Lack of trans-activation function for Maedi Visna virus and Caprine arthritis encephalitis virus Tat proteins

Stéphanie Villet, a, * Claudine Faure, a Baya Amel Bouzar, b Thierry Morin, b Gérard Verdier, a Yahia Chebloune, b and Catherine Legras a

a UMR 5534 INRA/CNRS/UCBL, “Rétrovirologie Animale et Vecteurs Rétroviraux,” Centre de Génétique Moléculaire et Cellulaire, Université Claude Bernard, 43 Boulevard du 11 novembre 1918, Villeurbanne, 69622, France
b UMR 754 INRA/ENVL/UCBL, Rétrovirus et pathologie comparée “Virologie Cellulaire, Moléculaire et Maladies Emergentes,” Université Claude Bernard, Lyon-I Batiment B, 50 avenue Tony Garnier, 69366 Lyon Cedex 07, France

Received 13 August 2002; returned to author for revision 16 October 2002; accepted 20 October 2002

Abstract

All lentiviruses contain an open reading frame located shortly upstream or inside of the env gene and encoding a small protein which has been designated Tat. This designation was mainly with respect to the positional analogy with the first exon of the trans-activator protein of the well studied human immunodeficiency virus type 1 (HIV-1). In this work we comparatively studied the trans-activation activity induced by Tat proteins of the small ruminant Maedi Visna virus (MVV) of sheep and Caprine arthritis encephalitis virus (CAEV) of goats on MVV and CAEV LTRs with that induced by the human lentivirus HIV-1 on its own LTR. The HIV-1 LTR alone weakly expresses the reporter GFP gene except when the HIV-1 Tat protein is coexpressed, the GFP expression is increased 60-fold. In similar conditions only minimal trans-activation increasing two- to three-fold the MVV and CAEV LTR activity was found with MVV Tat protein, and no trans-activation activity was detected in any used cell type or with any virus strain when CAEV Tat was tested. These results indicate that the small ruminant lentiviruses (SRLV) differ from the primate lentiviruses in their control of expression from the viral LTRs and put into question the biological role of the encoded protein named “Tat.”

Keywords: Tat; Lentivirus; CAEV; MVV; Trans-activation

Introduction

Maedi Visna virus (MVV) and Caprine arthritis encephalitis virus (CAEV) are members of the lentivirus genus that belongs to the retrovirus family. MVV and CAEV cause chronic progressive interstitial pneumonia, mastitis, arthritis, and encephalitis in sheep and goats (Narayan and Cork, 1985; Sigurdsson, 1954; Sigurdsson and Palsson, 1958; Sigurdsson et al., 1957). These inflammatory diseases are the result of interactions between mononuclear cells and the infected cells of monocyte/macrophage lineage, which were shown to be the main infected target cells in vivo. Differentiation of the monocyte into a macrophage is necessary to stimulate MVV and CAEV virus expression (Gendelman et al., 1985, 1986). Similar to other lentiviruses, MVV and CAEV cause persistent infection. However, unlike the human, simian, and feline lentiviruses, MVV and CAEV do not cause immune deficiency in their infected hosts and do not infect the CD4+ T-lymphocytes.

Their genomes contain genes encoding the structural proteins Gag, Pol, and Env, and three additional regulatory proteins Tat, Rev, and Vif. MVV and CAEV tat genes encode 94 and 86 amino acid (aa) products. MVV Tat protein, similar to HIV-1 Tat protein, was described as a trans-activator protein which upregulates viral expression via an increase of viral RNA synthesis (Hess et al., 1989) and RNA stability (Gdovin and Clements, 1992). However, this increase is very variable, between 1.6- (Gourdou et al., 1989) and 46-fold activation (Gdovin and Clements, 1992)
but lower than HIV-1 Tat trans-activation (100-fold) (Feinberg et al., 1991; Hauber et al., 1987). Moreover, the lack of expression of this protein following deletion of its coding sequences in CAEV-CO virus strain does not abolish the virus replication and no significant difference was observed between the wild-type and the CAEVΔTat virus (Harmache et al., 1995).

Tat-mediated transcription activation was shown to be dependent on the presence of AP-1 and AP-4 sites located in the U3 region of MVV long terminal repeat (LTR) (Gdovin and Clements, 1992). Although the proximal AP-1 site to the U3 region of MVV long terminal repeat (LTR) (Gdovin et al., 1989), MVV Tat protein does not bind the AP-1 site directly (Gdovin and Clements, 1992).

MVV Tat protein can be divided into the following three domains: a N-terminal acidic and hydrophobic domain, a central leucine-rich domain, and a C-terminal cysteine-rich domain. Comparative analysis of MVV Tat mutants, and search of cellular proteins interacting with Tat domains, have shown that (i) the N-terminal acidic is an activator domain (Carruth et al., 1994) and can interact in vitro with TATA binding protein (TBP) (Morse et al., 1999); (ii) the leucine-rich domain can interact in vitro with Fos- and Jun-specific proteins of AP-1 sites, via leucine-zipper domain (Morse et al., 1999). The cysteine-rich domain could be involved in protein dimerization.

CAEV Tat protein is structurally similar to MVV Tat protein, with a 100% identity in the leucine- and cysteine-rich domains; in contrast, the activation domain is very divergent between the two proteins. It was shown that CAEV Tat protein trans-activates viral LTR, but at a lower level than MVV Tat protein’s viral LTR (Jackson et al., 1991; Kalinski et al., 1994; Saltarelli et al., 1993).

Since it was shown that deletion of Tat in CAEV genome has no effect on virus replication, we were interested to determine if there is a correlation between the lack of interaction with viral replication and the lack of trans-activation. To test this hypothesis, we directly compared the trans-activation with MVV, CAEV, and human immunodeficiency virus type 1 (HIV-1) Tat proteins in similar conditions. The trans-activation was studied in various cell types including cells from species different from sheep and goats, and with Tat proteins from different MVV and CAEV strains. Finally, we studied the subcellular localization of MVV and CAEV Tat proteins.

Our results clearly show that MVV Tat protein can upregulate two to three times MVV and CAEV LTR promoters independently of used cell type or MVV strain, although HIV-1 Tat protein can trans-activate its own LTR 60-fold in the same conditions. We could not observe an increase of LTR expression by CAEV Tat protein, with any used cell type or CAEV strain, and this absence of trans-activation does not correlate with a lack of entry of CAEV Tat protein into the cell nucleus.

Results

Comparison of basal activity of MVV, CAEV, HIV-1, and CMV promoters

Four plasmids were constructed to study comparatively the promoter basal activity of MVV, CAEV, and HIV LTRs, with that of the CMV promoter (Fig. 1A). All these plasmid constructs contain the green fluorescent protein (GFP) reporter gene driven by (1) CMV viral promoter (pCMV); (2) LTR sequences from MVV KV-1772 virus (pLTR KV-1772); (3) LTR sequences from CAEV CO strain (pLTR CO); or (4) LTR sequences from HIV-1 virus (pLTR HIV).

Plasmid DNAs of these four constructs were introduced by transfection into HeLa cells. At 48 hours posttransfection, samples of transfected cells were used to quantify the GFP protein fluorescence level using the flow cytometry (FACS) analysis. The results of this analysis show that the basal activities of MVV and CAEV LTR promoters were very high, since no significant difference was observed between their fluorescence level and that of the constitutive CMV promoter (Fig. 1B). Moreover, the basal promoter activities of MVV and CAEV LTR sequences were found to be much higher (30- to 40-fold) than that of HIV-1 LTR sequences.

Comparison of trans-activation of MVV, CAEV, and HIV-1 LTRs with Tat proteins

To define the optimal experimental conditions, we used different amounts of pTat KV-1772 plasmid DNA to transf ect with the pLTR KV-1772 plasmid DNA in HeLa cells. The data reported in Fig. 2B show that the best results were obtained with 1 μg of pTat KV-1772 plasmid DNA. To quantify the trans-activation activity of Tat proteins of MVV and CAEV on their corresponding viral LTR promoter sequences, in comparison with the HIV-1 Tat/LTR system, three plasmids containing Tat-coding sequences isolated from these three viruses were constructed, pTat KV-1772, pTat CO, and pTat HIV. These constructs contain tat gene from MVV KV-1772, CAEV CO, and HIV-1 molecular clones, respectively, driven by the CMV promoter. A control plasmid pΔTat lacking the tat gene was also constructed (Fig. 2A).

HeLa cells were cotransfected with pLTR HIV-, pLTR KV-1772-, or pLTR CO-expressing GFP reporter gene driven by LTR promoter sequences (see Fig. 1A) and the respective Tat-expressing plasmids or pΔTat control plasmid (Fig. 2A). The fluorescence intensity in cotransfected cells was quantified by FACS analysis at 48 h posttransfection. The level of LTR trans-activation by its respective Tat protein was determined following comparison of the fluorescence intensity of cells cotransfected with pLTR and p-Tat plasmids to that cotransfected with pLTR and pΔTat control plasmid.
The data reported in Fig. 2B show that MVV KV-1772 Tat protein trans-activates two to three times MVV LTR, while no significant trans-activation was observed with CAEV Tat protein. In contrast, in similar conditions HIV-1 Tat protein was found to trans-activate at least 60-fold HIV-1 LTR.

These results are in agreement with those reported in the literature describing that MVV Tat protein trans-activates more efficiently MVV LTR than CAEV Tat protein does, but much less than HIV-1 Tat protein.

Cell type dependent Tat protein trans-activation of viral LTRs

The murine NIH-3T3 and caprine TIGEF fibroblasts, the human epithelial HeLa cells, and the ovine immortalized 4133 macrophages were cotransfected with pLTR and pTat plasmid DNAs from each of MVV KV-1772 and CAEV-CO infectious molecular clones, respectively. The pΔTat plasmid was used as negative control. Analysis of the intensity of fluorescence of GFP protein by FACS was used to evaluate the trans-activation activity of MVV and CAEV LTR by MVV and CAEV Tat proteins. The results are reported in Fig. 3A and B.

While no trans-activation activity was observed with CAEV-CO Tat protein on CAEV LTR in any of the used cell type (Fig. 3A), the trans-activation activity of MVV LTR by MVV KV-1772 Tat protein was found to upregulate 1.4 times in TIGEF cells and 2.2 times in NIH-3T3 cells (Fig. 3B). This last result indicates that MVV Tat protein may interact with cellular proteins from other species than goat to increase the transcription activity level. Surprisingly, the highest trans-activation activity of MVV Tat/LTR system was found in the murine fibroblasts NIH-3T3 cell line,
and the use of the natural target host cells of these viruses: macrophages (4133 ovine macrophage cell line) did not result in an increase of the trans-activation activity. Interestingly, the LTR trans-activation level seems to be inversely correlated to the promoter basal level since the highest activation was seen in NIH-3T3 cells in which the promoter basal activity was the weakest, and the weakest activation was seen in TIGEF cells in which the promoter basal activity was the highest.

**Influence of viral LTR and tat gene origin on the trans-activation level**

To determine whether the low trans-activation activity of MVV and CAEV Tat proteins was common in SRLV strains, we extended our study to LTRs and Tat proteins from various strains and isolates of MVV and CAEV in a Tat/LTR system. New pLTR plasmid was constructed with LTR from MVV LV1-KS1 strain in addition to pLTR KV-1772, pLTR CO and pLTR HIV plasmids; or by these same pLTR plasmids and the pΔTat control plasmid. Forty-eight hours later, transfected cells were submitted to FACS analysis to quantify the fluorescence intensity. The level of activation was calculated by the ratio of fluorescence intensity of each cell line to that of the pΔTat control cell line (B, C). The values shown at the top of each bar represent the value of activation over the basal LTR promoters. The data represent the mean value of at least three separate transfection experiments. The activities of basal promoters (pΔTat control plasmid) were used as controls.
1772 and pLTR CAEV-CO. New pTat plasmids were constructed with tat genes from MVV LV1-KS1 and Pe.01 strains in addition to pTat KV-1772, and tat gene from CAEV 93017 and 664 strains in addition to pTat CAEV-CO plasmid.

GFP-expressing plasmid DNAs under the transcriptional control of MVV and CAEV LTR promoter sequences were cotransfected with plasmids expressing either MVV or CAEV tat genes, or the control pΔTat plasmid, into NIH-3T3 cells. Transfected cells were studied by FACS analysis as above described at 48 h posttransfection.

The results of this analysis clearly show a very high basal activity of both CAEV and MVV LTR promoters. In addition, no significant difference was observed in terms of Tat protein activity between the various strains (Fig. 4B). Tat proteins from various strains of MVV trans-activate two- to three-fold MVV or CAEV LTR promoters. In contrast to these results, no trans-activation activity of CAEV Tat
Fig. 4. Analysis of the influence of LTR (A) or tat gene (B) origin on the trans-activation activity of MVV LTR by MVV Tat proteins. (A) Murine cells NIH-3T3 were cotransfected with either pLTR KV-1772, pLTR LV1-1KS1, or pLTR CO as first plasmid, and pTat KV-1772 or pΔTat control as second plasmid. Forty-eight hours later, the fluorescence intensity in transfected cells were analyzed and quantified by flow cytometry. The trans-activation level was calculated by the ratio of fluorescence intensity of each cell line expressing Tat compared to that transfected with the pΔTat control. (B) Murine cells NIH-3T3 were cotransfected with pLTR KV-1772 as the first plasmid and either pTat KV-1772, pTat LV1-1KS1, pTat Pe1.01, pTat CO, pTat 93017, and pTat 664 or by pΔTat control as second plasmid. Forty-eight hours posttransfection, the fluorescence intensity in transfected cells was analyzed and quantified by flow cytometry. The level of activation was calculated as in (A). The values shown at the top of each bar represent the value of activation over the basal LTR promoters. The data represent the mean value of at least three separate transfection experiments. The activities of basal promoters (pΔTat control plasmid) were used as internal controls.
proteins on MVV LTR (Fig. 4B) or CAEV LTR was observed (data not shown). To determine whether the absence of CAEV Tat protein trans-activation activity is related to a subcellular localization of Tat in cells, we analyzed the protein localization in Tat-expressing cells.

**Subcellular location of MVV and CAEV Tat proteins**

In previous in vitro studies, it was shown that (i) MVV Tat protein interacts with Jun protein via its leucine-rich domain and with TBP protein via its activator domain (Morse et al., 1999), and (ii) MVV Tat protein activator domain was located in the NH2-terminal region of the protein (Carruth et al., 1994, 1996). In contrast however, there are no data demonstrating such interactions in cell-culture conditions. Minimal studies were reported up-to-date for the trans-activation activity of CAEV Tat protein, but considering the very high homology between MVV and CAEV, the results obtained for MVV virus were often transposed to CAEV. However, the difference of LTR trans-activation activity between MVV and CAEV Tat proteins and the nucleotide divergence in their activator domain pushes us to consider these two proteins independently. We therefore have comparatively studied the cellular localization of these two proteins to investigate whether both Tat proteins enter equally in the nucleus.

The Flag epitope was used to generate fusion proteins in the NH2-terminal positions of MVV KV-1772 and CAEV CO Tat molecules named pTat MVV-Flag and pTat CO-Flag, respectively. The activity of pTat MVV-Flag was found to be unchanged compared to that of the nonfused pTat MVV (data not shown). Plasmid DNAs expressing pTat MVV-Flag and pTat CO-Flag were used to transfect NIH-3T3 cells in parallel with a control plasmid pΔTat (without tat gene) used as negative control. Western blot analysis using antibody directed against Flag epitope was performed on the different protein extracts at 48 h posttransfection (Fig. 5A). To exclude the possibility that the protein localization is due to the Flag epitope, a similar experiment was performed using plasmid DNA expressing MVV KV-1772 native Tat protein, and a specific antibody directed against MVV Tat protein epitope was used (Fig. 5B).

The results of this analysis show that both MVV and CAEV Tat proteins were found to be mainly in a nuclear localization. Therefore, the absence of LTR trans-activation by CAEV Tat protein cannot be explained by an inability of this protein to penetrate into the nucleus.

**Discussion**

Our main aim in this study was to determine whether the genetically well-conserved MVV and CAEV Tat coding sequences have an effective role in trans-activating their respective viral LTRs. As expected, our comparative study clearly showed that MVV and CAEV Tat proteins trans-activate their viral LTR at a very weak level compared to HIV-1 Tat protein’s viral LTR. One of the explanations could be the very high basal activity of MVV and CAEV LTR promoters since it reaches the activity level of the well-known constitutive CMV promoter and is at least 40 times higher than that to HIV-1 LTR promoter. One can suppose that the trans-activation by MVV or CAEV Tat
proteins is not yet possible as a consequence of expression level saturation. To evaluate this hypothesis, it would be of interest to study the trans-activation activity of MVV and CAEV Tat proteins in a cell type weakly expressing MVV and CAEV LTR promoters, or use LTR mutants that have a weak basal promoter activity. However, in all the cells we tested from various animal species and cells described in the literature, MVV and CAEV promoters are expressed at a very high level.

To determine whether the weak trans-activation activity observed for these proteins was not the consequence of an isolated case, we used LTR and Tat proteins from different MVV and CAEV strains in the Tat/LTR system. The use of these various strains helped us to analyze the influence of viral LTR trans-activation level with respect to the LTR promoter sequences or Tat protein origin.

We used promoter sequences from two MVV molecular clones LV1-1KS1 (Staskus et al., 1991) and KV-1772 (Andresson et al., 1993) and from a CAEV isolate, CO isolate (Saltarelli et al., 1990). It is interesting to note that the LV1-1KS1 is poorly pathogenic in vivo, while in contrast the KV-1772 strain was isolated for its neurovirulence. Moreover, the LV1-1KS1 replicated with a virus titer 2 log lower than that of KV-1772 in macrophage cultures (Torsteinsdottir et al., 1997). Sequence comparison of these two molecular clones reveals 1% divergence in nucleotides with 2 mutations in gag, 9 in pol, 12 in env, 3 in vif, 4 in tat, and 5 in rev genes.

In viral LTR, three mutations differentiate LV1-1KS1 from KV-1772 molecular clones. Two of these mutations affect the integrity of AP-1 site present in the U3 sequences. Similar to MVV K1514 the MVV prototype molecular clone, this AP-1 site (TGAGTCA) is located in MVV LV1-1KS1 LTR at position −52. This sequence was found to be mutated (G to A), transforming the canonic sequence (TGAGTCA) to (TAAGTCA) in MVV KV-1772 LTR; simultaneously, a canonic sequence was generated at position −75. In the LTR of CAEV CO strain, no canonic but only some putative AP-1 site sequences (such as the sequence in the position −52 (TGCTCA)) were identified. These three promoter sequences exhibit many differences in both the content and the location of their AP-1 sites, which have been shown to be essential for viral LTR trans-activation by MVV Tat protein (Gdovin and Clements, 1992).

Since MVV Tat protein trans-activates CAEV LTR at the same level as MVV viral LTR (Fig. 4A), these data suggest that these AP1 sites are not essential for the trans-activation activity of MVV Tat protein.

To determine whether SRLV Tat proteins trans-activate viral LTRs, we selected tat genes from three MVV (LV1-1KS1, KV-1772, and Pe101) and three CAEV strains (CO, 93017, and 664). Tat proteins of all these viral strains were 100% identical in the leucine-rich and the cysteine-rich domains, except for one mutation that substitutes a leucine by an isoleucine in the CAEV664 strain.

The activator domains are relatively well conserved among MVV strains despite the mutation of one phenylalanine that has been previously described to be more or less important for viral LTR trans-activation, depending on the cell type used (Carruth et al., 1994, 1996). On the other hand this activator domain is very different between MVV and CAEV Tat proteins. CAEV Tat protein activator domain does not contain the phenylalanine here abovementioned. The results of our study indicate that this amino acid is not critical for the activity of MVV Tat protein since MVV KV-1772 protein, lacking this phenylalanine residue, trans-activates the LTRs at the same level as MVV LV1-1KS1 Tat protein does (Fig. 4B). MVV LV1-1KS1 strain, known to induce weak pathogenesis in infected sheep, showed neither a lower LTR basal level (Fig. 4A) nor a lower Tat protein trans-activation activity (Fig. 4B). Therefore these data exclude the relationship between the trans-activation of LTR and the severity of induced pathogenesis.

Interestingly, none of the tested CAEV Tat proteins induced trans-activation of SRLV LTR promoters. This data indicate that the absence of trans-activation activity of CAEV Tat is common to many strains. In addition, our data demonstrated that the lack of this trans-activation activity of CAEV Tat proteins does not correlate with the inability of this protein to enter in the nucleus of Tat-expressing cells.

Altogether these data suggest that the primary function of these SRLV Tat proteins is not to trans-activate the viral LTR promoters as Tat proteins from other lentiviruses do (Cullen, 1992). EIAV (Albrecht et al., 2000), BIV (Barboric et al., 2000), SIV (Viglianti and Mullins, 1988), and HIV (Luo et al., 1993) Tat proteins were shown to have similar interactions with their target sequences, by attachment on TAR sequences (attachment site for Tat protein). In contrast, in MVV or CAEV LTRs no TAR sequence was identified. These data suggest that the LTR promoters of SRLV look to be Tat-independent. The lack of TAR sequence in FIV LTR suggests also that FIV Tat protein may act similar to SRLV Tat proteins (de Parseval and Elder, 1999).

To date, the biological role of SRLV Tat protein still remains unclear, and the only well-defined property is that SRLV Tat protein is not essential for efficient virus replication (Harmache et al., 1995). Since Tat is dispensable for virus replication for small ruminant lentiviruses, we speculate that this protein is rather an accessory than a regulator protein, as was previously suggested (Cullen, 1992). Further experiments must be done to help to better define the biological functions of these well-conserved open reading frames.

Materials and methods

Plasmid construction

pLTR plasmids were constructed by inserting the U3 promoter sequences from MVV and CAEV, and the U3R
sequences from HIV-1 in replacement of the SV40 promoter into the EcoRI and XhoI sites of pGFPemd-p promoter (Packard). U3 sequences were amplified from pLV1-RS1 plasmid (Staskus et al., 1991) for LV1-KS1 U3 region and p8Xsp5-RK1 plasmid (Andresson et al., 1993) for KV-1772 U3 region using V3 and V5 primers, and from pK9 Kb (Mselli-Lakhal et al., 2000; Turelli et al., 1996) plasmid for CAEV-CO U3 region using C3 and C5 primers. U3R sequences were amplified from pLTR HIV-CAT plasmid (kindly provided by L. Gazzolo) using U3-HIV-1 and R-HIV-1 primers.

The nucleotide sequences of used oligonucleotide primers are presented here as follows: V3: 5’ TGAATTCCAGCTTCCAAAAG 3’; V5: 5’ TCTCGAGTGGACTGTCAGGAC 3’; C3: 5’ GGAATTCAGGATCCAGGAC 3’; C5: 5’ TCCGAGGATCTGTTGAGCACCTG 3’; U3-HIV-1: 5’ GCTCGAGAAGGGCTAATTCACT 3’; R-HIV-1: 5’ GGAATTTCTATTGAGGCTTAAGCAG 3’. TAT01 and TAT02 primers, as follows: TAT02: 5’ GAATGCTGGGAAA 3’ and MVV KV-1772 from p8XSp5RK1, using TAT01 and (Mselli-Lakhal et al., 1999) using BR5 and BR3 primers: BR5: 5’ GCAAGCTTGATTATGTTCCC 3’; 93017 tat gene was amplified from pK9 Kb plasmid for CAEV, using a primer already containing the flag sequence, named V-Flag5’ and C-Flag5’, respectively, and a second primer, as above described and named TAT02 and TAT04, respectively: V-Flag5’: 5’ GGAATTCATGTTACATTAAAGGAC-GACGATGCAAGAGAAGTACCAAGAAGACAGC-CAGGAGGCTATTAGAAGGGAGGATTTTCAATTATGAGA 3’; C-Flag5’: 5’ GGAATTCATG-GACTACAAGGACGACATGCTGAAGAACTTGCTCAAGAAGGGAAGCACATCCACAGAAGACTT-GTAAGGGAACGTACGGGAAAGAGAAAG 3’. The obtained PCR products containing the Tat-Flag fusions were then inserted between EcoRI and HindIII sites of BSK-CMV-pA plasmid to generate pTat MVV-Flag and pTat CO-Flag plasmids.

Cells

Murine embryo fibroblasts NIH-3T3 and human epithelial carcinoma HeLa cells were maintained in DMEM medium supplemented with 10% fetal calf serum and incubated at 5% CO2 at 37°C. Large T immortalized goat embryo fibroblasts (TIGEF) (Da Silva Teixeira et al., 1997) were maintained in MEM medium supplemented with 10% fetal calf serum. Immortalized ovine macrophages 4133 (Olivier et al., 2001) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

Transfection procedures

To assay Tat-mediated trans-activation, cell lines were plated in six-well cluster dishes at a density of 2 x 10^4 cells per well 24 h before transfection. TIGEF, NIH-3T3, and 4133 cells were transfected by the Fugene-6 method (Roche) and used according to the manufacturer’s instructions. Plasmid DNAs (pTat: 1 μg, pLTRs: 1 μg) were mixed with 6 μl fuge in optimem serum-free medium. The DNA-fugene sample was allowed to incubate for 15 min at room temperature and was added to the cell monolayer. HeLa cells were transfected by the effectene method (Qiagen) and used according to the manufacturer’s instructions. Four microliters of enhancer was added to the DNA-EB buffer sample and incubated for 3 min at room temperature. Then 5 μl of effectene reagent was mixed with the DNA-enhancer sample and incubated for an additional 10 min at room temperature before adding to cells. Medium was changed 5 h after transfection.

Analysis of transfected cells

Forty eight hours posttransfection, cells were dissociated by trypsin treatment and analyzed by flow cytometry. Cell
fluorescence levels were determined by FACScan and the data analyzed by using the Cell Quest program (Becton–Dickinson). Each transfection was done in duplicate and repeated two to five times with at least three different plasmid preparations to ensure the reproducibility of the results. To avoid fluorescence saturation, a scale was performed with different amounts of plasmids and an equivalent quantity (1 μg) of pLTR and pTat plasmids was retained.

**Protein extracts and Western blot analysis**

For Western blot analysis, cells were harvested 48 h posttransfection. For total protein extracts, cells were scraped in 1 ml 1× PBS and then centrifuged for 10 s at 10,000 rpm and pellets were resuspended into 50 μl 1× PBS. Protein extracts were incubated at −20°C before quantification by Bradford assay (Bio-Rad) and gel analysis.

For cytoplasmic and nuclear extracts, cells were dissociated by trypsin, washed with 1× PBS, resuspended in 200 μl of A lysis solution (HEPES pH 7.9 10 mM; MgCl2 1.5 mM; KCl 10 mM; NP-40 0.1%; protease inhibitor 50 μg/ml; DTT 0.5 mM; ZnCl2 0.1 mM), and then ice-incubated for 15 min. The resulting solution was centrifuged for 1 min at 12,000 rpm, and the supernatant was named the cytoplasmic fraction. Fifty microliters of B lysis solution was added to the pellet and the mixture was incubated for 45 min at 4°C before centrifugation for 15 min at 12,000 rpm. The supernatant was called the nuclear fraction.

Proteins from cell lysates were separated on a sodium dodecyl sulfate (SDS)–16.5% polyacrylamide gel. MVV Tat protein and Flag fusion Tat proteins were detected by using a rabbit polyclonal antiserum specific to Visna virus Tat and a monoclonal antibody directed against flag epitope, respectively. Blots were developed by enhanced chemiluminescence (ECL kit; Amersham, France) according to the manufacturer’s instructions.

**Acknowledgments**

This work was supported by a research grant from the Centre National de la Recherche Scientifique, the Institut Nationale de la Recherche Agronomique. We thank the Ministère de la Recherche for the fellowship of (S.V.). We thank L. Gazzolo for kindly providing the pLTR HIV-CAT and pSG5 Tat plasmids, and G. Cordier for kindly providing the pPe1-01, p93017.02, and p664.03 plasmids.

**References**


species encoding the Tat protein of caprine arthritis encephalitis virus. Virology 204 (2), 828–834.