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Genetics

The transcriptional transactivator of human foamy virus maps to the *bel* 1 genomic region

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ABSTRACT **The human foamy virus (HFV) genome possesses three open reading frames (*bel* 1, 2, and 3) located between *env* and the 3′ long terminal repeat. By analogy to other human retroviruses this region was selected as the most likely candidate to encode the viral transactivator. Results presented here confirmed this and showed further that a deletion introduced only into the *bel* 1 open reading frame of a plasmid derived from an infectious molecular clone of HFV abolished transactivation. In contrast, deletions in *bel* 2 and *bel* 3 had only minor effects on the ability to transactivate. The role of the *bel* 1 genomic region as a transactivator was further investigated by eukaryotic expression of a genome fragment of HFV spanning the *bel* 1 open reading frame. A construct expressing *bel* 1 under control of a heterologous promoter was found to transactivate the HFV long terminal repeat in a dose-dependent fashion. Furthermore, it is shown that the U3 region of the HFV long terminal repeat is sufficient to respond to the HFV transactivator.**

Human foamy virus (HFV) is a member of the spumaviruses, the third subfamily of Retroviridae (1). It was isolated in 1971 from nasopharyngeal carcinoma cells, originating from Kenya (2). So far, HFV is the only foamy virus isolate completely sequenced and for which an infectious molecular clone exists (3–6). The genome organization and nucleotide sequence analysis of HFV reveal striking similarities to human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV). In addition to *gag*, *pol*, and *env*, the HFV genome has three open reading frames (ORFs) that are located between the *env* gene and the 3′ long terminal repeat (LTR), and these are designated *bel* 1, 2, and 3 (ref. 4; Fig. 1). In addition, there is limited but significant homology between the deduced amino acid sequences of *bel* 1 and *bel* 3 and HIV-2 *tat* and *nef*, respectively, which suggests that the *bel* ORFs might have regulatory functions as shown for HIV and HTLV (6–8). It has been reported recently that the activity of the HFV LTR is markedly enhanced in HFV-infected cells (9). Similar findings were described for the closely related simian foamy virus 1 (SFV-1) (10). In both studies evidence has been presented that the foamy virus transactivator acts on the LTR at the transcriptional level (9, 10).

The aim of this study was to identify the gene encoding the HFV transactivator. For this purpose we constructed deletion mutants of an infectious molecular clone, and plasmids expressing the *bel* 1 ORF were tested for their ability to transactivate the HFV LTR.

MATERIALS AND METHODS

Plasmid Constructions. pHSRV is an infectious molecular clone for HFV (6). $pHFV_{Af}$ was made by Klenow fill-in of an *Afl* II site in the integrase domain of the *pol* gene of pHSRV. Nucleotide sequence analysis revealed a premature stop codon at the modified *Afl* II site. Transfection with $pHFV_{Af}$ does not lead to progeny infectious virus, but expression of structural viral proteins can be observed as judged from indirect immunofluorescence using anti-HFV antiserum (data not shown). Plasmid pΔgpe was generated by removing a 2.3-kilobase-pair (kbp) *Nco* I fragment comprising *gag* and *pol* sequences and a 1.6-kbp *Hpa* I/*Pvu* II fragment comprising *env* sequences from pHSRV. $p\Delta gpe_B$ was generated by deleting a 208-base-pair (bp) *Bam*HI fragment from the *bel* 1 ORF of pΔgpe. $p\Delta gpe_{Bg}$ was designed as a *Bgl* II frameshift mutant in the *bel* 2 ORF. After blunt ending with Klenow enzyme and religation, nucleotide sequence analysis revealed an 89-bp deletion in the *bel* 2 ORF in $p\Delta gpe_{Bg}$. $p\Delta gpe_H$ has a 93-bp *Hin*dIII deletion in *bel* 2 and *bel* 3, and the deletion in $p\Delta gpe_{H\text{-}H}$ comprises two *Hin*dIII fragments and a total of 0.55 kbp in *bel* 2, *bel* 3, and the 3′ LTR. The deletion mutants of pHSRV are shown in Fig. 1. The genome organization of HFV depicted here is slightly different from that published previously (4). We resequenced the region of the putative *S*1 ORF and found an additional C at nucleotide position 6337 of the HFV genome. Furthermore, when resequencing the *bel* 1 genomic region, we found only two A instead of three A at nucleotide position 9560, a C instead of a T at nucleotide position 10146, a G instead of a T at nucleotide position 10156, and a G instead of a T at nucleotide position 10189. As a consequence, the *S*1 ORF is not present in the HFV genome, but the *pol* ORF is longer and overlaps the *env* ORF, and the *bel* 1 ORF is 46 codons longer in the 5′ and 59 codons longer in the 3′ direction.

The *bel* 1 expression plasmids were derived by deleting the chloramphenicol acetyltransferase (CAT) gene in pSV2cat (11) from the *Hin*dIII to the *Bal* I site, blunt ending, and inserting a 1048-bp *Ssp* I fragment of pHSRV comprising the complete *bel* 1 ORF in sense (pSbel1-S) and antisense (pSbel1-A) orientations. The genomic region expressed in these plasmids is shown in Fig. 3.

p3′cat (−1177/+319), which harbors the 3′ LTR of HFV in front of the CAT gene, has been described recently, when it was designated pHSRVcat (9). p3′cat (+319/−1177) was constructed the same way with the LTR in reverse orientation. p5′cat (−777/+351) was derived by inserting an *Asp*718/*Nar* I fragment from pHSRV comprising the complete 5′ LTR into p0cat-Bg (9) by blunt-end ligation. Deletions were created from the 5′ LTR subcloned into a pUC19 vector by BAL-31 digestion and *Bgl* II linker insertion. Appropriate *Bam*HI/*Bgl* II fragments were then cloned into p0cat-Bg, and the length of the deletion was determined by sequencing, using a CAT gene-specific reverse primer. The LTR constructs are depicted in Fig. 5. All plasmid manipulations were made according to established methods (12) and confirmed by extensive restriction enzyme analysis and/or

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Abbreviations: HFV, human foamy virus; SFV, simian foamy virus; HTLV, human T-cell leukemia virus; HIV, human immunodeficiency virus; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; ORF, open reading frame.

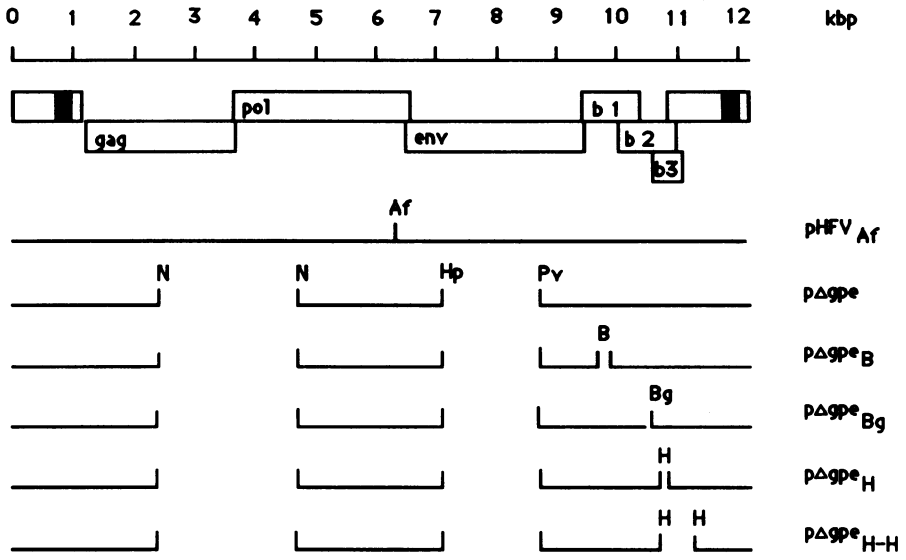

FIG. 1. Genome organization and deletion mutants of HFV. Af, *Afl* II; B, *Bam*HI; Bg, *Bgl* II; H, *Hin*dIII; Hp, *Hpa* I; N, *Nco* I; Pv, *Pvu* II. The *Afl* II mutant created a premature stop in the integrase domain after 923 of 970 codons of *pol*. The *Nco* I deletion leaves the first 383 codons of *gag* intact, and the *Hpa* I/*Pvu* II deletion leaves the first 197 codons of *env* intact. In $p\Delta gpe_B$ only the first 85 of 301 codons of the *bel* 1 ORF and in $p\Delta gpe_{Bg}$ only the first 193 of 364 codons of the *bel* 2 ORF can be transcribed. The *Hin*dIII deletion in $p\Delta gpe_H$ comprises the internal codons 298–329 of *bel* 2 and the internal codons 38–69 of *bel* 3 (total of 167 codons). In $p\Delta gpe_{H-H}$ the C-terminal 66 codons of *bel* 2, the C-terminal 129 codons of *bel* 3, and 414 nucleotides of the 3′ LTR are deleted.

nucleotide sequence analysis using the dideoxy chain-termination method (13).

Transfections and CAT Assays. Baby hamster kidney cells (BHK-21), human glioblastoma cells (U 251-MG), human hepatoma cells (Hep G2), human lung fibroblastoid cells (C16), and human epithelial cells (HeLa) were cultured in modified Eagle's medium supplemented with 5% fetal bovine serum and antibiotics. Unless otherwise stated, equimolar amounts of effector plasmids and indicator plasmids were transfected by the calcium phosphate coprecipitation method (14), with the DNA concentration in the transfection cocktail adjusted to 20 μg/ml using herring sperm DNA. After overnight incubation with the precipitate, cells were shocked for 2.5 min with 15% glycerol in transfection buffer and refed. After 48 hr the cells were harvested and processed as described (11). One-hundred micrograms of protein of the BHK-21 cell lysates, as determined with a commercial protein assay (Bio-Rad), was assayed for 60 min at 37°C in 0.25 M Tris·HCl (pH 8.0) with 1 mM acetyl-CoA (Sigma) and 0.1 μCi of [^{14}C]chloramphenicol (1 Ci = 37 GBq; Amersham). After thin-layer chromatography the spots on the chromatography plates were excised and radioactivity was assayed by liquid scintillation spectroscopy to determine the amount of acetylated chloramphenicol as a percentage of total input chloramphenicol. All values represent the mean of three to six independent transfections. To compare transactivation in different human cell lines the β-galactosidase expression plasmid pCH110 (15) was cotransfected, and protein amounts applied in CAT assays were normalized for equal β-galactosidase activity determined as previously described (15).

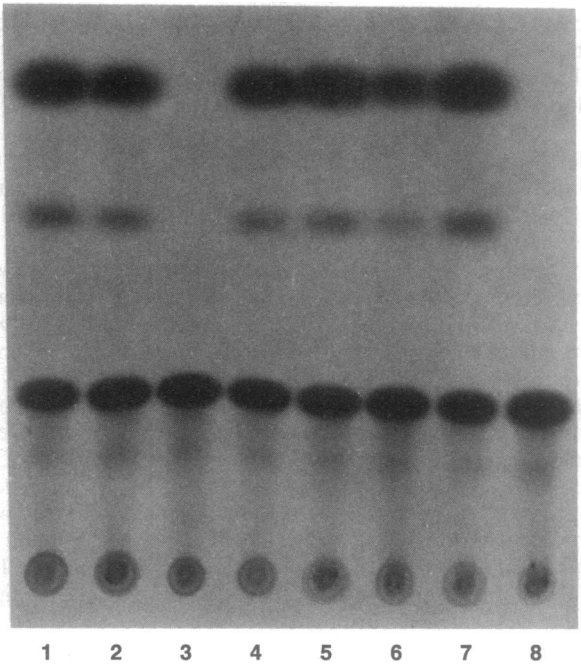

FIG. 2. Transactivation of p5′cat (−777/+194) by HFV mutants and *bel* 1 expression plasmid. Lanes: 1, $pHFV_{Af}$; 2, pΔgpe; 3, $p\Delta gpe_B$; 4, $p\Delta gpe_{Bg}$; 5, $p\Delta gpe_H$; 6, $p\Delta gpe_{H-H}$; 7, pSbel1-S; and 8, pSbel1-A.

RESULTS

Transactivation of the HFV LTR by Deletion Mutants. To map the gene(s) responsible for transactivation we designed a set of deletion mutants of the infectious plasmid pHSRV,

Table 1. Quantitative analysis of transactivation by HFV deletion mutants

Plasmid	p5′cat (−777/+351)	p3′cat (−1177/+319)	pSV2cat	p0cat-Bg
$pHFV_{Af}$	32.5	15.6	83.1	1.0
pΔgpe	29.4	22.4	77.5	0.4
$p\Delta gpe_B$	0.3	0.5	79.6	0.8
$p\Delta gpe_{Bg}$	13.5	14.2	80.8	0.4
$p\Delta gpe_H$	14.5	9.7	75.4	0.9
$p\Delta gpe_{H-H}$	10.0	13.5	74.6	0.2
pUC19	0.6	0.8	73.5	0.8

Equimolar amounts of indicator plasmid (equalized to 2.5 μg of pSV2cat) and of effector plasmid (equalized to 2.5 μg of pΔgpe) were transfected into BHK-21 cells. CAT assays were performed with equal amounts of protein from the prepared lysates. Values represent the percentage of acetylated chloramphenicol.

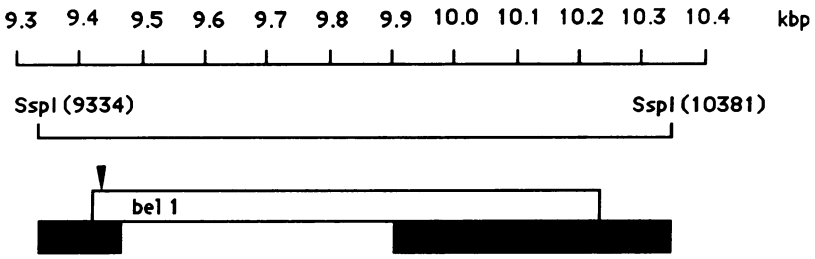

FIG. 3. *bel* 1 genomic region expressed in the CAT gene deleted vector pSV2cat (11). The scale indicates the map of the HFV provirus. The arrow indicates the first ATG codon of the *bel* 1 ORF. *env* and *bel* 2 ORFs are marked black.

which was previously shown to transactivate the HFV LTR (6). Details of the constructs are given in Fig. 1. Effector plasmids and indicator plasmids were transfected into BHK-21 cells and CAT assays were performed. A representative example is shown in Fig. 2. Quantitative analysis of the CAT assays is summarized in Table 1. Relative to the negative control, transfections with the promoterless CAT construct p0cat-Bg or with pUC19 vector as effector plasmid, transactivation was observed using $pHFV_{Af}$ as effector and either p5'cat (−777/+351) or p3'cat (−1177/+319) as indicator. Thus, transactivation with $pHFV_{Af}$ was taken as the positive reference in our experiments. Plasmid pΔgpe was designed to map the transactivator to the 3' ORFs of HFV. With pΔgpe as effector, transactivation was clearly demonstrable with either indicator. Transactivation was completely abolished by removing 70 codons solely in the *bel* 1 ORF ($p\Delta gpe_B$), indicating that this distinct genomic region is essential for expression of the HFV transactivator. Furthermore, introduction of the *Bam*HI deletion of $p\Delta gpe_B$ into the infectious molecular clone renders pHSRV replication incompetent (data not shown). In contrast, the 30-codon deletion solely in the *bel* 2 ORF in plasmid $p\Delta gpe_{Bg}$ resulted only in a reduction of about 50% in CAT values as compared with pΔgpe. When the *bel* 2 mutant of $p\Delta gpe_{Bg}$ was introduced into pHSRV, the resulting plasmid, $pHFV_{Bg}$, still retained infectivity (data not shown). This indicated that this genomic region was not essential for viral replication. When deletions were introduced into the 3' region of the *bel* 2 ORF and into the *bel* 3 ORF ($p\Delta gpe_H$) or extending into the 3' LTR ($p\Delta gpe_{H\text{-}H}$), only marginal effects on the CAT values were observed in relation to $p\Delta gpe_{Bg}$. The promoter activities of the control plasmids pSV2cat and p0cat-Bg remained relatively unchanged after cotransfection with either effector plasmid. In conclusion, the results from these experiments suggest that the *bel* 1 ORF plays the major role in transactivating the HFV LTR.

Transactivation of the HFV LTR by *bel* 1. To prove that the *bel* 1 genomic region encodes the HFV transactivator, we cloned the genomic fragment of pHSRV comprising the *bel* 1 ORF in both orientations into a eukaryotic expression vector (Fig. 3). When these plasmids were used as effectors, transactivation in BHK-21 cells was observed only with pSbel1-S expressing the *bel* 1 ORF in the sense orientation but not with pSbel1-A expressing the fragment in antisense orientation (Table 2 and Fig. 2). Similar results were obtained when the *bel* 1 gene fragment was expressed under control of various eukaryotic promoters such as the mouse major histocompatibility complex class I (H2) promoter (16) (data not shown), which indicated that the *bel* 1-mediated transactivation is not restricted to this special construct. When various human cell lines were tested, transactivation by pSbel1-S was found to be independent of the cell type used. In the fibroblastoid cell line C16 the transactivation of p5'cat (−777/+194) obtained with pSbel1-S was 63-fold higher than with pSbel1-A. The pSbel1-S/pSbel1-A ratio was of the same order of magnitude in glioblastoma cells (U251-MG; 86-fold), in epithelial cells (HeLa; 107-fold), and in hepatoma cells (Hep G2; 91-fold). As shown in Fig. 4 the transactivation of the HFV LTR by pSbel1-S is dose dependent. In our assay the transactivation of p5'cat (−777/+194) increased linearly with the amount of cotransfected pSbel1-S and approached a plateau with 1 μg of the effector plasmid, whereas no transactivation was observed with up to 10 μg of pSbel1-A.

Table 2. Transactivation of HFV LTR CAT constructs by *bel* 1 expression plasmid to BHK-21 cells

Plasmid	pSbel1-S	pSbel1-A	Fold induction*
p5'cat (−777/+351)	48.1	0.9	53.4
p5'cat (−777/+194)	87.0	0.9	96.6
p5'cat (+194/−777)	44.0	2.5	17.6
p5'cat (−777/+4)	78.6	1.3	60.5
p3'cat (−1177/+319)	41.0	0.7	58.5
p3'cat (+319/−1177)	31.4	2.5	12.6
pSV2cat	68.2	67.5	1.0
p0cat-Bg	0.4	0.6	0.7

*Ratio pSbel1-S/pSbel1-A.

A Putative Transactivator-Responsive Element Is Located in U3. To determine whether LTR sequences downstream of the start of transcription are essential for transactivation we investigated the responsiveness of deletion mutants of the 5' LTR to pSbel1-S and pSbel1-A. In p5'cat (−777/+351) the complete LTR was placed 5' to the CAT gene, in p5'cat (−777/+194) only U3R was cloned 5' to the CAT gene and in p5'cat (−777/+4) only U3 and four bases of R were located in front of the indicator gene (Fig. 5). As shown in Table 2, all constructs corresponded well to the *bel* 1 expression plasmid. Although the R region was not found to be essential

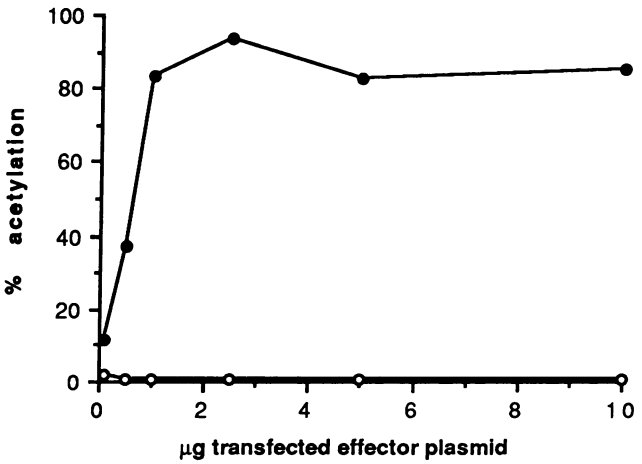

FIG. 4. Dose-dependent transactivation by *bel* 1. Two and one-half micrograms of p5'cat (−777/+194) and 0.1, 0.5, 1.0, 2.5, 5.0, and 10.0 μg of either expression plasmid pSbel1-S or pSbel1-A were cotransfected into BHK-21 cells. The DNA amount in the transfection cocktail was adjusted to 20 μg with herring sperm DNA. ●, pSbel1-S; ○, pSbel1-A.

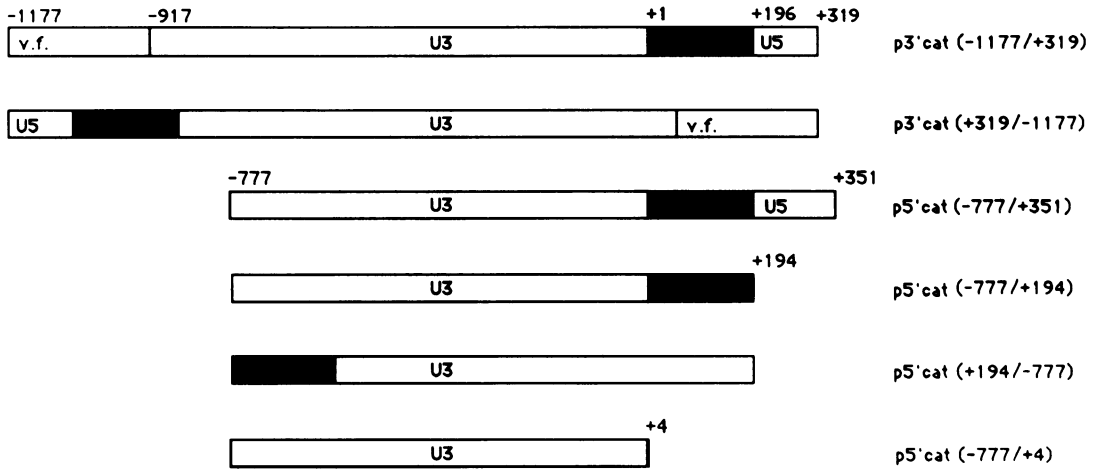

FIG. 5. HFV LTR portions placed 5′ to the CAT gene in vector p0cat-Bg (9). Different lengths of U3 in 5′ and 3′ LTRs are due to an insertion of nonviral DNA into the 3′ LTR of the infectious plasmid pHSRV (6). The R region of the LTR is marked black. v.f., viral flanking.

for transactivation, the U3R construct responded best to pSbel1-S. The 2-fold increase in transactivation with the U3R construct compared to the U3RU5 construct argues for a negative regulatory element in U5. When U3R in p5′cat (−777/+194) and U3RU5 in p3′cat (−1177/+319) were placed in antisense orientation in front of the CAT gene, responsiveness to pSbel1-S was not abolished but decreased significantly. Furthermore, we observed a 2- to 3-fold increase in basal promoter activity with these plasmids, as deduced from cotransfection with pSbel1-A (Table 2).

DISCUSSION

The experiments reported here were designed to map the transactivator function to one of the HFV genes. Two lines of evidence indicated that the *bel* 1 ORF encodes the HFV transactivator: (*i*) deletions in *gag*, *pol*, *env*, *bel* 2, and *bel* 3 had only minor, if any, effect on transactivation, whereas a deletion in *bel* 1 completely abolished transactivation, and (*ii*) the eukaryotic expression of the *bel* 1 genomic region was found to be sufficient to transactivate the HFV LTR in a variety of cell lines tested. Whether the HFV transactivator is translated from a single or multispliced mRNA is not yet known and must be studied by functional analysis of cDNA clones. In HTLV and HIV the transactivator genes are translated from double or multispliced mRNAs (17, 18), and, in the case of HIV, only the second exon is essential for the expression of functional active *tat* (19, 20).

The mechanism of transactivation in HTLV and HIV is different. Whereas *tax* in HTLV acts indirectly on a repeated 21-bp enhancer in the U3 region of the LTR (21, 22), in HIV *tat* acts directly on the nascent mRNA in the R region of the LTR (23, 24). To obtain a first hint concerning the transactivator mechanism in HFV we tested LTR deleted constructs for their responsiveness to pSbel1-S. The observed transactivation of a U3cat construct, p5′cat (−777/+4), by pSbel1-S indicates that the putative transactivator-responsive region resides in U3 and confirms previous results that transactivators of foamy viruses function transcriptionally (9, 10). However, alignment of LTR sequences from closely related foamy viruses revealed great divergence in U3 compared to 85% homology in R (10). Examination of cross-transactivation between different foamy viruses, as conducted for HIV and simian immunodeficiency virus (25), and mapping of a transactivator-responsive element in the HFV LTR should permit identification of the mechanism of transactivation more precisely.

Surprisingly, we found that the LTR placed in reverse orientation 5′ to the CAT gene responded to pSbel1-S. This result may be explained if one assumes that cryptic initiation sites are used in these constructs and that the mechanism of transactivation is independent of the orientation of the target sequence. When examining the inverted LTR sequence we found one TATATAA motif that might initiate transcription from the inverted strand. Furthermore, with these constructs a weak increase in basal promoter activity was observed, which suggested that the HFV LTR (in sense orientation) might be controlled in a complex manner by up- and down-regulating factors and/or sequences. This should be addressed in further studies.

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