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Functional Organization of the Bel-1 *trans* Activator of Human Foamy Virus

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Human foamy virus encodes a 300-amino-acid nuclear regulatory protein termed Bel-1 that is required for human foamy virus replication in culture. Bel-1 is a potent *trans*-activator of gene expression directed by the homologous HFV long terminal repeat as well as the long terminal repeat of human immunodeficiency virus type 1. We have used mutational analysis to define several discrete functional domains within Bel-1. The C-terminal ~50 amino acids of Bel-1 are shown to be essential for Bel-1 activity but can be effectively substituted by the C-terminal activation domain of VP16. We therefore conclude that the Bel-1 C terminus forms part of an activation domain. Mutations within a central, ~100-amino-acid segment of Bel-1 preclude *trans*-activation by either Bel-1 or the Bel-1/VP16 chimera. These sequences are therefore proposed to direct the interaction of Bel-1 with its viral DNA target sequences. A short Bel-1 segment located between the activation and binding domains is shown to mediate the nuclear localization of this regulatory protein. Although the functional organization of Bel-1 therefore appears comparable to that reported for other eukaryotic transcriptional activators, Bel-1 does not contain sequences homologous to known transcriptional activation or DNA-binding motifs.

Human foamy virus (HFV), also known as human spumaretrovirus, is the prototype of the foamy virus subgroup of the family *Retroviridae* (7, 8, 23). Although recent data suggest that HFV infections may be common in certain human populations (20), HFV has not yet been clearly associated with any disease state (8, 23, 35). However, the marked cytopathic effect of HFV in culture (8, 23) and the pathogenic effects reported in transgenic animals bearing HFV genes (4) suggest that HFV may have the potential to cause human disease.

Like other complex human retroviruses, including human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus Type I (HTLV-I), HFV encodes a potent transcriptional *trans*-activator of its long terminal repeat (LTR) promoter element (7, 15, 30, 34). This 300-amino-acid regulatory protein, termed Bel-1, is localized to the nucleus of expressing cells (15, 19) and is essential for viral replication in culture (19). The major target sequence for Bel-1 within the HFV LTR promoter is located ~130 bp 5′ to the site of transcription initiation and is therefore believed to be a DNA sequence (15, 34). However, the precise sequence requirement for Bel-1 function remains unknown, as does whether Bel-1 interacts directly with this DNA target element.

Recently, it was demonstrated that the Bel-1 protein can also *trans*-activate gene expression directed by the HIV-1 LTR (14, 17). The target site for Bel-1 in the HIV-1 LTR is again located in U3 at ~120 bp 5′ to the transcription initiation site. This response element is therefore immediately adjacent to, but has been shown to be distinct from, the duplicated HIV-1 LTR NF-κB enhancer sequence (14, 17). Surprisingly, inspection of the DNA target sites for Bel-1 in the HFV and HIV-1 LTRs has failed to reveal any evident sequence similarity, thus raising the possibility that Bel-1 can activate these two promoter elements via distinct mechanisms.

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In this communication we report the identification and approximate delineation of amino acid sequences within Bel-1 that regulate the DNA sequence specificity, the activation potential, and the subcellular localization of this potent viral *trans*-activator. These observations strongly suggest a direct interaction between Bel-1 and its two known DNA target sites and are consistent with the hypothesis that Bel-1 activates transcription from the HFV and HIV-1 LTRs via similar mechanisms.

MATERIALS AND METHODS

Construction of molecular clones. We have described the cytomegalovirus immediate-early (CMV-IE) promoter-based expression vector pBC12/CMV as well as its derivatives, pBel-1, containing the HFV *bel-1* gene, and pBel-1+2+3, containing all three Bel open reading frames (ORFs) (15). Missense mutations were introduced into the *bel-1* gene present in pBel-1 by using the polymerase chain reaction (26). In each case, flanking oligonucleotide primers that spanned a unique 5′ *Sac*I site, located within the CMV-IE promoter, or a unique 3′ *Eco*RI site, located within the plasmid backbone, were used with oligonucleotide primers that introduced *Bgl*II sites at various locations within the Bel-1 ORF (Fig. 1B). The specific amino acid changes introduced by these primers are listed in Table 1. All mutations were confirmed by sequencing. Deletions of *bel-1* sequences located between introduced point mutations were performed by standard recombinant DNA technology, as previously described (21). Derivatives of pΔ247/296 containing the 78-amino-acid carboxy-terminal activation domain of the herpes simplex virus VP16 *trans* activator (33) were obtained by insertion of a *Bgl*II-*Bam*HI fragment containing this VP16 sequence at the introduced *Bgl*II site of pΔ247/296. This herpes simplex virus-derived DNA sequence contains a translation termination codon and therefore precludes expression of the C-terminal 5 amino acids of Bel-1.

A cDNA copy of the HFV *bet* gene was isolated by

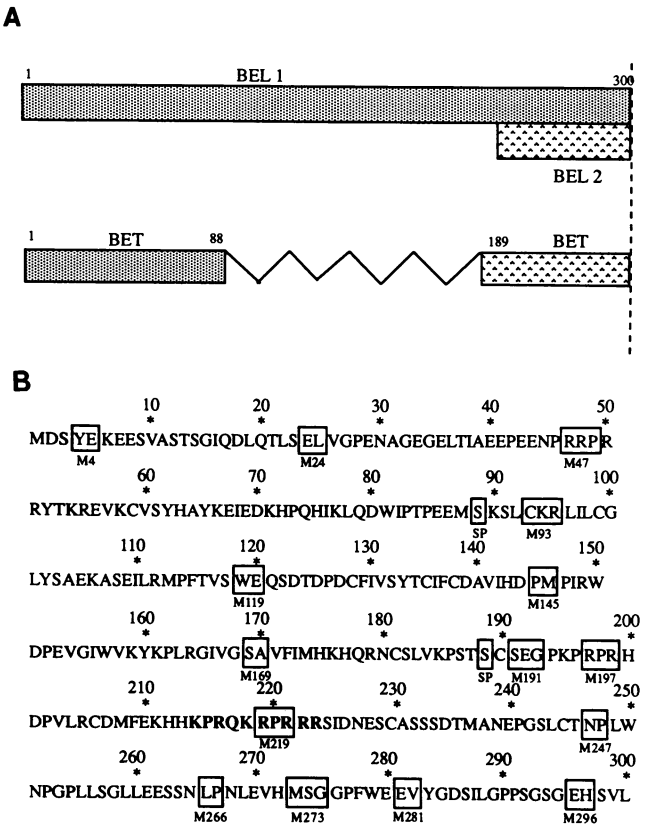

FIG. 1. Structure of HFV *bel-1* expression plasmids. (A) The pBel-1+2+3 expression plasmid contains the entire *bel-1* gene as well as all of the overlapping *bel-2* gene. The major gene product encoded by this HFV sequence, termed Bet, is derived by splicing the 88 N-terminal amino acids of *bel-1* in frame with the *bel-2* ORF (19, 27). In the pBel-1 plasmid, Bel-2, and hence Bet, is truncated immediately 3′ to the *bel-1* translation termination codon. The pBel-1 expression vector therefore encodes a full-length Bel-1 protein and a truncated form of Bet termed ΔBet. The pcBet and pcΔBet plasmids express the identical HFV sequences as pBel-1+2+3 and pBel-1, respectively, but in a prespliced or cDNA form. (B) Predicted primary sequence of the HFV Bel-1 protein. The locations of the various missense (M) mutations introduced into Bel-1 are indicated, as are the locations of the splice (SP) sites used in the formation of Bet. The basic sequence shown to form the Bel-1 NLS is indicated in boldface type.

reverse transcription of total RNA isolated from COS cells transfected with the pBel-1+2+3 expression construct (15) followed by amplification by polymerase chain reaction (26) with the oligonucleotide primers previously used in the derivation of the pBel-1 expression plasmid (15). Expression plasmids pcBet, containing the full-length Bet cDNA sequence, and pcΔBet, containing a *bet* cDNA truncated at the end of the Bel-1 ORF, were derived from pBel-1+2+3 and pBel-1, respectively, after cleavage at an *Apa*I site previously introduced immediately 5′ to the Bel-1 initiation codon (15) and at a *Cla*I site located immediately 3′ to the Bet splice acceptor sequence.

The indicator constructs pBC12/HSRV/SEAP, pMκB/SEAP, and pBC12/CMV/SEAP are all based on the secreted alkaline phosphatase (SEAP) indicator gene and have been described previously (3, 14, 15). These plasmids contain, respectively, the full-length HFV LTR, an HIV-1 LTR lacking functional NF-κB sites, and the CMV-IE promoter element.

TABLE 1. Characteristics of Bel-1 missense mutants

Name[a]	Mutation	Activity[b]	Subcellular localization[c]
Bel-1		++	N
M4	YE → RS	++	nt
M24	EL → RS	++	nt
M47	RRP → QIS	++	nt
M93	CKR → LDL	−	N > C
M119	WE → RS	−	N > C
M145	PM → RS	−	N
M169	SA → DL	−	N
M191	SEG → LRS	++	N
M197	RPR → DLG	−	N
M219	RPR → QIW	+	N = C
M247	NP → RS	++	nt
M266	LP → RS	++	nt
M273	MSG → KIC	++	nt
M281	EV → RS	++	nt
M296	EH → RS	++	nt

[a] The specific missense (M) mutations introduced into Bel-1 are indicated.
[b] Biological activity is derived from Table 2 and is given as ++ (>50% of wild-type), + (~50% of wild-type) and − (not detectably active).
[c] The subcellular location of Bel-1 proteins is given as nuclear (N), predominantly nuclear (N > C), or diffuse (N = C) (see Fig. 3 for representative examples). Most fully active Bel-1 mutants were not tested (nt) for their subcellular distribution.

The pCMV/β-gal plasmid contains the full-length prokaryotic β-galactosidase (β-gal) gene expressed under the control of the CMV-IE promoter present in the pBC12/CMV expression construct. The initiation codon (underlined) of the β-gal gene has, however, been replaced with a consensus eukaryotic translation initiation codon (16) that also introduces a unique *Nco*I site (5′-CCATGG-3′). This site facilitated the insertion, between the first and second amino acids of β-gal, of short sequences that were predicted to encode the Bel-1 nuclear localization signal (NLS). Specifically, these synthetic oligonucleotides encoded the inserted peptide sequence NH_2-KGPKPRPRHDPVLRCDM-COOH in the pCMV/β-gal(193–208) construct and the sequence NH_2-FEKHHKPRQKRPRRRSIDM-COOH in the pCMV/β-gal (209–226) construct. The origin of these Bel-1-derived sequences is indicated by the name of the resultant plasmid (Fig. 1B).

Cell culture and DNA transfection. COS cells were maintained as previously described (15) and were transfected with DEAE-dextran and chloroquine (6). Equal amounts of the relevant indicator and effector constructs were transfected unless otherwise stated. SEAP assays were performed with COS cell culture medium harvested at ~70 h posttransfection, as previously described (3).

Immunoprecipitation and immunofluorescence. The previously described (15, 19) rabbit antiserum specific for the Bel-1 ORF of HFV was raised against a recombinant fusion protein containing amino acids 16 to 149 of Bel-1. A rabbit antiserum specific for β-gal was obtained from Cappel Inc., West Chester, Pa. The method used for [^{35}S]cysteine labeling of transfected COS cell cultures and for specific immunoprecipitation of labeled proteins has been described elsewhere (6, 21). In these experiments, the Bel-1 antiserum was used at a 1:750 dilution.

The technique of indirect immunofluorescence, used to localize proteins within transfected COS cell cultures, was performed as previously described (6, 21). The primary Bel-1- or β-gal-specific rabbit antisera were used at a 1:750 dilution, and the secondary antibody, rhodamine-conjugated

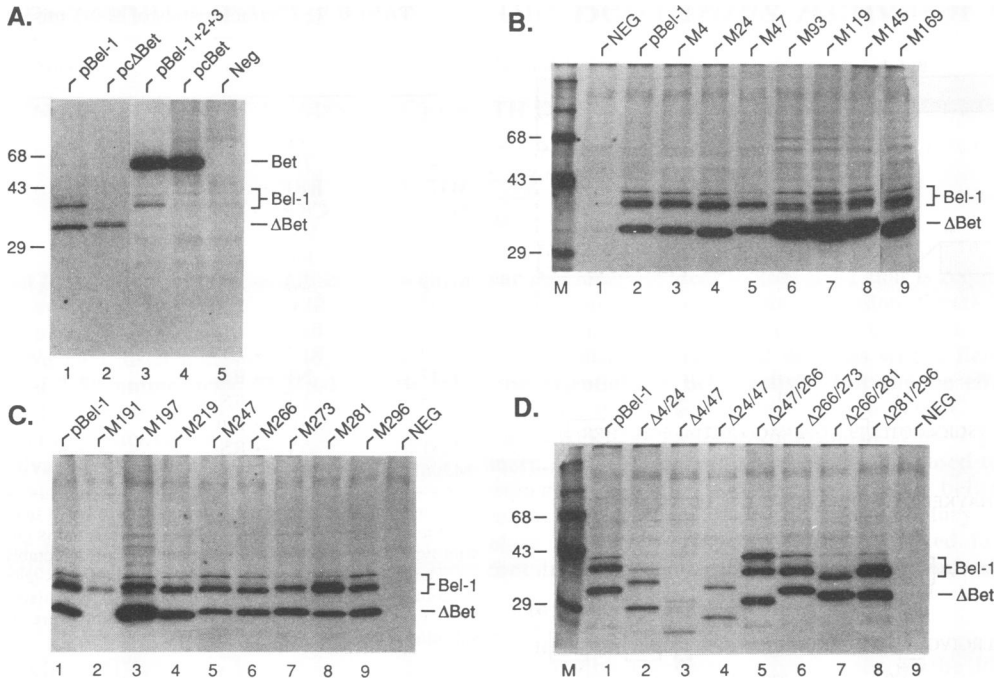

FIG. 2. Immunoprecipitation analysis of COS cells transfected with mutant or wild-type forms of Bel-1. COS cell cultures (35-mm) were transfected with the indicated wild-type or mutant Bel-1 or Bet expression plasmids or with the negative (Neg) control vector pBC12/CMV. At ~70 h posttransfection, cultures were labeled with [^{35}S]cysteine, lysed, and immunoprecipitated with rabbit polyclonal antisera raised against a recombinant Bel-1 protein (6, 19). Precipitated proteins were resolved by electrophoresis on discontinuous sodium dodecyl sulfate–10% acrylamide gels and visualized by autoradiography. This rabbit antiserum recognizes not only the ~36-kDa Bel-1 protein but also the full-length (~54-kDa) Bet protein and the truncated (~31-kDa) ΔBet protein. The protein molecular mass markers (M) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) are of the indicated size in kilodaltons. (A) Cells transfected with pBel-1 (lane 1), pcΔBet (lane 2), pBel-1+2+3 (lane 3), and pcBet (lane 4). (B) Cells transfected with pBel-1 (lane 2) and mutations introduced in the N-terminal region (lanes 3 to 9). (C) Cells transfected with pBel-1 (lane 1) and mutations introduced in the C-terminal region (lanes 2 to 8). (D) Cells transfected with pBel-1 (lane 1) and mutations formed by excision of sequences between missense mutants (lanes 2 to 8).

goat anti-rabbit immunoglobulin G (Boehringer Mannheim Inc., Indianapolis, Ind.) was used at a 1:50 dilution.

RESULTS

The *bel-1* gene of HFV is located between the viral envelope gene and the 3′ LTR and overlaps extensively with a second HFV ORF termed *bel-2* (Fig. 1A) (8, 27). In HFV-infected cells, this region of the viral genome is expressed both as an unspliced mRNA that encodes the ~36-kDa Bel-1 protein and as a spliced mRNA that encodes a ~54-kDa protein, termed Bet, consisting of the first 88 amino acids of Bel-1 attached to 392 amino acids derived from the Bel-2 ORF (Fig. 1A) (8, 19, 27). Although the role of the Bet protein in the life cycle of HFV remains unclear, it is known that Bet is a cytoplasmic protein that is expressed at high levels in HFV-infected cells (7, 19).

The pBel-1+2+3 plasmid directs expression of a segment of the HFV genome that precisely spans the Bel-1 and Bel-2 ORFs as well as a third potential ORF, termed Bel-3, that overlaps with the C-terminal domain of Bel-2 (15). This expression plasmid is therefore predicted to encode both Bel-1 and the full-length Bet protein. An expression plasmid, termed pcBet, containing a "prespliced" or cDNA form of the same region of the HFV genome is, in contrast, predicted to express exclusively the Bet gene product. The pBel-1 expression plasmid also contains the entire *bel-1* gene but truncates Bet immediately 3′ to the Bel-1 translation termination codon (Fig. 1A). The parental pBel-1 plasmid is therefore predicted to encode the full-length, 300-amino-acid Bel-1 protein as well as a presumably defective, truncated Bet (ΔBet) protein consisting of the first 88 amino acids of Bel-1 joined to 112 amino acids derived from *bel-2* and 40 amino acids contributed by flanking vector sequences (Fig. 1A). A similar cDNA expression construct, termed pcΔBet, is predicted to express ΔBet exclusively.

Immunoprecipitation analysis of COS cells transfected with these four expression plasmids, using a rabbit antiserum specific for the shared N-terminal region of Bel-1, provided data consistent with these predictions (Fig. 2A). In particular, both pBel-1+2+3 and pBel-1 induced the expression of a ~36 kDa protein that was not detected in cells transfected with pcBet or pcΔBet. Both pBel-1+2+3 and pcBet also gave rise to high levels of expression of the ~54-kDa Bet protein whereas both pBel-1 and pcΔBet induced the expression of a protein that exhibited a mobility consistent with that predicted for the truncated ΔBet protein (~31 kDa). A minor ~40-kDa species observed only in the pBel-1+2+3- and pBel-1-transfected cell cultures appears likely to represent a posttranslationally modified form of Bel-1.

Mutational analysis of Bel-1. As a first step toward defining the functional organization of the HFV Bel-1 *trans* activator, we introduced 15 distinct missense mutations at various sites within the *bel-1* gene present in pBel-1 (Fig. 1B; Table 1). Each mutant was named according to the position of the first Bel-1 amino acid changed by the introduced mutation. The

TABLE 2. Relative activity of wild-type and mutant Bel-1 and Bet proteins

Protein	Activity[a] when used with indicator construct:	
	HFV LTR	HIV-1 LTR
Bel-1	100	100
Bet	<1	<1
ΔBet	<1	<1
M4	70 ± 10	96 ± 20
M24	118 ± 19	76 ± 11
M47	133 ± 7	74 ± 30
M93	<1	<1
M119	<1	<1
M145	<1	<1
M169	<1	<1
M191	90 ± 25	80 ± 50
M197	<1	<1
M219	40 ± 9	77 ± 48
M247	84 ± 7	93 ± 19
M266	70 ± 13	97 ± 33
M273	84 ± 9	94 ± 28
M281	81 ± 12	102 ± 21
M296	96 ± 16	77 ± 30

[a] The relative activity of each Bel-1 or Bet protein was assayed by transfection into COS cells together with constructs containing either the entire HFV LTR (pBC12/HSRV/SEAP) or an HIV-1 LTR lacking NF-κB sites (pMκB/SEAP) linked to the SEAP indicator gene (3, 14). *trans* activation is given relative to the activity of the wild-type Bel-1 expression vector, pBel-1, which is arbitrarily set at 100. These data represent the average of three transfection experiments. In a representative experiment, the supernatant SEAP activity of the basal HFV LTR promoter was 1.51 mOD/min (OD is optical density unit) and the Bel-1 *trans*-activated activity was 1,547 mOD/min while the basal activity of the NF-κB⁻ HIV-1 LTR was 1.06 mOD/min and the *trans*-activated activity was 175.4 mOD/min.

TABLE 3. Relative activity of Bel-1 deletion and substitution mutants

Protein	Activity[a] when used with indicator construct:	
	HFV LTR	HIV-1 LTR
N-terminal deletions		
Δ4/24	26 ± 5	72 ± 22
Δ24/47	107 ± 33	60 ± 29
Δ4/47	2.5 ± 0.7	13 ± 10
C-terminal deletions		
Δ247/266	20 ± 2	25 ± 17
Δ266/273	64 ± 15	104 ± 52
Δ266/281	11 ± 2	3.8 ± 3.9
Δ281/296	94 ± 22	86 ± 38
Δ247/296	<1	<1
Chimeric proteins		
Δ247/VP16	2.2 ± 0.4	178 ± 34
Δ247/VP16/M93	<1	<1
Δ247/VP16/M119	<1	<1
Δ247/VP16/M145	<1	<1
Δ247/VP16/M169	<1	<1
Δ247/VP16/M197	<1	<1

[a] The relative activity of each Bel-1 mutant was assayed as described in Table 2, footnote *a*.

first 88 amino acids of Bel-1 are shared with Bet, whereas the sequence encoding the last 111 amino acids of Bel-1 also encodes part of Bet in a different reading frame (Fig. 1A). Mutations introduced into either of these sequences are therefore predicted to affect both Bel-1 and Bet. One of these mutations, termed M191, was in fact designed to truncate Bet at the very beginning of the Bel-2 ORF (see below). In contrast, mutations introduced between Bel-1 positions 89 and 188 are predicted to affect Bel-1 only.

We have previously reported (14, 15) that Bel-1, when expressed from either the pBel-1+2+3 or the pBel-1 expression plasmid, can activate the expression of indicator genes linked to the HFV LTR by 500- to 1,000-fold and can activate the expression of indicator genes linked to an HIV-1 LTR lacking functional NF-κB sites by between 100- and 200-fold, when assayed by transient transfection in COS cells. In contrast, neither the full-length Bet protein encoded by pcBet nor the truncated Bet protein encoded by pcΔBet was able to exert any detectable effect on gene expression directed by these two retroviral promoter elements (Table 2).

This cell culture-based assay system was used to assess the ability of the various missense mutants of Bel-1 to *trans* activate gene expression directed by the HFV or the NF-κB⁻ HIV-1 LTR promoter elements. Data derived from three distinct transfection assays are presented in Table 2 and summarized in Table 1. Mutations introduced into the N-terminal (M4, M27, and M47) or C-terminal (M247, M266, M273, M281, and M296) region of Bel-1 had little or no effect on Bel-1 function. In contrast, mutations introduced into the Bel-1 specific central region (M93, M119, M145, and M169) completely inhibited Bel-1 function on either LTR target sequence, as did mutation M197. The M191 mutation, which is predicted to preclude expression of the Bel-2 component of Bet, failed to affect Bel-1 function detectably. A final missense mutant, M219, was unusual in that it displayed partial activity on the HFV LTR. However, M219 displayed essentially wild-type Bel-1 activity when tested on the HIV-1 LTR indicator construct (Table 2).

We next generated a series of N-terminal or C-terminal deletion mutants by excision of Bel-1 sequences between adjacent missense mutants that displayed wild-type phenotypes (Table 3). Deletion of sequences located between M4 and M24 (Δ4/24) or between M24 and M47 (Δ24/47) had a relatively minor effect on Bel-1 function. However, removal of this entire sequence (Δ4/47) did reduce Bel-1 activity significantly. A series of deletion mutations targeted to the C-terminal domain of Bel-1 produced contrasting results (Table 3). Although two mutants, Δ266/273 and Δ281/296, displayed essentially wild-type activity, two other deletions produced significant (Δ247/266) or marked (Δ266/281) reductions in Bel-1 activity. Interestingly, this latter mutant lacks only seven more amino acids than the active Δ266/273 construct. Deletion of a large part of the Bel-1 C-terminal region, in Δ247/296, abrogated Bel-1 activity entirely.

Immunoprecipitation analysis was used to assess whether the mutations introduced into Bel-1 significantly affected the level of Bel-1 protein expression in transfected cells. With the exception of M191 (see below), all plasmids expressing active Bel-1 missense mutants were observed to induce a protein expression pattern closely comparable to that observed with the wild-type pBel-1 plasmid (Fig. 2B and C). However, there was some variation in the relative level of expression of the ~36-kDa and the ~40-kDa form of Bel-1. The M191 mutation, which is predicted to truncate Bet but not Bel-1, prevented expression of the ~31-kDa ΔBet protein but did not affect the migration of the Bel-1 protein (Fig. 2C, lane 2). However, this mutation did reduce the level of expression of this fully active Bel-1 protein. Remarkably, all the plasmids expressing negative Bel-1 missense mutants yielded a distinct pattern marked by the significant overexpression of ΔBet and the appearance of a novel protein

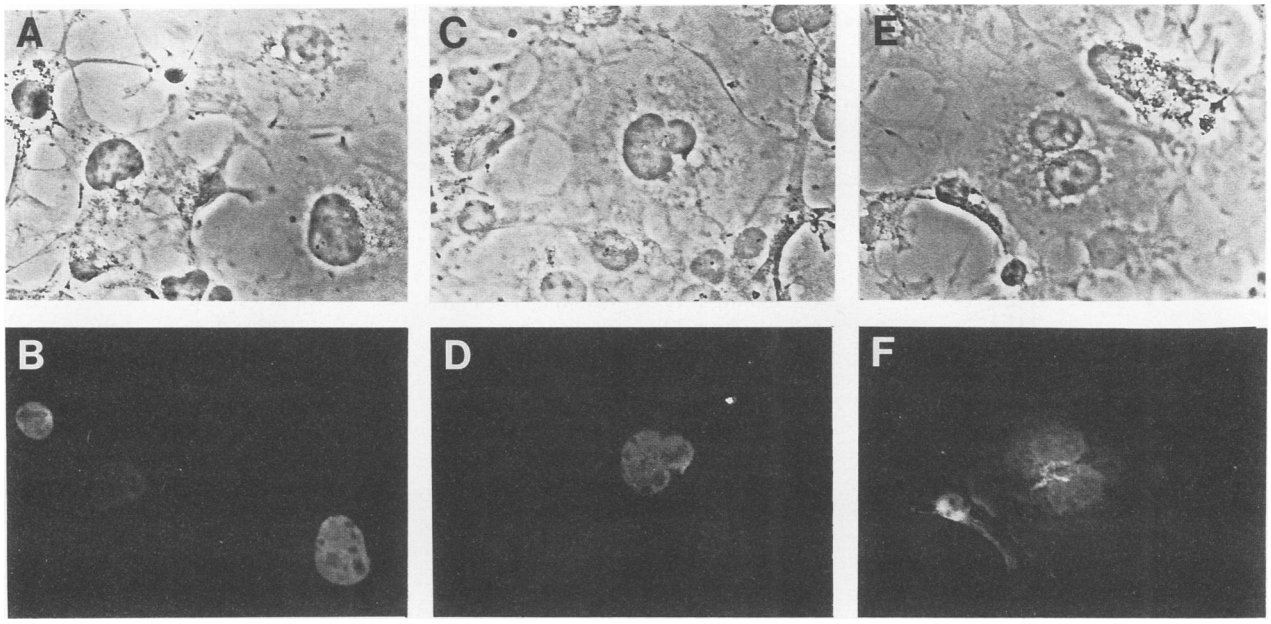

FIG. 3. Subcellular localization of selected Bel-1 mutants. Phase-contrast (A, C, and E) and corresponding immunofluorescence (B, D, and F) photographs of COS cells transfected with plasmids expressing the M197 (A and B), M93 (C and D) or M219 (E and F) mutants of Bel-1. Cells were fixed, permeabilized, and stained at ~70 h after transfection with a rabbit polyclonal antiserum specific for Bel-1, as previously described (6, 21). The M197 mutant is localized to the nucleus and yields a pattern indistinguishable from wild-type Bel-1 (panel B). The M93 mutant, although concentrated in the nucleus, nevertheless gives rise to clearly detectable cytoplasmic fluorescence (panel D). The M219 mutant is diffusely distributed throughout expressing cells (panel F).

species that migrated slightly more rapidly than the 36-kDa form of Bel-1 (Fig. 2B, lanes 5 to 8, and 2C, lane 3).

Deletions introduced into the N terminus of Bel-1, and particularly the Δ4/47 mutation, markedly reduced the level of immunoprecipitated Bel-1 and ΔBet protein (Fig. 2D). Although this may suggest that these HFV proteins are significantly destabilized by these deletion mutations, it is also possible that these truncated proteins are simply poorly recognized by the Bel-1-specific rabbit antiserum. Deletions within the C-terminal region of Bel-1 had, in contrast, only a minimal effect on the level of recovery of the major form of Bel-1. However, some of these mutations did affect the relative synthesis of the more slowly migrating form of Bel-1 (Fig. 2D, lanes 5 to 8).

Subcellular localization of Bel-1 mutants. It has previously been shown that Bel-1 is localized to the nucleus of expressing cells (15, 19). We therefore examined all negative Bel-1 missense mutants, as well as selected positive mutants, to see whether they continued to display nuclear localization and to confirm that they could accumulate to an intracellular level comparable to that seen with the wild-type protein. Representative immunofluorescence photographs of transfected COS cells are shown in Fig. 3, and these data are summarized in Table 1.

Most of the inactive Bel-1 mutants displayed a subcellular localization indistinguishable from that of the wild type (Fig. 3B). However, both M93 and M119 gave a low level of cytoplasmic fluorescence (Fig. 3D). Interestingly, M219, which retains a high level of Bel-1 activity, displayed a marked cytoplasmic fluorescence, although the M219 protein did remain detectable in the cell nucleus (Fig. 3E).

Identification of the Bel-1 NLS. Two sequences within Bel-1, localized between positions 193 and 200 and between positions 214 and 223, display the highly basic character typical of an NLS (12, 31) (Fig. 1). The observation that M219 was biologically active but aberrant in its subcellular localization whereas the inactive M197 mutant retained a wild-type distribution pattern (Fig. 3) suggested that the more C-terminal of these sequences was likely to be the Bel-1 NLS.

The prokaryotic enzyme β-gal localizes to the cytoplasm when expressed in eukaryotic cells (12). However, β-gal will localize to the nucleus if it is fused to a functional NLS sequence (12). To identify the Bel-1 NLS directly, we fused Bel-1 sequences derived from positions 193 to 208 or from positions 209 to 226 to the N terminus of the β-gal ORF present in the eukaryotic expression vector pCMV/β-gal to give, respectively, pCMV/β-gal(193–208) and pCMV/β-gal(209–226). We then used immunofluorescence analysis to compare the subcellular localization of these chimeric β-gal derivatives with that observed for the wild-type β-gal. As shown in Fig. 4, both β-gal and the β-gal(193–208) proteins were preferentially localized to the cytoplasm of transfected COS cells. In contrast, the β-gal(209–226) protein (Fig. 4D) gave a pattern of nuclear fluorescence closely similar to that observed with Bel-1 itself (Fig. 3B). We therefore conclude that the basic amino acid sequence located between positions 209 and 226 in Bel-1 is both necessary and sufficient for the nuclear localization of Bel-1.

***trans*-dominant Inhibition of Bel-1 Function.** Inactive mutant forms of transcriptional regulatory proteins can exert a *trans*-dominant negative effect on the function of the wild-type protein (11). To test whether the negative Bel-1 mutants described in Table 1 could exert such an inhibitory effect, we transfected COS cells with plasmids containing the HFV LTR, the NF-κB$^-$ HIV-1 LTR, or, as a control, the CMV-IE promoter, linked to the SEAP indicator gene. These indicator constructs were cotransfected with a low level of the wild-type Bel-1 expression vector pBel-1 together with a 10-fold excess of a wild-type or defective mutant Bel-1

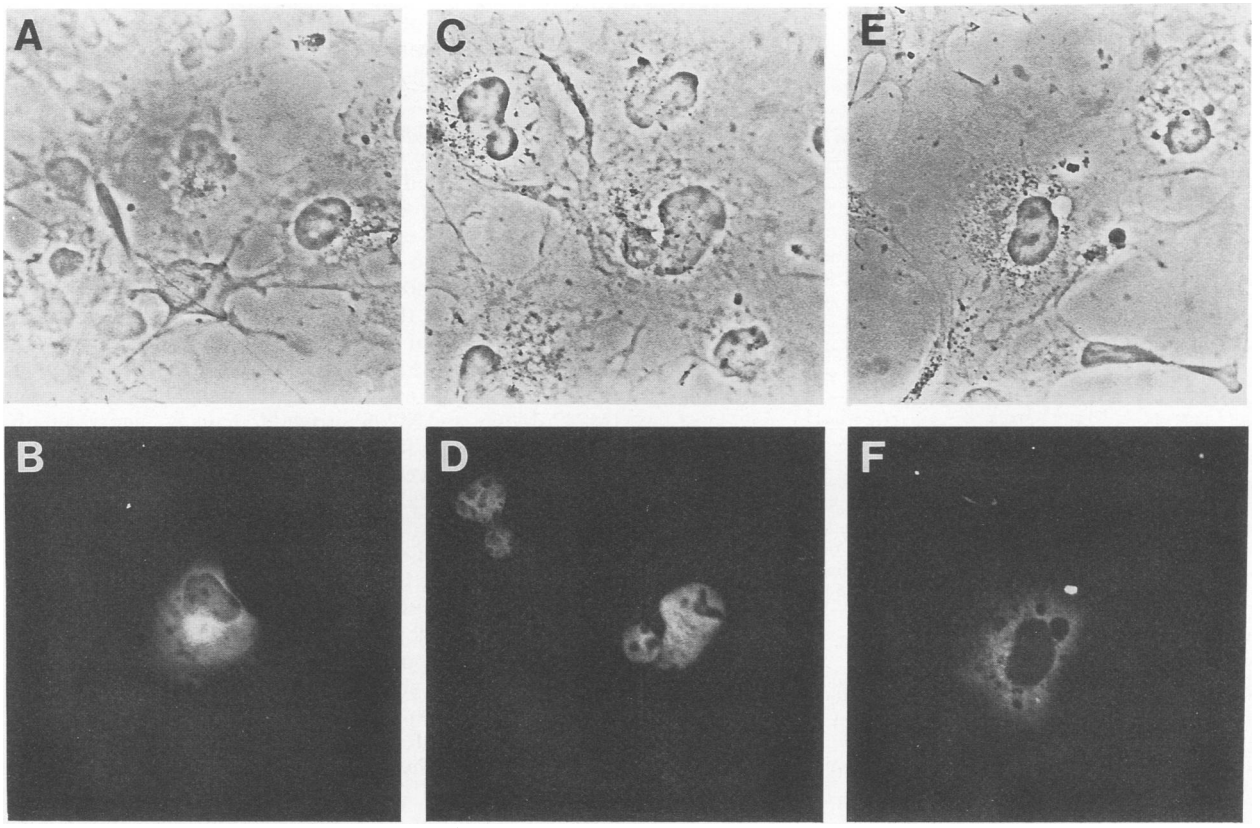

FIG. 4. Subcellular localization of β-gal derivatives. Phase-contrast (A, C, and E) and corresponding immunofluorescence (B, D, and F) photographs of COS cells transfected with plasmids expressing wild-type β-gal (A and B) or expressing β-gal fused to Bel-1 amino acids 209 to 226 (C and D) or 193 to 208 (E and F). Cells were fixed, permeabilized, and stained at ~70 h after transfection with an affinity-purified rabbit anti-β-gal antiserum. Both wild-type β-gal and the β-gal(193–208) proteins are localized predominantly to the cell cytoplasm (panels B and F). The β-gal(209–226) protein, in contrast, yields a nuclear pattern of fluorescence (panel D) that is very similar to that seen with the wild-type protein Bel-1 (Fig. 3B).

expression plasmid (Table 4). This approach was also used to examine whether the full-length or truncated forms of the HFV Bet protein encoded by the pcBet and pcΔBet expression plasmids would affect *trans* activation by Bel-1.

Expression of additional wild-type Bel-1 protein enhanced HFV LTR-driven expression by ~sixfold and HIV-1 LTR-specific gene expression by ~twofold. In contrast, all the negative Bel-1 missense mutants exerted an inhibitory effect. M93 and M119 inhibited gene expression from either LTR by 2- to 3-fold, whereas M145, M169, and M197 inhibited Bel-1 *trans* activation of the HFV LTR by 3- to 5-fold and activation of the HIV-1 LTR by ~10-fold. However, these mutant Bel-1 expression vectors had no significant effect on gene expression driven by the CMV-IE promoter, thus demonstrating that this *trans*-dominant inhibitory effect was specific for Bel-1. The possibility that modulation of the level of expression of the truncated Bet (ΔBet) protein encoded by these vectors (Fig. 2) contributed to this inhibition was excluded by the demonstration that neither ΔBet nor the full-length Bet protein was able to affect Bel-1 function significantly (Table 4).

We also analyzed the ability of a ~10-fold excess of the negative Bel-1 deletion mutant Δ247/296 to inhibit the wild-type Bel-1 protein. Δ247/296 proved able to inhibit Bel-1 activation of the HFV LTR ~5-fold and of the HIV-1 LTR ~10-fold. However, this deletion mutant also inhibited the CMV-IE promoter by ~2-fold, raising the possibility that at least part of this inhibitory effect is nonspecific.

Activity of Bel-1/VP16 chimeras. DNA sequence-specific *trans* activators can frequently be dissected into two discrete functional domains (13, 25, 33). Sequence specificity is conferred by a "binding domain" that mediates the direct or indirect interaction of the transcription factor with the appropriate DNA target sites. A second domain, the transcription activation domain, permits the functional interaction of the *trans* activator with a component(s) of the cellular transcription machinery. One of the most potent activation domains has been mapped to the C-terminal 78 amino acids of the VP16 transcriptional *trans*-activator encoded by HSV (33).

For reasons discussed in more detail below, we hypothesized that the C-terminal domain of Bel-1 was also likely to be a transcription activation domain. As a test of this hypothesis, we asked whether the inactive Δ247/296 mutant could be rescued by substitution of the 78-amino-acid C terminus of VP16 in place of the C-terminal 54 amino acids of Bel-1. For the HFV LTR, the resultant Δ247/VP16 fusion protein induced a ~18-fold *trans* activation that, although readily detectable, was markedly lower than the ~800-fold increase observed for the wild-type Bel-1 protein. In contrast, for the HIV-1 LTR, Δ247/VP16 induced a level of *trans* activation (~280-fold) that was actually significantly higher than seen with the wild-type Bel-1 protein. We therefore conclude that a Bel-1 protein lacking the C-terminal ~54 amino acids retains the ability to specifically interact with its HFV and HIV-1 LTR target sequence but lacks the ability to

TABLE 4. Analysis of *trans* dominance of various Bel-1 and Bet proteins

Clone	Activity[a] when used with indicator construct:		
	HFV LTR	HIV-1 LTR	CMV-IE
Bel-1 + BC12/CMV	100	100	100
Bel-1 + Bel-1	626 ± 95	214 ± 41	74
Bel-1 + Bet	145 ± 49	141 ± 48	136
Bel-1 + ΔBet	161 ± 57	140 ± 56	176
Bel-1 + M93	47 ± 4	47 ± 13	116
Bel-1 + M119	50 ± 9	31 ± 2	109
Bel-1 + M145	27 ± 8	11 ± 2	118
Bel-1 + M169	36 ± 5	11 ± 4	132
Bel-1 + M197	22 ± 1	12 ± 5	96
Bel-1 + Δ247/296	26 ± 6	8 ± 2	44

[a] COS cell cultures (35 mm) were transfected (6) with 250 ng of a plasmid containing the HFV LTR (pBC12/HSRV/SEAP), an NF-κB⁻ HIV-1 LTR (pMκB/SEAP), or the CMV-IE promoter (pBC12/CMV/SEAP) linked to the SEAP indicator gene (3). Each culture was also transfected with 25 ng of the pBel-1 plasmid and with a 10-fold excess (250 ng) of the negative control plasmid pBC12/CMV or the indicated Bel-1 or Bet protein expression plasmid. These data represent the average of three (HFV and HIV-1 LTR) or two (CMV-IE promoter) separate experiments.

activate once bound. Activation can be partly (HFV) or completely (HIV-1) restored by substitution of the heterologous VP16 activation domain in *cis*.

The demonstration that the N-terminal 250 amino acids of Bel-1 do not contain an effective activation domain but do retain a functional binding domain suggested that the phenotypically similar mutants of Bel-1 derived by introduction of missense mutations between positions 93 and 197 might well represent the converse, i.e., might retain a functional activation domain but lack a binding domain. To test this hypothesis, we introduced each of these point mutations (i.e., M93, M119, M145, M169, and M197) into the context of the Δ247/VP16 chimera. As shown in Table 3, these proteins were all found to be completely inactive on both the HFV and the HIV-1 LTRs.

DISCUSSION

We have used mutational analysis to define sequence elements within the HFV Bel-1 protein that contribute to the biological activity of this novel retroviral regulatory protein. Our observations have led to the identification and partial delineation of three functional domains within Bel-1, i.e., the transcription activation domain, the binding or specificity domain, and the NLS (Fig. 5).

Bel-1 activation domain. Sequence comparisons between the HFV Bel-1 protein and the equivalent *trans* activators encoded by simian foamy virus types 1 and 3 reveal only a modest level of conservation (24, 29). By far the most highly conserved sequence is a ~15-amino-acid motif located proximal to the C terminus of each protein (Fig. 6). Bel-1 is known to be active in a wide range of both mammalian and nonmammalian cell types (15). This suggested that the cellular proteins involved in mediating Bel-1 function and, by extension, the activation domain of Bel-1 itself were likely to have been highly conserved during evolution. We therefore hypothesized that this conserved C-terminal motif was likely to form the core of a Bel-1 activation sequence.

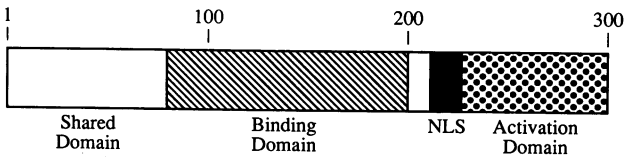

FIG. 5. Functional organization of the HFV Bel-1 *trans* activator. The approximate domain organization of the Bel-1 protein is indicated. Given the resolution of the mutational analysis presented here, the borders of each domain should be considered no more than approximate. "Shared Domain" refers to the fact that the first 88 amino acids of Bel-1 also form the N terminus of Bet and may well serve a critical function in this latter protein.

Despite the tight conservation of this C-terminal Bel-1 sequence, missense mutations introduced either directly into this motif (M273 and M281) or into adjacent sequences (M247, M266, and M296) proved to have no significant effect on Bel-1 function (Table 2). However, deletion of the major part of this conserved sequence, in Δ266/281, or of this entire C-terminal domain, in Δ247/296, did severely reduce Bel-1 activity (Table 3).

If the C-terminal domain of Bel-1 indeed forms a discrete activation domain, the nonfunctional Bel-1 deletion mutant, Δ247/296, should retain the ability to interact with its DNA target sites but lack the ability to activate transcription once bound (13, 25). A prediction of this hypothesis is that the Δ247/296 mutant should prove capable of inhibiting the wild-type Bel-1 protein when present in *trans* (11, 21); this was indeed found to be the case (Table 4). A second prediction of this hypothesis is that substitution of a heterologous activation domain in place of the deleted Bel-1 C terminus should at least partly reconstitute the biological activity of the inactive Δ247/296 deletion mutant. As shown in Table 3, attachment of the 78-amino-acid C-terminal activation domain of VP16 at Bel-1 amino acid 247 did in fact partially (on the HFV LTR) or completely (on the HIV-1 LTR) restore *trans* activation. Because the VP16 activation domain must be bound to a promoter element to activate transcription (25, 28, 33), we can also deduce from this observation that Bel-1 either binds directly to its DNA target sequences or is indirectly bound by the action of a cellular cofactor. The hypothesis that Bel-1 sequences proximal to the C terminus include a discrete activation domain has now been further validated by our recent observation that a fusion protein derived by attachment of this sequence to the DNA-binding motif of GAL4 can efficiently activate a promoter that contains GAL4 DNA-binding sites (10, 13). The observation that complete abrogation of Bel-1 function requires the deletion of a large part of this C-terminal sequence (Table 3) implies that the Bel-1 activation domain displays partial internal functional redundancy, a property previously reported for the C-terminal activation domain of VP16 (5).

Bel-1 specificity domain. The observation that the Δ247/VP16 fusion protein can activate both HFV and HIV-1 LTR-specific gene expression demonstrates that the N-terminal 246 amino acids of Bel-1 are sufficient to target this chimeric protein to these DNA sequences. It should be noted, however, that these data do not address the relative affinity of the Δ247/VP16 and wild-type Bel-1 proteins for these LTR target sites. A reduction in the efficiency of binding of the Δ247/VP16 mutant to the HFV LTR, but not to the HIV-1 LTR, might well explain the relatively low level of activity of this chimeric protein when tested on the former. However, it is also possible that the HFV LTR is simply less responsive than the HIV-1 LTR to the VP16 activation domain (28).

The mutational analysis of Bel-1 described in Tables 1 and

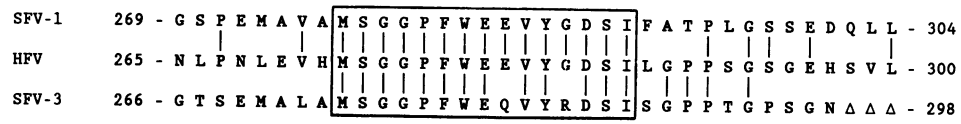

FIG. 6. Sequence comparison of the C-terminal domains of foamy virus transcriptional activators. The Bel-1 protein of HFV shows only limited sequence homology to the equivalent proteins encoded by simian foamy virus type 1 (~39% homology) or type 3 (~34% homology) (24, 29). However, a highly conserved stretch of amino acids is located toward the C terminus of each protein and is indicated here. Although our observations are consistent with the hypothesis that this conserved sequence forms the core of the activation domain of these related viral regulatory proteins, it should also be noted that this sequence does not resemble known eukaryotic transcription activation motifs (25).

2 identified five missense mutations, located between Bel-1 amino acids 93 and 198, that lacked detectable biological activity. We believe that these mutations all lie within a single Bel-1 functional domain, i.e., the specificity domain. In particular, the observation that each of these mutations abolishes the ability of the Δ247/VP16 fusion protein to activate gene expression from both the HFV and HIV-1 LTRs (Table 3) clearly demonstrates that these mutations can prevent the appropriate targeting of the intact VP16 activation motif present in Δ247/VP16.

An interesting feature of the five inactive missense mutants, M93, M119, M145, M169, and M197, is that they all induce a novel pattern of HFV gene expression from the pBel-1 plasmid (Fig. 2B and C). Of note, these mutations each led to the overexpression of the ~31-kDa ΔBet protein and to the appearance of a novel, presumably Bel-1-related, protein species of ~35 kDa. Because four of these five mutations are not included in Bet (Fig. 1A), this cannot be a direct effect on the stability of this truncated protein. Bet is localized exclusively to the cytoplasm of expressing cells (7, 19) and does not play any evident role in mediating Bel-1 function (Tables 2 and 4). A direct interaction between Bel-1 and Bet, although possible, therefore seems unlikely. Although the origin of this interesting phenomenon is therefore unclear, this shared phenotype does appear consistent with the hypothesis that these mutations are each affecting Bel-1 function in a similar manner.

A mutant transcriptional *trans* activator that retains a functional activation domain but has lost the ability to interact with its DNA target site can inhibit the wild-type protein in *trans* by sequestering cellular cofactors off the DNA, a phenomenon termed squelching (28). Analysis of the five inactive missense mutants demonstrates that they are indeed able to specifically inhibit Bel-1 function when present in *trans* (Table 4). It is of interest that the M93 and M119 mutants, which do not localize efficiently to the cell nucleus (Table 1), also inhibit the wild-type Bel-1 protein less effectively than the entirely nuclear M145, M169, and M197 mutants do. This may suggest that these two mutants are interacting with a nuclear cofactor that is important for transcriptional activation by Bel-1.

Bel-1 NLS. NLS sequences are normally highly basic and are also frequently proline rich (12, 31). On this basis, two sequences within Bel-1, located between positions 193 and 200 and between positions 214 and 223, were identified as candidate NLS sequence. Missense mutations introduced into these sequence elements proved to have distinct phenotypes. Disruption of the 193/200 sequence, in M197, produced an inactive Bel-1 protein displaying a normal subcellular location. In contrast, disruption of the 214/223 sequence, in M219, produced a partly active Bel-1 protein displaying a diffuse pattern of subcellular immunofluorescence (Table 1; Fig. 3). Direct confirmation that the 214/223 sequence did indeed form the Bel-1 NLS was provided by the demonstration that a peptide that included this sequence element, but not an equivalently sized peptide that included the 193/200 sequence, was sufficient to confer nuclear localization on the normally cytoplasmic β-gal protein (Fig. 4). The observation that mutations introduced into the NLS had only a moderate effect on Bel-1 activity suggests that the NLS is unlikely to serve a second essential role in mediating Bel-1 function, for example as part of the binding domain of Bel-1.

N-terminal sequences in Bel-1. It has previously been noted (24) that the N-terminal region of Bel-1 displays an unusually high concentration of acidic amino acids, a property also observed in the "acidic-blob" family of activation domains exemplified by VP16 (5, 25, 28). However, the observation that the inactive Δ247/296 mutant, which retains the Bel-1 N terminus, could be rescued by the addition of the VP16 acidic activation domain demonstrates that the Bel-1 N terminus is not functionally analogous. Indeed, the resilience of Bel-1 activity in the face of extensive deletions of this N-terminal sequence (Table 3) may suggest that this region does not perform a critical role in Bel-1 *trans* activation. We note that the first 88 amino acids of Bel-1 are shared with Bet, a cytoplasmic protein that is expressed at very high levels in HFV-infected cells (19) and that presumably serves an important function in the HFV replication cycle. It is therefore possible that this N-terminal sequence is primarily important for Bet function.

Mechanism of *trans* activation by Bel-1. As noted above, *trans* activation of the HFV and HIV-1 LTRs by Bel-1 is mediated by LTR sequence elements that display no evident sequence homology (14, 17). Similarly, activation of transcription from the LTR promoters of HTLV-I and HIV-1 by the HTLV-I Tax protein requires DNA target sequences that are clearly distinct (1, 2, 9, 18, 32). It has been shown for the HTLV-I LTR that a complex consisting of Tax and a cellular cofactor binds directly to this DNA target sequence (2, 9, 22, 36). In contrast, Tax *trans*-activation of the HIV-1 LTR appears to result from the posttranslational activation of the cellular transcription factor NF-κB (1, 18). It has proven possible to functionally segregate these distinct biological activities by introducing specific mutations into the Tax protein (32). We therefore asked whether any of the mutations described here could segregate the ability of Bel-1 to *trans* activate these distinct LTR promoter elements. As shown in Tables 2 and 3, this has not proven possible. In particular, all partially active Bel-1 mutants were equivalently active (≤twofold variation) when tested on either the HFV or HIV-1 LTR. Similarly, all negative Bel-1 mutants displayed *trans* dominance over Bel-1 when measured on either LTR target.

The single exception to the equivalent activity of the various Bel-1 mutants on the HFV and HIV-1 LTRs occurred with the Δ247/VP16 mutant. This chimeric protein was ~40-fold less active than Bel-1 on the HFV LTR but actually more active than Bel-1 on the HIV-1 LTR. As noted above, this could result from the inefficient interaction of the

truncated Δ247/VP16 protein with the HFV (but not the HIV-1) LTR or might reflect a lack of responsiveness of the basal HFV promoter to an acidic-blob *trans* activator such as VP16 (28). Despite this discrepancy, the Δ247/VP16 mutant actually represents the strongest argument for an equivalent mechanism of action of Bel-1 on both targets. In particular, it is known that the VP16 activation domain is functional only when bound, either directly or indirectly, to a DNA target (25, 28, 33). The fact that the Δ247/VP16 chimera is active on both the HFV and HIV-1 LTRs therefore demonstrates that Bel-1 itself is bound, either directly or indirectly, to these DNAs. These data are therefore most consistent with the hypothesis that Bel-1 binds to, and activates, the HFV and HIV-1 LTRs by similar mechanisms.

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