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Analysis of the Role of the *bel* and *bet* Open Reading Frames of Human Foamy Virus by Using a New Quantitative Assay

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We have constructed a BHK-21-derived indicator cell line containing a single integrated copy of the β-galactosidase (β-Gal) gene under control of the human foamy virus (HFV) long terminal repeat promoter (from −533 to +20). These foamy virus-activated β-Gal expression (FAB) cells can be used in a quantitative assay to measure the infectious titer of HFV. Our results show that the FAB assay is 50 times more sensitive than determination of the virus titer by the end-point dilution method. Using the FAB assay, we have found that HFV can productively replicate in several erythroblastoid cell lines as well as in the Jurkat T-cell line. We have also examined the roles of *bel2*, *bet*, and *bel3* in viral replication by constructing proviral HFV clones in which the reading frame of Bel2, Bet, or Bel3 is disrupted by placement of translation stop codons. Analysis of these mutants reveals that while the *bel3* gene is not required for viral replication in vitro, mutations in the *bel2* or *bet* gene decrease cell-free viral transmission approximately 10-fold.

The prototype spumavirus, human foamy virus (HFV) or human spumaretrovirus, was originally isolated from a patient with nasopharyngeal carcinoma (1). In culture, spumavirus is highly cytopathic, inducing multinucleated syncytia with a characteristic vacuolated appearance in the cytoplasm. Although it has been shown that in vitro HFV replicates productively in fibroblastic diploid cells but poorly in epithelium-like cells (23, 24), viral replication in other cell types is still unclear. There have been numerous searches for diseases associated with HFV infection (37). The finding of both virus-related sequences and antigens in patients with Graves' disease suggests an association with this autoimmune disease (19, 38). It has also been reported that expression of spumaviral genes in neurons and brain cells in transgenic mice is correlated with neurological disorders (2, 3, 5).

The genome of HFV contains *gag*, *pol*, and *env*, as well as several other genes, located between *env* and the 3′ long terminal repeat (LTR) (10, 25). At least four open reading frames (ORFs) are predicted to exist, including *bel1*, *bel2*, *bet*, and *bel3*, which result from singly and/or multiply spliced mRNAs (28). Analysis of viral proteins in infected cells has shown that while Bel1 accumulates in the nucleus, Bel2 is found in the cytoplasm (11, 21). Bet, an abundant viral protein in the cytoplasm, is encoded by a multiply spliced mRNA in which the N-terminal portion of Bel1 is spliced in frame with the Bel2 ORF. Thus far, expression of the Bel3 ORF has not been reported, although *bel3* transcripts have been found in infected cells (28).

The *bel1* gene, like human immunodeficiency virus (HIV) *tat* and human T-cell leukemia virus *tax*, encodes a potent transcriptional transactivator of its own LTR promoter (17, 32). Deletion of *bel1* completely abolishes the infectivity of HFV (21). The primary target sequence for Bel1 within the HFV LTR has been mapped to ~130 bp 5′ to the cap site (36). A second region located ~400 bp 5′ to the cap site also contributes to optimal reactivity to Bel1 (27). Bel1 can also *trans* activate gene expression directed by the HIV type 1 LTR, through a target site different from that of HIV Tat (16, 20). In contrast to *bel1*, the roles of *bel2*, *bet*, and *bel3* in HFV replication remain unknown.

Titers of foamy virus stocks have routinely been determined by examination of cytopathic effects (CPE) by the end-point dilution method. Here, we present a quantitative method for titration of HFV using an indicator cell line in which expression of β-galactosidase (β-Gal) can be activated by HFV infection. This foamy virus-activated β-*gal* expression (FAB) assay exploits the requirement for *trans* activation of the HFV LTR promoter by the viral Bel1 protein. It provides a simple and rapid method for titration of HFV, which can be completed in 2 days and is 50-fold more sensitive than titration of virus by the end-point dilution method. Using this assay, we have reexamined the spectrum of HFV cell tropism. We also report results of mutational analyses of the *bel2*, *bet*, and *bel3* genes and conclude that while *bel3* is dispensable for viral replication, the *bel2* or *bet* gene is required for optimal cell-free viral transmission in vitro.

MATERIALS AND METHODS

Cells. Human embryonic lung (HEL) cells (ATCC CCL 137) and human foreskin primary fibroblastic cells (obtained from Adam Geballe, Fred Hutchinson Cancer Research Center) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS). BHK-21 (baby hamster kidney cells, ATCC CCL 10) and FAB cells were grown in Dulbecco's modified Eagle medium with 5% FBS. Human cell lines, including B/N (an Epstein-Barr virus-transformed B lymphocytic cell line obtained from Mark Groudine, Fred Hutchinson Cancer Research Center), 174xCEM (a T-B lymphoblastic hybrid cell line, NIH AIDS Reagent Program catalog no. 272), Jurkat (a $CD4^+$ T-cell line, ATCC TIB 152), U937 (a histiocytic lymphoblastoid cell line, ATCC CRL 1593), K562 (an erythroblastoid cell line, ATCC CCL 243), and H92.1.7 (an erythroblastoid cell line originally called

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HEL92.1.7, ATCC TIB 180), were grown in RPMI medium supplemented with 5% FBS.

Preparation of virus stocks. Virus stocks were originally obtained by transfection of BHK-21 or HEL cells with the full-length HFV proviral DNA. By the modified calcium phosphate method (6), the transfection efficiency of BHK-21 cells was much higher than that of HEL cells (data not shown). Transfected cells were then scraped from culture dishes and removed along with the culture medium, and virus was released by three cycles of freezing and thawing. These total cell lysates were then added to BHK-21 or HEL cells to obtain high-titer virus stocks. Since the virus titer obtained from propagation of HFV in HEL cells was usually slightly higher than in BHK-21 cells, HEL cells were used in all the infection experiments. To prepare cell-free virus stocks, culture supernatants were cleared by low-speed centrifugation (4,000 × *g* for 10 min) and filtered through a 0.2-μm-pore-size filter membrane.

Plasmids. The infectious molecular clone of HFV, pHSRV13, and plasmids pdbel1 and pBCbel1 have been described previously (21) and were kindly provided by M. Löchelt and R. Flügel (German Cancer Research Center, Heidelberg, Germany). Plasmid pdbel1 is derived from pHSRV13 but contains a 208-bp deletion in the *bel1* gene. Plasmid pBCbel1 is a eukaryotic *bel1* expression vector using an immediate-early promoter from cytomegalovirus. Plasmid pHSRVΔGPE was constructed by inserting the 2,488-bp *Cla*I fragment of pHSRV13 into the 2,958-bp *Cla*I-digested fragment of phagemid pBluescriptII SK$^+$ (Stratagene). Plasmid pCMVhph, as described previously (4), encodes resistance to hygromycin B driven by the cytomegalovirus promoter.

Plasmid pEQJK was constructed by ligating a 2,065-bp *Hin*dIII-*Sac*I fragment of pEQ3 (33), a promoterless β-Gal vector, with the 5,057-bp *Hin*dIII-*Sac*I fragment of pJK2. pJK2 is an HIV LTR–β-Gal expression plasmid in which a nuclear localization signal from simian virus 40 T antigen has been inserted upstream of the β-Gal gene (18). A DNA fragment from nucleotides −533 to +20 (+1 represents the first nucleotide of the R region) of pHSRV13 was amplified by the polymerase chain reaction using primers 5′-CTCCCGGGATC AGAACATTGAC-3′ and 5′-TGCACTGCAGCGAGTAGT GAAG-3′ and inserted into the *Sma*I-*Pst*I site of pEQJK. The resultant plasmid, pHSRV5LG, thus contains the HFV LTR promoter, followed by a nuclear localization signal, the β-Gal gene, and the poly(A) signal from simian virus 40 (see Fig. 1A).

Virus assays. For the FAB assay, the indicator cells were plated in 12-well plates (22-mm diameter) at 4 × 10^4 cells per well in Dulbecco's modified Eagle medium with 5% FBS the day before infection. The following day, the medium was removed and the cells were incubated with dilutions of a virus stock for 2 h at 37°C. Virus was then removed from each well and replaced with growth medium. Two days later, the monolayer was fixed at room temperature for 5 min with 2 ml of fixation solution (1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline [PBS]). Cells were subsequently washed three times with PBS and incubated at 37°C for 30 min with 0.5 ml of staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] per ml in PBS). The reaction was stopped by removing the staining solution and then washing each well with PBS. Blue cells were then counted under a light microscope.

To determine the virus titer by CPE development by the end-point dilution method, serial dilutions of the HFV stock were used to infect HEL, BHK-21, and FAB cells. Cultures were monitored daily for a period of 2 weeks for the appearance of CPE. In cocultivation experiments, HEL cells were first infected with HFV at a multiplicity of infection (MOI) of 3. Three days later, the cells were treated with 10 μg of mitomycin per ml for 2 h to inhibit cell division. After extensive washes, the treated HEL monolayers were trypsinized and cocultivated with different hematopoietic suspension cells at a ratio of 1 to 20. The suspension cells were maintained in RPMI medium containing 5% FBS and subcultured every 3 to 5 days. After the second passage, adherent HEL cells would no longer be observed in the cultures. The virus titer in the culture medium was periodically monitored by the FAB assay.

Site-directed mutagenesis. Translation stop codons were introduced into the reading frame of *bel2*, *bet*, or *bel3* without affecting the coding sequence of the other overlapping genes (see Fig. 3), by using an oligonucleotide-directed mutagenesis kit (Amersham) according to the manufacturer's protocol. The single-stranded DNA template derived from pHSRVΔGPE was used in the mutagenesis reaction. A 21-mer, 5′-AGGGCT ACTAGAAGAGTCCAG-3′, was used to create the Δ*bel2* mutant, in which the Leu codon (TTG) at residue 34 was replaced with TAG. For the Δ*bel2C* mutant, the Arg (AGA) and Gln (CAG) codons at residues 246 and 248 were converted to TGA and TAG by a 30-mer, 5′-TGGAATGTCACTTGA AACTAGGGAAAACAA-3′. A 22-mer, 5′-CATAGAACCA TAATGGCAGACT-3′, was used to generate the Δ*bel3* mutant, in which the Leu codon (TTA) at residue 90 was replaced with TAA. The Ser codon (TCA) at residue 130 was changed to TAA in the Δ*bel3C* mutant with a 23-mer, 5′-GGTGGAAT GTAACTAGAAACCAG-3′. After confirmation by DNA sequencing, each mutation was introduced into the full-length proviral cDNA by inserting the 2,488-bp *Cla*I fragment from each mutagenized pHSRVΔGPE into the 12,919-bp *Cla*I fragment of pHSRV13.

Immunoprecipitation. HEL cells were infected with either wild-type or mutant virus at an MOI of 20. Four days after infection, cells were labeled with 10 μl of [^{35}S]methionine per ml for 24 h and lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 0.5% aprotinin, 10 mM iodoacetamide, 0.5% Nonidet P-40, 0.5% sodium dodecyl sulfate (SDS), and 0.5% sodium desoxycholate. Two microliters of serum from chimpanzees naturally infected with foamy virus (a gift from Krishna Murthy, Southwestern Foundation for Biochemical Research, San Antonio, Tex.) was mixed with the cell lysates at 4°C overnight and incubated with protein A-Sepharose for 1 h. Samples were then washed four times with RIPA buffer (10 mM Tris-Cl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% sodium desoxycholate, 0.1% SDS, and 0.5% aprotinin) and analyzed with an SDS–15% polyacrylamide gel. Gels were then dried and exposed to Kodak XAR-5 film.

RESULTS

Construction of an indicator cell line for titration of HFV. In order to develop a quantitative assay for titration of HFV, BHK-21 cells were cotransfected with plasmids pCMVhph and pHSRV5LG (Fig. 1A). Although HFV grows to a slightly higher titer in HEL cells, HEL cells are not suitable for construction of an indicator line since they do not clone well. Hygromycin-resistant clones were randomly picked to screen for expression of β-Gal. Aliquots of each clone were either mock infected or infected with HFV. Two days after infection, cultures were stained with X-Gal and examined for blue cells. If cells contain an integrated LTR–β-Gal gene, expression of HFV *bel1* after infection would be expected to *trans* activate the expression of β-Gal, which could then react with the

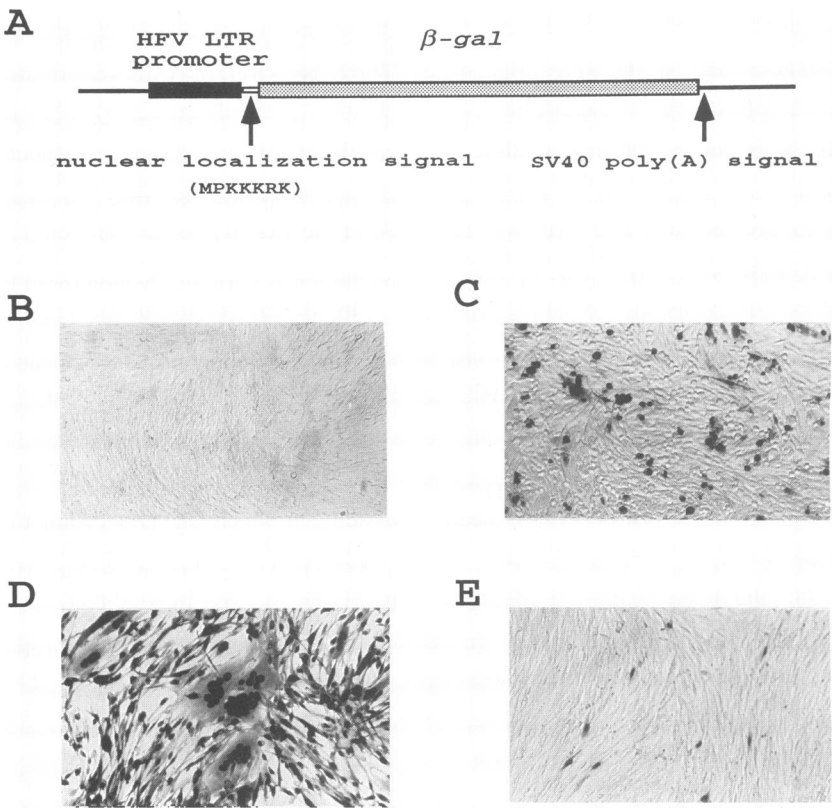

FIG. 1. (A) Schematic diagram of plasmid pHSRV5LG. The solid box indicates the HFV LTR promoter (from nucleotides −533 to +20; +1 represents the first nucleotide of the R region), and the hatched box indicates the β-Gal gene. The nuclear localization sequence consists of seven amino acid residues (MPKKKRK) derived from the T antigen of simian virus 40 which are sufficient to direct heterologous fusion proteins to the nucleus (15). The FAB cells in panels B to E were fixed and stained as described in Materials and Methods. (B) Mock infection of FAB cells; (C) transfection of FAB cells with the eukaryotic *bel1* expression vector pBCbel1 DNA (21); (D) infection of FAB cells with HFV at an MOI of 50; (E) infection of FAB cells with HFV at an MOI of 0.005.

specific chromophor, X-Gal. Because a nuclear localization signal was inserted upstream of the β-Gal gene, an intensive blue color should be found in cell nuclei where β-Gal was concentrated.

Of 22 clones screened, three expressed a high level of β-Gal activity upon HFV infection but showed no β-Gal expression after mock infection. An intensive blue color could be found in nuclei after 30 min of incubation with X-Gal. One of these clones, termed FAB, was further characterized. The basal level of β-Gal activity in these FAB cells was extremely low. No cells turned blue after mock infection (Fig. 1B) or transfection with DNA of a *bel1* mutant (pdbel1) (data not shown), even after 48 h of incubation with X-Gal. In contrast, β-Gal expression was activated after transfection with the eukaryotic *bel1* expression vector, pBcbel1 DNA (Fig. 1C). Results from Southern blot analysis demonstrated that these cells contained only a single copy of the LTR–β-Gal gene (data not shown). When infected with HFV at a high MOI, cells often formed syncytia containing more than 10 nuclei (Fig. 1D). At a low MOI, however, syncytia were rare and singly infected cells were observed (Fig. 1E).

Sensitivity of the FAB assay. Experiments were performed using the end-point dilution of virus stocks to compare the infectious titer of HFV determined by appearance of CPE to that obtained by staining infected FAB cells. A series of dilutions of either total HFV-infected cell lysates or cell-free virus supernatants (prepared as described in Materials and Methods) were used to infect FAB, BHK-21, and HEL cells. Two days later, FAB cells were stained with X-Gal and virus titers were determined by counting the number of blue cells. Only those wells containing between 20 and 100 blue cells were counted. The virus titer of total infected cell lysates determined by the FAB assay was 1×10^9, while that of the cell-free virus stock was 7.5×10^6 (Fig. 2A). This difference is not surprising, since it is known that HFV is highly cell associated (14). However, when virus titers were determined by CPE development on HEL, BHK-21, or FAB cells, after a period of 2 weeks, only 0.5×10^7 to 2×10^7 infectious units were obtained for the total cell lysate and 0.5×10^5 to 2×10^5 infectious units were obtained for the cell-free virus stock (Fig. 2B). We thus conclude that not only is the FAB assay more rapid, but it is more sensitive than the end-point dilution method. The cell-free virus stock was also serially diluted to determine the linear range of the FAB assay. At a high dilution, the number of blue cells counted was proportional to the amount of virus added and only single blue cells were found (Fig. 1E).

Viral replication in different human hematopoietic cell lines. Determination of the in vivo target cells of HFV is important for understanding the pathogenic potential of spumavirus. It has been shown that in culture HFV grows preferentially in fibroblastic cells but not in epithelium-like cells (23, 24). Yet little is known about HFV growth in hematopoietic cells, which are often the primary target cells for the other

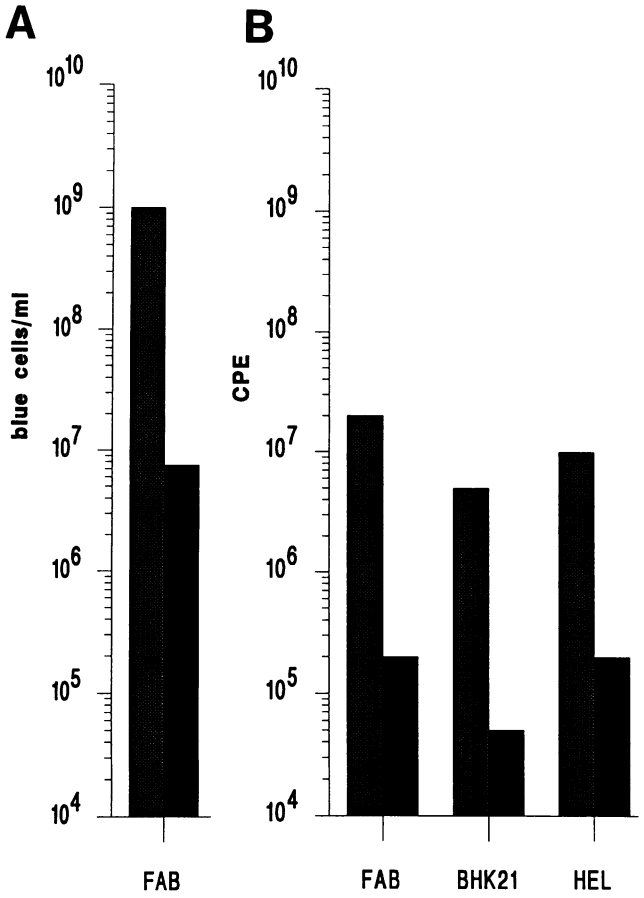

FIG. 2. Comparison of the infectious titers of an HFV stock of either total infected cell lysates (dark grey stipple) or cell-free virus supernatants (black) determined by the FAB assay scored 2 days postinfection (A) or by the appearance of CPE in FAB, BHK-21, and HEL cells by the end-point dilution method scored 2 weeks postinfection (B).

families of retroviruses. Using the FAB assay, we have examined HFV growth in different human hematopoietic cell lines. Since several attempts to infect these suspension cells with cell-free virus stocks failed, we decided to infect cells using a cocultivation method (described in Materials and Methods). Mitomycin-treated HFV-infected HEL cells were cocultivated with different hematopoietic suspension cells in RPMI medium with 5% FBS. The virus titer in each culture was periodically monitored by the FAB assay. As shown in Table 1, while U937

TABLE 1. HFV growth in human hematopoietic cell lines measured by the FAB assay

Cell line	BCFU[a]/ml of culture medium at wk:			
	3	4	5	6
B/N	200	32	7	<0.1
174xCEM	70	7	<0.1	<0.1
Jurkat	1,350	16,000	12,000	12,000
U937	10,000	8,000	1,200	11
K562	5,000	350	60	70
H92.1.7	59,000	4,000	280	<0.1

[a] BCFU, blue-cell-forming unit.

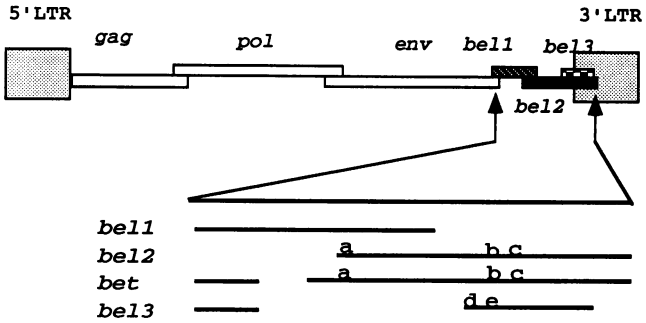

FIG. 3. Properties of the *bel2*, *bet*, and *bel3* mutants. Letters a to e indicate the positions of the translation stop codons inserted into each reading frame of *bel1* genes. a, TAG in Δ*bel2*; b, TGA in Δ*bel2C*; c, TAG in Δ*bel2C*; d, TAA in Δ*bel3*; e, TAA in Δ*bel3C*.

(histiocytic lymphoblastoid cells), K562 (erythroblastoid cells), and H92.1.7 (erythroblastoid cells) produced the maximal amount of viruses within the 3 weeks of cultivation, a delayed and prolonged viral replication was observed in Jurkat T cells, peaking at the 4th week. In contrast, HFV growth in B/N (B cells) and 174xCEM (T-B hybrid cells) was relatively poor. It is possible that the virus titer detected at the early time point represents residual virus from the mitomycin-treated infected HEL cells.

Construction of *bel2-bet* and *bel3* mutants. Except for *bel1*, functions of the *bel* genes are still unknown. To examine the roles of *bel2-bet* and *bel3* in HFV replication, translation stop codons were introduced into the reading frames of these genes by site-directed mutagenesis without affecting the coding sequences of the other overlapping genes (Fig. 3). Wild-type proviral DNA as well as DNA from each of the four mutants was transfected into BHK-21 cells. In every case, CPE was found as early as 2 to 3 days posttransfection. In the wild type and *bel3* mutants (Δ*bel3* or Δ*bel3C*), extensive CPE and cell

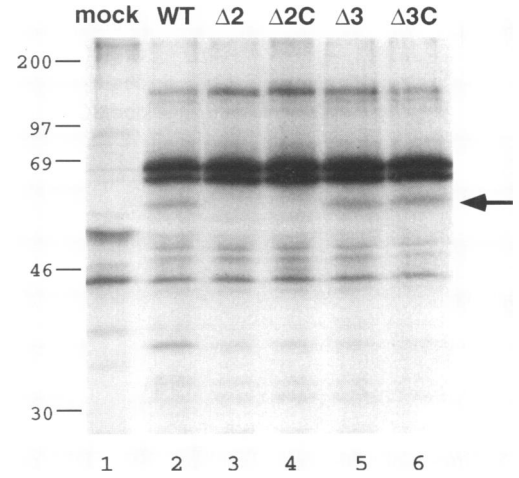

FIG. 4. Analysis of virus-specific proteins in HFV-infected cells by immunoprecipitation. HEL cells (10^6) were either mock infected (lane 1) or infected with wild-type (lane 2) or mutant HFV virus (lanes 3 to 6) at an MOI of 20. [^{35}S]methionine-labeled cell lysates were then immunoprecipitated with chimpanzee serum and analyzed in an SDS–15% polyacrylamide gel (see Materials and Methods). The positions of molecular mass markers (in kilodaltons) are shown on the left, and the arrow indicates the position of 56-kDa viral Bet protein.

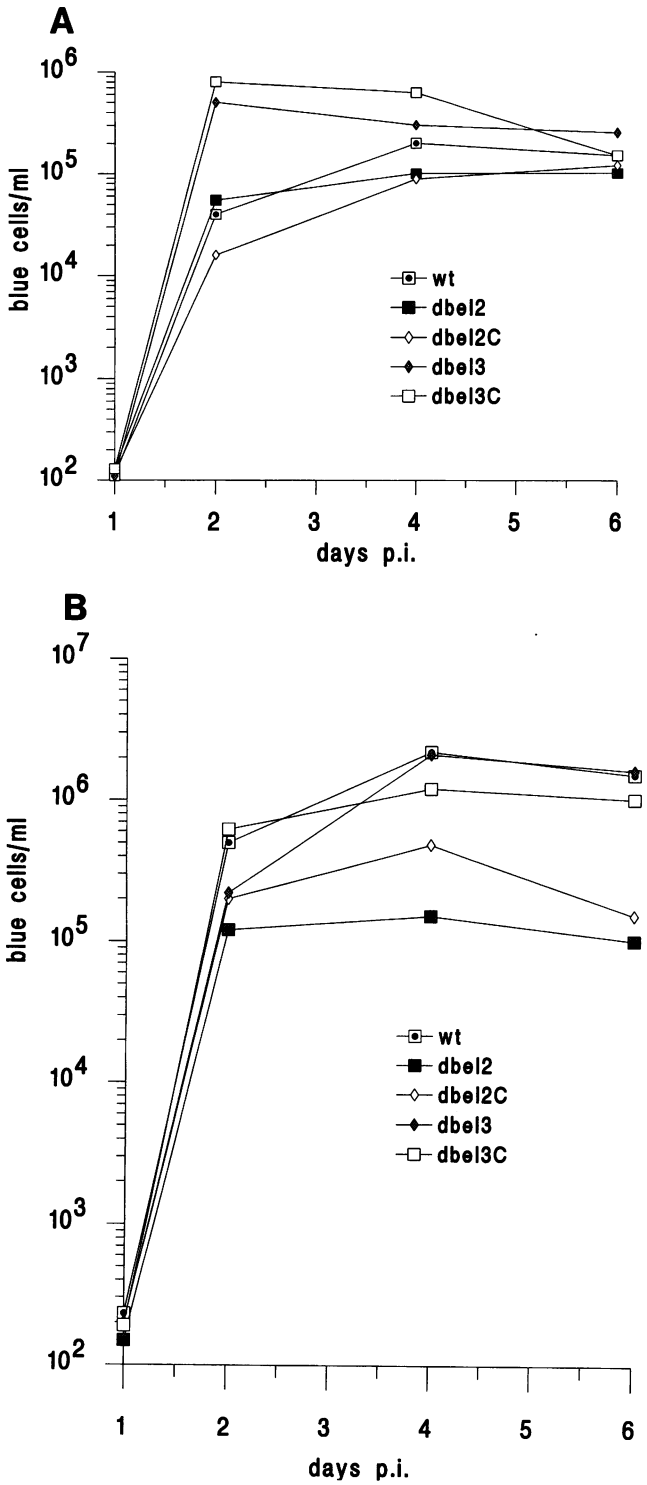

FIG. 5. Growth of wild-type and mutant HFV in HEL cells. HEL cells were infected with wild-type (wt) or mutant (d, Δ) virus at an MOI of 0.03. At different time intervals postinfection (p.i.) cells (A) and cell-free media (B) from each culture were collected separately and their virus titers were measured by the FAB assay. The data shown here were taken from one of the three repeated experiments. Although the exact number of titers varied slightly from one experiment to another, the slopes and shapes of each growth curve were similar.

death were observed at 5 to 6 days posttransfection. However, CPE spread was slightly slower in cells transfected with either of the *bel2-bet* mutants (Δ*bel2* or Δ*bel2C*), peaking at 7 to 8 days. The delay of viral spread in *bel2-bet* mutants was more evident when cell-free rather than cell-associated virus stocks were used to infect fresh HEL cells (data not shown).

To detect viral proteins in infected cells, labeled HFV-infected cell lysates were immunoprecipitated with serum from chimpanzees naturally infected with foamy virus, which is known to contain virus-specific antibody (29). The 56-kDa Bet protein, an abundant viral protein in HFV-infected cells (11, 21), was detected in cells infected with the wild type or *bel3* mutants (Fig. 4, lanes 2, 5, and 6) but was not present in cells infected with either of the *bel2-bet* mutants (Fig. 4, lanes 3 and 4), which contained translation stop codons predicted to disrupt the Bel2 and Bet open reading frames.

Characterization of *bel2-bet* and *bel3* mutants. To further explore the phenotype of delayed viral spread observed in *bel2-bet* mutants, growth curves of wild-type HFV and the four mutants were compared. HEL cells were first infected with wild-type or mutant viruses at an MOI of 0.03 (as determined by the FAB assay). Titers of cell-associated virus or free virus released into culture media were measured separately by the FAB assay. Interestingly, virus progeny carrying mutations in the *bel3* gene replicated more efficiently than wild-type parental virus, especially at the early rounds of infection (Fig. 5). A plateau in the level of virus titers occurred rapidly with both wild-type and mutant viruses. This is probably because of the rapid development of cell cytopathicity which prevents further rounds of infection and leads to killing of the infected cells. While no apparent difference was detected in cell-associated virus titers (Fig. 5A), the titer of non-cell-associated virus of *bel2-bet* mutants was about 10 times less than that of the wild type (Fig. 5B). This 10-fold reduction of viral replication in *bel2-bet* mutants was also observed in human primary fibroblastic cells (data not shown). Indeed, the same effect was also found when HEL cells were inoculated with the same amount of cell-free virus normalized by the reverse transcriptase assay (data not shown).

DISCUSSION

Biological studies of HFV have been hindered by the lack of a sensitive and quantitative assay system. Although the reverse transcriptase assay measures the physical number of virus particles, it does not accurately reflect the infectious titer of viruses (31). Titration of foamy viruses is commonly done by either monitoring CPE development or plaque formation by end-point dilution (22, 30). Both methods rely entirely on efficient virus spread and require an extended incubation period, ranging from 8 to 14 days. An immunofluorescence assay has been developed as a semiquantitative method to detect the presence of viral antigens (8); however, the results can be severely affected by the conditions of cell growth and the concentration of virus added (12). Therefore, this system may not be reliable for titration of very dilute virus stocks.

We have constructed an indicator cell line that provides a quantitative assay for titration of virus derived from the proviral clone of HFV. This system is based on activation of an integrated β-Gal gene driven by the HFV LTR in BHK-21 cells, which are permissive for HFV infection. The specific response of HFV LTR to its *trans* activator, Bel1, tightly controls the expression of β-Gal in these cells. No activation of β-Gal was detected when DNA from a *bel1* deletion mutant (pdbel) was transfected into FAB cells, confirming the requirement for a specific interaction of HFV LTR with Bel1 in this

assay. Any HFV virion that has completed the early steps of viral replication and expressed the *bel1* gene should be detectable by the FAB assay, since this system requires only intracellular *bel1* expression. In addition to detecting replication-competent HFV, the FAB assay could also be used for determining the effects of mutations on various stages of the viral life cycle.

Our results have indicated that the FAB assay is about 50-fold more sensitive than determination of the titer of a virus stock by CPE development by end-point dilution. This is not unexpected, as foamy virus often becomes latent in infected cells (14) and formation of CPE depends heavily on continual productive viral replication. In a routine examination, 1 infected cell in 10^6 uninfected cells can be easily detected by our in situ assay after 2 days. This indicator system thus provides a biological assay for HFV which is simple, rapid, sensitive, and quantitative.

We have examined viral growth in different hematopoietic cell lines to further explore the cell tropism of HFV. Surprisingly, HFV grew well in all cell types tested except for the B-cell line. More interestingly, the delayed and prolonged viral replication in Jurkat cells, a $CD4^+$ T-cell line, is reminiscent of the persistent foamy virus infections observed in their natural and experimental animal hosts (35). It will be of interest to test FAB cells with primary viral isolates from individuals suspected of foamy virus infection.

The roles of the *bel2*, *bet*, and *bet* ORFs in viral replication remain unknown. Flügel (9) and others (13) have observed a pattern of conserved His and Cys residues in Bel2 similar to those seen in HIV Vif and picornaviral cysteine proteases and thus suggested a similar role for HFV Bel-2 and HIV Vif. We have analyzed the role of *bel2* and *bet* by constructing proviral genomes of HFV in which the reading frame of Bel2 and Bet is disrupted by insertion of translational stop codons. Analysis of two such mutants has revealed that although each clone could produce infectious virus following transfection, the *bel2* and *bet* mutants are less infectious than the wild type in either fibroblastic diploid cell lines or primary fibroblastic cells. The 10-fold reduction of infectivity in these mutants is observed only when cell-free virus is used to infect cells. Therefore, as suggested for HIV *vif* (7, 34), *bel2* and *bet* may play a role in efficient cell-free viral transmission. Our results also indicate that the N-terminal portion of the *bel2* and *bet* gene products is probably not functionally important since the mutant (Δ*bel2C*) with the 3′-truncated *bel2-bet* behaves similarly to the mutant (Δ*bel2*) in which the Bel2-Bet ORF is completely removed.

It has been noted that the *bel3* gene, whose sequence is found in HFV but not in simian foamy virus, contains a leucine heptad repeated motif (25). Interestingly, our experiments with site-specific mutagenesis have indicated that interruption of the Bel3 ORF actually enhances viral replication in vitro. Although analysis of the deduced amino acid sequences has revealed some homology between Bel3 and HIV type 2 Nef (26), the significance of this similarity is unclear. Despite the fact that *bel3* is not required for HFV infection in vitro, the in vivo roles of the *bel2*, *bet*, and *bel3* ORFs remain to be determined.

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REFERENCES

1. **Achong, B. G., W. A. Mansell, M. A. Epstein, and P. Clifford.** 1971. An unusual virus in cultures from a human nasopharyngeal carcinoma. J. Natl. Cancer Inst. **46:**299–307.
2. **Aguzzi, A., K. Bothe, I. Anhauser, I. Horak, A. Rethwilm, and E. F. Wagner.** 1992. Expression of human foamy virus is differentially regulated during development in transgenic mice. New Biol. **4:**225–237.
3. **Aguzzi, A., E. F. Wagner, K.-O. Netzer, K. Bothe, I. Anhauser, and A. Rethwilm.** 1993. Human foamy virus proteins accumulate in neurons and induce multinucleated giant cells in the brain of transgenic mice. Am. J. Pathol. **142:**1061–1071.
4. **Aronoff, R., and M. L. Linial.** 1991. Specificity of retroviral RNA packaging. J. Virol. **65:**71–80.
5. **Bothe, K., A. Aguzzi, H. Lassmann, A. Rethwilm, and I. Horak.** 1991. Progressive encephalopathy and myopathy in transgenic mice expressing human foamy virus genes. Science **253:**555–557.
6. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
7. **Fisher, A. G., B. Ensoli, L. Ivanoff, M. Chamberlain, S. R. Petteway, Jr., L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1987. The *sor* gene of HIV-1 is required for efficient virus transmission in vitro. Science **237:**888–893.
8. **Fleming, W. A., and J. K. Clarke.** 1970. Fluorescence assay of foamy virus. J. Gen. Virol. **6:**277–284.
9. **Flügel, R. M.** 1992. Spumaviruses: a group of complex retroviruses. J. Acquired Immune Defic. Syndr. **4:**739–759.
10. **Flügel, R. M., A. Rethwilm, B. Maurer, and G. Darai.** 1987. Nucleotide sequence analysis of the *env* gene and its flanking regions off the human spumaretrovirus reveals two novel genes. EMBO J. **6:**2077–2084.
11. **Giron, M.-L., F. Rozain, M.-C. Debons-Guillemin, M. Canivet, J. Peries, and R. Emanoil-Ravier.** 1993. Human foamy virus polypeptides: identification of *env* and *bel* gene products. J. Virol. **67:**3596–3600.
12. **Gould, E. A., and J. Hartley.** 1979. Factors affecting the growth and titration by immunofluorescence of simian foamy virus. Arch. Virol. **62:**63–70.
13. **Guy, B., M. Geist, K. Dott, D. Spehner, M. P. Kieny, and J. P. Lecocq.** 1991. A specific inhibitor of cysteine proteases impairs a *vif*-dependent modification of human immunodeficiency virus type 1 *env* protein. J. Virol. **65:**1325–1331.
14. **Hooks, J. J., and C. J. Gibbs.** 1975. The foamy viruses. Bacteriol. Rev. **39:**169–185.
15. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39:**499–509.
16. **Keller, A., E. D. Garrett, and B. R. Cullen.** 1992. The *bel1* protein of human foamy virus activates human immunodeficiency virus type 1 gene expression via a novel DNA target site. Virology **66:**3946–3949.
17. **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.
18. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:**2232–2239.
19. **LaGaye, S., P. Vexiau, V. Morozov, V. Guenebaut-Claudet, J. Tobaly-Tapiero, M. Canivet, G. Cathelineau, and J. Peries.** 1992. Human spumavirus-related sequences in the DNA of leukocytes from patients with Graves disease. Proc. Natl. Acad. Sci. USA **89:**10070–10074.
20. **Lee, A. H., K. J. Lee, S. Kim, and Y. C. Sung.** 1992. 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. J. Virol. **66:**3236–3240.

21. **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 *bel1* gene. Virology **184:**43–54.
22. **Loh, P. C., B. C. Achong, and M. A. Epstein.** 1977. Further biological properties of the human syncytial virus. Intervirology **8:**204–217.
23. **Loh, P. C., and K. S. Ang.** 1981. Replication of human syncytium forming virus in human cells: effect of certain biological factors and selective chemicals. J. Med. Virol. **7:**67–73.
24. **Loh, P. C., and F. M. Matsuura.** 1984. Human spumavirus replication in human cells. J. Med. Virol. **14:**247–254.
25. **Maurer, B., H. Bannert, G. Darai, and R. M. Flügel.** 1988. Analysis of the primary structure of the long terminal repeat and the *gag* and *pol* genes of the human spumaretrovirus. J. Virol. **62:**1590–1597.
26. **Maurer, B., and R. F. Flügel.** 1987. The 3′ orf protein of human immunodeficiency virus 2 shows sequence homology with the *bel3* gene of the human spumaretrovirus. FEBS Lett. **222:**286–288.
27. **Maurer, B., E. Serfling, V. ter Meulen, and A. Rethwilm.** 1991. Transcription factor AP-1 modulates the activity of the human foamy virus long terminal repeat. J. Virol. **65:**6353–6357.
28. **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.
29. **Netzer, K. O., A. Rethwilm, B. Maurer, and V. ter Meulen.** 1990. Identification of the major immunogenic structural proteins of human foamy virus. J. Gen. Virol. **71:**1237–1241.
30. **Parks, W. P., and G. J. Todaro.** 1972. Biological properties of syncytium-forming (foamy) viruses. Virology **47:**673–683.
31. **Parks, W. P., G. J. Todaro, E. M. Scolnick, and S. A. Aaronson.** 1971. RNA dependent DNA polymerase in primate syncytium-forming (foamy) viruses. Nature (London) **229:**258–260.
32. **Rethwilm, A., O. Erlwein, G. Baunach, B. Maurer, and V. ter Meulen.** 1991. The transcriptional transactivator of human foamy virus maps to the *bel1* genomic region. Proc. Natl. Acad. Sci. USA **88:**941–945.
33. **Schleiss, M. R., R. D. Catherine, and A. P. Geballe.** 1991. Translational control of human cytomegalovirus gp48 expression. J. Virol. **65:**6782–6789.
34. **Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin.** 1987. The HIV 'A' (*sor*) gene product is essential for virus infectivity. Nature (London) **328:**728–730.
35. **Swack, N. S., and G. D. Hsiung.** 1975. Pathogenesis of simian foamy virus infection in natural and experimental hosts. Infect. Immun. **12:**470–474.
36. **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. **19:**3661–3666.
37. **Weiss, R. A.** 1988. Foamy retroviruses. A virus in search of a disease. Nature (London) **333:**497–498.
38. **Wick, G., B. Grubeck-Loebenstein, K. Trieb, G. Kalishnig, and A. Aguzzi.** 1992. Human foamy virus antigens in thyroid tissue of Graves' disease patients. Int. Arch. Allergy Immunol. **99:**153–156.