Feline foamy virus Tas protein is a DNA-binding transactivator

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Foamy viruses (FVs) harbour a transcriptional transactivator (Tas) and two Tas-responsive promoter regions, one in the 5’ long terminal repeat (LTR) and the other an internal promoter (IP) in the envelope gene. To analyse the mechanism of transactivation of the FVs, the specificity of feline FV (FFV) Tas protein, which is more distantly related to the respective proteins of non-human primate origin, were investigated. FFV Tas has been shown specifically to activate gene expression from the cognate promoters. No cross-transactivation was noted of the prototype foamy virus and human immunodeficiency virus type 1 LTR. The putative transactivation response element of FFV Tas was mapped to the 5’ LTR U3 region (approximately nt −228 to −195). FFV Tas binds to this element in addition to a previously described sequence (position −66 to −51). It is therefore concluded that FFV Tas is a DNA-binding transactivator that interacts with at least two regions in the virus LTR.

The genome of the foamy viruses (FVs) contains three open reading frames (ORFs) encoding the gag, pol and env structural genes; the regulatory tas and bel-2 genes are located between the env gene and the 3’ long terminal repeat (LTR), which are under the control of both the 5’ LTR and an internal promoter (IP) (Lo¨chelt et al., 1993). The tas gene encodes the Tas protein, which transactivates virus transcription from both promoters (Lo¨chelt, 2003). The Coleman strains of feline FV (FFV) were previously isolated and sequenced (Hatama et al., 2001a, b). As experimental infection by FFV caused no clinical signs in cats, FFV has been considered to be apathogenic (Zenger et al., 1993; Winkler et al., 1999), thus confirming on the virus the status of a useful retroviral vector (Schwantes et al., 2003). Transactivation of the FFV LTR by FFV Tas leads to the synthesis of viral genomic RNA (Winkler et al., 1997; Bodem et al., 1998). However, the mechanism of transactivation of the promoters by FFV Tas is yet to be characterized.

Previously, it has been reported that the human immunodeficiency virus type 1 (HIV-1) LTR is transactivated by many regulatory proteins of other viruses, including the prototype FV (PFV) Tas (Gendelman et al., 1986; Siekevitz et al., 1987; Seto et al., 1988; Horvat et al., 1989; Twu et al., 1989; Keller et al., 1992; Lee et al., 1992). Thus it is important to investigate the cross-transactivation properties and transactivation-response element (TRE) of FFV Tas. We report here highly specific FFV Tas transactivation of the FFV LTR and IP promoter, and the identification of the FFV Tas TRE in the U3 region of FFV LTR. While our results show that FFV binds to this element, FFV Tas did not generally induce HIV-1 or feline immunodeficiency virus (FIV) LTR promoters.

To construct FFV, PFV and FIV LTR reporter plasmids, each LTR fragment was amplified by PCR with primers A (5’-TATCATGGGCACAAAGAGAATTC-3’) and B (5’-AATTTCACCTAATCGAAGCC-3’); C (5’-TGTTGGTGAAATGGCCACTAG-3’) and D (5’-ATTGTCAATGGAATTGTGA-3’); and E (5’-TGGGATGAGTATTGGAACCC-3’) and F (5’-ccctgtggcggccaatctg-3’) using pSKY3.0, pHS007 and pFIV14, respectively, as templates (Olmsted et al., 1989; Hatama et al., 2001b; Fujii et al., 2004). To prepare IP reporter plasmids, FFV and PFV IP fragments were also amplified by PCR with primers G (5’-TAAAGAGTTTAAAGGCCCAACC-3’), and H (5’-GAGCTGCAGCCACTGCTG-3’); and I (5’-ACTGGGACCTTTAAAAGGCCAC-3’) and J (5’-CTGTGGTATCCAGGCAGG-3’) using pSKY3.0 and pHS007, respectively, as templates. These fragments were ligated into the Smal site up-stream of the firefly luciferase (Luc) reporter gene in pGL3-basic plasmids (Promega). The HIV-1 LTR-Luc reporter plasmid has been described previously (Yamamoto et al., 2002). To construct FFV and PFV Tas expression vectors for mammalian cells, full-length FFV and PFV tas genes were amplified by PCR with primers K (5’-ATGGCCTCAATCACTCCGGC-3’) and L (5’-ATGGATTAGCTGCTATTCG-3’); and primers M (5’-ATGGATTACCAGGAAAG-3’) and N (5’-TTATAAAACTGAATGTTCC-3’), respectively.

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Each fragment was cloned into the pCR2.1 TA cloning vector (Invitrogen) and digested with HindIII and XbaI and then fragments were inserted into pcDNA3.1/Hygro (Invitrogen) digested with HindIII and XbaI. The HIV-1 Tat expression plasmid, pCD-Tat, was constructed by introducing the KpnI–BamHI fragment of pCV-1 (Yamamoto et al., 2002) into pcDNA3.1/Hygro digested with KpnI and BamHI.

To study the activity of FFV Tas protein, we investigated the transactivation specificity of FFV Tas in human or feline cells. The FFV Tas-, PFV Tas- and HIV-1 Tat-expressing plasmids were co-transfected with FFV, PFV, FIV and HIV-1 LTR-Luc or FFV and PFV IP-Luc plasmids into HeLa or CRFK cells. Luc activities were measured at 48 h post-transfection as described previously (Otake et al., 2004), and

### Table 1. Specific transactivation of FFV Tas against the FFV LTR

Cells were co-transfected with the indicated LTR or IP reporter plasmid and the effector plasmid and the Luc assay was performed at 48 h post-transfection. Luc activity was measured and normalized as Luc values (Luc/β-gal). The absolute mean level of Luc activity in the sample of the FFV LTR-Luc plasmid plus FFV Tas effector plasmid transfected was 194 ± 6. Data are means of three independent experiments. −, Not done.

<table>
<thead>
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<th>Target</th>
<th>HeLa Relative Luc activity</th>
<th>CRFK Relative Luc activity</th>
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<tr>
<td>FFV LTR</td>
<td>FFV Tas: 64</td>
<td>FFV Tas: 95</td>
</tr>
<tr>
<td></td>
<td>PFV Tas: &lt;1</td>
<td>PFV Tas: 70</td>
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<tr>
<td></td>
<td>HIV-1 Tat: 22</td>
<td>HIV-1 Tat: 3</td>
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<tr>
<td>FFV IP</td>
<td>FFV Tas: –</td>
<td>PFV Tas: 9</td>
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<tr>
<td></td>
<td>–</td>
<td>HIV-1 Tat: &lt;1</td>
</tr>
<tr>
<td>FIV LTR</td>
<td>FFV Tas: &lt;1</td>
<td>PFV Tas: &lt;1</td>
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<tr>
<td>PFV LTR</td>
<td>PFV Tas: 593</td>
<td>HIV-1 Tat: 198</td>
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<tr>
<td>PFV IP</td>
<td>–</td>
<td>PFV Tas: 2280</td>
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<td>FFV Tas: &lt;1</td>
<td>HIV-1 Tat: 6576</td>
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<tr>
<td></td>
<td>1570</td>
<td>700</td>
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Fig. 1. Identification of cis-regulatory elements in the FFV 5′ LTR of the FFV Tas.
(a) Luc reporter assay with hybrid promoter constructs. Numbers represent regions of the 5′ LTR and IP. HeLa cells were co-transfected with the truncated LTR or the IP-SV40 promoter plasmids in the presence or absence of the FFV Tas-expressing plasmid. Luc activity was measured 48 h post-transfection. Data represent Luc activity relative to the sample of the SV40 promoter plasmid without FFV Tas. Data are given as means ± SD of three independent experiments. (b) EMSA. The [γ-32P]-ATP-labelled DNA probes designated #1–4 from the LTR regions are underlined. The complex of DNA with purified FFV Tas protein was subjected to 5% native polyacrylamide gel electrophoresis. Unlabelled probe #1–4 DNAs were also used for competition assay.
found to be highest for the combination of FV Tas and its own LTR in each cell (Table 1). Interestingly, although PFV Tas transactivates not only PFV LTR but also HIV-1 LTR, as reported previously (Keller et al., 1992; Lee et al., 1992), FFV Tas enhanced HIV-1 LTR-driven promoter activity to a lesser extent compared with PFV Tas (Table 1). On the whole, while FFV and PFV Tas activated FFV and PFV IP transcription, respectively, FFV and PFV Tas did not enhance FIV LTR promoter activity (Table 1). HIV-1 Tat did not transactivate FFV LTR promoter activity in CRFK cells, but had a weak stimulating effect on the FFV LTR in HeLa cells (Table 1). These results indicate that the presence of FFV Tas is essential for effective transcription of FFV genes and that the FFV Tas protein specifically activates both LTR and IP promoters.

Similar experiments were also performed in HIV-1 IIIB persistently infected U937 cells. As expected, HIV-1 LTR was dramatically induced in its transcriptional activity (approximately 22-fold) compared with the pGL3-basic control plasmid because of the activity of endogenous Tat. In contrast, the transcriptional activity of neither FFV nor PFV LTR-Luc was induced significantly. Next, to examine whether FFV Tas transactivates the HIV-1 LTR promoter in HIV-1-infected cells, these were co-transfected with FFV Tas or PFV Tas expression plasmid along with HIV-1 LTR-Luc plasmid. Interestingly, although PFV Tas transactivated the HIV-1 LTR promoter (approximately 9-fold) compared with pcDNA3.1/Hyg control plasmid-transfected cells, FFV Tas did not enhance HIV-1 LTR promoter activity (approximately 9-fold). Similar results were obtained with persistently HIV-1 IIIB-infected MT-4 T cells (data not shown). These results suggest that, in HIV-1-infected U937 or MT-4 T cells, the promoter activity of FFV LTR is not influenced significantly and that FFV Tas transactivates the HIV-1 LTR promoter much less efficiently compared with PFV Tas.

To define cis-acting elements of the FFV LTR involved in transactivation by FFV Tas, several deletion mutants in the U3 region were constructed. HeLa cells were co-transfected with the mutated FFV LTR-Luc reporter plasmid in the presence or absence of the FFV Tas-expressing plasmid and Luc activity was measured. Deletion of the upstream region between nt −268 and −171 in the U3 region dramatically reduced the level of transactivation by FFV Tas (see Fig. 2a).

To map the FFV Tas TRE further, we examined the effects of truncated LTR mutants upstream of the SV40 promoter (Fig. 1a). In the presence of FFV Tas, Luc plasmids containing nt −417 to −171 or −268 to −39 of the U3 region showed markedly higher transactivation than in its absence. Luc activity of the plasmid harbouring an inverted orientation of nt −417 to −171 was also enhanced by FFV Tas, indicating that the TRE might function as an orientation-independent enhancer. These results indicate that the region from nt −268 to −171 of the 5′ LTR is required for efficient transactivation by FFV Tas.

To test for direct interactions between the TRE and FFV Tas,
a gel electrophoretic mobility-shift assay (EMSA) was undertaken with a purified glutathione S-transferase (GST)-fused FFV Tas protein (amino acids 1–209). The GST fusion proteins were expressed in *Escherichia coli* BL21 and purified with Bulk GST purification modules (Pharmacia). For these assays, the fusion protein was mixed with each of the following [γ-32P]-ATP-labelled DNA probes: #1, nt 268 to 240; #2, nt 251 to 218; #3, nt 228 to 195; and #4, nt 204 to 171 (Fig. 1b) in binding buffer (10 mM Tris/HCl pH 7.5, 65 mM KCl, 2.5 mM MgCl2, 1 mM dithiothreitol and 5% glycerol) containing 200 ng poly(dI-dC) and 40 μg BSA for 30 min at room temperature. The reaction products were analysed on a 5% native polyacrylamide gel and positive bands were visualized by autoradiography. The fusion protein formed a discrete complex with probe #3 that could be specifically inhibited by competition with unlabelled probe #3 DNA (Fig. 1b), but not with probes #1, 2 or 4 (data not shown). The control GST and GST-FFV Tas (aa 1–300) did not show any complex formation in this assay. It can be inferred from these results that FFV Tas can bind directly to nt 228 to 195 of the LTR U3 region and that FFV Tas-binding sites. To investigate the relative contributions of each of these putative FFV LTR TREs to the FFV Tas-mediated transactivation, comparative analyses were performed. In the presence of FFV Tas, Luc plasmids were co-transfected into HeLa cells with pG5-luc plasmid (Promega). Although the region of aa 1–161 of FFV Tas did not show transactivation activity, the region of aa 162–209 enhanced the transactivation activity approximately 3000-fold compared with control Gal4. The expression of mutant Tas proteins was analysed by RT-PCR and found to be of similar levels to the wild-type construct (data not shown), indicating that the transactivation domain of FFV Tas is located in the C-terminal domain, aa 162–209.

To determine FFV Tas TRE besides nt 228 to 195 in the FFV LTR, additional mutants of the LTR-Luc plasmid were constructed. Deletion of the upstream region between nt 170 and 38 in the U3 region completely abolished promoter activity by FFV Tas (Fig. 2a), indicating that another TRE is located in this region (Bodem et al., 2004). Thus there is a possibility that FFV LTR contains at least two FFV Tas-binding sites. To investigate the relative contributions of each of these putative FFV LTR TREs to the FFV Tas-mediated transactivation, comparative analyses were performed. In the presence of FFV Tas, Luc plasmids driven by the SV40 promoter containing nt 268 to 171 of the FFV LTR showed higher transactivation activity than that of nt 101 to 39, compared with the control SV40 promoter-Luc plasmid (Fig. 2b). Next, we performed EMSA by using probes nt 228 to 195 and 70 to 50 of the FFV LTR to compare the affinity of the FFV Tas to each TRE. Each probe similarly formed a specific complex with FFV Tas, indicating that each TRE appears to have a similar affinity to the FFV Tas (Fig. 2c). The implication of these data is that FFV Tas similarly binds to the two TREs.

In the current study, we have provided a description of highly specific FFV Tas transactivation activity against FFV promoters. Our results are consistent with those of previous studies demonstrating that FFV Tas does not activate the PFV LTR and IP and that PFV Tas does not transactivate FFV LTR (Winkler et al., 1997), even though PFV Tas has been reported earlier to transactivate HIV-1 LTR (Keller et al., 1992; Lee et al., 1992). Furthermore, we have demonstrated that FFV Tas did not influence HIV-1 transcription in HIV-1-infected MT-4 T or U937 and uninfected HeLa cells, although it slightly enhanced the HIV-1 LTR promoter activity in CRFK cells. In CRFK cells, feline-specific transcriptional factors upregulated by FFV Tas might indirectly influence HIV-1 LTR promoter activity. Additionally, FFV and PFV Tas did not transactivate the FIV LTR and HIV-1 Tat did not transactivate the LTR and IP of FFV and PFV in CRFK cells. In preliminary experiments we observed that the putative transactivator of FIV, OrfA (Parseval & Elder, 1999) also did not transactivate FFV LTR and IP promoters. These novel findings strongly support previous reports that characterization of transactivation of FFV shows features distinct from those of PFV and HIV-1. In addition, we have demonstrated that FFV Tas is a DNA-binding protein. Although PFV Tas has been reported to be a DNA-binding protein (He et al., 1996; Kang et al., 1998), it has been suggested that FFV Tas contributes to DNA binding (Bodem et al., 2004), which strongly supports the results observed in our present study. Previously it was reported that a region highly homologous to the TRE of PFV Tas was found in HIV-1 LTR (Lee et al., 1992). However, a region highly homologous to the two TREs of FFV Tas was not found in the HIV-1 LTR in our study. In conclusion, our results suggest that there are some distinctive features in the transcriptional mechanism of FFV that differentiate it from those of either PFV or HIV-1.

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**References**


