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Transactivation of Human Immunodeficiency Virus Type 1 Long Terminal Repeat-Directed Gene Expression by the Human Foamy Virus *bel1* Protein Requires a Specific DNA Sequence

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Human foamy virus (HFV) encodes the transcriptional transactivator *bel1*. The *bel1* protein transactivates HFV long terminal repeat (LTR)-directed gene expression by recognizing a region in U3. It also transactivates human immunodeficiency virus type 1 (HIV-1) LTR-directed gene expression in transient transfection assays. To identify the specific region in HIV-1 LTR responsible for *bel1* action, we examined the effect of *bel1* on chloramphenicol acetyltransferase (CAT) gene expression in transfected cells with a series of mutant HIV-1 LTR/CAT plasmids. The region between −158 and −118 from the transcription initiation site, immediately upstream of the core enhancer element, was identified as responsible for the transactivation by *bel1*. In addition, *bel1* transactivated a heterologous promoter when this region was positioned upstream of it in the sense and antisense orientations. Optimal transactivation of the HIV-1 LTR by *bel1* did not require an intact TAR sequence, suggesting that the binding of *tat* to the TAR sequence is not a prerequisite for *bel1* function in HIV-1 LTR-directed gene expression. In the region of the HIV-1 LTR that is necessary for the *bel1*-mediated transactivation, we have found a sequence which is conserved between HIV-1 and HFV. Our results suggest that the *bel1* action on HIV-1 seems to be mediated by a specific DNA sequence which is shared by both the HIV-1 LTR and HFV LTR.

The family *Retroviridae* consists of three subfamilies grouped according to biological and pathogenic properties: *Oncovirinae*, *Lentivirinae*, and *Spumavirinae* (9, 30). Oncoviruses are associated with malignant tumors, and lentiviruses are associated with slowly progressing inflammatory and degenerative disorders with long latency periods. Spumaviruses cause soapsudslike degeneration in infected cells. In humans, human T-cell leukemia virus type I (HTLV-I), a member of the *Oncovirinae*, causes T-cell leukemia and tropical spastic paraparesis. Human immunodeficiency virus type 1 (HIV-1), a member of the *Lentivirinae* subfamily, is the causative agent of AIDS. Human foamy virus (HFV), the genome of which has been molecularly cloned (8, 19, 24, 25), belongs to the *Spumavirinae* subfamily.

These human retroviruses encode transactivators required for their long terminal repeat (LTR)-specific gene expression. HTLV-I *tax*, encoded by the pX region, transactivates HTLV-I LTR-directed gene expression via a 21-bp direct repeat in U3 (6, 10, 14, 31, 34). HIV-1 *tat* is an RNA-specific transactivator which has been shown to interact directly with its target sequence, TAR (4, 5, 7, 28, 33, 36). TAR is located at the 5′ end of the HIV-1 transcript. HFV *bel1* is still poorly understood, but it appears to transactivate HFV LTR-directed gene expression mediated by a putative *bel1*-responsive element (BRE) located in the U3 region (16, 26, 27).

Gene expression of HIV-1 is regulated by cellular factors and by its own viral gene products, including the *tat* and *rev* proteins (reviewed in references 3 and 22). Also, transcriptional control sequences in the LTR interact with several cellular DNA-binding proteins, including SP1, NF-κB, LBP-1, NFAT-1, and TF-IID (reviewed in reference 15). HIV-1 gene expression is also regulated by exogenous factors, such as T-cell mitogens (35, 37), DNA damage (39), and heterologous viral transactivators (1, 11, 13, 20, 32, 35, 38), including HTLV-I *tax*. *tax* has been reported to induce the expression of certain cellular proteins which in turn transactivate HIV-1 LTR-directed gene expression by binding to the enhancer (1, 35). Here we report that HFV *bel1* transactivates HIV-1 LTR-directed gene expression, and the response requires the specific region in U3 of the HIV-1 LTR.

To determine whether any HFV gene product transactivates HIV-1 LTR-directed gene expression, BHK-21 cells were cotransfected with HFV proviral DNA and an HIV-1 LTR/chloramphenicol acetyltransferase (CAT) construct which contains the −453 to +80 region of the HIV-1 LTR upstream of the indicator CAT gene. A marked stimulation of HIV-1 LTR-directed CAT gene expression was observed in the cotransfection of HIV-1 CAT with pHSRV but not with mock DNA (Fig. 1). This finding indicates that HFV encodes a *trans*-acting factor that stimulates HIV-1 LTR-directed gene expression.

To investigate which gene product of HFV is necessary for HIV-1 LTR transactivation, deletions were introduced into pHSRV to inactivate *bel1* or *bel2* and *bel3* open reading frames (ORFs) (Fig. 1). A small deletion in the *bel1* ORF of pHSRVΔB completely abolished the transactivating ability. However, pHSRVΔB2-3, which has a small deletion in the *bel2* and *bel3* ORFs, showed nearly the same transactivating activity as did pHSRV. Furthermore, the *bel1* expression plasmid pS-bel1-S, which contains the entire *bel1* ORF downstream of the simian virus 40 (SV40) early promoter, retained the ability to stimulate HIV-1 LTR-directed gene expression at a somewhat lower level compared with pHSRV. The difference of the transactivation level between

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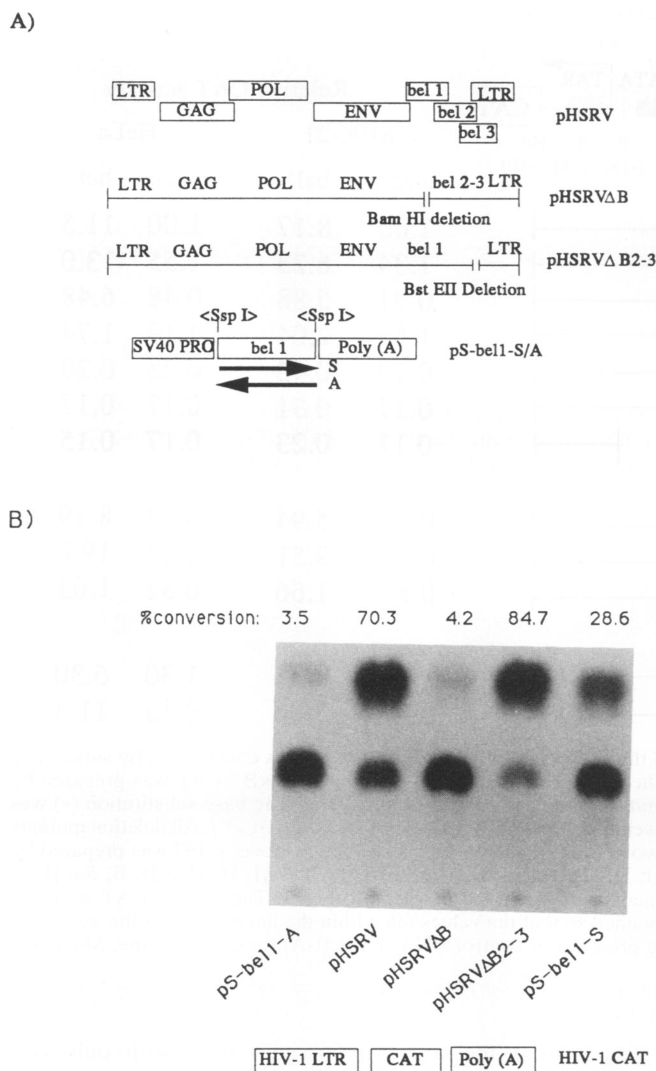


FIG. 1. Structures and transactivation abilities of the HFV deletion mutants and *bel1* expression plasmid. (A) pHSRV, which contains the full genome of HFV, is described elsewhere (24). Deletion mutants were made with available restriction endonuclease cleavage sites: pHSRVΔB and pHSRVΔB2-3 were constructed by deleting the 209-bp *Bam*HI-*Bam*HI fragment to inactivate the *bel1* ORF and the 263-bp *Bst*EII-*Bst*EII fragment to inactivate both *bel2* and *bel3* ORFs, respectively. pS-bel1-S and pS-bel1-A were constructed by inserting a 1,048-bp *Ssp*I fragment of pHSRV comprising the entire *bel1* ORF downstream of the SV40 early promoter in the sense and antisense orientation, respectively (26). (B) HIV-1 CAT, the HIV-1 3′ LTR-directed CAT gene expression plasmid, was cotransfected into BHK-21 cells with each plasmid indicated at the bottom. Cotransfections of HIV-1 CAT and the plasmids to be tested for transactivating ability were carried out with 2 μg of both DNAs. One million BHK-21 cells were transfected by using the DEAE-dextran method (23). Cells were treated with chloroquine and subjected to dimethyl sulfoxide shock as described previously (16). The percent conversion of chloramphenicol to its acetylated form was measured 48 h after transfection as described previously (12). A 30-min time point and equivalent amounts of protein lysates were used for all reactions. Data from at least two experiments done on different days were found to be reproducible within the error range of 20% standard deviation.

pHSRV and pS-bel1-S could be due to the different level of *bel1* gene expression (SV40 versus native HFV promoter and enhancer). HIV-1 LTR transactivation by cotransfection with the *bel1* expression plasmid was also observed in Jurkat human T cells and in COS monkey kidney cells at about a fivefold-higher level (data not shown). As a consequence, we concluded that the *bel1* protein among HFV gene products is responsible for the transactivation of HIV-1 LTR-directed gene expression. These results agree with the recent report by Keller et al. (16), who showed that HIV-1 LTR-directed gene expression is transactivated by cotransfection with a *bel1* expression plasmid in COS cells.

To determine which LTR sequences are critical to transactivation by *bel1*, we generated a series of deletions in the LTR and tested their ability to direct *bel1*-responsive transcription (Fig. 2). These deletion mutants were transfected into BHK-21 or HeLa cells in the presence or absence of a *bel1* expression vector, and transient expression of the CAT enzyme was measured. The ability of the LTR to respond to *bel1* was not affected significantly until the deletion reached 158 bp upstream from the RNA start site. Deletion of an additional 40 bp (to −118) did not lower basal CAT activity, but the LTR was not activated by *bel1*. Further deletions extending downstream from −118 resulted in considerably lower basal CAT activity relative to the wild-type plasmid and resulted in no response to *bel1*. Examination of the coordinates of these deletions indicates that an essential part of BRE is located between −158 and −118, the region just upstream from the nucleotide sequence interacting with the cellular transcription factor NF-κB (κB sites). To our knowledge, there are no known consensus regulatory sequences identified in this region.

We also tested whether NF-κB is needed for transactivation of the HIV-1 LTR by *bel1* (Fig. 2). For this experiment, BHK-21 and HeLa cells were transfected with HIV-1 LTR/CAT fusion constructs containing wild-type or mutated κB sites. It was previously shown that the mutations used in these constructs abolish NF-κB binding (21). Mutations in these sites lowered basal LTR activity two- to fourfold, but when a *bel1* expression vector was cotransfected, levels of CAT activity from these mutant LTR constructs were comparable to that from the wild-type LTR. Therefore, *bel1* apparently activates the HIV-1 LTR containing defective κB sites more efficiently than the wild-type LTR, in terms of magnitude of transactivation. When mutated κB sites were combined with the deletion mutations constructed as described above, the same region of the LTR (−158 to −118) was found to harbor the element interacting with the *bel1* protein (Fig. 2).

To test whether the TAR region of the HIV-1 LTR interacting with *tat* is necessary for transactivation by *bel1*, BHK-21 and HeLa cells were transfected with LTR/CAT constructs containing the deletion in the TAR region (ΔTAR; +34 to +80) (Fig. 2). The same deletion was previously shown to abolish transactivation of the LTR by *tat*. In both cell lines, the ΔTAR LTR construct was transactivated by *bel1* as efficiently as was the wild type. The same results were obtained when defective κB sites were combined with the ΔTAR LTR. These data indicate that TAR is not required for transactivation of the LTR by *bel1*. We were also interested in how *bel1* and *tat* interact with each other. In HeLa cells, *bel1* and *tat* transactivated LTR-directed expression 11- and 35-fold, respectively (Table 1). When both plasmids were transfected, CAT activity was increased more than 170-fold. Similar observations were made for BHK-21 cells. These results suggest that *bel1* and *tat* pro-

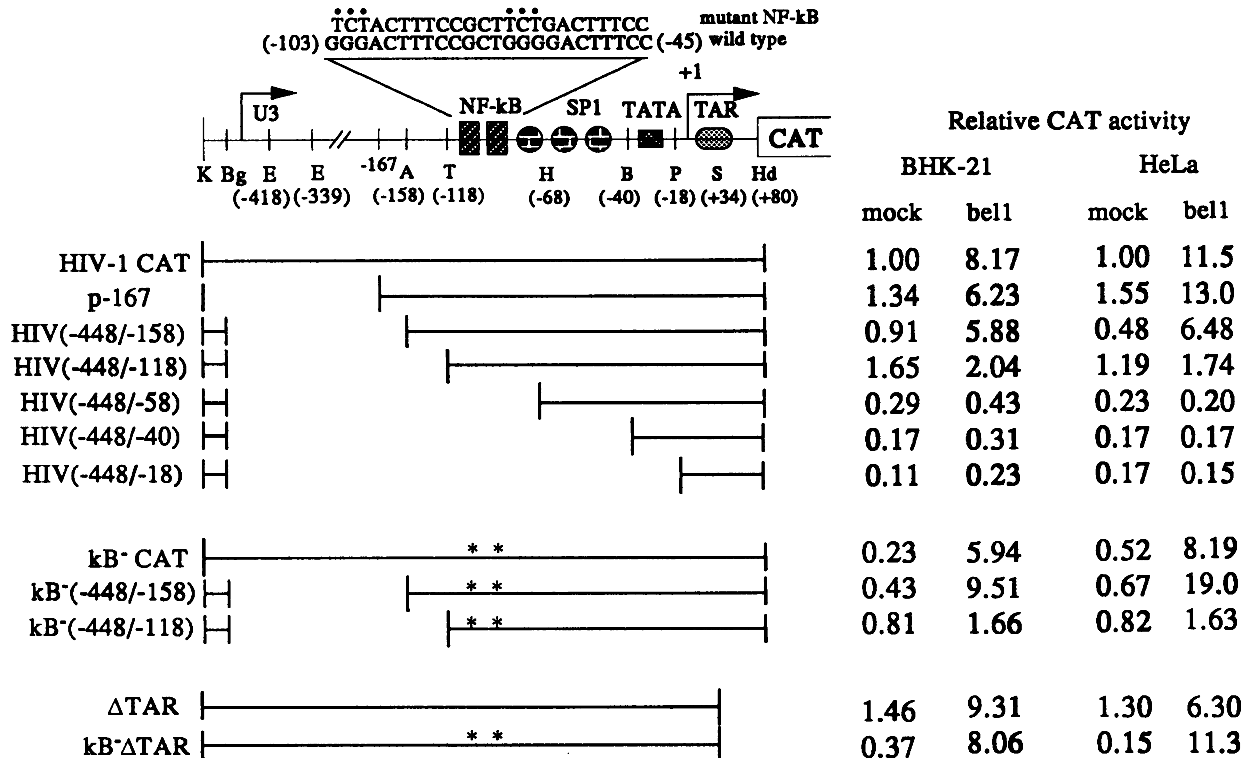


FIG. 2. Schematic diagram of wild-type and mutant HIV-1 LTRs and their responses to *bel1*. HIV-1 CAT was constructed by subcloning the *Kpn*I-*Hin*dIII fragment comprising the HIV-1 3′ LTR upstream of the CAT gene and SV40 poly(A) signal. κB⁻ CAT was prepared by changing the two NF-κB binding sites (GGG → TCT) by site-directed mutagenesis (21), as shown at the top. The base substitution (*) was confirmed by the dideoxy sequencing method (29) with the CAT gene antisense primer (5′-GGCCGTAATATCCAGC-3′). All deletion mutants except p-167 were constructed by deleting the sequences between the two restriction enzyme sites as shown above. p-167 was prepared by *Bal* 31 deletion of pU3RIII CAT (28). Restriction enzyme sites: K, *Kpn*I; Bg, *Bgl*II; E, *Eco*RV; A, *Ava*I; T, *Taq*I; H, *Hae*III; B, *Ban*II; P, *Pvu*II; S, *Sac*I; Hd, *Hin*dIII. Transfection and CAT assays were performed as described in the legend to Fig. 1. The relative CAT activities (percent conversion of chloramphenicol into its acetylated form) were obtained so that the values fell within the linear range of the assay and were normalized to the activity obtained with plasmid HIV-1 CAT in the presence of control DNA (pS-bel1-A) for each cell line. Mock and bel1 refer to pS-bel1-A and pS-bel1-S, respectively.

teins have an additive or cooperative effect in transactivation.

The foregoing studies identified the −158 to −118 region of the HIV-1 LTR as containing the nucleotide sequence interacting with *bel1*. To be certain that this region is indeed BRE, we fused the −158 to −118 region of the HIV-1 LTR upstream from the TATA box of the herpes simplex virus *tk* gene (Fig. 3A). In addition, we placed the −339 to −118 region of the HIV-1 LTR in the sense or antisense orientation. This truncated *tk* promoter contains only 37 bp upstream from the transcription start site, with only one transcription element, a TATA box (2). As a negative control, the identical plasmid lacking the putative BRE was used. The plasmids were transfected into BHK-21 cells with or without a *bel1* expression plasmid, and transient expression of the CAT enzyme was measured to test whether this DNA fragment indeed harbors BRE (Fig. 3). In the absence of the *bel1* expression vector, all four plasmids gave a background conversion of chloramphenicol to its acetylated forms of around 1.0% (Fig. 3B, lanes 2, 4, 6, and 8). When cells were cotransfected with a *bel1* expression vector, CAT activity was increased 5- to 10-fold with the test plasmids containing the putative BRE in either orientation (Fig. 3B, lanes 3, 5, and 7). Only a background level was detected in the absence of this nucleotide sequence. These data suggest that the −158 to −118 region of the HIV-1 LTR is sufficient for transactivation by *bel1* and that BRE functions independently of its orientation. Interestingly, when nucleotide sequences of the LTR from HIV-1 and HFV were aligned, the −124 to −116 region of the HIV-1 LTR (TGA CATCGA) was very similar to the −134 to −126 region of the HFV LTR (TGAGATCGA). Therefore, the HIV-1 LTR region that we identified as BRE contains this homologous sequence. No other distinct homology was found between the LTR sequences of HIV and HFV (our unpublished data).

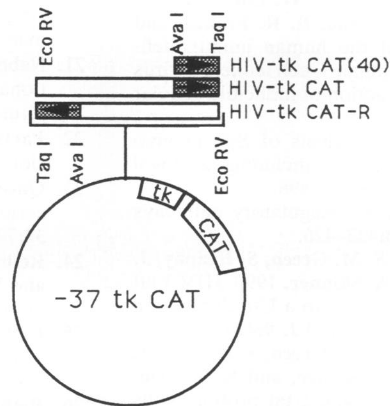
TABLE 1. Additive or cooperative activation of the HIV-1 LTR by *bel1* and *tat*[a]

Infection	Relative CAT activity[b]	
	BHK-21	HeLa
Mock	1.00	1.00
bel1	8.17	11.5
tat	5.31	35.7
bel1 + *tat*	30.6	172

[a] BHK-21 and HeLa cells were cotransfected with HIV-1 CAT and the indicated plasmids. Mock, *bel1*, and *tat* represent cotransfection with pS-bel1-A, pS-bel1-S, and cytomegalovirus *tat*, respectively.

[b] Values were obtained as described in the legend to Fig. 2.

A)



B)

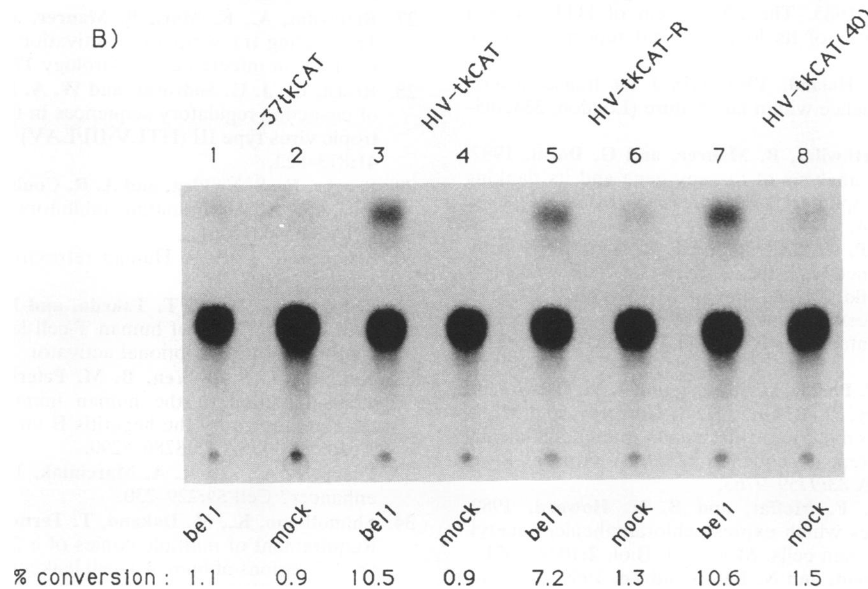


FIG. 3. Transactivation by *bel1* of the truncated herpes simplex virus *tk* promoter containing the putative BRE. (A) The *Eco*RV (−339)-*Taq*I (−118) fragment of the HIV-1 LTR was filled with Klenow enzyme and inserted into the *Sal*I site of −37tkCAT (2) in the sense or antisense orientation. HIV-tkCAT(40) was constructed by deleting the *Eco*RV-*Ava*I fragment from HIV-tkCAT. The inserted DNA sequence was confirmed by the dideoxy DNA sequencing method (29). The arrow indicates the orientation of the insert with respect to the CAT gene. (B) The indicated reporter plasmids (1 μg) were transfected into BHK-21 cells with 3 μg of pS-bel1-A (mock infection; lanes 2, 4, 6, and 8) or *bel1* expression plasmid pS-bel1-S (lanes 1, 3, 5, and 7). The CAT assay was performed subsequently as described in the legend to Fig. 1.

We are currently investigating whether this nanomer sequence is indeed a key element interacting with the *bel1* protein.

It has previously been shown that the HIV-1 LTR is transactivated by many regulatory proteins of other viruses (1, 11, 13, 20, 32, 35, 38). However, the action of *bel1* appears to be distinct from those of other viral proteins in that the target element is a specific DNA sequence located upstream from the κB sites. For example, the early gene products of herpes simplex virus, cytomegalovirus, and hepatitis B virus transactivate the HIV-1 LTR, but their responsive sequences have not been identified or have been mapped around the TATA box. We do not yet know whether the *bel1* protein interacts with the DNA sequence directly or indirectly via other cellular proteins. Whatever the mechanism, it is clear that *bel1* transactivates the HIV-1 LTR as efficiently as does *tat* in some cell types. Given the broad range of cells for HIV-1, including epithelial cells and fibroblasts, that are also susceptible to HFV, it would be important to determine the prevalence of HFV among HIV-1-infected individuals.

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