cis-Acting Regulatory Regions in the Long Terminal Repeat of Simian Foamy Virus Type 1

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Simian foamy virus type 1 (SFV-1), a member of the *Spumavirinae* subfamily of retroviruses, encodes a transcriptional transactivator (*taf*) that strongly augments gene expression directed by the viral long terminal repeat (LTR) (A. Mergia, K. E. S. Shaw, E. Pratt-Lowe, P. A. Barry, and P. A. Luciw, *J. Virol.* 65:2903–2909, 1991). This report describes *cis*-acting regulatory elements in the LTR that control viral gene expression. A series of LTR mutants and hybrid promoter constructs have been analyzed in transient expression assays for responsiveness to *Taf*. The targets for transactivation have been mapped to two regions of the U3 domain of the LTR, between positions –1196 and –880 and between positions –403 and –125 (+1 represents the transcription initiation site). No significant nucleotide sequence homology between these two regions is noted; thus, the SFV-1 *taf* gene acts through at least two distinct sequence elements in the LTR. The target contained between positions –403 and –125 acts independently of orientation, in different cell types and species, and in the context of a heterologous promoter. Thus, the target element between positions –403 and –125 has properties of a transcriptional enhancer. The observation that two distinct elements in the SFV-1 LTR are targets for transcriptional transactivation is novel with respect to observations for other retroviral systems. The R-U5 region of the SFV-1 LTR down-regulates transactivation by severalfold. Computer analysis of the R-U5 region revealed a secondary structure with a free-energy level of –74 kcal (ca. –310,000 J); this structural feature may account for the inhibitory effect on gene expression directed by the LTR. *Taf* of SFV-1 had no effect on gene expression directed by the LTR of the related human foamy virus, whereas *Taf* transactivates gene expression directed by the LTRs of the human and simian immunodeficiency viruses. Comparative functional analysis of *Taf* on homologous and heterologous LTRs may facilitate elucidation of the mechanism of transactivation of foamy viruses.

Retroviruses are classified into seven genera, one of which contains the spumaviruses. Spumaviruses, also known as foamy viruses, have been recovered from several mammalian species, including human, simian, bovine, feline, and murine species (13, 32). The simian foamy virus type 1 (SFV-1), an isolate from a rhesus macaque (14), has been molecularly cloned, and the genome has been completely sequenced (Fig. 1) (20, 22–24). In addition to the genes *gag*, *pol*, and *env*, the genome of SFV-1 contains two open reading frames (ORFs) at the 3' end of *env*, extending into the 3' long terminal repeat (LTR). SFV-1 encodes three species of viral mRNA: an 11.5-kb RNA representing the full-length transcript, a 6.5-kb RNA representing the *env* message, and a 2.8-kb RNA from the ORF region (24). The ORF region of SFV-1 is specified by messages that involve multiple splicing events (24). The first ORF, designated *taf* (transcriptional transactivator of foamy virus), activates gene expression directed by the homologous LTR (23, 24). A related human foamy virus (HFV) encodes the virion *gag*, *pol*, and *env* proteins, as well as three large ORFs (*bel-1*, *bel-2*, and *bel-3*) which extend from the 3' end of *env* into the 3' LTR (7, 19). Transcripts for the *bel* region of HFV are also generated by complex splicing events (25). The *bel-1* gene of HFV also has a transactivator function (15, 37). *bel-1* of HFV (also designated *taf*) shows 39% homology with the predicted amino acid sequence of SFV-1 *taf* (24). *bel-1* has been shown to be essential for virus replication *in vitro* (18). Translation frames encoding *bel-2* and ORF-2 have 38%

homology at the amino acid level (24); the potential function of these genes is not known. SFV-1 does not specify a translation frame that corresponds to *bel-3* of HFV (24). LTR sequences of these two primate foamy viruses differ significantly in the U3 domain (24% nucleotide homology); this domain encompasses the *cis*-acting target element for the viral *taf* genes (15, 23, 24, 37, 41). In contrast, the R-U5 domains of both viral LTRs show 85% homology (23). The fact that the U3 domains of the LTRs and the *taf* genes diverge greatly implies that target sequences for transactivation in the two viruses may be distinct.

To define the target for *Taf*, several deletion mutants of the SFV-1 LTR were constructed. Also, hybrid promoters containing portions of the SFV-1 LTR and both the herpes simplex virus (HSV) thymidine kinase (*tk*) promoter and the simian virus 40 (SV40) early promoter were made. By using transient expression assays, the target for transactivation was mapped to two regions in the U3 domain of the LTR. In addition, the R-U5 region of the LTR was shown to down-regulate transactivation.

MATERIALS AND METHODS

Cell cultures. COS-7 (African green monkey fibroblast), CV-1 (African green monkey fibroblast), and L929 (murine fibroblast) cell lines were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Plasmid constructions. The plasmid pSFV-1 LTR/CAT, which contains the SFV-1 LTR placed upstream from the

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— = 1kb

FIG. 1. Genomic organization of SFV-1 showing the LTRs and the ORFs. *taf* represents the transcriptional transactivator of foamy virus. The designation for ORF-2 may be changed when its role in the viral life cycle is defined.

chloramphenicol acetyltransferase (CAT) gene, has been described previously (23). Deletion mutants of the SFV-1 LTR were constructed by using appropriate restriction enzymes and placed upstream from the CAT gene in an expression vector. The constructions of pSFV-1 LTR/CAT 41, pSFV-1 LTR 46, pSVORF-1, pHFV 5'LTR/CAT, pH TLV-1 LTR/CAT, pHIV LTR/CAT, pSIV LTR/CAT, pS Vtat(HIV), pSVtat(SIV), pHTLV LTR/tax, and ptk/CAT are described elsewhere (3, 24, 28, 34, 36). pSFV LTR/CAT 61, pSFV-1 LTR/CAT 62, pSFV-1 LTR/CAT 63, pSFV-1 LTR/CAT 48, and pSFV-1 LTR/CAT 50 contain nucleotide sequences from -270, -403, -501, -593, and -754, respectively, to +334; +1 represents the transcription initiation site. pSFV-1 LTR/CAT 49 was generated by removing the *Hind*III fragment from positions -593 to -880 from pSFV-1 LTR/CAT wt. pSFV-1 LTR 51, pSFV-1 LTR 52, and pSFV-1 LTR 53 contain nucleotide sequences from -270, -403, -501, -593, and -754, respectively, to +6.

Hybrids of SFV-1 LTR regions and the HSV tk gene promoter were generated by placing the SFV-1 LTR sequences upstream from the tk promoter in the CAT expression vector. pLTR51tk/cat, pLTR52tk/cat, and pLTR53tk/cat contain nucleotide sequences from positions -270, -403, and -501, respectively, to position -125 of the SFV-1 LTR cloned upstream from the tk promoter. pLTR53Rtk/cat and pLTR56Rtk/cat contain nucleotide sequences from positions -501 to -125 and -1196 to -880 of the SFV-1 LTR, respectively, cloned in reverse orientations upstream from the tk promoter.

In pSVRU5/CAT and pSVU5R/CAT, the R-U5 region of the SFV-1 LTR was placed downstream from the SV40 promoter in the sense and opposite orientations, respectively, in the CAT expression vectors. pSV300/CAT contains the SFV-1 LTR sequence from positions -593 to -880 (similar in size to that of R-U5) downstream from the SV40 promoter in the CAT expression vector.

DNA transfection. Cells were seeded at a density of 3×10^5 /35-mm-diameter well in six-well dishes 24 h prior to transfection. Transfection was carried out by the DEAE-dextran method on COS-7, L929, and CV-1 cells as previously described (2). For each experiment, cells were transfected in duplicate at least twice with 2 μ g of plasmid expressing the CAT gene (pSFV-1 LTR/CAT or pSFV-1 LTR/CAT mutants or hybrids of SFV-1 LTR regions and the tk promoter or hybrids of the SV40 promoter and the R-U5 region of the SFV-1 LTR) and with 3 μ g of carrier DNA (pSP65; Promega) or of plasmid expressing the *taf* gene (pSVORF-1). Cell extracts were prepared at 48 h after transfection, and CAT levels were measured as previously described (2).

RESULTS

cis-acting regulatory elements in the U3 domain of the SFV-1 LTR. Transient expression assays with cell lines representing several species indicated that the SFV-1 genome encodes a transactivator that functions on the LTR at the transcriptional level (23, 24). To define *cis*-acting elements of the SFV-1 LTR involved in transactivation, several SFV-1 LTR deletion mutants were constructed and placed upstream from the CAT gene in an expression vector. Plasmids containing the mutant forms of the LTR as well as the wild-type LTR were cotransfected with carrier plasmid or with plasmid expressing the *taf* gene into COS-7 cells. Cell lysates were prepared 48 h after transfection and assayed for CAT activity. The basal promoter activity of the SFV-1 LTR in the transient expression assay was very low, whereas in cotransfected cells the *taf* gene greatly increased LTR-directed gene expression (28-fold) (Fig. 2). All deletion mutants show the same basal-level CAT activity; thus, the LTR does not appear to contain *cis*-acting negative regulatory elements. Transactivation was totally abolished by removing most of the upstream U3 region (positions -125 to -1196), even though the TATA box was retained. Deletions from the 5' end of the LTR to position -880 in the U3 region showed transactivation levels threefold lower than that of the wild-type LTR; thus, the region upstream from -880 is partially required for transactivation. Successive deletions from the 5' end to positions -754, -593, -501, -403, and -270 demonstrated CAT activities 9.6-, 10.8-, 8.0-, 8.2-, and 3.5-fold higher, respectively, than basal levels. Deletion from -880 to -593 showed CAT transactivation equivalent to that of the wild-type LTR. In summary, analysis of deletion mutants revealed that the regions between positions -403 and -125 and between -1196 and -880 in the SFV-1 LTR contain *cis*-acting sequences required for transactivation of viral gene expression.

To confirm that two regions in the SFV-1 LTR are required for transactivation, DNA fragments that contain the sequences between positions -1196 and -880, -501 and -125, -403 and -125, and -270 and -125 were subcloned upstream from the HSV tk promoter (pLTR56tk/cat, pLTR53tk/cat, pLTR52tk/cat, and pLTR51tk/cat, respectively) and tested for CAT expression in two cell lines (COS-7 and L929). pSFV-1 LTR/CAT and the HSV tk promoter expressing CAT (ptk/cat) were controls to compare levels of transactivation. Plasmids containing the hybrid promoters demonstrated CAT activities severalfold higher than that of the tk promoter directing CAT; thus, the regions between positions -1196 and -880 and between -403 and -125 are necessary and sufficient for transactivation by *taf* (Fig. 3). The sequence between positions -1196 and -880 functions only in the sense orientation, whereas the latter sequence (-403 to -125) responds to *Taf* irrespective of orientation (Fig. 3).

Transactivation of heterologous LTRs. The U3 regions of the LTRs of SFV-1 and HFV have only 24% nucleotide sequence identity, and the *taf* genes of the two viruses show 38% homology at the level of predicted amino acids. To determine whether *taf* of SFV-1 transactivates gene expression directed by the HFV LTR, transfection experiments with the COS-7 cell line were performed. CAT expression directed by the HFV LTR remains the same in both the presence and the absence of SFV-1 *taf*; thus, there is no cross-transactivation between SFV-1 and HFV (Table 1). The LTRs of additional primate retroviruses were also tested; these include human T-cell lymphotropic virus type I

FIG. 2. Transient expression assays of deletion mutants of the SFV-1 LTR. Lines represent SFV-1 LTR regions remaining after deletion. Shaded areas in the U3 domain encompass the targets for transactivation. Cells were cotransfected with the LTR plasmids and pORF-1 (pSVtaf) or control plasmid pSP65. CAT assays were performed on cell extracts 48 h later as described in Materials and Methods. The values shown are from reactions measuring the conversion of [3 H]acetyl coenzyme to [3 H]acetyl chloramphenicol. Generally, less than 10% variation in replicate samples was observed. -TAF, cotransfection of the reporter plasmid (pSFV-1 LTR/CAT) with pSP65; this value represents basal promoter activity. +TAF, cotransfection of the reporter plasmid (pSFV-1 LTR/CAT) and the transactivator plasmid (pORF-1). Restriction enzyme sites are as follows: Xh, *Xho*I; Hi, *Hind*III; Sp, *Spe*I; Nd, *Nde*I; Ss, *Ssp*I; RI, *Eco*RI; and Sa, *Sac*I. wt, wild type.

(HTLV-I), human immunodeficiency virus type 1 (HIV-1), and simian immunodeficiency virus (SIV). *taf* had no effect on the HTLV-I LTR, whereas *taf* transactivates CAT expression directed by the SIV LTR and the HIV-1 LTR 11-fold and 12-fold, respectively (Table 1). Tat of SIV and HIV-1 and Tax of HTLV-I did not transactivate gene expression directed by the LTR of SFV-1 (Table 1).

Negative regulatory effect of the R-U5 sequences. Additional deletion mutants were constructed in the R-U5 domain of the LTR to determine whether other *cis*-acting regulatory regions are present in the SFV-1 LTR. Deletion of the R-U5 region of the LTR shows basal-level CAT activity similar to that found in the wild-type LTR. The LTR devoid of the R-U5 sequences directed CAT expression to a level 218-fold higher in cells cotransfected with the plasmid expressing *taf* (Fig. 4). This value is at least 11-fold more than the level of transactivation observed with the wild-type LTR. Thus, the R-U5 of SFV-1 has a negative regulatory function. Deletion of the LTR from the 5' end up to position -270 and the R-U5 region showed transactivation levels greater than that of the wild-type LTR (Fig. 4).

To determine whether the R-U5 region of SFV-1 LTR has an inhibitory role on a heterologous promoter, this region was cloned in both orientations downstream from the SV40 promoter in an expression vector. Both orientations of R-U5 lowered the levels of CAT by at least 10-fold with two different cell lines (COS-7 and CV-1) (Fig. 5). A control DNA sequence about the size of the R-U5 region showed no inhibition of CAT expression directed by the SV40 promoter; thus, the negative effect of R-U5 is not due to the increased distance of the CAT gene from the promoter.

DISCUSSION

A previous report revealed that the *taf* gene of SFV-1 encodes a transcriptional transactivator which augments gene expression directed by the viral LTR (23, 24). In this report, we have mapped the target for Taf to two regions in the U3 domain of the LTR, between positions -1196 and -880 and between positions -403 and -125; transactivation is mediated by the second region in both orientations. Furthermore, we have shown that the R-U5 domain of the SFV-1 LTR down-regulates transactivation. Studies with heterologous LTRs revealed that *taf* has no effect on the HFV and HTLV-I LTRs. However, the *taf* gene of SFV-1 transactivates gene expression directed by the SIV and HIV-1 LTRs.

Lentiviruses (e.g., HIV-1, HIV-2, SIV, visna maedi virus, and equine infectious anemia virus) and certain oncoviruses (e.g., HTLV-I, HTLV-II, and bovine leukemia virus) also encode transactivators which require target elements in the LTR (9, 27, 33, 39). Transactivation by the HIV *tat* gene product regulates accumulation of viral transcripts through a target element in the LTR located immediately downstream from the cap site; efficient transactivation depends on the specific sequence, secondary structure, location, and orientation of the target element (4, 5, 16, 33, 39). In HTLV-I, a transactivator gene that encodes a protein, designated p40^{tax}, regulates viral replication at the transcriptional level through three 21-bp direct repeats in the LTR (8, 31, 40). The fact that SFV-1 has no significant homology with lentivirus and oncovirus LTRs and with transactivators suggests that the mechanism of the transcriptional transactivator is different from that of other retroviruses.

FIG. 3. Transient expression assays of hybrid promoters in COS-7 and L929 cell lines. Numbers represent the regions of the LTRs cloned upstream from the HSV tk promoter (TK-prm). + and -, presence or absence, respectively, of Taf.

Several experiments have implicated the involvement of host cell DNA-binding proteins in transactivation in the HIV and HTLV systems (1, 6, 8, 29, 30, 38). The precise role of host proteins in transactivations by *tat*, *tax*, and *taf* remains to be determined. Inspection of the transactivator target regions of SFV-1 revealed an AP-1 motif (TGACTCA) at position -225. In visna maedi virus, the AP-1 binding sites in the U3 domain of the LTR are critical for transactivation by visna maedi virus Tat (12). The AP-1 motif(s) mediates transcription induction by phorbol esters (17). Interestingly, studies with Jurkat cells of cell activation by both protein

kinase C and the cyclic AMP-dependent protein kinase pathways revealed no activation of SFV-1 LTR (our unpublished results). In addition, deletion from the 5' end to position -270 substantially reduced transactivation, compared with deletion from the 5' end to position -403; accordingly, the role of the putative AP-1 site, if any, is minor. Furthermore, the lack of known *cis*-acting regulatory sequences, other than the putative AP-1 binding sequence at -225, in the target regions implies a novel transactivation mechanism. Since both orientations of the major *cis*-acting region are active, one possible mechanism of transactivation

TABLE 1. Transient expression assays with Taf and heterologous LTR plasmids

Cotransfecting plasmid	CAT activity (relative activity) ^a				
	-Taf	+Taf	+Tat (HIV)	+Tat (SIV)	+Tax
pSFV-1 LTR/CAT	1,800	24,000 (13)	1,800 (1.0)	1,600 (0.9)	1,900 (1.1)
pHFV 5'LTR/CAT	1,700	2,700 (1.6)			
pHIV LTR/CAT	1,300	22,000 (12)	101,000 (77)		
pSIV LTR/CAT	1,100	13,000 (11)		111,000 (100)	
pHTLV-I LTR/CAT	1,300	1,200 (0.9)			18,000 (14)

^a Cells were cotransfected with the indicated LTR plasmids and pORF-1 (pSVtaf); CAT assays were performed 48 h later as described in Materials and Methods. Conversion of [³H]acetyl coenzyme A to [³H]acetylated chloramphenicol is shown as counts per minute. Less than 10% variation in replicate samples was observed. The relative activity was obtained by comparing the levels of CAT activity of each transfection with basal levels observed with the control pSP65. - and +, in the absence and presence, respectively, of the indicated protein.

FIG. 4. CAT expression assays of R-U5 deletion mutants in COS-7 in the presence (+) or absence (-) of Taf. Lines represent SFV-1 LTR regions remaining after deletion. Restriction enzyme sites are abbreviated as in the legend to Fig. 2.

in SFV-1 may be similar to that of transcriptional enhancer elements.

Studies of Taf activity on heterologous LTRs may shed light on the mechanism of transactivation. The *taf* genes of both SFV-1 and HFV activate the gene expression-directed HIV LTR in the COS-7 cell line. However, Taf of SFV-1 has no effect on the HFV LTR; also, HFV Taf does not activate the SFV-1 LTR (35). The lack of cross-transactivation is not surprising, because of a high degree of sequence divergence between the U3 domains of the LTRs; the *taf* genes also show a very low level of homology. The mechanism by which Taf transactivates SIV and HIV LTRs remains to be determined. Taf acts through a target in the U3 domain of the

HIV LTR, whereas the target for Tat is in the R-U5 portion of the HIV LTR (21). The observation that Taf transactivates gene expression directed by the primate lentiviruses implies that foamy viruses may play a role as a cofactor in progression of disease caused by the immunodeficiency viruses (26).

Lentiviruses and oncoviruses encode posttranscriptional transactivators, *rev* and *rex*, respectively, that act on *cis*-acting viral sequences, designated *rev* or *rex* response elements (RREs) (11). Both *rev* and *rex* regulate splicing and/or transport of mRNA for viral structural polypeptides; RREs for *rev* and *rex* are RNA stem-loop structures (11). In HTLV-II, the RRE is located within the R-U5 region of the

FIG. 5. Effects of the R-U5 domain of the SFV-1 LTR on the SV40 early promoter (SV40-prm). CAT assays were performed after transfection of COS-7 and CV-1 cells with plasmid constructs containing hybrids of the SV40 promoter and the R-U5 region of the SFV-1 LTR in the presence or absence of Taf.

FIG. 6. Computer analysis of the predicted R-U5 region of the SFV-1 LTR. The structure was determined by using the Genetics Computer Group Fold Program (University of Wisconsin, Madison, Wis.). Position 1 represents the cap site.

5' LTR; removal of the RRE results in significant increases of LTR-linked gene expression in the absence of Rex (10). The observation that the R-U5 region of the SFV-1 LTR has an inhibitory effect in both orientations suggests that the mechanism of down-regulation may involve secondary-structure features of viral RNA. In fact, computer analysis of the R-U5 region revealed a secondary structure with a free-energy level of -74 kcal (ca. $-310,000$ J) (Fig. 6). In transient expression assays, plasmids expressing ORF-2 or *taf*/ORF-2 or a cDNA from SFV-1-infected cells that is equivalent to *bet* of HFV (25) failed to relieve the inhibitory action of the R-U5 region on transactivation (our unpublished results). The significance and mechanism of down-regulation of transactivation by R-U5 in SFV-1 replication remain to be determined. Alternatively, the polyadenylation signal or some other *cis*-acting element in the 5' LTR may be involved in premature transcription termination, possibly via the secondary structure of R-U5. Mutational analysis is required to determine whether the secondary structure and/or the polyadenylation signal plays a role in negative regulation of gene expression directed by the SFV-1 LTR.

The investigations described in this report provide a basis for understanding the mechanism of transactivation of SFV-1. Comparisons of regulation of primate foamy virus with that of other retroviruses may have significance for understanding mechanisms by which pathogenic retroviruses establish latency and cause disease. Observations made in the course of SFV studies will also enhance the understanding of mechanisms controlling expression of cellular genes and may uncover new (cellular) transcription factors.

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