. RNase protection analysis did not show the presence of the 103-base fragment, which corresponded to transcripts that span RU5, thus suggesting the absence of transcripts initiating at the 3′ LTR.

3.2. Evaluation of the level of read-through transcription in the absence of selective forces

To exclude the possibility that the selection of a dominant marker, Neo^r, might be responsible for the detected read-through effect, an additional strategy was approached. Constructs were designed, in which the bacterial *lacZ* gene, deprived of its promoter-enhancer sequences, was inserted downstream of the 3′ LTR (Fig. 1), while the *neo* gene expression was independently driven by the SV40 promoter. The resulting plasmids, pTat-LTR-LacZ and pΔTat-LTR-LacZ, were stably transfected into Cf2Th cells. Pooled G418-resistant colonies were grown, and then the level of β-D-galactosidase (β-Gal) expression was histochemically evaluated. Data showed that the number of β-Gal-expressing cells was higher in pTat-LTR-LacZ- than in pΔTat-LTR-LacZ-transfectants (Fig. 5A). To measure the level of β-Gal activity more accurately, a spectrophotometric assay was performed. Data showed that the level of β-Gal activity in pTat-LTR-LacZ-transfectants was threefold higher than that obtained in pΔTat-LTR-LacZ-transfectants (Fig. 5B). To confirm that the increase in *lacZ* expression in pTat-LTR-LacZ-transfectants originated from augmented read-through transcription rather than from stimulation of the overall transcription in the presence of Tat or from higher copy number, the steady-state transcription level was measured by RT-PCR. To evaluate the level of the overall transcription, transcripts that spanned U3R were semi-quantitated by RT-PCR. RT was performed in the presence of an R-specific primer [E239(as)], and then PCR was performed in the presence of the primer pair E1(s)/E239(as) bordering the U3-R region. To measure the level of read-through transcripts that spanned the entire 3′ LTR region, E319(as)-primed cDNA was subjected to PCR in the presence of the primer pair E1(s)/E319(as) bordering the U3-R-U5 domain. RT-PCR was performed in the presence of actin primers. Data showed that whereas both pTat-LTR-LacZ- and pΔTat-LTR-LacZ-transfectants gave rise to similar levels of U3R-containing transcripts (Fig. 5C, lanes a and b), the level of 3′ LTR-spanning transcripts was 3.2-fold higher in pTat-LTR-LacZ- (Fig. 5C, lane d) than in pΔTat-LTR-LacZ- (Fig. 5C, lane c) transfectants.

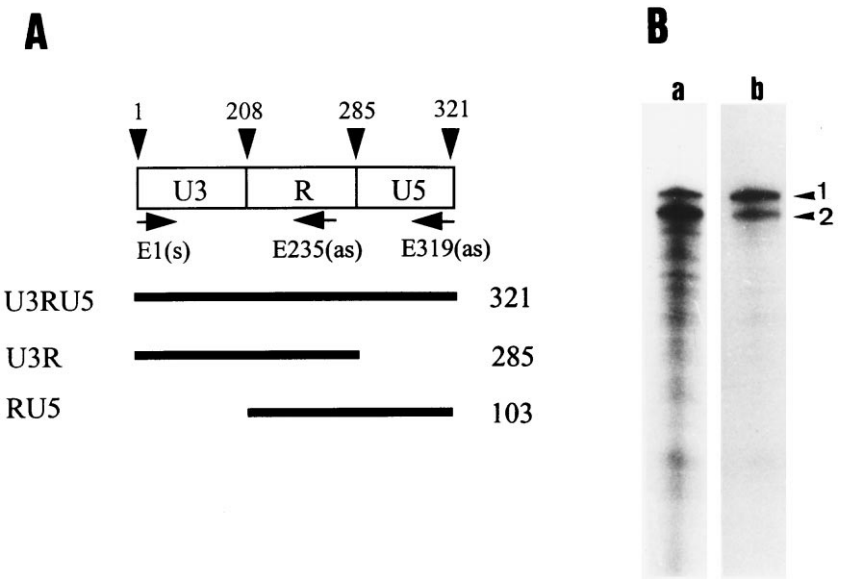

Fig. 4. Determination of the level of read-through transcription by RNase protection analysis. (**A**) Schematic presentation of the antisense LTR (U3RU5) riboprobe, location of primers used to amplify the eluted RNase protected bands by RT-PCR (see text) and the expected sizes (in bases) of RNase-resistant fragments (filled-in boxes). (**B**) pCell-LTR-neo or pTat-LTR-neo was transfected into Cf2Th cells as described in the legend to Fig. 2. Cell were grown in the presence of G418, and 3 weeks later, colonies were isolated. The U3RU5 riboprobe (10^6 cpm) was hybridized with 20 μg of total RNA obtained from three colonies of (a) pCell-LTR-neo or with (b) pTat-LTR-neo transfectants, and RNase digestion was performed. A representative assay is shown. The 321 (1) and the 285 (2) -base RNase-protected fragments are indicated by arrowheads. The proportional intensity of the RNase-protected bands was calculated and is indicated as a percentage. pCell-LTR-neo: 1, 35.7%; 2, 64.3%; pTat-LTR-neo: 1, 54.8%; 2, 45.2%.

3.3. Regulation of the pre-mRNA 3′ processing by EIAV TAR

Recent in-vitro polyadenylation assays suggested that similar to the TAR of HIV-1 (Gilmartin et al., 1992), also the EIAV TAR potentiates 3′ processing of viral transcripts (Graveley and Gilmartin, 1996). We have thus postulated that if the TAR secondary structure is responsible for regulating EIAV polyadenylation also in-vivo, then abolishment of its conformation should result in bypassing the poly(A) signal, thus giving rise to increased read-through transcription. Experiments were thus performed to elucidate whether read-through transcription was augmented as a result of abrogating the EIAV TAR secondary structure. pLTR-neo was subjected to site-directed mutagenesis, which deleted the C at nt 8131 of EIAV genome (Kawakami et al., 1987), thus resulting in a plasmid designated pLTR(TAR*)-neo (Fig. 1). Such a deletion was shown to abrogate EIAV TAR secondary structure and to abolish Tat activity (Carvalho and Derse, 1991). Data showed that a higher number of G418-resistant colonies developed following transfection of pLTR(TAR*)-neo, as compared to pLTR-neo-transfectants (Fig. 6A). To more accurately evaluate the contribution of TAR to polyadenylation efficiency, the level of read-through transcription in mutated TAR reporter constructs in the absence of selective forces was measured. pLTR-neo or pLTR(TAR*)-neo was co-transfected with pBSpac, which conferred resistance to puromycin. Puromycin-resistant colonies were assayed for the presence of the reporter plasmids using PCR (not shown), and then RNase-protection assays were performed on total RNA of several puromycin-resistant colonies (Fig. 6B). Quantitation of the labelled RNase-protected bands showed that the proportional level of the 321-base RNase-protected U3RU5 band as compared to the 285-base U3R protected band, was twofold higher in pLTR(TAR*)-neo- (Fig. 6B, lane d) than in pLTR-neo-transfectants (Fig. 6B lane c). The 103-base fragment that would represent the RU5-spanning transcripts was not observed in the RNase protection analysis, suggesting the absence of transcripts initiating at the 3′ LTR. Next, the efficiency of read-through transcription was evaluated by the amount of RT-PCR products obtained in the presence of a U3/U5 primer pair, E1(s)/E319(as) (Fig. 6C, lanes c, d) as compared to that obtained in the presence of a U3/R primer pair, E1(s)/E239(as) (Fig. 6C lanes a, b). RT-PCR in the presence of actin primers was performed in parallel(Fig. 6C, lanes e, f). Data showed that pLTR(TAR*)-neo-transfectants (Fig. 6C, lane d) exhibited read-through transcription that was sixfold more efficient than that exhibited by pLTR-neo-transfectants (Fig. 6C, lane c).

3.4. The supply of recombinant Tat in trans *augments read-through transcription*

Our data suggested that the Tat protein encoded by the various *tat*-containing reporter plasmids stimulated

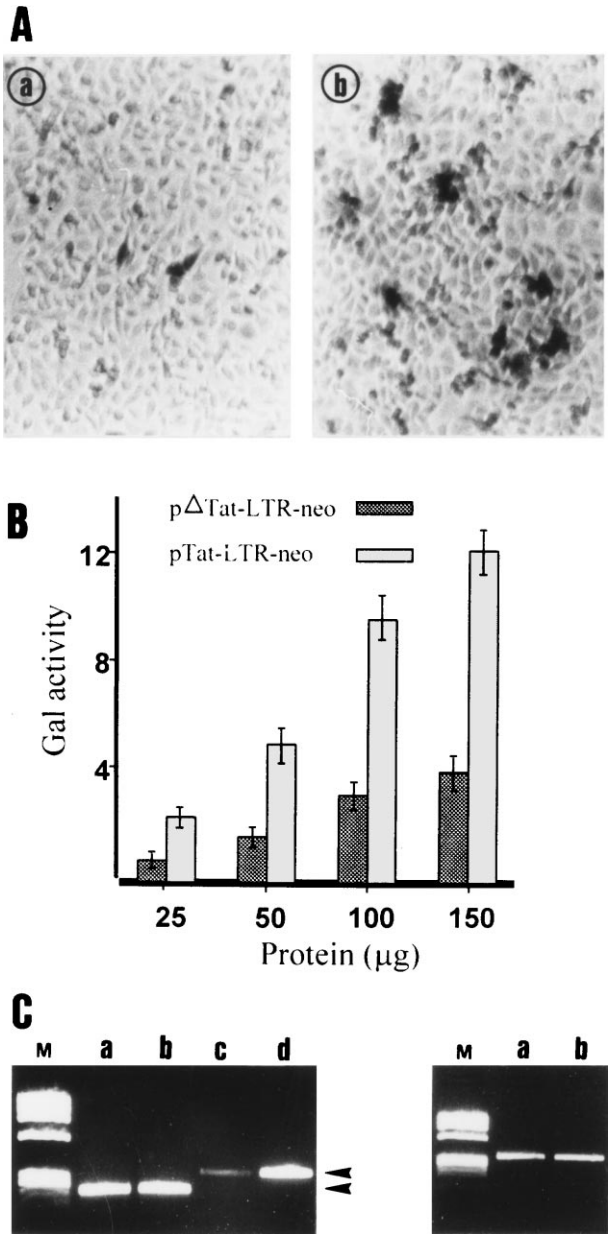

Fig. 5. Determination of the level of read-through transcription using *lacZ* reporter constructs. (**A**) Histochemical assay. (a) pΔTat-LTR-LacZ- and (b) pTat-LTR-LacZ- pooled G418-resistant transfectants were stained with X-Gal. (**B**) Spectrophotometric assay. The pΔTat-LTR-LacZ- and pTat-LTR-LacZ- G418-resistant transfectants shown in A were harvested, and the level of β-Gal activity was measured using cell lysates containing increasing amounts of cell proteins. The results are the average of five experiments. (**C**) Left panel: total RNA obtained from pTat-LTR-LacZ (lanes b, d) or from pΔTat-LTR-LacZ- (lanes a, c) G418-resistant transfectants were reverse-transcribed in the presence of oligo d(T) primer, and subsequent PCR was performed in the presence of E1(s)/E239(as) (a, b) or E1(s)/E319(as) (c, d). The 319- and 239-bp RT-PCR products are indicated by arrowheads. The intensity of the ethidium-bromide-stained RT-PCR products, calculated in arbitrary units are: a, 33.1; b, 39; c, 15.3; d, 48.9. Right panel: in parallel, oligo d(T)-primed cDNA of (a) pΔTat-LTR-LacZ- or (b) pTat-LTR-LacZ transfectants was PCR-amplified in the presence of actin-specific primers. RT-PCR products were analyzed by 2.5% agarose gel electrophoresis. *Hae*III-digested ΦX174 DNA served as size markers (M).

read-through transcription of downstream genes. However, it could be argued that the presence of viral sequences in *cis*, rather than the supply of the Tat protein in *trans*, is responsible for either transcript stability or more efficient transcript utilization. Experiments were thus performed in which a recombinant Tat protein was introduced into cells stably transfected with pCell-LTR-LacZ- in which a heterologous cellular sequence replaced the *tat* coding region. Data showed that incorporation of increasing amounts of the recombinant Tat protein up to 2 μg/ml, increased β-Gal activity up to a maximal level of 2.2-fold as compared to the level of β-Gal activity in the presence of bovine serum albumin (BSA) (Fig. 7).

4. Discussion

Similar to mammalian mRNAs (Proudfoot, 1991), the core poly(A) site of several primate lentiviruses (Guntaka, 1993), as well as EIAV (Graveley and Gilmartin, 1996), contain the conserved AAUAAA hexamer, located 16 nt upstream of the cleavage site, and a GU-rich element residing 6 nt downstream of the cleavage site. Recent experiments suggested that similar to HIV-1 (Guntaka, 1993), also, the U3 domain of EIAV contains an enhancer element that regulates efficient pre-mRNA 3′ processing at the viral 3′ core poly(A) site, presumably by enhancing the stability of binding of the cleavage and polyadenylation specificity factor (CPSF) to the poly(A) core site (Graveley and Gilmartin, 1996). In HIV-1, it was shown that TAR functions in regulating 3′ processing of viral pre-mRNAs (Valsamakis et al., 1991; Gilmartin et al., 1992). Similar to the primate lentiviruses, the EIAV TAR is located downstream of the initiation site for transcription, between residues +1 and +25 (Carvalho and Derse, 1991). Although the EIAV TAR can presumably form a stem–loop structure, its direct binding to Tat could not be conclusively demonstrated (our unpublished results). Nevertheless, a point mutation that was suggested to perturb the presumed TAR secondary RNA structure abolished Tat activity (Carvalho and Derse, 1991), thus suggesting the relevance of its secondary structure to function. Recently, it was shown that, similar to the TAR of HIV-1, abrogation of the EIAV TAR presumed secondary structure decreased the polyadenylation efficiency when assayed in a cell-free system (Graveley and Gilmartin, 1996). Hence, our data, showing that perturbation of the presumed TAR stem–loop structure stimulated read-through transcription, suggest that the TAR of EIAV functions also in transfected cells, in potentiating the correct utilization of the viral 3′ processing. Interestingly, in reporter constructs in which the entire TAR region was deleted, no G-418 resistant colonies developed (data not shown). Based on

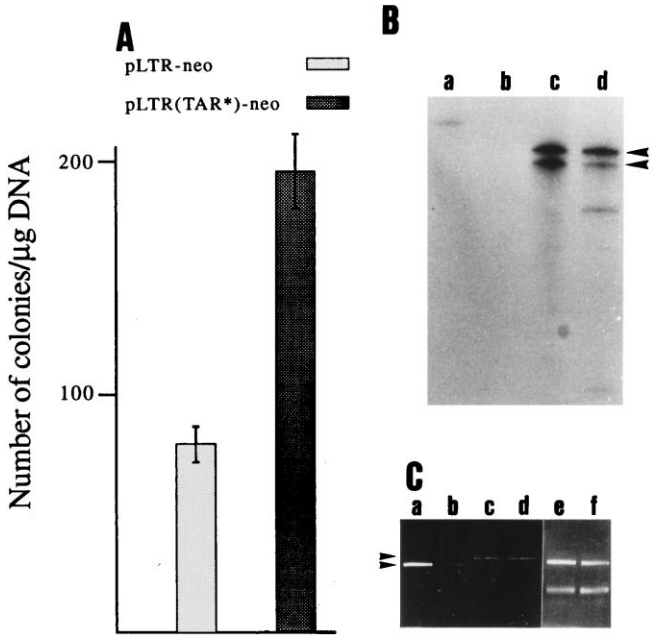

Fig. 6. Determination of the level of read-through transcription in *neo*-reporter constructs containing point-mutated TAR. (**A**) pLTR-neo or pLTR(TAR*)-neo were transfected into Cf2Th cells as described in the legend to Fig. 2. Cells were grown in the presence of G418, and 3 weeks later, colonies were Giemsa-stained and enumerated. The histograms present the average numbers of colonies of five experiments. (**B**) pLTR-neo or pLTR(TAR*)-neo (5 μg DNA) were co-transfected into Cf2Th cells, together with 0.5 μg of pBSpac. Cells were grown in the presence of puromycin (2.5 μg/ml), and 14 days later, puromycin-resistant colonies were isolated and assayed for the amount of the reporter plasmid sequences using PCR. Total RNA obtained from three colonies containing similar levels of reporter plasmid DNA was subjected to RNase protection analysis. The U3RU5 riboprobe (10^6 cpm) was hybridized with 20 μg of total RNA, and then RNase-digestion was performed. Representative gel electrophoresis of an RNase-protection assay. The 321- and 285-base RNase protected fragments are indicated by arrowheads. The additional smaller bands seen in lane d could not be amplified by PCR following their extraction from the gel using various internal primers (see text) and thus were regarded as non-specific background. The proportional intensity of the 321- and 285-base RNase-protected bands was calculated and is indicated as a percentage. (a) Undigested riboprobe; (b) mock-transfected cells; (c) cells transfected with pLTR-neo: 321 base—49.6%; 285 base—50.4%, or (d) pLTR(TAR*)-neo: 321 base—64%; 285 base—36%. (**C**) Representative RT-PCR assay. Total RNA obtained from pLTR-neo (a, c), or pLTR(TAR*)-neo (b, d) puromycin-resistant transfectants was reverse-transcribed in the presence of (a, b) E235(as) or (c, d) E319(as), and subsequent PCR was performed in the presence of E1(s)/E235(as) (a, b) or of E1(s)/E319(as) (c, d). The 319- and 235-bp RT-PCR products are indicated by arrowheads. The intensity of the ethidium-bromide-stained RT-PCR products indicated in arbitrary units are: a, 10.4; b, 1.67; c, 2.03; d, 2.15. In parallel, total RNA of (e) pLTR-neo or (f) pLTR(TAR*)-neo puromycin-resistant transfectants was RT-PCR-amplified in the presence of actin-specific primers. RT-PCR products were analyzed by 2.5% agarose gel electrophoresis. *Hae*III-digested ΦX174 DNA served as size markers (not shown).

the notion that TAR functions by spatially juxtaposing the U3 enhancer element and the core poly(A) site (Valsamakis et al., 1991; Gilmartin et al., 1992; Graveley and Gilmartin, 1996), it is reasonable to assume that

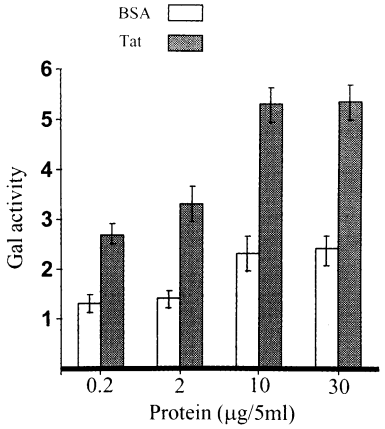

Fig. 7. Determination of the level of *LacZ* read-through expression in the presence of recombinant Tat protein. pCell-LTR-lacZ G418-resistant transfectants were scrape loaded (Rosin-Arbesfeld et al., 1994) with increasing amounts (0.2–30 μg/5 ml) of recombinant Tat protein or bovine serum albumin (BSA). After 18 h of incubation, the level of β-Gal activity was spectrophotometrically measured using cell lysates containing increasing amounts of cell proteins. The results are the average of β-Gal activity of five experiments obtained with 16 μg of cell proteins.

the TAR deletion, which artificially juxtaposed U3 and the core poly(A) site, potentiated cleavage and polyadenylation to such a level as to completely prevent read-through transcription into the downstream *neo* gene.

Based on observations suggesting that the TAR of HIV-1 and EIAV function in potentiating the 3′ core poly(A) site (Gilmartin et al., 1992; Graveley and Gilmartin, 1996) and that the supply of HIV Tat in *trans* can occlude terminator sequences in a cell-free system when placed downstream of the 5′ LTR (Graeble et al., 1993), we sought to investigate the possible effect of the EIAV Tat on read-through transcription. Interestingly, although the HIV-1 core poly(A) site was suggested to function efficiently (Ashe et al., 1995), the occurrence of read-through transcription at a frequency of 1.8% was reported (Raineri et al., 1995). To facilitate analysis of the genetic structure of EIAV read-through transcripts, a strategy was adopted in which G-418 selection was dependent on read-through transcription. Data showed that the number as well as the size of the G418-resistant colonies were larger in the presence of Tat, thus suggesting Tat-dependent occlusion of the EIAV 3′ poly(A) site. Moreover, our data, showing that under non-selective conditions, the presence of Tat increased the read-through efficiency by 3.2-fold, argue against selection of a dominant marker (Neo^r), being responsible for the observed 3′ core poly(A) occlusion.

In the Oncovirinae subfamily of retroviruses, it was shown that transcription of downstream-located cellular genes can initiate at the viral promoter located at the 3′ LTR [reviewed by Katz and Skalka (1990)]. The RNase protection analyses performed in the present study using pTat-LTR-neo-transfectants (Fig. 4) or pLTR(TAR*)-

neo- and pLTR-neo-transfectants (Fig. 6) did not reveal the presence of a 103-base fragment that would represent transcripts initiating at the 3′ LTR. Although our data cannot conclusively exclude the presence of such transcripts, it is suggested that such a mechanism is much less effective than EIAV Tat-mediated read-through transcription.

The present study indicates that downstream cellular genes can be activated by a read-through mechanism through the EIAV 3′ LTR. The possibility that integration of retroviruses occurs predominantly in the vicinity of transcriptionally active genes (King et al., 1985; Rohdewohld et al., 1987; Mooslehner et al., 1990), supported by data demonstrating that proviral insertion is not absolutely random (Shih et al., 1988), might suggest a possible biological relevance for the activation of adjacent cellular genes in lentiviral pathogenesis.

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