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The Carboxy-Terminal Transcription Enhancement Region of the Human Spumaretrovirus Transactivator Contains Discrete Determinants of the Activator Function

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The *bel1* gene of human spumaretrovirus (HSRV) encodes a 300-amino-acid nuclear protein termed Bel1 that is a potent activator of transcription from the cognate long terminal repeat (LTR). Bel1 can also efficiently activate the human immunodeficiency virus type 1 (HIV-1) LTR. We have previously shown that the amino-terminal 227-residue region (minimal activator region) of Bel1 can activate the HSRV LTR at low levels and that two distinct domains within the carboxy-terminal 73 residues, from residues 255 to 266 and 272 to 300, that bear little sequence homology can independently enhance the activity of the minimal activator domain (L. K. Venkatesh, C. Yang, P. A. Theodorakis, and G. Chinnadurai, J. Virol. 67:161–169, 1993). We now report on the further characterization of these two transcriptional enhancement regions. Mutational analysis of the region comprising residues 255 to 266 indicates that a cluster of leucine residues is critical to the function of this region. Also, residues 273 to 287, which are identical in sequence to a 15-amino-acid segment near the carboxy terminus of the simian foamy virus transcriptional activator Taf, can independently enhance the activity of the minimal activator region. To delineate the region(s) of Bel1 that could function autonomously as an activator domain, we tested the activity of chimeric proteins comprising either wild-type or functionally defective forms of Bel1 fused to the DNA binding domain, Gal4(1-147), of the yeast transcriptional activator Gal4 on a synthetic promoter comprising Gal4 DNA binding sites linked to the adenovirus E1B TATA box (minimal promoter). Gal4-Bel1 was found to activate basal transcription from the E1B TATA box at least 35-fold, and the region responsible for this activation function was localized to the carboxy-terminal 73 amino acids. When the transcriptional enhancement regions were tested for autonomous activator function as Gal4(1-147) chimeras, residues 272 to 300, but not 255 to 266, were found to activate transcription efficiently when targeted to the E1B TATA motif and also to HSRV and HIV-1 LTRs. The highly conserved region between amino acids 273 and 287 alone was found to activate transcription efficiently when targeted to the HSRV LTR but not to the E1B TATA box or the HIV-1 LTR. Thus, our results demonstrate that the carboxy-terminal 29-amino-acid region (residues 272 to 300) contributes to Bel1 transactivation by functioning as an autonomous activator of TATA motif-directed transcription in a manner similar to that of other modular transcriptional activators. In contrast, the domain comprising residues 255 to 266 functions to augment transcription only in conjunction with the minimal activator region, which suggests that the two distinct carboxy-terminal activation-enhancing domains of HSRV Bel1 may function by different mechanisms.

Human spumaretrovirus (HSRV), also known as human foamy virus, is a complex retrovirus belonging to the spumavirus subfamily of retroviruses, which has been poorly characterized in terms of pathogenic potential and genetic regulatory mechanisms operational during viral multiplication (3, 17, 27). In contrast, the other complex human retrovirus representatives of the lentivirus and oncovirus subfamilies, human immunodeficiency viruses (HIVs) and human T-cell leukemia viruses, respectively, have been intensely scrutinized, primarily because they have been linked with various pathogenic states in humans. While a causative effect for HSRV infection in any diseased state remains to be unambiguously established, the demonstration, from genome sequence analysis and structural mapping of HSRV transcripts in infected human cells (4, 20), that HSRV exhibits a genetic complexity comparable to that of other complex human retroviruses of proven pathogenicity has prompted a reevaluation of its pathogenic potential. It has been shown that expression of the entire HSRV genome or of a subgenomic region can lead to neurodegenerative effects in transgenic mice (1), and the more recent demonstration of a statistically significant correlation between the occurrence of Graves' disease and the presence of HSRV-related infection markers (10) suggests that HSRV may indeed have the potential to induce human disease.

In addition to the virion structural proteins, the HSRV genome encodes a 300-amino-acid nuclear protein termed Bel1, the product of the *bel1* gene, that is a potent activator of transcription from the cognate long terminal repeat (LTR) (9, 15, 22, 25). The major site for Bel1 interaction in the HSRV LTR maps to the region between −136 and −62 relative to the transcription initiation site in the U3 region, although the precise sequence requirement remains undefined. We have also shown that the R-U5 region of the HSRV LTR can negatively regulate Bel1-mediated transactivation (25). Bel1 can also stimulate HIV-1 LTR expression (9, 25); maximal levels of activation, however, can be obtained only upon functional inactivation of binding sites in the core enhancer for the cellular transcription factor NF-κB (8, 11). The HIV-1 LTR region between −140 and −116 relative to the cap site has been identified as the Bel1-

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responsive element and contains a TGACATCGA motif between −124 and −116 that is almost identical in sequence to the HSRV LTR region between −134 and −126, which suggests that the two LTRs contain similar Bel1 target sequences.

We have recently demonstrated by site-directed mutagenesis that distinct regions of Bel1 are involved in subcellular localization and transcriptional activation of the HSRV and HIV-1 LTRs (26). The highly acidic 55-residue region at the amino terminus was dispensable for transactivation. Two distinct domains between residues 56 and 300 were found to cooperate to confer full activator function. The region comprising residues 56 to 227, inclusive of an arginine-rich nuclear localization signal between amino acids 211 and 222, was sufficient to minimally activate the HSRV but not the HIV-1 LTR. The carboxy-terminal 73 residues were found to contain two functionally redundant regions bearing little sequence homology between residues 255 and 266 and between residues 272 and 300 that could independently enhance the activity of the minimal activator domain on the HSRV LTR to near wild-type (wt) Bel1-induced levels. Of the two functionally redundant regions at the carboxy terminus, only that between residues 272 and 300 exhibited significant activation enhancement function with respect to HIV-1 LTR expression.

In this study, by tagging a series of amino acid segments from heterologous proteins as well as from the carboxy-terminal 73-amino-acid region of Bel1 to the minimal activator domain comprising residues 1 to 227, we identified residues and motifs that contribute specifically to the transcription enhancement function of residues 255 to 266 and 272 to 300. Also, by designing protein fusion strategies to target different segments of Bel1 upstream of diverse promoters, we demonstrate that the Bel1 region between residues 272 and 300 but not that between residues 255 and 266 serves as an autonomous activator of transcription directed by the TATA motif (minimal promoter). Thus, the activation-augmenting functions of these two carboxy-terminal Bel1 domains are likely to involve different mechanisms.

MATERIALS AND METHODS

Recombinant plasmids. Various reporter plasmids, based on the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene, were constructed as follows. −710/+4 HSRV LTR CAT, which expresses the CAT gene under the transcriptional control of the HSRV 3′ LTR sequences between −710 and +4 with respect to the transcription initiation site (25), and G5BCAT, which contains five DNA binding sites for the yeast transcriptional activator Gal4 inserted immediately upstream of the adenovirus E1B TATA box (minimal promoter [13]), have been previously described. Plasmid G5BCAT-SP was derived from G5BCAT by positioning three consensus binding sites for the cellular transcription factor Sp1 at the *Xba*I site 5′ to the E1B TATA box (7). G5 HSRV LTR CAT was constructed by cloning a *Xho*I (blunt-ended)-*Nco*I fragment carrying the Bel1-responsive −136/+4 region from the HSRV 3′ LTR (25), inclusive of the TATA motif, and part of the CAT coding sequences between the *Xba*I (blunt-ended) site downstream of the Gal4 DNA binding sites and *Nco*I site present in the CAT coding sequence in G5BCAT. G5 ΔκB HIV LTR CAT contains HIV-1 LTR sequences between −159 and +77 with functionally inactivated binding sites for the inducible transcription factor NF-κB (21) positioned between the Gal4 DNA binding sites and the CAT gene in G5BCAT (6).

The phenotypically wt *bel1s* background, which is identical in amino acid sequence to the genomic *bel1* open reading frame but contains a T→C transition at codon 89, thereby eliminating the production of a spliced mRNA species and hence the expression of a truncated *bel1*-related *bet* gene product from the genomic *bel1* transcription unit, has been described previously (26). *bel1s* gene expression in COS cells, under the transcriptional control of the powerful cytomegalovirus immediate-early promoter, results in the high-level expression of a Bel1-specific protein of ~36 kDa from full-length mRNA. All Bel1 proteins, both wt and mutant, examined in this study were investigated for expression and transactivation potential in the *bel1s* background. Mutants *bel1s*-227, -254, and -266, coding for the amino-terminal 227, 254, and 266 residues, respectively, of Bel1, and *bel1s*Δ228-271 and -Δ194-200, lacking the indicated amino acid regions, have been described in our initial study (26). *bel1s*-254/*tat* 67-101 and *bel1s*-254/*rev* 58-116 contain amino acids 67 to 101 and 58 to 116 from HIV-1 Tat and Rev, respectively, fused in frame to the amino-terminal 254 residues of Bel1. *bel1s*-227/255-266 s* and *bel1s*-227/255-266 l* contain the Bel1 sequence between amino acids 255 to 266 (LLSGLLEESSNL) with either the serines or leucines mutated to alanines, respectively, fused in frame with the amino-terminal 227 residues of Bel1 and were constructed by using corresponding double-stranded oligonucleotides. The *bel1s*Δ228-271/287t coding region carries a fusion in frame of Bel1 amino acids 272 to 287 with the amino-terminal 227 residues and was synthesized by the polymerase chain reaction, using *bel1s*Δ228-271 DNA as the template and a 3′ primer designed to terminate translation at codon 287. *bel1s*Δ83-150 lacks residues 83 to 150 and was constructed by deletion of the region between the two *Bam*HI sites in the *bel1s* open reading frame, blunt ending with T4 DNA polymerase, and religation of vector ends. *bel1s*Δ166-167, -Δ176-177, and -Δ185-186 were constructed by oligonucleotide-directed mutagenesis of the indicated residues and carry a substitution of a missense Ala-Ser sequence as previously described (26).

The parental vector for the construction of Gal4-Bel1 chimeric proteins was pCMV-Gal4. pCMV-Gal4 was constructed by cloning a *Hin*dIII (blunt-ended)-*Xba*I fragment from pSG424 (24) that contains, in sequence, coding information for the DNA binding domain (amino acids 1 to 147) of the yeast transcriptional activator Gal4 [Gal4(1-147)], a polylinker with multiple cloning sites for fusion to heterologous proteins, and a DNA sequence that specifies translation termination codons in all three reading frames, between the cytomegalovirus immediate-early promoter and the interleukin-2 poly(A) addition signal in pCMV. All Gal4-*bel1s** proteins were constructed by cleaving either wt *bel1s* or mutant *bel1s*Δ83-150, -Δ166-167, -Δ176-177, -Δ185-186, -Δ194-200, -Δ211-222, -Δ228-271, -254, and -266 constructs at the *Eco*RI site overlapping residues 15 to 17 in Bel1, filling in with T4 DNA polymerase, recutting with *Xba*I downstream of the Bel1 translation termination codon, and cloning the various *bel1* gene fragments thus obtained between the *Sma*I and *Xba*I sites downstream of the coding sequence for Gal4(1-147) in pCMV-Gal4. The resultant chimeric proteins comprise the Gal4 DNA-binding domain fused in frame upstream of *bel1s* residues 16 to 300, containing either the wt or mutant Bel1 sequences. For the construction of Gal4(1-147) fusions with Bel1 residues 226 to 300, 272 to 300, 226 to 254, 226 to 266, and 272 to 287, expression vectors containing coding sequences for wt *bel1s*, and *bel1s*Δ228-271, -254, -266, and -Δ228-271/287t, respectively, were cleaved at the

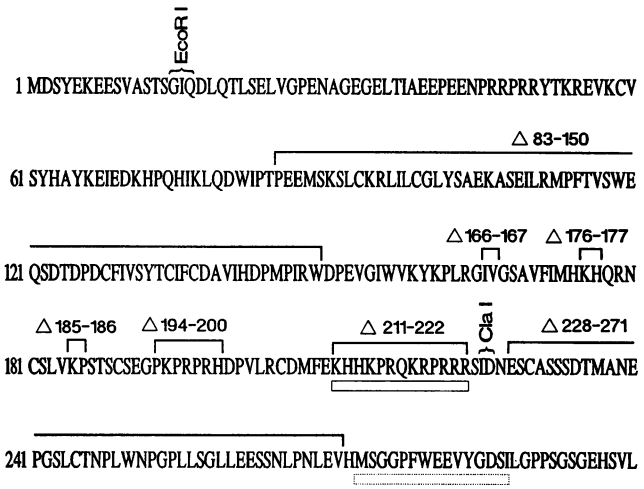

FIG. 1. Complete amino acid sequence of the HSRV Bel1 protein. Amino acids that were altered by deletion or oligonucleotide-directed substitution mutagenesis in the various Bel1 mutants analyzed in this study are indicated by brackets. An *Eco*R1 site and a *Cla*I site overlapping residues 15 to 17 and 225 to 226, respectively, used in the construction of Bel1 fusion proteins with Gal4(1-147), are shown. The nuclear localization sequence comprising residues 211 to 222 is boxed with solid lines. Also, the region between residues 273 and 287 that is completely homologous to a 15-amino-acid segment near the carboxy terminus of SFV Taf (18) is boxed with broken lines.

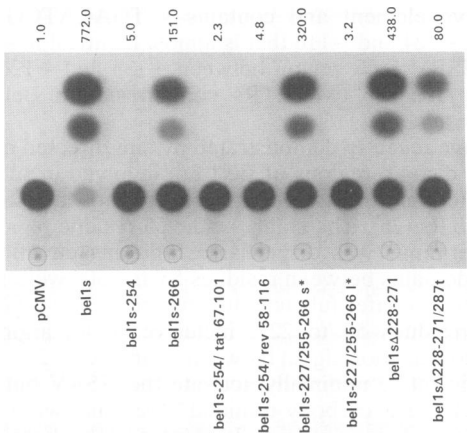

FIG. 2. Identification of amino acids important to the function of the carboxy-terminal activation enhancement domains of Bel1. COS cells were transfected with 2 μg of the reporter −710/+4 HSRV LTR CAT and 2 μg of either pCMV (parental expression vector, negative control) or wt and various Bel1 mutants as indicated. CAT activities indicated above the lanes are averages of three separate transfection experiments and are expressed relative to basal LTR activity.

unique *Cla*I site coincident with Bel1 residues 225 and 226; after filling in of overhangs with T4 DNA polymerase and cleavage with *Xba*I, the various carboxy-terminal Bel1 fragments were isolated and cloned between the *Sma*I and *Xba*I sites in pCMV-Gal4 as described above. Sequences of the wt and mutant Bel1 proteins are shown in Fig. 1.

Cell culture, DNA transfection, and CAT assays. COS cells were maintained in culture and transfected with combinations of various reporter and Bel1 expression plasmid DNAs along with a plasmid expressing *Escherichia coli* β-galactosidase (control indicator for normalizing transfection efficiencies) as a calcium phosphate coprecipitate. Cell extracts were assayed for CAT activity 48 h after transfection, and patterns of CAT expression were confirmed for reproducibility by performing each experiment at least three times. CAT values in different repetitions of the same experiment ranged between 5 and 15% of the average value indicated for each transfection. Unless otherwise stated, experimental procedures adopted were identical to those reported previously (26).

Immunoprecipitation. Specific immunoprecipitation of various Bel1 proteins from transfected COS cell cultures labeled with a mixture of [^{35}S]cysteine and [^{35}S]methionine, using a rabbit antiserum directed against Bel1 residues 1 to 20 at a 1:200 dilution, and resolution of precipitated proteins on 10% discontinuous sodium dodecyl sulfate-polyacrylamide gels were performed exactly as described previously (26).

RESULTS

Specificity of the carboxy-terminal activation enhancement domains. We have previously reported that the amino-terminal 227 (or 254)-residue minimal essential region of Bel1 can cause a low but reproducible 5-fold activation of the reporter construct −710/+4 HSRV LTR CAT, whereas the carboxy-terminal 73 residues contain two distinct regions lacking obvious sequence homology between residues 255 and 266 and between residues 272 and 300 that can independently enhance the activity of this amino-terminal minimal essential domain by about 30- and 90-fold (equivalent to approximately 25 and 75% of wt Bel1-induced transactivation), respectively (26). A series of mutations was therefore introduced downstream of the minimal essential region to assess the specificity of and to identify the amino acid residues and motifs critical to the activation enhancement function of these two carboxy-terminal domains. The results of these experiments are illustrated in Fig. 2.

As demonstrated previously, wt *bel1s* activated the −710/+4 HSRV LTR construct very efficiently, by at least 750-fold, in transfected COS cells, whereas *bel1s*-254, coding for the amino-terminal 254 residues (or *bel1s*-227 expressing the amino-terminal 227 residues; not shown) induced activation by only 5-fold above background levels induced in the presence of the parental expression vector pCMV. Also, expression of Bel1 residues 1 to 266 (*bel1s*-266, containing the transcription enhancement region between residues 255 to 266 downstream of the minimal activator domain between residues 1 to 254) resulted in efficient activation of the HSRV LTR, albeit at one-fifth the wt Bel1-induced level, whereas *bel1s*Δ228-271 (expressing the activation enhancement region between residues 272 and 300 downstream of the minimal activator domain between residues 1 and 227) exhibited about one-half of wt-induced activity. Amino acid segments from heterologous proteins were tested for the ability to substitute for Bel1 residues 255 to 300 to determine whether this region functioned merely in the structural stabilization of the amino-terminal 254 residues. Substitution of the carboxy-terminal 255- to 300-residue region of Bel1 with either residues 67 to 101 or residues 58 to 116 from the HIV-1 Tat or Rev protein (*bel1s*-254/*tat* 67-101 or *bel1s*-254/*rev* 58-116, respectively) did not augment the level of activation induced by *bel1s*-227 or *bel1s*-254, suggesting that the carboxy-terminal domains

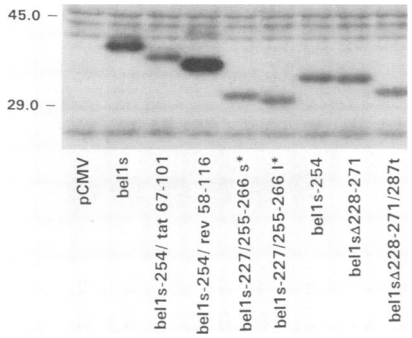

FIG. 3. Immunoprecipitation analysis of Bel1 proteins in transfected COS cells. COS cell cultures were transfected with 2 μg of either pCMV or the various Bel1 constructs indicated, labeled with 100 μCi each of [^{35}S]cysteine and [^{35}S]methionine, and subjected to immunoprecipitation analysis as described in the text. Immunoprecipitated proteins were resolved on discontinuous sodium dodecyl sulfate–10% acrylamide gels. The mobilities (in kilodaltons) of protein markers of known molecular weight are shown at the left.

comprising residues 255 to 266 and 272 to 300 functioned specifically to augment the activity of the Bel1 minimal activator domain. We have observed that a Bel1 protein consisting of the amino-terminal 227 residues linked to the domain comprising residues 255 to 266 activates HSRV LTR expression as efficiently as does *bel1s*-266, demonstrating that the region between amino acids 228 and 254 is dispensable for transactivation (data not shown). To determine whether the abundance of serine and leucine residues in the Bel1 sequence LLSGLLEESSNL between residues 255 and 266 reflected the importance of these amino acids to the function of this carboxy-terminal domain, we transferred this region, with either the serine or the leucine residues mutated to alanines, downstream of residue 227 in Bel1 to yield *bel1s*-227/255-266 s* and *bel1s*-227/255-266 l*, respectively. As shown in Fig. 2, the mutation of leucine but not serine residues abrogated the activation enhancement function of Bel1 residues 255 to 266. Finally, construct *bel1s*Δ228-271/287t, carrying a translation termination codon after residue 287 in the *bel1s*Δ228-271 background, was assessed for activator function to determine whether the highly conserved region between residues 273 and 287 (Fig. 1) could enhance the activity of the amino-terminal 227-residue minimal activator domain. HSRV LTR expression activated by *bel1s*Δ228-271/287t was approximately 16-fold higher than that activated by *bel1s*-227 or *bel1s*-254 but was still 5-fold less than the level activated by *bel1s*Δ228-271, which retains the entire 29-amino-acid activation enhancement domain.

These results demonstrate that the leucine-rich motif between residues 255 and 266 and the highly conserved 15-amino-acid segment comprising residues 273 to 287 can contribute specifically to the function of the carboxy-terminal activation enhancement domains of Bel1.

Expression of Bel1 mutant proteins in transfected COS cells. ^{35}S-labeled protein extracts from COS cells transfected with the Bel1 constructs used for Fig. 2 were subjected to immunoprecipitation analysis to assess the levels of expression of the various mutant proteins relative to wt Bel1. As shown in Fig. 3, the Bel1-specific rabbit antiserum (directed against residues 1 to 20) precipitated a protein of ~36 kDa in *bel1s*-expressing cells that was not detected in a transfection of the parental expression vector pCMV. All of the Bel1 mutants examined were found to express proteins at levels comparable to that of wt Bel1. The various mutant proteins migrated with mobilities consistent with their estimated molecular masses with the exception of the *bel1s*-254/*rev* 58-116 protein, which migrated more rapidly than expected for a protein predicted to contain 313 amino acids.

These data suggest that the mutations introduced into the carboxy-terminal 73-amino-acid region of Bel1 did not significantly affect the relative levels of expression of the resultant Bel1 proteins and further demonstrate that the variation in the levels of activation exhibited by the various mutants are primarily a consequence of functional differences in their carboxy-terminal sequences.

Activity of Gal4-Bel1 mutants on a synthetic promoter. To determine whether Bel1 could activate transcription from a core or minimal promoter comprising the TATA box and its associated general transcription factors that mediate basal levels of transcription, Bel1 amino acids 16 to 300 (in the *bel1s* background) were fused in frame downstream of the DNA binding domain of the yeast Gal4 transcriptional activator, Gal4(1-147), to yield Gal4-*bel1s**. The product of the Gal4-*bel1s** gene, Gal4-Bel1, was found to activate expression of −710/+4 HSRV LTR CAT efficiently (not shown), indicating that Bel1 amino acids 16 to 300 are sufficient for stimulation of HSRV LTR expression. Gal4-Bel1 was then tested for its ability to activate transcription from the minimal promoter construct G5BCAT, which contains the canonical adenovirus E1B TATA box inserted between five Gal4 DNA binding sites and the CAT gene. Gal4-*bel1s** was found to activate transcription from G5BCAT at least 35-fold (see Fig. 6). However, the uninduced level of CAT activity elicited from G5BCAT was remarkably low, making an accurate estimate of the actual extent of Gal4-Bel1-mediated transactivation somewhat difficult. To circumvent this problem, we instead used a synthetic promoter, G5BCAT-SP, that contains three consensus binding sites for the cellular transcription factor Sp1 inserted immediately upstream of the E1B TATA box in G5BCAT. G5BCAT-SP exhibited a readily detectable basal level of CAT expression (~5-fold higher than that of G5BCAT), permitting accurate quantitation of the level of activation mediated by Gal4-Bel1 and mutants thereof, as described below.

As shown in Fig. 4, Gal4-*bel1s** activated G5BCAT-SP expression to a level about 60-fold higher than that activated by a construct expressing Gal4(1-147) alone. The Bel1 coding region between residues 16 and 300 from a series of previously described functionally defective (*bel1s*Δ194-200, -Δ211-222, and -254) and positive (*bel1s*-266 and -Δ228-271) mutants as well as from the newly characterized mutants *bel1s*Δ83-150, -Δ166-167, -Δ176-177, and -Δ185-186 (see Materials and Methods for construction and Table 1 for analysis of transactivation and dominant suppressor function) were built into the Gal4-*bel1s** background to assess the effect of these mutations on the activation function of Gal4-Bel1. All mutations in the minimal essential domain between amino acids 1 to 227 that were functionally defective for HSRV LTR stimulation in the wt *bel1s* background were found to be efficient in transactivation of G5BCAT-SP when expressed as Gal4(1-147) fusion proteins; however, among the carboxy-terminal mutations analyzed, Gal4(1-147) fusions with Bel1 residues 16 to 254 or 266 failed to induce G5BCAT-SP expression, whereas Gal4-*bel1s**Δ228-271 alone exhibited activity sixfold higher than that of Gal4(1-147). A similar pattern of CAT expression was obtained when the Gal4-Bel1 constructs were tested on the minimal promoter construct G5BCAT (data not shown).

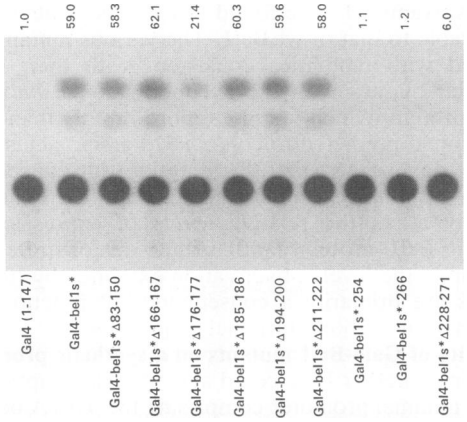

FIG. 4. Transactivation potential of Gal4(1-147)–Bel1 fusion proteins. COS cells were transfected with 2 μg of G5BCAT-SP and 2 μg of pCMV-Gal4(1-147) (negative control) or the various Gal4-Bel1 fusion gene (carrying Bel1 sequences, either wt or mutant, between residues 16 to 300 in the *bel1s* background) plasmids as indicated. CAT activities indicated above the lanes represent averages of three independent experiments.

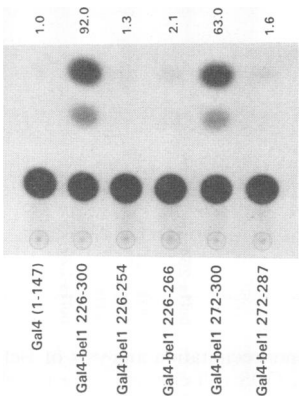

FIG. 5. Transactivation potential of Bel1 carboxy-terminal fragments fused to Gal4(1-147). COS cells were transfected with 2 μg of G5BCAT-SP and 2 μg of either pCMV-Gal4(1-147) (negative control) or plasmids expressing various Bel1 carboxy-terminal amino acid segments fused to Gal4(1-147). CAT activities indicated above the lanes represent averages of four separate DNA transfection experiments.

The activation phenotypes of the various Gal4-Bel1 constructs suggest that the region between Bel1 residues 1 and 227 is dispensable for activation of TATA motif-dependent transcription and that Bel1 amino acids 272 to 300 may potentially encode an activator of minimal promoter function.

Localization of the Bel1 carboxy-terminal activator domain. On the basis of the information gleaned from the above-described experiments, we constructed a series of Gal4(1-147) fusions with various Bel1 carboxy-terminal segments to precisely localize the region in the carboxy-terminal activation enhancement region of Bel1 between residues 228 and 300 that contains the activator of minimal promoter function. The activity of each of these constructs was tested on the reporter G5BCAT-SP. As shown in Fig. 5, a Gal4(1-147) fusion protein expressing Bel1 residues 226 to 300 activated G5BCAT-SP expression to a level about 92-fold higher than the uninduced level dictated by Gal4(1-147). The region between Bel1 residues 226 and 254 or 266 was unable to stimulate the activity of this synthetic promoter significantly. However, the transcription enhancement region comprising amino acids 272 to 300 activated CAT expression 63-fold, although the highly conserved region between residues 272 and 287 was, by itself, deficient in transactivation. Similar results were obtained with the reporter G5BCAT (see Fig. 6).

Taken together, these results suggest that the activation enhancement region between Bel1 residues 272 and 300 contains a strong activator of basal promoter transcription and that mere expression of the highly conserved region between residues 272 and 287 alone is not sufficient for this function. Further, Bel1 residues 254 to 266 seem incapable of stimulating the transcriptional activity of the E1B TATA box, and its activation enhancement function is therefore likely to involve a different mechanism.

Bel1 activator domain sequences required for stimulation of E1B TATA, HSRV, and HIV promoter expression. We next examined whether the amino acid sequence requirements in the Bel1 activator region between residues 272 and 300 for stimulation of transcription from the adenovirus E1B TATA box, the HSRV LTR, and the HIV-1 LTR were identical. Accordingly, Gal4(1-147)–Bel1 hybrid constructs containing various Bel1 sequences in the activator region comprising residues 228 to 300 were assessed for transactivation potential on the CAT gene-based reporter plasmids G5BCAT, G5 HSRV LTR CAT, and G5 ΔκB HIV LTR CAT, which contain, respectively, the adenovirus E1B TATA motif, Bel1-responsive HSRV LTR sequences between −136 and +4 relative to the cap site, and Bel1-responsive HIV-1 LTR sequences (with functionally inactive NF-κB binding sites) between −157 and +77 with respect to the cap site, inserted downstream of five DNA binding sites for Gal4(1-147).

As already mentioned, Gal4-*bel1s** containing Bel1 residues 16 to 300 fused to Gal4(1-147) activated G5BCAT expression about 35-fold over the basal level (Fig. 6a, G5BCAT). Remarkably, Gal4-*bel1* 272-300 expressing the carboxy-terminal 29-amino-acid activator domain induced a level of activation ~5-fold higher than that induced by Gal4-*bel1s**, which contains a fusion of Bel1 residues 16 to 300. In contrast, Gal4-*bel1* 272-287 expressing the highly conserved Bel1 region was severely defective, about 50-fold

TABLE 1. Analysis of additional mutants in the Bel1 minimal essential region for transactivation and dominant suppressor function

bel1 mutant[a]	Relative CAT activity[b]	Dominant inhibition[c]
pCMV	1.0	
bel1s	720.0	
*bel1s*Δ83-150	1.2	−
*bel1s*Δ166-167	1.3	+++
*bel1s*Δ176-177	1.1	+
*bel1s*Δ185-186	708.2	−

[a] All Bel1 proteins, both wt and mutant, were found to be expressed at similar levels in transfected COS cells, as determined by immunoprecipitation analysis.

[b] Average of two independent experiments. Activation of the reporter −710/+4 HSRV LTR CAT by various Bel1 constructs was assayed as described in the legend to Fig. 2.

[c] The ability of functionally defective Bel1 proteins to suppress wt Bel1-mediated activation of the HSRV LTR was assayed by transfecting COS cells with wt and mutant Bel1 plasmids at 1:10 molar ratios. +++, +, and − denote 90, 70, and 0% inhibition of wt activity, respectively.

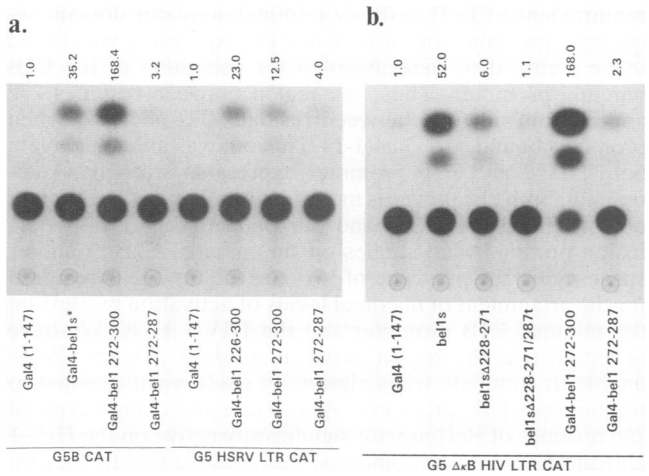

FIG. 6. Differences in the requirement of Bel1 carboxy-terminal activator domain sequences for activation of the minimal adenovirus E1B promoter (G5BCAT), the HSRV LTR, and the HIV-1 LTR. (a) COS cells were transfected with 2 μg of the reporter plasmid G5BCAT or G5 HSRV LTR CAT along with 2 μg of the various Gal4-Bel1 fusion constructs as indicated. (b) COS cell cultures were transfected with 2 μg of the reporter G5 ΔκB HIV LTR CAT (lacking functional NF-κB binding motifs) along with 2 μg of the indicated effector plasmids. CAT activities indicated above the lanes represent averages of three independent experiments.

less than Gal4-*bel1* 272-300, for transactivation. When the various Bel1 carboxy-terminal segments were tested, as Gal4(1-147) chimeras, for activation of the HSRV LTR (Fig. 6a, G5 HSRV LTR CAT), residues 272 to 300 were somewhat less efficient than residues 226 to 300 (as also observed with G5BCAT-SP; Fig. 5), inducing about 13-fold stimulation. The region comprising residues 272 to 287 proved to be an efficient activator, only 3-fold less in magnitude than residues 272 to 300, of HSRV LTR expression (compared with the 50-fold decline observed in the activity of Gal4-*bel1* 272-287 relative to Gal4-*bel1* 272-300 on E1B TATA motif-directed transcription).

We next tested whether the highly conserved Bel1 sequence between amino acids 272 and 287 could, paralleling its action on −710/+4 HSRV LTR (see Fig. 2), function to enhance the activity of the amino-terminal 227 residues on the HIV-1 LTR. We have previously shown that Bel1 (*bel1s*) can activate the HIV-1 LTR about 50- to 60-fold and that whereas *bel1s*-227, coding for residues 1 to 227, is functionally inactive, *bel1s*Δ228-271, which contains the amino-terminal 227 residues fused to the carboxy-terminal activator domain comprising amino acids 272 to 300, exhibited 5- to 6-fold activation (26). This activity of *bel1s* and *bel1s*Δ228-271 was reproducible on the reporter G5 ΔκB HIV-1 LTR CAT (Fig. 6b). However, *bel1s*Δ228-271/287t, expressing a fusion of Bel1 residues 1 to 227 with the highly conserved amino acids 272 to 287, was unable to activate this HIV-1 LTR reporter. The activator domain encompassing residues 272 to 300 and the conserved motif between residues 272 and 287 were also tested for transactivation as Gal4(1-147)-tagged proteins. Gal4-*bel1* 272-300 activated CAT expression about 168-fold, whereas Gal4-*bel1* 272-287 was almost totally defective, exhibiting an ~50-fold lower level of induction.

These results demonstrate that Bel1 residues 272 to 300 contain an efficient autonomous activator of transcription from the adenovirus E1B TATA box, the HIV-1 LTR, and the HSRV LTR. The overall integrity of this 29-amino-acid segment is essential for high-level activation of the E1B TATA box and the HIV-1 LTR. In contrast, the strongly conserved 15-amino-acid segment spanning residues 273 to 287 alone could function as a relatively efficient activator of HSRV LTR transcription.

DISCUSSION

We have previously reported on the approximate organization of functional domains in the HSRV Bel1 protein through the analysis of an extensive panel of mutants. Two distinct regions between residues 56 and 300 were found to cooperate to confer full activator function; the more amino-terminal minimal essential region spanning residues 56 to 227 or 254 contains an amino acid sequence between residues 88 and 110 that is strongly conserved between HSRV Bel1 and the simian foamy virus (SFV) transactivator Taf (18) and an arginine-rich nuclear localization sequence comprising residues 211 to 222. The minimal essential region was able to stimulate −710/+4 HSRV LTR expression about fivefold but was without activity on the HIV-1 LTR. Mutants in the distal part of the minimal essential region, particularly Δ194-200 lacking a proline-rich region and Δ211-222, were found to possess a dominant negative phenotype. Functional analysis of a new series of mutants, Δ83-150, Δ166-167, Δ176-177, and Δ185-186, in this study indicates that Δ166-167 and Δ176-177, particularly the former, can also function as dominant suppressors of wt Bel1 function. In contrast, mutants in the proximal half of the minimal essential domain such as Δ92-93, Δ94-98, Δ135-136, and Δ83-150 failed to act as dominant suppressors. By analogy to other modular transcriptional activators with distinct target specificity and activator domains, the proximal half of the minimal essential region of Bel1 exhibits the characteristics of a promoter specificity domain, interacting directly or through another cellular transcription factor with the Bel1-responsive element in the HSRV LTR. The distal half of the minimal essential domain may contribute to the action of the specificity domain but, because of the high yield of dominant negative mutants in this region, is also likely to serve a different function, perhaps contributing to the low activator function observed with residues 1 to 227.

We have reported that the carboxy-terminal 73 residues of Bel1 contain two functionally redundant regions between residues 255 and 266 and between residues 272 and 300 that can independently augment the activity of the amino-terminal 227- or 254-residue minimal essential region. These two carboxy-terminal sequences do not exhibit any obvious homology between themselves or with other known activators. As previously demonstrated, Bel1 residues 1 to 227 or 254 can activate HSRV LTR expression about fivefold but lack the ability to stimulate the HIV-1 LTR. The regions between amino acids 254 and 266 and between amino acids 272 and 300 were found to enhance the activity of the amino-terminal minimal essential region to about 20 to 25 and 60 to 75%, respectively, of the level displayed by wt Bel1 on the HSRV LTR, compared with the much weaker augmenting effects of these two regions (about 3 and 8% of wt activity) on the HIV-1 LTR. The activities of these two regions are clearly specific and may not be limited to a relatively generalized function such as structural stabilization of the minimal essential domain, as revealed by the inability of amino acid segments from heterologous proteins, HIV-1 Tat and Rev, to functionally substitute for Bel1

residues 254 to 300 in activation of the HSRV LTR. The activation enhancement region comprising residues 255 to 266 contains three serine and five leucine residues. Since Bel1 is phosphorylated and the importance of this posttranslational modification to the transactivation function remains uncertain, these serines were mutated to alanines; the mutation, however, had no discernible effect on the activation-augmenting function of this region. In striking contrast, the substitution of leucines with alanines resulted in complete loss of the activation enhancement function of this region. It is unlikely that a high concentration of leucine residues alone is sufficient to mediate this function, since the substitution of this region with a leucine-rich sequence from HIV-1 Rev (*bel1s*-254/*rev* 58-116) was unable to restore the activation enhancement function. The leucines at residues 259 and 266 constitute a heptad repeat and therefore may specify a mini-leucine zipper, a two-stranded parallel α-helical coiled-coil structure that has been shown to function as a dimerization motif in a number of eukaryotic transcriptional activators (19). Whether Bel1 transactivation requires dimerization, and the possible involvement of the leucine-rich sequence in this dimerization function, are issues that remain to be addressed. Alternatively, the leucines (possibly in conjunction with other residues) in this region of Bel1 may function by a different mechanism, perhaps as an activator or activator-recruiting motif in the context of the HSRV LTR. The second activation-enhancing domain constituting residues 272 to 300 contains a region between amino acids 273 and 287 that is identical in sequence to residues 277 to 291 of the SFV-1 transcriptional activator Taf (18). This highly conserved sequence can enhance the activity of the amino-terminal 227 residues on the HSRV LTR by 16-fold, thus exhibiting about one-fifth the enhancing activity of residues 272 to 300. This highly conserved sequence in the foamy virus transactivators does not resemble the sequence of any eukaryotic activator domain thus far characterized.

We have used a protein fusion strategy to target various segments of the Bel1 protein to the minimal adenovirus E1B TATA promoter as well as the HSRV and HIV-1 LTRs through the DNA binding domain, Gal4(1-147), of the yeast transcriptional activator Gal4 in an effort to distinguish the Bel1 promoter specificity sequences from those that confer activator function. This Gal4(1-147)-based approach has been elegantly used to study the mechanisms of transactivation, by various transcriptional activators such as VP16 (23) and adenovirus E1A (13, 16), of promoters containing upstream DNA binding sites for Gal4(1-147). A Gal4-Bel1 construct expressing Bel1 residues 16 to 300 was found to activate transcription from the adenovirus E1B TATA motif at least 35-fold, thus suggesting that Bel1, like E1A and VP16, can interact either directly or through an adaptor (coactivator; reviewed in reference 12) with a component of the general transcription apparatus such as TFIID or TFIIB. Using a series of Gal4(1-147)–Bel1(16-300) fusion constructs carrying mutations that render Bel1 defective in transactivation of the HSRV LTR, we demonstrated that the region between amino acids 16 and 227 was dispensable for activation of transcription from the adenovirus E1B TATA motif. On the other hand, the carboxy-terminal regions between residues 226 and 300 and between residues 272 and 300 were found to strongly activate E1B TATA motif-directed transcription. In contrast, neither residues 226 to 266, which include the leucine-rich activation enhancement region between residues 255 and 266, nor the highly conserved sequence between residues 272 and 287 was able to stimulate the activity of the E1B TATA motif significantly. The requirement of Bel1 carboxy-terminal activator domain sequences for induction of HIV-1 LTR expression was found to be identical to that observed for activation of the E1B minimal promoter. Thus, the region between residues 272 and 300 but not that between residues 272 and 287, when promoter bound as a Gal4(1-147) fusion, was able to activate both HIV-1 and E1B promoter expression strongly. Additionally, both our previous mutational analysis of Bel1 action on the HIV-1 LTR (26) and our present Gal4(1-147)–Bel1 fusion protein-based studies on the minimal E1B promoter suggest that the presence of residues 228 to 271 is required for the attainment of maximal levels of activation by Bel1 on the minimal E1B promoter and the HIV-1 LTR. We have observed that the region between residues 228 and 266 is unable to stimulate transcription by itself when targeted to the minimal E1B promoter. Also, since the amino-terminal 266 residues of Bel1 are not significantly active on the HIV-1 (26) and E1B TATA promoters, it is possible that this region contains a cryptic transcriptional activator function in the context of these promoters that is realized only in cooperation with residues 272 to 300.

Significant differences were observed when the carboxy-terminal 28 amino acids of Bel1 were assayed for activator function on the HSRV LTR. Specifically, this amino acid sequence when targeted to the HSRV LTR was found to induce only about 12-fold activation, compared with the much stronger activating effects (90- to 170-fold) on the minimal E1B promoter and the HIV-1 LTR. This low level of activity may reflect the nature of the HSRV basal transcription apparatus or the presence of negative regulatory elements in the HSRV LTR. However, the highly conserved sequence between residues 272 and 287 could also efficiently activate transcription from the HSRV LTR, at one-third the level observed with the entire 29-amino-acid activator. These results clearly demonstrate that this conserved 15-amino-acid segment in the carboxy-terminal region of foamy virus transactivators forms the core sequence of an activator of HSRV LTR expression. Recent experiments involving domain substitution with the HSV VP16 activation domain also provide indirect evidence for the existence of an activator domain in the carboxy-terminal 50-amino-acid region of Bel1 (5).

The results from our previous studies and from the present analysis clearly demonstrate that the Bel1 transactivator of HSRV contains at least two distinct activator domains (Fig. 7). The more proximal of these is contained within the amino-terminal 266 residues and can activate the HSRV LTR, but not the HIV-1 LTR or the canonical TATA motif contained in the adenovirus E1B promoter, efficiently; this region also includes the promoter specificity domain between residues 1 and 227 and the leucine motif-dependent activation enhancement function conferred by residues 255 to 266. The second activator domain is located within the carboxy-terminal 29 residues and can activate E1B TATA, HIV-1 LTR, and HSRV LTR expression efficiently. The entire 29-amino-acid segment is required for strong activation of E1B TATA and HIV-1 LTR expression; in contrast, the highly conserved sequence between residues 272 and 287 alone is able to induce efficient HSRV LTR expression. Similar results have also been obtained for human HeLa cells, suggesting that the observed differences in activation of these promoters are independent of cell type (24a). We have now observed that the region between Bel1 residues 228 and 300 is a potent activator of transcription in the yeast *Saccharomyces cerevisiae* (24b). This finding argues for a high degree of evolutionary conservation in the mechanism

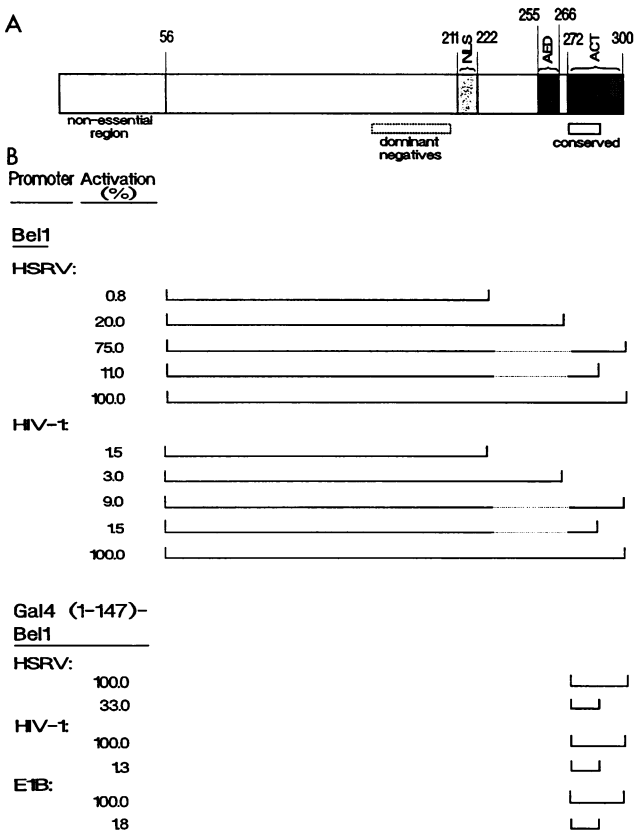

FIG. 7. Organization of functionally important regions in the Bel1 transactivator and data summarizing the contribution of various carboxy-terminal regions to Bel1 transactivation. (A) Locations of some functionally important domains of Bel1. The nonessential amino-terminal 55-residue region and the minimal essential region comprising residues 1 to 227 have been previously described (26). The amino acid sequence between residues 273 and 287 (boxed with solid lines) that is highly conserved between HSRV Bel1 and SFV Taf and the region encompassing residues 165 to 200 in which mutants with a dominant negative phenotype have been isolated (boxed with broken lines) are indicated. NLS, nuclear localization sequence; AED, activation enhancement domain; ACT, autonomous activator of E1B TATA, HIV LTR, and HSRV LTR expression. (B) Summary of results on the functional activities of various carboxy-terminal segments important to Bel1 induction of the HSRV, HIV-1, and adenovirus E1B promoters, both in the 300-amino-acid Bel1 background and as Gal4(1-147) fusion constructs. Under Bel1, activities of *bel1s*-227, *bel1s*-266, *bel1s*Δ228-271, *bel1s*Δ228-271/287t, and wt *bel1s* are shown; under Gal4(1-147)–Bel1, activities of Bel1 residues 272 to 300 and 272 to 287, as Gal4(1-147) fusions, are indicated.

of Bel1-mediated transactivation and further supports our argument that Bel1 interacts, directly or through a coactivator, with a general transcription factor. The association of general transcription factors with common promoter elements such as the TATA and initiator motifs is an ordered process in which TFIID first associates with the TATA motif and provides a recognition site for the association of other factors (TFIIA, -IIB, -IIE, -IIF, -IIH, and -IIJ) and RNA polymerase II (reviewed in reference 28). Given that Bel1 residues 272 to 300 can directly activate TATA motif-directed transcription, Bel1-mediated activation of promoters may involve the facilitated assembly of one or more of these general transcription factors by this region. The ability of the activator sequence in residues 1 to 266 and that between residues 272 and 287 to efficiently activate HSRV but not HIV-1 or adenovirus E1B TATA expression may reflect variations in the nature of common promoter elements such as the TATA motif and/or cooperativity with other transcription factors assembled on these promoter templates as has been demonstrated in other systems (2, 14).

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REFERENCES

1. **Bothe, K., A. Aguzzi, H. Lassman, A. Rethwilm, and I. Horak.** 1991. Progressive encephalopathy and myopathy in transgenic mice expressing human foamy virus genes. Science **253**:555–557.
2. **Carey, M., Y.-S. Lin, M. R. Green, and M. Ptashne.** 1990. A mechanism for synergistic activation of a mammalian gene by Gal4 derivatives. Nature (London) **345**:361–364.
3. **Cullen, B. R.** 1991. Human immunodeficiency virus as a prototypic complex retrovirus. J. Virol. **65**:1053–1056.
4. **Flügel, R. M., A. Rethwilm, B. Maurer, and G. Darai.** 1987. Nucleotide sequence analysis of the *env* gene and its flanking regions of the human spumaretrovirus reveals two novel genes. EMBO J. **6**:2077–2084.
5. **He, F., J. D. Sun, E. D. Garrett, and B. R. Cullen.** 1993. Functional organization of the Bel-1 *trans* activator of human foamy virus. J. Virol. **67**:1896–1904.
6. **Kamine, J., and G. Chinnadurai.** 1992. Synergistic activation of the human immunodeficiency virus type 1 promoter by the viral Tat protein and cellular transcription factor Sp1. J. Virol. **66**:3932–3936.
7. **Kamine, J., T. Subramanian, and G. Chinnadurai.** 1991. Sp1-dependent activation of a synthetic promoter by human immunodeficiency virus type 1 tat protein. Proc. Natl. Acad. Sci. USA **88**:8510–8514.
8. **Keller, A., E. D. Garrett, and B. R. Cullen.** 1992. The Bel-1 protein of human foamy virus activates human immunodeficiency virus type 1 gene expression via a novel DNA target site. J. Virol. **66**:3946–3949.
9. **Keller, A., K. M. Partin, M. Löchelt, H. Bannert, R. M. Flügel, and B. R. Cullen.** 1991. Characterization of the transcriptional *trans* activator of human foamy retrovirus. J. Virol. **65**:2589–2594.
10. **Lagaye, S., P. Vexiau, V. Morozov, V. Guénebaut-Claudet, J. Tobaly-Tapiero, M. Canivet, G. Cathelineau, J. Périès, and R. Emanoil-Ravier.** 1992. Human spumaretrovirus-related sequences in the DNA of leukocytes from patients with Graves disease. Proc. Natl. Acad. Sci. USA **89**:10070–10074.
11. **Lee, A. H., K. J. Lee, S. Kim, and Y. C. Sung.** 1992. Transactivation of the human immunodeficiency virus type 1 long terminal repeat-directed gene expression by the human foamy virus *bel1* protein requires a specific DNA sequence. J. Virol. **66**:3236–3240.
12. **Lewin, B.** 1990. Commitment and activation at Pol II promoters: a tail of protein-protein interactions. Cell **61**:1161–1164.
13. **Lillie, J. W., and M. R. Green.** 1989. Transcriptional activation by the adenovirus E1a protein. Nature (London) **338**:39–44.
14. **Lin, Y.-S., M. Carey, M. Ptashne, and M. R. Green.** 1990. How different eukaryotic transcriptional activators can cooperate promiscuously. Nature (London) **345**:359–361.
15. **Löchelt, M., H. Zentgraf, and R. M. Flügel.** 1991. Construction of an infectious DNA clone of the full-length human spumaretrovirus genome and mutagenesis of the *bel*1 gene. Virology **184**:43–54.
16. **Martin, K. J., J. W. Lillie, and M. R. Green.** 1990. Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. Nature (London) **346**:147–152.

17. **Mergia, A., and P. A. Luciw.** 1991. Replication and regulation of primate foamy viruses. Virology **184:**475–482.
18. **Mergia, A., K. E. S. Shaw, E. Pratt-Lowe, P. A. Barry, and P. A. Luciw.** 1991. Identification of the simian foamy virus transcriptional transactivator gene (*taf*). J. Virol. **65:**2903–2909.
19. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:**371–378.
20. **Muranyi, W., and R. M. Flügel.** 1991. Analysis of splicing patterns of human spumaretrovirus by polymerase chain reaction reveals complex RNA structures. J. Virol. **65:**727–735.
21. **Nabel, G., S. A. Rice, D. A. Knipe, and D. Baltimore.** 1988. Alternative mechanisms for activation of human immunodeficiency virus enhancer in T cells. Science **239:**1299–1302.
22. **Rethwilm, A., K. Mori, B. Maurer, and V. ter Meulen.** 1990. The transcriptional transactivator of human foamy virus maps to the *bel*1 genomic region. Proc. Natl. Acad. Sci. USA **88:**941–945.
23. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. Gal4-VP16 is an unusually potent transcriptional activator. Nature (London) **335:**563–564.
24. **Sadowski, I., and M. Ptashne.** 1989. A vector for expressing Gal4(1-147) fusions in mammalian cells. Nucleic Acids Res. **18:**7539.
24a. **Venkatesh, L. K., and G. Chinnadurai.** Unpublished data.
24b. **Venkatesh, L. K., and G. Chinnadurai.** Unpublished data.
25. **Venkatesh, L. K., P. A. Theodorakis, and G. Chinnadurai.** 1991. Distinct cis-acting regions in U3 regulate *trans*-activation of the human spumaretrovirus long terminal repeat by the viral *bel1* gene product. Nucleic Acids Res. **9:**3661–3666.
26. **Venkatesh, L. K., C. Yang, P. A. Theodorakis, and G. Chinnadurai.** 1993. Functional dissection of the human spumaretrovirus transactivator identifies distinct classes of dominant-negative mutants. J. Virol. **67:**161–169.
27. **Weiss, R. A.** 1988. Foamy retroviruses. A virus in search of a disease. Nature (London) **333:**497–498.
28. **Zawel, L., and D. Reinberg.** 1993. Initiation of transcription by RNA polymerase II: a multi-step process. Prog. Nucleic Acid Res. Mol. Biol. **44:**67–108.