

Functional Domains of the Simian Foamy Virus Type 1 Transcriptional Transactivator (Taf)

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The genome of simian foamy virus type 1 encodes a transcriptional transactivator (Taf) that dramatically elevates gene expression directed by the viral long terminal repeat. In this report, we describe the functional domains of simian foamy virus type 1 Taf. Several *taf* mutants and fusion proteins of Taf and the DNA-binding domain of the *Saccharomyces cerevisiae* transcriptional transactivator GAL4 were used in this study. Taf contains two potent activation domains. One of the activation domains is located at the amino terminus (positions 1 to 48, with position 1 representing the initiator amino acid methionine) and contains several acidic amino acids. The second activation domain was mapped to a region at the carboxy terminus (positions 277 to 300). These two domains activate gene expression directed by the viral long terminal repeat independently of each other. No significant amino acid sequence homology between the activation domains is noted. Thus, Taf belongs in part to the family of acidic transcriptional transactivators. The activation domain at the carboxy terminus is conserved among foamy virus transactivators but is not related to other known transcriptional activators. Therefore, the mechanism of gene activation by the carboxy terminus of Taf may be novel. In addition, a potential binding domain rich in basic amino acids (positions 179 to 222) and a highly conserved sequence among foamy virus transactivators (positions 93 to 109) were found to be critical for Taf activity.

Foamy viruses are members of a distinct subfamily of retroviruses that possess a complex genome organization as well as complex means of regulating gene expression (21). Although these viruses induce extensive cytopathology in cell culture, the pathogenic potential of foamy viruses has not been clearly defined. Recently, a portion of the genome of a human foamy virus (HFV) was shown to cause neurodegenerative disease in transgenic mice (1). These findings suggest a biological role for foamy virus, perhaps with certain neurological diseases. Simian foamy virus type 1 (SFV-1), isolated from a rhesus macaque, and type 3 (SFV-3), isolated from an African green monkey, have been molecularly cloned and sequenced (21, 23-25, 30). In addition to *gag*, *pol*, and *env*, the genomes of SFV-1 and SFV-3 contain two open reading frames (ORFs) between *env* and the long terminal repeat (LTR). In contrast, the genome of HFV contains three additional ORFs (*bel-1*, *bel-2*, and *bel-3*) in the corresponding region (4, 20). Complex splicing events are required to generate messages that encode the ORF regions of foamy viruses (25, 26). The first ORF encodes a transcriptional transactivator designated Taf (transcriptional transactivator of foamy virus) that activates gene expression directed by the homologous LTR (13, 25, 31, 32, 38). *cis*-acting elements for transactivation have been mapped to several distinct regions of the U3 domain of the LTR (13, 15, 22, 31, 32, 38). Comparative studies show that the U3 domain of the LTRs and the predicted amino acid sequences of the *taf* genes of the two SFVs (SFV-1 and SFV-3) have diverged greatly from that of HFV, with 38% and 34% identities, respectively (21, 30). Consequently, Taf of either SFV-1 or HFV does not cross-transactivate gene expression directed by the heterologous LTR (22). The HFV Taf protein has been shown to be critical for viral replication (17). The

functions of the potential genes encoded by other ORFs of foamy viruses remain to be determined.

The LTRs and *taf* genes of HFV and SFVs lack significant homology with lentivirus and oncovirus LTRs and transactivators. Thus, the mechanisms of transcriptional transactivation in the foamy virus system may be fundamentally different. To gain insights into the mechanisms of transcriptional transactivation of foamy viruses, several mutant SFV-1 *taf* genes were constructed and tested in transient expression assays for their ability to transactivate SFV-1 LTR-directed gene expression. Furthermore, functional domains were defined by domain swapping with the *Saccharomyces cerevisiae* transcriptional transactivator GAL4. We have identified two distinct activating domains, one located at the amino terminus and the second at the carboxy terminus, that function independently of each other. The activation domain at the amino terminus contains several negatively charged amino acids similar to those of the acidic transcriptional transactivators. A region that contains several clusters of positively charged amino acids (potential binding domain) and a conserved region among the transactivators of foamy viruses are necessary and required for Taf protein activity.

MATERIALS AND METHODS

Cell culture. L-929 (murine fibroblast) and COS-7 (African green monkey fibroblast) cell lines were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmid constructions. Plasmids were generated by standard techniques (35). Plasmids with the U3 domain placed upstream of the chloramphenicol acetyltransferase (CAT) gene (pSFV-1 LTR/CAT41) and with the SFV-1 *taf* gene cloned downstream from the simian virus 40 early promoter (pSVORF-1 or pSVTAFwt) have been described previously

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(22). Plasmids pSG424, pGAL4-VP16, pGAL4-IE, and pGAL4_s/E1bCAT, were kindly provided by Michael R. Green of Harvard University, Cambridge, Mass. Plasmid pSG424 contains the DNA-binding domain of the yeast transcriptional transactivator GAL4 (34). pGAL4-VP16 and pGAL4-IE contain activation domains of the herpes simplex virus protein VP16 and pseudorabies virus immediate-early protein fused to the DNA-binding domain of GAL4, respectively (19, 33). The reporter plasmid pGAL4_s/E1bCAT contains the adenovirus E1b TATA box and five tandem 17-mer repeats of the GAL4 targets inserted immediately upstream of the CAT gene (16). Hybrids of the DNA-binding domain of the yeast GAL4 transcription factor and the activation domains of Taf were constructed by cloning the DNA sequences that contain the appropriate regions in frame with GAL4 into plasmid pSG424. pGAL4-TAF/NH2, pGAL4-TAF/MID, and pGAL4-TAF/CO contain the DNA-binding domain of GAL4 fused to the first 88 amino acids, amino acid positions 51 to 270, and the last 68 amino acids of Taf, respectively.

Deletion mutations of the *taf* gene used for transient expression assays were generated by cloning the appropriate restriction fragments in an expression vector that contains the simian virus 40 early promoter. pSVTAFΔ92-108 contains the *taf* sequence with amino acids 92 to 108 deleted. pSVTAFΔ27-57 was constructed by removing the *taf* sequence with amino acids 27 to 57. Successive carboxyl-terminus deletion mutations of *taf* were generated by taking advantage of the appropriate restriction enzyme sites. pSVTAF/PvuII, pSVTAF/BamHI, pSVTAF/NcoI, and pSVTAF/SspI contain amino acid sequences from 1 to 302, 270, 261, and 132, respectively. Site-specific mutations of *taf* were constructed by replacing appropriate regions with synthetic oligonucleotides or by introducing mutations by polymerase chain reaction with pairs of oligonucleotides containing altered sequences (10). The mutated *taf* sequences were placed downstream of the simian virus 40 early promoter for expression; plasmids with the altered amino acids are shown in Fig. 1 and 2. Combinations of site-specific and deletion mutations were created by ligating the appropriate DNA fragments (Fig. 1). Mutations were confirmed by sequencing double-stranded DNA templates using the dideoxy-chain termination method (36) with [α -³⁵S]dATP and Sequenase polymerase (U.S. Biochemicals, Cleveland, Ohio).

DNA transfection. Transfections were performed by the DEAE-dextran method on COS-7 and L-929 cells as described previously (2). For each experiment, duplicate cell cultures were transfected with 2 μ g of CAT expression plasmid (pSFV-1LTR/CAT41 or pGAL4_s/E1bCAT) and 3 μ g of plasmid DNA of the pSVTAFwt or Taf mutant or GAL4 fusions or carrier DNA (pSP65). CAT assays, performed on whole-cell extracts prepared 48 h after transfection, measured the conversion of [³H]acetyl coenzyme A to ³H-acetylated chloramphenicol (2).

RESULTS

Acidic region. The amino termini of the predicted transactivator genes of foamy viruses have a region with a high content of acidic amino acids (21). Of the first 48 amino acids encoded by the SFV-1 *taf* gene, 15 are glutamates and aspartates. This feature of foamy viruses' transactivators appears to be reminiscent of eukaryotic acidic transcriptional activators (29). To determine whether this acidic region is the activation domain of SFV-1 Taf, a chimeric

TABLE 1. Transient expression assays with target pGAL4_s/E1bCAT and activator plasmids

Activator plasmid	CAT activity (cpm) [relative activity] ^a	
	COS-7 cells	L-929 cells
pSG424	2,100 [1]	2,500 [1]
pGAL4-VP16	12,000 [6]	141,000 [56]
pGAL4-IE	9,600 [5]	66,000 [26]
pGAL4-TAF/NH2	85,000 [40]	191,000 [76]
pGAL4-TAF/CO	63,000 [30]	67,000 [26]
pGAL4-TAF/MID	2,800 [1]	1,900 [1]

^a Cells were cotransfected with the indicated effector plasmids and pGAL4_s/E1bCAT; 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. Relative activity is obtained by comparing the level of CAT activity of each transfection with the basal level observed with the control pSG424.

protein containing the DNA-binding domain of the yeast transcription factor GAL4 fused to the first 88 amino acids of Taf (pGAL4-TAF/NH2) was constructed. Transcriptional activation by this GAL4-Taf fusion protein was assayed after cotransfection with a CAT reporter plasmid whose promoter contained GAL4-binding sites upstream of a TATA box. For comparison, plasmid-encoding chimeras between the GAL4 DNA-binding domain and the potent activation domain of the human herpes simplex virus VP16 protein (GAL4-VP16) or the immediate-early protein of pseudorabies virus (GAL4-IE) were cotransfected with the reporter plasmid bearing the GAL4 target. Table 1 shows that the acidic region of Taf activates transcription by 40- and 76-fold in COS-7 and L-929 cells, respectively, when bound artificially to the promoter through the DNA-binding region of another activator. These levels of transactivation by GAL4-Taf fusion protein are comparable to the level of CAT activity in L-929 cells cotransfected with plasmid expressing the GAL4-VP16 or GAL4-IE fusion protein; the activities of GAL4-VP16 and GAL4-IE fusion proteins were significantly lower in COS-7 cells (Table 1). Therefore, the amino-terminal region rich in acidic amino acids, similar to the acidic transcriptional transactivators, is the activation domain of Taf.

We tested whether activation by Taf is related to the net negative charge by replacing, in various combinations, the acidic amino acids within the acidic region. The ability of each Taf derivative to activate SFV-1 LTR-directed CAT expression was measured. All site-specific mutants show a level of transactivation severalfold higher than that of the basal promoter activity (Fig. 1). Replacing less than five acidic amino acids (pSVTAF/A1, pSVTAF/A2, and pSVTAF/A11) had no effect on the activity of Taf. Although substituting 11 of the acidic amino acids (pSVTAF/A111) reduced CAT expression by at least fourfold, Taf still maintained potent activation activity. Furthermore, deletion of amino acids 27 to 57 (pSVTAFΔ27-57) did not affect the function of Taf. Substitution of the remaining acidic amino acids in the acidic region of this mutant (pSVTAF/A112) resulted in Taf activity with one-seventh of the wild-type activity. Replacement of increasing numbers of acidic residues, thus, led to progressive decreases in transcriptional activation as with other acidic transcriptional transactivators (5, 6). The observation that all the mutations tested resulted in transcriptional activation severalfold higher than the basal level suggests that there may be a second activation domain that works independently of the acidic region.

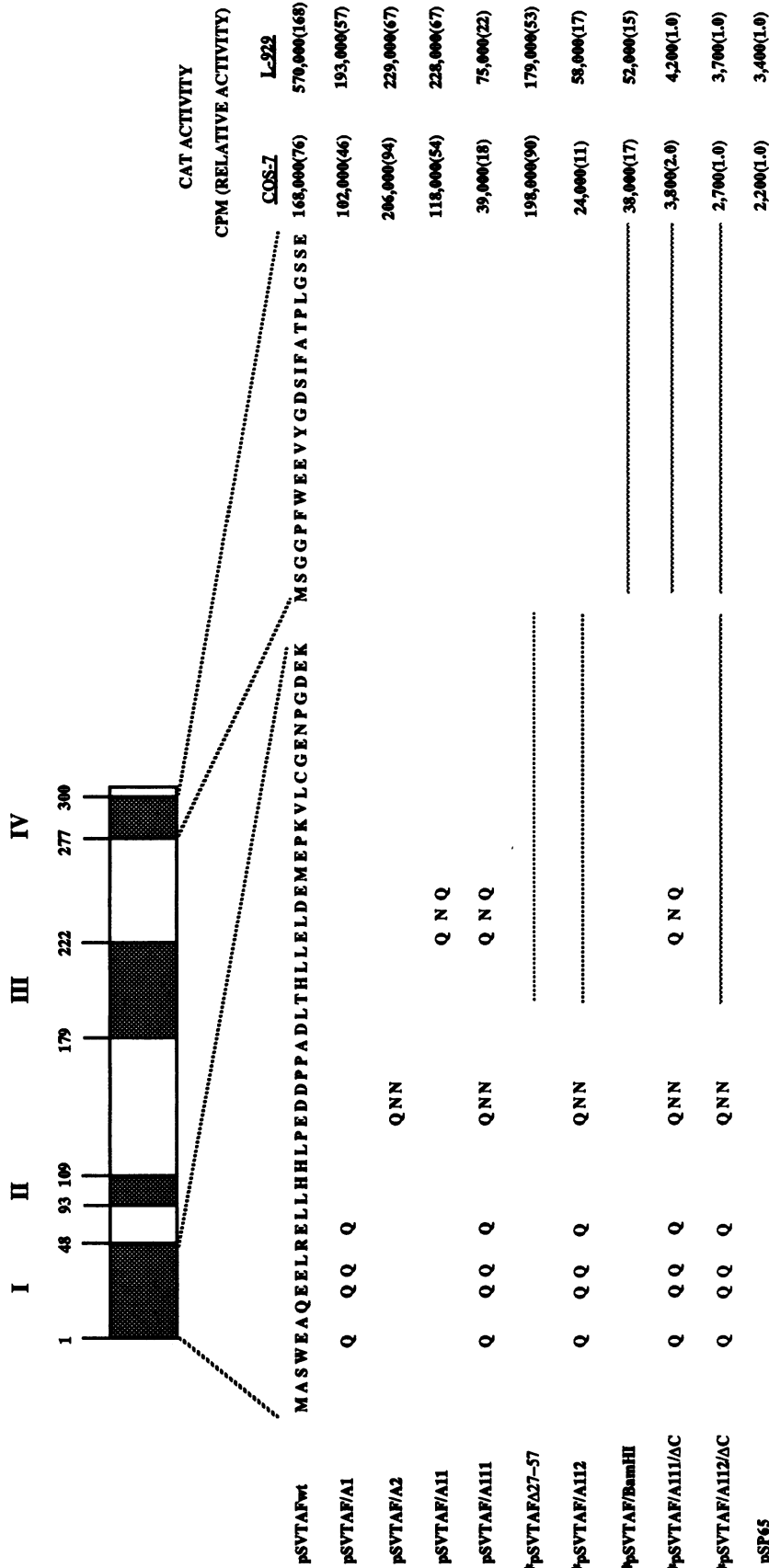


FIG. 1. Transient expression assays of site-specific and/or deletion mutants of SFV-1 *taf* in COS-7 and L-929 cells. The numbers over the map indicate the positions of amino acids of SFV-1 Taf in regions I, II, III, and IV. The sequences shown in one-letter amino acid code represent the amino-terminal acidic region and the carboxy-terminal conserved region of Taf. Substituted amino acids for each mutant are indicated below the wild-type amino acid sequence. Lines represent deleted regions of SFV-1 *taf*. Cells were transfected with the indicated plasmids and pSFV-1/CAT41. CAT assays were performed 48 h later, as described in Materials and Methods. The values shown, counts per minute (cpm), are from reactions measuring the conversion of ³H-acetyl coenzyme A to ³H-acetylated chloramphenicol. Generally, less than 10% variation in replicate samples was observed. Cotransfection of the reporter plasmid pSFV-1LTR/CAT41 with pSP65 represents basal promoter activity. The asterisks indicate that for these plasmids, the lines representing the deleted region are not drawn to scale. The line at the amino-terminal region represents deletion of Taf at amino acid positions 27 to 57, and the line at the carboxy terminus indicates deletion of Taf from the carboxy terminus to amino acid position 270.

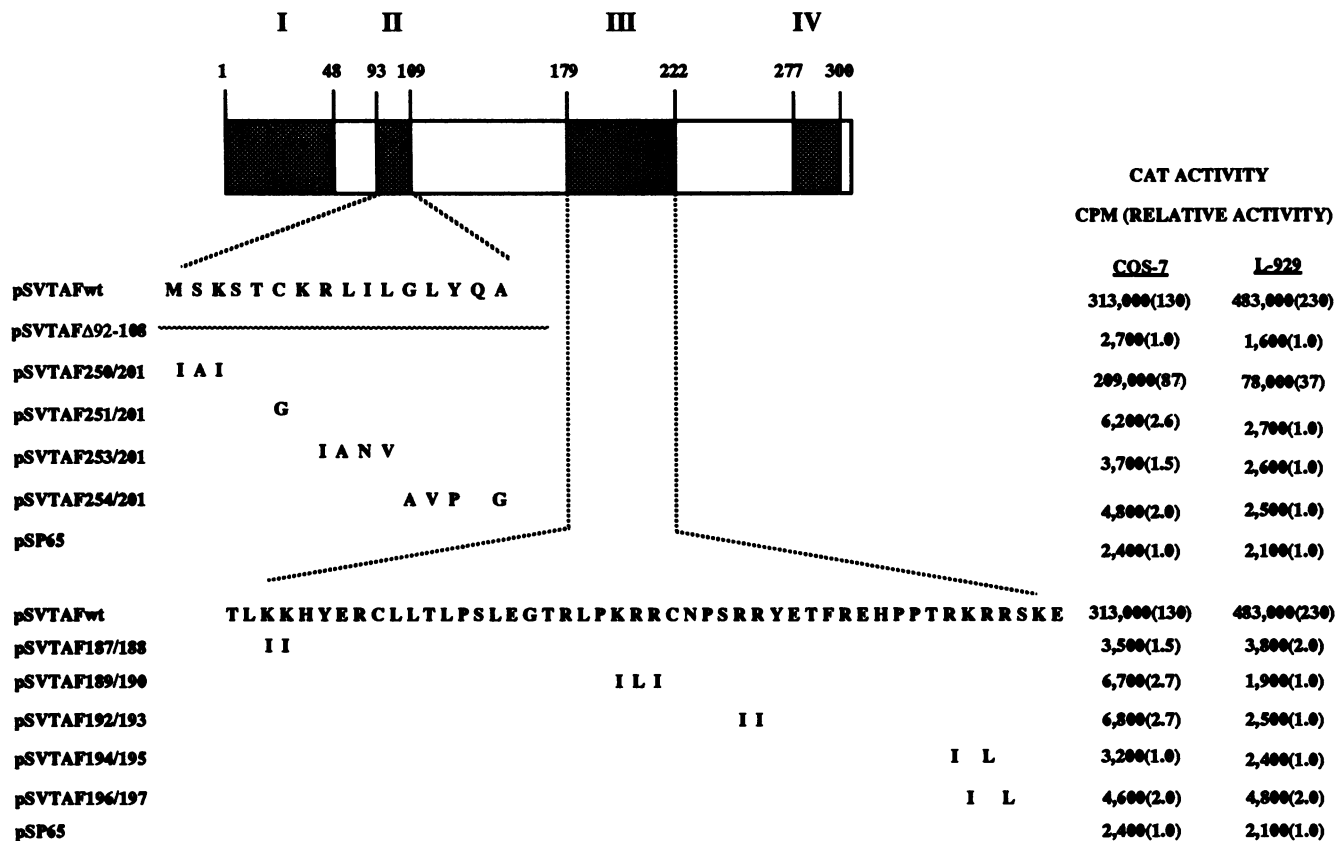


FIG. 2. Mutational analysis of the basic region (region III) and a highly conserved region II of *taf* in COS-7 and L-929 cells. Substituted amino acids for each mutant are shown below the wild-type amino acid sequence. Lines represent deleted regions of SFV-1 *taf*.

Conserved regions. The predicted Taf protein sequences of the SFVs and HFV show a high degree of sequence divergence; they show only 38% homologies (21, 30). There are two regions (regions II and IV [Fig. 1 and 2], however, which are highly conserved among the *taf* genes of foamy viruses; 12 of 17 amino acids in region II (positions 93 to 109) and 18 of 24 amino acids in region IV (positions 277 to 300) are identical. These highly homologous clusters in genes with low sequence homologies overall indicate that the conserved region may define critical functional domains of Taf; one of these regions may be the second activation domain that functions independently of the acidic region. To test this hypothesis, we have introduced deletions in regions II and IV and tested the mutations for the ability to transactivate SFV-1 LTR-directed CAT expression. Taf with a deletion at the carboxy terminus that includes region IV (pSFTAF/BamHI) showed transactivation activity 17 and 15 times higher than the basal level in COS-7 and L-929 cells, respectively (Fig. 1). These transactivation levels are substantially lower than that of the wild-type Taf (76-fold in COS-7 and 168-fold in L-929 cells compared with basal levels). Only mutations in both regions I (acidic region) and IV (pSVTAF/A111/ΔC and pSVTAF/A112/ΔC) resulted in CAT expression that is equivalent to the basal promoter activity; this result implicates these two regions as the activating domains of Taf that can independently augment gene expression directed by the LTR (Fig. 1). Furthermore, we cloned the carboxy terminus of Taf in frame with the DNA-binding domain of GAL4 (pGAL4-TAF/CO) and

tested this construct for the ability to transactivate gene expression directed by a promoter element containing the GAL4 target. The fusion protein augmented CAT expression by 30-fold in COS-7 cells and 26-fold in L-929 cells, validating the notion that the conserved region at the carboxy terminus is a second activator domain of Taf (Table 1). A fusion protein that contains the DNA-binding domain of GAL4 and the Taf amino acid sequence positions 51 to 270 (pGAL4-TAF/MID) did not transactivate CAT expression directed by a promoter element containing the GAL4 target, indicating that there are no other activation domains.

Removal of amino acids from positions 93 to 109 (pSVTAFΔ93-109) completely abolished Taf activity (Fig. 2, region II). To define the exact sequences required for activity in the conserved region II, *taf* mutations were generated by oligonucleotide-directed mutagenesis (Fig. 2). Substitution of amino acids MSK (amino acid positions 93 to 95) to IAI (pSVTAF250/201) had no effect on Taf activity, whereas single substitution of C (position 98) to G (pSVTAF251/201) completely abolished the ability of Taf to transactivate gene expression directed by SFV-1 LTR. Replacement of amino acids RLIL (positions 100 to 103) with IANV (pSVTAF253/201) and amino acids GLYQA (positions 105 to 109) with AVPQG (pSVTAF254/201) also nullified Taf activity. These results indicate that within conserved region II, the amino acids between positions 96 to 109 are critical for Taf function.

Basic regions. The predicted amino acid sequence of *taf* shows clusters of basic amino acids (region III) that may be

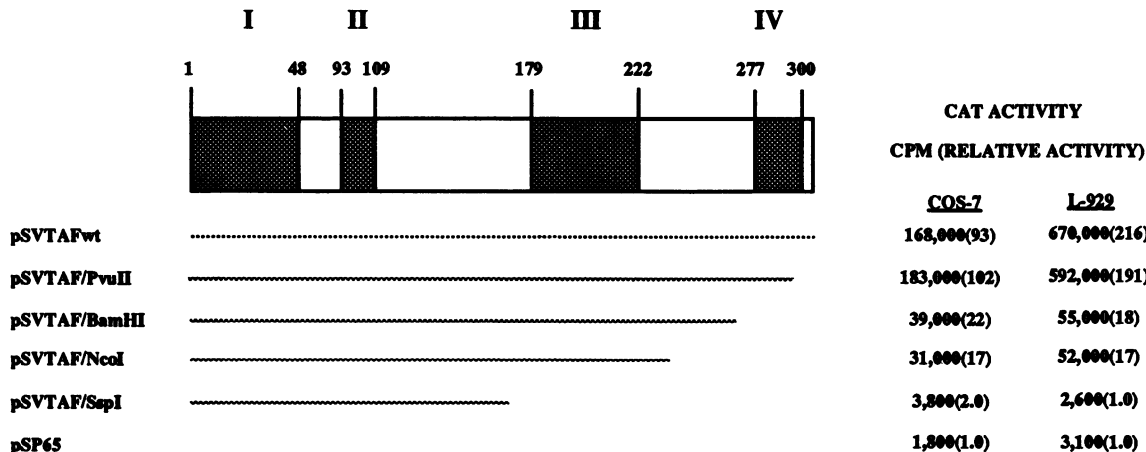


FIG. 3. Effects of successive deletion mutations at the carboxy terminus of Taf on transactivation. Lines represent SFV-1 LTR regions remaining after deletion. CAT assays were performed after transfection of COS-7 and L-929 cells with plasmid constructs containing the mutant *taf* genes and pSFV-1LTR/CAT41.

essential for nuclear localization and DNA binding. To determine whether these basic amino acids are important determinants of SFV-1 Taf activity, successive deletions were introduced in the carboxy terminus of Taf and tested for the ability to transactivate gene expression directed by the LTR. The last six amino acids at the carboxy terminus (positions 303 to 308) of Taf (pSVTAF/PvuII) are dispensable (Fig. 3). Removal of sequences from the carboxy terminus to amino acid 261 (pSVTAF/NcoI) reduced transactivation to 17-fold above the basal level in both COS-7 and L-929 cells from 93- and 216-fold, respectively. A *taf* mutation with a deletion that included the basic region (pSVTAF/SspI), lacked the ability to transactivate gene expression directed by the LTR. These results were further validated by site-specific mutations of the basic amino acids. All substitutions of amino acids KK (positions 179 and 180) to II (pSVTAF187/188), KRR (positions 200 to 202) to ILI (pSVTAF189/190), RR (positions 207 and 208) to II (pSVTAF192/193), RR (positions 119 and 121) to IL (pSVTAF194/195), and KR (positions 120 and 122) to IL (pSVTAF196/197) reduced Taf activity to the basal level, suggesting that the basic region is critical for Taf function (Fig. 2). A corresponding region of the human Taf protein (Bel-1) was also shown to be essential for transactivation (9, 39).

DISCUSSION

This report investigates the functional domains of the transcriptional transactivator (Taf) protein of SFV-1 that activates gene expression directed by the viral LTR. The amino-terminal acidic region and a highly conserved sequence, among the *taf* genes of foamy viruses, located at the carboxy terminus were identified to be the activation domains of the Taf protein (regions I and IV) (Fig. 4). These two domains activate gene expression directed by the viral LTR independently of each other. A second highly conserved region (region II) located downstream from the acidic region is a critical determinant of Taf activity. The region in the middle of Taf (region III) that contains several discrete stretches of positively charged amino acids is required and necessary for transactivation.

Several of the activation domains of the acidic transcrip-

tional transactivators do not show any strong amino acid sequence homology to each other, except for the fact that they all contain a number of negatively charged amino acids (28). One-third of the first 48 amino acids of the Taf protein of SFV-1 as well as SFV-3 and HFV are acidic amino acids. Although a high proportion of negatively charged amino acids are present in the amino termini of the Taf proteins of foamy viruses, the acidic regions are highly divergent (Fig. 5). Activation domains of several transcriptional regulators have been mapped by fusing segments of those proteins to the DNA-binding domain from heterologous protein (8, 16, 19, 33). Similarly, the acidic region of SFV-1 Taf protein is a potent activator when fused to the DNA-binding domain of the yeast transcriptional transactivator GAL4. Therefore, SFV-1 Taf in part falls into the class of acidic transcriptional transactivators. Chimeric protein of the GAL4 and the acidic region of the human foamy virus *taf* gene, however, does not transactivate gene expression directed by a promoter element containing the GAL4 target sequence (39). The negatively charged residues in acidic activators are shown to be displayed as an amphipathic helix in which the acidic amino acids are positioned on one side of the helix, presumably to allow them to come into contact with another protein (7, 18). Since the *taf* genes of SFV-1 and HFV have significantly different acidic domains, the discrepancies of the GAL4 fusion experiments may reflect structural differences, implying that Taf of HFV may not be an acidic activator.

Analyses of transcriptional activation for a number of activators have revealed that certain proteins contain more than one activation domain. The human glucocorticoid re-

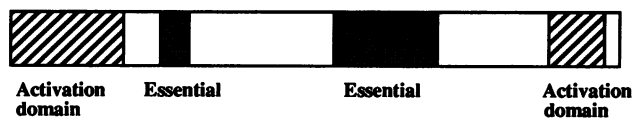


FIG. 4. Schematic representation of the different functional domains of the SFV-1 transactivator Taf. The hatched boxes indicate the activation domains of Taf located at the amino and carboxy termini. The two essential regions are the highly conserved region (region II [Fig. 2]) among foamy transactivators and the potential binding domain (region III [Fig. 2]) of SFV-1 Taf.

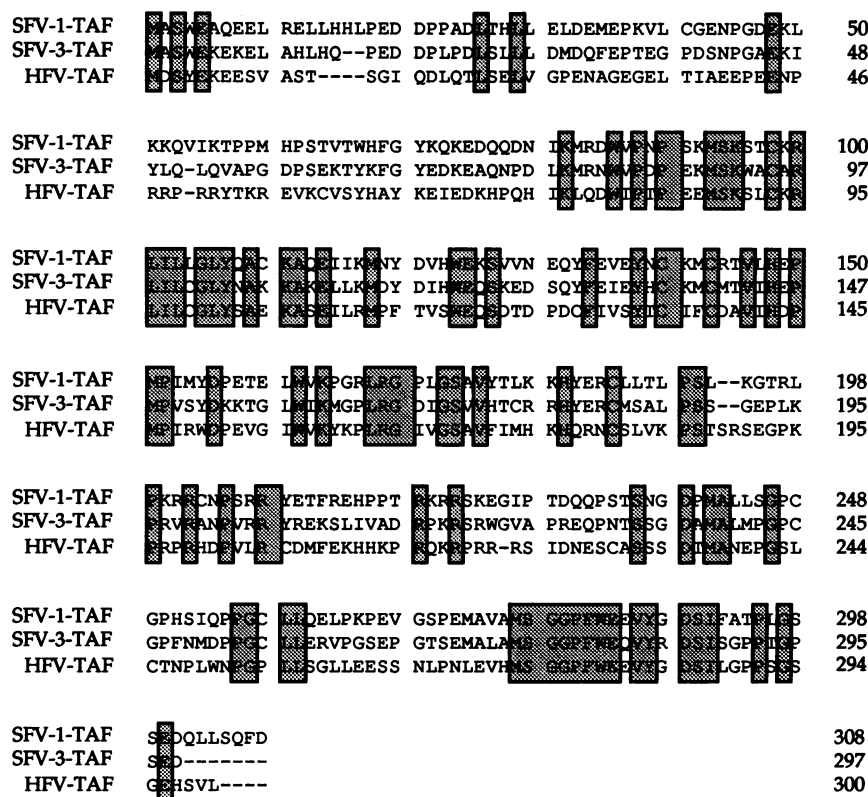


FIG. 5. Predicted amino acid sequences of the Taf proteins of SFV-1, SFV-3, and HFV. The one-letter amino acid code is used, and dashes represent gaps introduced to align sequences. Amino acids identical to those in the three proteins are shaded and boxed. Alignment was determined by Sequence Analysis Software Package, version 7.2, provided by the Genetics Computer Group (University of Wisconsin).

ceptor has two acidic activation domains (8, 11). Activation domains in certain transcriptional transactivators are not confined to a single factor. Two unrelated potent activation domains, one at the amino terminus rich in glutamine and the other one at the carboxy terminus rich in proline, are identified for the transcriptional activator of Oct-2 (37). Similarly, SFV-1 Taf has a second activation domain at the carboxy terminus that is structurally distinct from the amino-terminal acidic region (Fig. 4). The carboxy terminus of HFV Taf was also shown to be the activation domain and is highly conserved among the three *taf* genes of foamy viruses sequenced so far (9, 39). The mechanism of gene activation by the carboxy terminus of Taf remains to be determined; it may be a novel mechanism that identifies a new class of transcriptional gene activators.

The importance of specific protein domains with respect to Taf function can be deduced both from Taf amino acid sequence comparisons with other transcription factors and from experimental manipulation of the Taf protein. Studies on a variety of transcription factors have revealed distinct modular structures that mediate DNA binding and gene activation (28). In general, DNA-binding domains are characterized by sequences that are rich in basic amino acids. Mutational analysis of the basic amino acids of region III (essential region [Fig. 4]) of SFV-1 Taf revealed that this region is functionally critical. A corresponding region in the *taf* gene of HFV has also been shown to be required for transactivation (9, 39). Although the basic domain of Taf is important for activity, it is not clear whether Taf binds to DNA directly or indirectly through cellular proteins. Fur-

thermore, the role of region II (essential region [Fig. 4]) for transactivation is not clear. It appears this highly conserved region is absolutely required for Taf activity; whether region II is part of the binding domain of Taf remains to be determined. The fact that Taf can activate gene expression directed by heterologous human and simian immunodeficiency virus LTRs that have no sequence similarities to foamy virus LTRs indicates the potential for this protein to interact with different cellular factors for binding to DNA (12, 14, 22). Binding studies using Taf alone or in combination with cellular extracts will be useful in determining whether host proteins are required for its interaction with DNA.

Nuclear targeting sequences are essential for the transport of proteins into the nucleus. The targeting sequences are usually short stretches with many positively charged amino acids (3, 27). Although several basic amino acids are present in the nuclear localization signals, protein sequences that bear low degrees of sequence homology have been reported to manifest identical biological functions (3, 27). The sequence KHHKPRQKRPRRR (positions 211 to 223 [Fig. 5]) in the basic region of HFV Taf has been identified as the nuclear localization signal (9, 39). Mutation analysis of the basic amino acids in the corresponding region (positions 213 to 222 [Fig. 5]) of SFV-1 revealed that the region is critical for activity, implicating the sequence REHPPTRKRRSK as the potential counterpart of the nuclear localization signal.

Studies presented in this report provide information about the locations and properties of the transactivation domains and insights into the functional importance of basic amino acids in the binding domain of Taf. The only known *cis*-

acting elements in the LTR regions where the targets for Taf have been mapped are AP-1 sites that have been shown to have little, if any, effect on transactivation (15, 22). Furthermore, *taf* does not show any significant degrees of homology with other transcriptional transactivators. Therefore, the mechanisms of transactivation in foamy viruses may be novel, and future studies may lead to the discovery of new cellular transcriptional factors that are involved in regulation of foamy virus gene expression.

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