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Cells Expressing the Human Foamy Virus (HFV) Accessory Bet Protein Are Resistant to Productive HFV Superinfection

Michael Bock,* Martin Heinkelein,* Dirk Lindemann,* and Axel Rethwilm*,†,[1]

*Institut für Virologie und Immunbiologie, Universität Würzburg, Versbacher Str.7, 97078, Würzburg, Germany; and †Institut für Virologie, Medizinische Fakultät Carl Gustav Carus, Dresden, Germany

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Bet is a foamy virus (FV) accessory protein not required for virus replication. The function of Bet is not understood. We report on the generation of cell lines stably expressing the HFV Bet protein. In Bet$^+$ cells, HFV replication was reduced by ~3–4 orders of magnitude compared with control cells. The HFV Bet-expressing cells only partially resisted infection by the distantly related feline FV (FFV). Pseudotyping experiments, using murine retroviral vectors with an HFV envelope, revealed that the resistance was not due to downregulation of the unknown HFV receptor. In transfection experiments, using proviral reporter gene constructs and infectious proviruses, no significant differences were detected between Bet$^+$ and control cells. In infection experiments, HFV vectors expressing an indicator gene under control of the HFV promoters showed no activity in Bet$^+$ cells. The results are best compatible with the hypothesis that the main block to productive superinfection of Bet$^+$ cells occurs at an early stage of replication between virus entry and provirus establishment. We suggest that inhibition of provirus integration by Bet protein may serve a distinct function in the unique foamy virus replication cycle. © 1998 Academic Press

Key Words: retroviruses, foamy viruses, accessory proteins, Bet.

INTRODUCTION

The foamy viruses (FVs) have gained increasing scientific interest because of their unusual replication strategy, which is without precedent among animal viruses (Rethwilm, 1996; Weiss, 1996). Although in its proviral form the FV genome organization is similar to known retroviruses, there exist significant functional dissimilarities in the way of replication, which have been addressed in recent studies (Baldwin and Linial, 1998; Bodem *et al.*, 1996; Enssle *et al.*, 1996; Fischer *et al.*, 1998; Goepfert *et al.*, 1995, 1997; Heinkelein *et al.*, 1998; Jordan *et al.*, 1996; Konvalinka *et al.*, 1995; Löchelt and Flügel, 1996; Moebes *et al.*, 1997; Schliephake and Rethwilm, 1994; Yu *et al.*, 1996a). The finding that FVs can reverse transcribe their RNA pregenome late in the replication cycle, which results in DNA as the functional virion nucleic acid (Moebes *et al.*, 1997), highlights their unique properties among retroviruses.

The FV genome is complex (Cullen, 1991; Rethwilm, 1995). In addition to the canonical retroviral *gag*, *pol*, and *env* genes, two accessory open reading frames (ORFs) are located between the *env* gene and the 3′ long terminal repeat (LTR) (Rethwilm, 1995). ORF 1 encodes for a DNA binding protein, Tas (for transactivator of spumaviruses), which is a potent activator of gene expression from the LTR and the internal promoter (IP) (He *et al.*, 1996; Kang *et al.*, 1998; Rethwilm, 1995; Zou and Luciw, 1996). The IP is located in the env gene upstream of the accessory genes of FVs and directs their gene expression in the initial phase of replication (Löchelt *et al.*, 1993, 1994). Tas is required for FV replication (Baunach *et al.*, 1993; Yu and Linial, 1993). ORF 2 encodes for the main part of the so-called Bet protein. Bet is translated from a multispliced mRNA, which uses a very efficient splicing event to fuse an ORF 1 exon to an ORF 2 exon (Fig. 1)(Hahn *et al.*, 1994; Muranyi and Flügel, 1991). In the case of HFV Bet, 88 amino acids are contributed by ORF 1, whereas 394 amino acids are contributed by ORF 2 (Hahn *et al.*, 1994; Muranyi and Flügel, 1991). The 60-kDa Bet protein is highly expressed in lytically FV-infected cells; it can be easily detected by antisera generated against recombinant ORF 1 or ORF 2 proteins; and a robust humoral immune response against Bet is generally seen in FV-infected primates (Baunach *et al.*, 1993; Hahn *et al.*, 1994; Rösener *et al.*, 1996). All this points to an important role of Bet for virus replication. However, the phenotypical analysis revealed that Bet$^-$ viruses replicate in cell culture to titers only 10-fold less, if reduced at all, compared with wild-type virus, and the function of Bet is not understood (Adachi *et al.*, 1995; Baunach *et al.*, 1993; Lee *et al.*, 1994; Schmidt and Rethwilm, 1995; Yu and Linial, 1993).

Interestingly, a Tas defective FV genome (ΔFV) has

[1] To whom reprint requests should be addressed. Fax: +49–931-2013934. E-mail: Rethwilm@vim.uni-wuerzburg.de.

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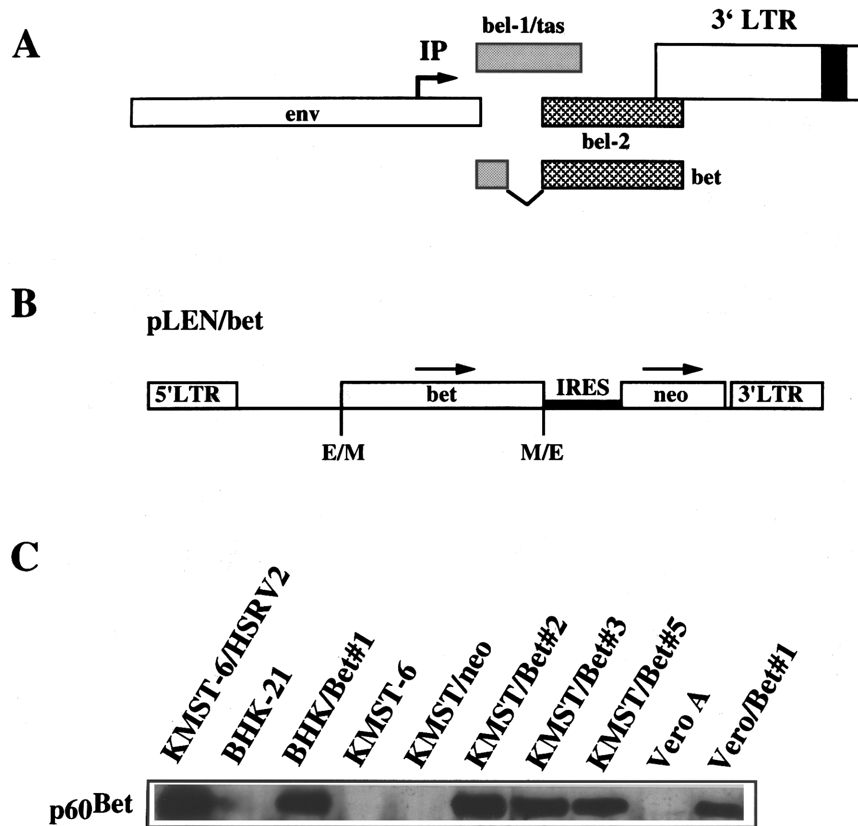


FIG. 1. Generation of Bet-expressing cell lines. (A) 3′ HFV genomic region. (B) The bet cDNA was inserted into the retroviral vector pLEN. After transfection of retroviral packaging cells, the supernatant was used to transduce KMST-6, BHK-21, and Vero A cells. KMST-6 cells were also transduced with vector only (KMST/neo). Single-cell clones were established by limiting dilution, and lysates from the cells (50 μg of protein) were probed by immunoblot using a Bet reactive rabbit serum (C). A lysate from lytically HFV infected KMST-6 cells (5 μg of protein) was run as a positive control. (IP) Internal promoter. (IRES) Internal ribosomal entry site present in the pLEN retroviral vector. (M) *Mun*I restriction site. (E) *Eco*RI restriction site.

been identified that resulted from reverse transcription of a pregenomic RNA from which the Bet intron was spliced out (Bodem *et al.*, 1998; Herchenröder *et al.*, 1996; Saib *et al.*, 1993). ΔHFV was shown to behave like a defective interfering (DI) virus, and cell lines with integrated copies of ΔHFV were found to be resistant to lytic HFV superinfection (Saib *et al.*, 1995). This resistance strongly depended on the number of integrated ΔHFV copies. No protein expression from the transactivator defective provirus could be detected. However, low levels of the 2.2-kb Bet mRNA were found in resistant cells, which harbored ≥15 copies of ΔHFV. Because ΔHFV cell lines with a mutated Bet failed to show the resistant phenotype, a role of Bet in mediating the resistance was suggested (Saib *et al.*, 1995).

To extend these studies and to get a better understanding of Bet function, we analyzed whether cells expressing only Bet are resistant to FV superinfection and, in the case of a positive result, to determine the nature of this block. For this study, we used the so-called HFV isolate because of the availability of HFV-related reagents. However, it is worth noting that HFV most likely represents a chimpanzee virus isolate of FVs (Bieniasz *et al.*, 1995; Herchenröder *et al.*, 1994) and that there is so far a lack of evidence for naturally occurring human FV infections (Ali *et al.*, 1996; Rösener *et al.*, 1996; Schweizer *et al.*, 1995).

RESULTS

Establishment of HFV Bet-expressing cell lines

The *bet* cDNA was introduced into the pLEN retroviral vector, and following packaging in ecotropic and amphotropic retroviral packaging cells, BHK-21, KMST-6, and Vero A cells were stably transduced (Fig. 1). Indirect immunofluorescence using a rabbit antiserum generated against recombinant Tas protein revealed strong cytoplasmic staining of the transduced cells (data not shown). Single-cell clones were established and probed by immunoblot. As shown in Fig. 1, the 60-kDa Bet protein was detected in lysates from the individual cell clones, which were used in further studies. Only one tenth of a lysate from lytically HFV-infected cells was necessary to produce on reaction with the antibody in an immunoblot a signal of the

strength comparable with the lysates from the Bet$^+$ cells (Fig. 1). Therefore the LEN/bet transduced stable cell clones expressed approximately one tenth the amount of Bet protein compared with lytically HFV-infected cells.

Resistance of Bet$^+$ cells to FV superinfection

Bet$^+$ cells and control cells were infected with HSRV2 (Schmidt and Rethwilm, 1995) at an m.o.i. of 0.2 and 1.0, and the cell-free virus was determined over time in the supernatant using a sensitive blue cell assay (Schmidt and Rethwilm, 1995). As shown in Fig. 2A, although Bet$^-$ control cells produced appreciable amounts of HFV, Bet$^+$ cells produced on the average 3–4 orders of magnitude lower levels of virus. The extent of this reduction appeared to correlate with the amount of input virus. However, even after prolonged (6 weeks) incubation of Bet$^+$ cells infected with an m.o.i. of 1.0, cell-free virus titers never exceeded 100/ml. To analyze whether the HFV Bet-expressing cells were resistant to infection by other FVs, the cells were infected with FFV. Compared with HFV, FFV is the most distantly related FV described to date; its gag, pol, and env genes show only ~45%, ~65%, and ~55% nucleotide sequence identity, respectively (Helps and Harbour, 1997; Winkler *et al.*, 1997). Replication of FFV was determined on FK/LTR(FFV)lacZ cells by a blue cell assay similar to HFV replication on BHK/LTR(HFV)lacZ cells. As shown in Fig. 2B, HFV *Bet*$^+$ cells, which were infected at m.o.i. of 0.2 and 1.0, initially resisted FFV replication. However, ~2 weeks after inoculation, the cell-free FFV titers produced in Bet$^+$ cells approached those of the control cells. This breakthrough indicated that HFV Bet is unable to keep FFV replication under control.

Bet$^+$ cells retain the FV receptor

It has been suggested that the function of Bet for FV replication may be similar to the function of Nef for the replication of primate lentiviruses (Rethwilm, 1995). CD4 receptor downregulation is one of the functions that have been associated with Nef (Cullen, 1994). The FV receptor is currently not known. To address the question of receptor downregulation by Bet protein, Bet$^+$ and Bet$^-$ cells were infected with a green fluorescent protein (GFP) expressing murine leukemia virus (MLV) vector that was pseudotyped either with the vesicular stomatitis virus (VSV) G protein or with the chimeric HFVΔ2MuLV envelope (Lindemann *et al.,* 1997). Except for the cytoplasmic tail of the transmembrane protein (TM), which is derived from MLV, the HFVΔ2MuLV envelope protein is made up of the HFV surface and TM domains (Lindemann *et al.,* 1997). Therefore, this chimeric envelope must use the FV receptor to gain entry into cells. In the event that Bet downregulates the FV receptor, *Bet*$^+$ cells should not allow efficient infection by HFVΔ2MuLV pseudotyped virus, whereas the virus pseudotyped with the VSV-G envelope should not be specifically affected by Bet. As shown in Fig. 3, no significant differences to infect Bet$^+$ or Bet$^-$ cells were found when using either of the two envelopes. This indicated that receptor downregulation was probably not responsible for the resistance of Bet$^+$ cells to HFV superinfection.

HFV promoter activity after transfection of Bet$^+$ cells

We next investigated whether the HFV promoters may be silent in Bet$^+$ cells. The cells were transfected with pHSRV2 together with the indicator plasmid p5′cat (−777/+4) (Erlwein and Rethwilm, 1993). Although some differences between individual cell clones were observed, the overall result obtained on transfection of these plasmids indicated that these differences were insignificant (Fig. 4). This suggested that Bet has no major influence on the activity of the HFV promoters when these are delivered to the cells as plasmids, which is equivalent to a provirus.

Analysis of virus production in Bet$^+$ cells after transfection of infectious genomes

The infection of cells by wild-type virus indicates that Bet is expressed to high levels throughout the replication cycle (Hahn *et al.*, 1994; Löchelt *et al.*, 1994; Muranyi and Flügel, 1991). Therefore it appears unlikely that Bet inhibits virus replication by interfering with late viral functions, such as particle assembly or viral export. However, to determine more precisely whether late viral functions were affected in *Bet*$^+$ cells, we transiently transfected Bet$^+$ and Bet$^-$ BHK-21 cells with infectious HFV genomes and analyzed the cell-free viral titers 48 h after transfection. As shown in Fig. 5, Bet$^+$ BHK-21 cells produced ~10–20 times less infectious virus compared with the control cells. To exclude the possibility that the observed difference may result from secondary rounds of infection, we also transfected the pMH9 plasmid into Bet$^+$ and Bet$^-$ BHK-21 cells. The plasmid pMH9 can only perform one round of replication because it has a large deletion in the U3 region of the 3′ LTR. As shown in Fig. 5, we still observed an ~4-fold difference in titers between Bet$^+$ and Bet$^-$ BHK-21 cells. The virus production after transient transfections of KMST-6 and Vero A cells was so low that differences between cell-free virus titers produced by these cells and the corresponding Bet$^+$ cells were insignificant (data not shown). We therefore generated Bet-expressing 293 human fibroblasts cell clones as we had done with BHK-21, KMST-6, and Vero A cells. Our 293 cells did not support efficient HFV replication (data not shown). However, transient virus production after transfection of 293T cells (a derivative of 293 cells) with infectious HFV genomes is very

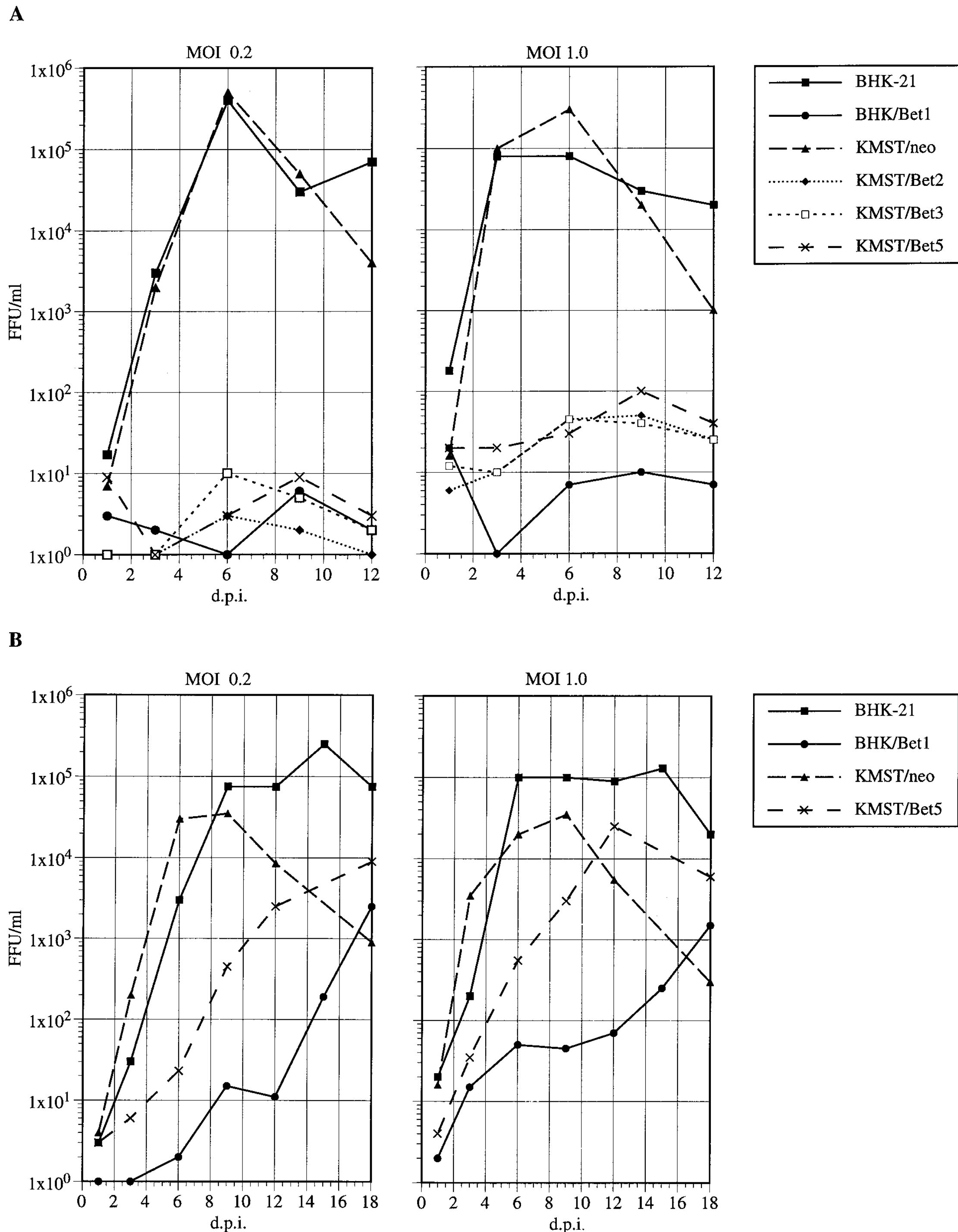


FIG. 2. Development of cell-free virus titers after infection of Bet^- and Bet^+ cells with foamy viruses. The Bet^- and Bet^+ cells were infected with HFV (A) and FFV (B) at the indicated m.o.i., and the cell-free supernatant was monitored for infectious virus on LTRlacZ indicator cells. Although control cells produced appreciable amounts of virus, Bet-expressing cells resisted a productive HFV infection even after prolonged cultivation (6 weeks). Vero A cells, in particular when infected at low m.o.i., produced considerably lower amounts of cell-free HFV compared with BHK-21 and KMST-6 cells. For this reason, the difference in the development of viral titers between Vero A and Vero/Bet1 cells was less pronounced than that with the other cells (data not shown). In contrast, Bet^+ cells resisted FFV replication only in the initial phase. The development of viral titers in the supernatant of these cells was retarded compared with the control cells by 1–2 weeks.

efficient (Lindemann and Rethwilm, 1998; Moebes *et al.,* 1997). When 293 cells were transfected with pHSRV2 or pcHSRV2, no significant differences in extracellular titers were observed between Bet^+ or Bet^- cells (Fig.5).

Although a minor effect on the late phase of virus

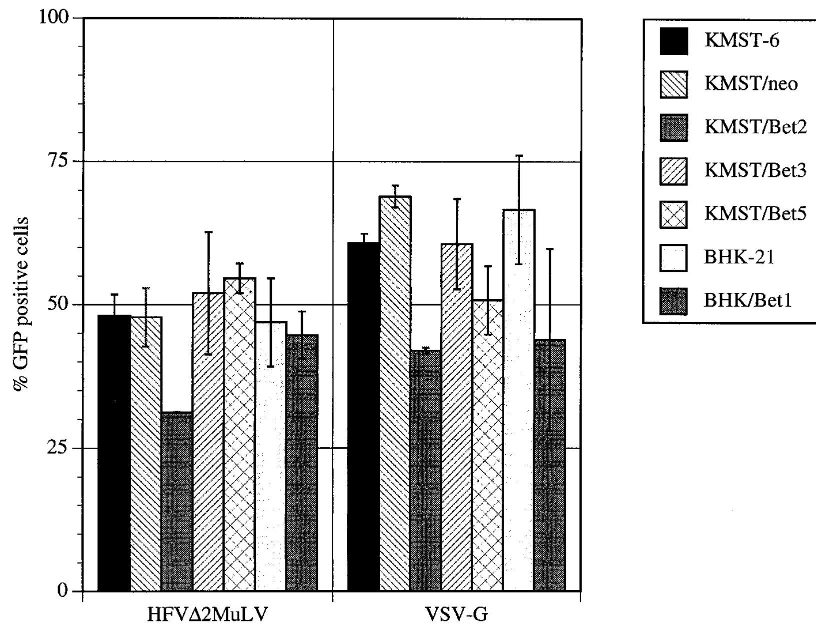


FIG. 3. Transduction efficiencies of Bet^- and Bet^+ cells infected with an MLV retroviral vector pseudotyped with different envelope proteins. The cells (2×10^4) were exposed to a GFP-encoding MLV retroviral vector pseudotyped with either the chimeric HFVΔ2MuLV or the VSV-G glycoprotein. HFVΔ2MuLV env uses the HFV receptor. No differences in transduction efficiencies between the two envelope proteins were observed, indicating that the HFV receptor expression on Bet^+ cells is not reduced compared with Bet^- cells. The mean and standard error of three independent experiments are shown.

replication cannot be excluded, these results argue that the resistant phenotype of Bet^+ cells to HFV superinfection probably has other causes.

HFV promoter activity after infection of Bet^+ cells

Recently, evidence as been presented indicating that the functionally active genome of extracellular HFV consists of largely double-stranded linear DNA (Moebes *et al.*, 1997). Because the effector plasmids in the transfection experiments presented in Figs. 4 and 5 delivered the virus as naked viral DNA integrated into the plasmid backbone, we wanted to know how viral DNA behaved on infection of Bet^+ cells. To analyze this and to dissect more precisely the problem of Bet-mediated resistance to FV superinfection, we performed infection experiments with an indicator gene expressing HFV vector, as illustrated in Fig. 6.

pFOV-7/gfp is a replication competent pFOV vector (Schmidt and Rethwilm, 1995) directing the expression of GFP as a fusion protein to a truncated Bet protein (Nestler *et al.*, 1997). The fusion partners are cleaved by an autocatalytically active protease encoded at the junction of the two proteins (Schmidt and Rethwilm, 1995). The expression of the indicator gene is dependent on the activity of the HFV promoters (Schmidt and Rethwilm, 1995). Infection of Bet^- cells resulted in a high percentage of GFP positive cells as detected by FACScan analysis after infection with FOV-7/gfp. However, when Bet^+ cells were infected, no GFP-expressing cells above background were detected (Fig. 6).

DISCUSSION

The results presented in this study showed that Bet-expressing cells are resistant to productive HFV replication. However, whether the resistant phenotype of Bet^+ cells is identical to that observed for ΔHFV-bearing cells (Saib *et al.*, 1995) cannot be deduced from our study because different methods were applied to analyze virus production. The inhibition of replication appeared to be rather specific for the homologous virus because the distantly related FFV was found able to replicate at least partially in HFV Bet^+ cells, and gene expression of an MLV vector genome was not impaired. Although other regions of the genomes are reasonably conserved, the weak homology of ~20% between HFV and FFV Bet (Bodem *et al.*, 1998; Winkler *et al.*, 1997) is likely responsible for the lack of resistance of the HFV *Bet*$^+$ cells to FFV replication.

Analysis of the late phase of viral replication, that is, after provirus integration, revealed only a minor difference between Bet^- and Bet^+ cells, which we do not consider to be the reason for the resistance of Bet^+ cells to HFV superinfection. Furthermore, it has been reported that Bet has a positive rather than a negative effect on viral export (Yu and Linial, 1993). In addition, we did not find a significant difference between Bet^+ and Bet^- cells in HFV promoter activity, when the effector plasmids were transfected into the cells. Interestingly, cells harboring human immunodeficiency virus (HIV) genes have been reported that resisted productive HIV superinfec-

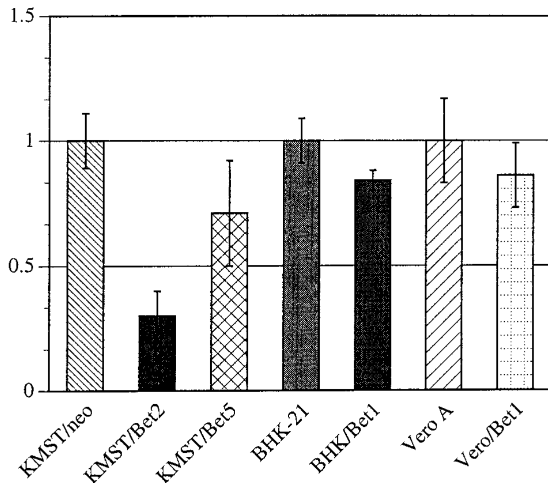


FIG. 4. Relative HFV promoter activity in Bet$^+$ and control cells. The cells were cotransfected with the infectious proviral clone pHSRV2 (Schmidt and Rethwilm, 1995), the indicator gene construct p5′cat(−777/+4)(Erlwein and Rethwilm, 1993), and the β-galactosidase-encoding plasmid pCH110. CAT values were normalized for differences in transfection efficiencies. Values obtained for the control cells were arbitrarily set to 1. Some differences between Bet$^-$ and Bet$^+$ cells can be seen, such as, between KMST/neo and KMST/Bet2 cells, which may reflect clonal variation. However, taking all results together, the differences between Bet-expressing and nonexpressing cells are not significant and cannot explain the differences observed in the development in viral titers (Fig. 2).

tion (Federico *et al.*, 1995). In this case, the block of replication was recently shown to result from an impairment in the generation of infectious progeny virus (D'Aloja *et al.*, 1998).

The most drastic difference between our Bet$^+$ and Bet$^-$ cells was found on infection with an indicator gene encoding virus. Although FOV-7/gfp expressed the indicator gene in Bet$^-$ cells, Bet$^+$ cells resisted GFP expression. How can the apparent paradox of active HFV promoters after transfection and the inactivity of these promoters after infection be explained? Retroviral LTRs require provirus integration for transcriptional activity (Coffin, 1996). Furthermore, it has been shown recently that HFV gene expression and replication require an intact viral integrase (Enssle *et al.*, 1998). Transfected plasmid DNA harboring retroviral genomes behaves like proviral DNA (Sakai *et al.*, 1993). Therefore our results strongly indicate that the Bet-mediated resistance to HFV superinfection is mainly due to an inhibition in the early phase of viral replication before provirus establishment. However, we do not know at what specific stage before integration Bet inhibits HFV replication. This may be at the stage of completion of reverse transcription, transport of the preintegration complex to specific sites of the nuclear matrix, or provirus integration itself.

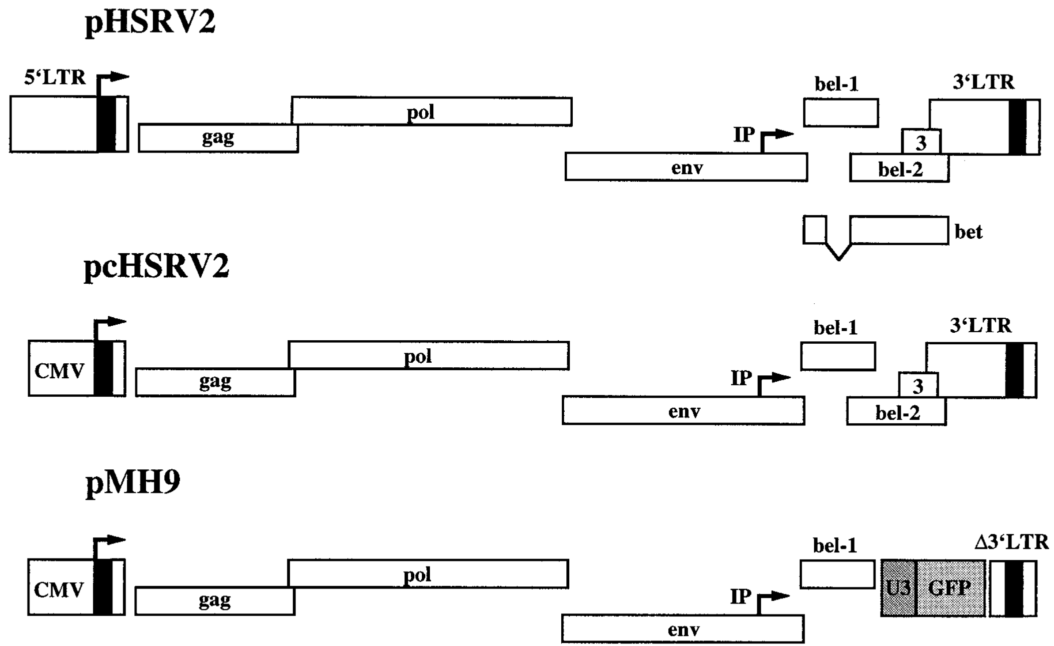
What might be the function of Bet in the FV replication cycle? We regard the integration of multiple ΔFV genomes into the same cell (Saib *et al.*, 1995) or of one or a few copies into transcriptionally active genomic regions, which may result in Bet expression, as too rare an event in the infected host to have any major effect on the survival and spreading of these horizontally transmitted viruses, which can infect many different cell types (Hooks and Gibbs, 1975). Evidently, such cells would be resistant to FV superinfection (Saib *et al.*, 1995). However, from the viewpoint of the virus, such a replication block would be rather late because the superinfecting virus could enter a cell and would be inhibited from replication. This is in contrast to the situation found for the primate lentiviral Nef protein, which is believed to prevent superinfection by receptor downregulation and thereby provide a selective advantage for the virus to replicate *in vivo* (Cullen, 1994).

Bet is expressed from the provirus after integration. This can lead to the idea that the phenomenon we investigated may be a cell culture epiphenomenon not related to any *in vivo* functions of Bet. Although we cannot formally exclude this possibility, we suggest a different explanation of our results, which may shed light on one aspect of Bet function. Investigations on the FV replication cycle have so far uncovered a variety of novel aspects (Rethwilm, 1996; Weiss, 1996). The finding of Gag protein recycling into the nucleus of already infected cells (Schliephake and Rethwilm, 1994; Yu *et al.*, 1996b) has led to the suggestion that FVs may perform an intracellular replication cycle similar to that demonstrated for hepadnaviruses (Ganem, 1996; Rethwilm, 1996; Schliephake and Rethwilm, 1994). Furthermore, it was observed that reverse transcription of the FV pregenomic RNA already occurs late in the replication cycle, resulting in a considerable amount of unintegrated copies of virus DNA (Moebes *et al.*, 1997). We recently obtained genetic evidence for such an intracellular HFV replication cycle (unpublished results). Although the efficiency of this replicative short cut is currently under investigation, we assume that the inhibition of multiple reintegrations into the host cell genome would be advantageous for the virus to avoid damage to the host cell genome. Thus, controlled regulation of an intracellular replication cycle would result in a higher virus release.

MATERIALS AND METHODS

Cells and viruses

Cell lines from baby hamster kidney (BHK-21), African green monkey kidney (Vero A), feline kidney (CRFK), human kidney (293 and 293T) (DuBridge *et al.*, 1987), and human skin (KMST-6) (Namba *et al.*, 1985) were cultivated in Eagle's minimal essential medium (MEM) or Dulbecco's modified Eagle's medium (DMEM) containing 5–10% FCS and antibiotics. BHK/LTR(HFV)lacZ cells (Schmidt and Rethwilm, 1995) were cultivated in MEM containing 5% FCS and 1 mg/ml G418 (PAN Systems). The retrovirus vector packaging cell lines GP+E-86 (Markowitz *et al.*, 1988) and PA317 (Miller and Buttimore,



cell line	plasmid	titer
BHK-21	pHSRV2	$7.8 \times 10^2 \pm 28$
	pcHSRV2	$8.2 \times 10^2 \pm 24$
	pMH9	$1.5 \times 10^2 \pm 17$
BHK/Bet1	pHSRV2	29 ± 4.9
	pcHSRV2	70 ± 11
	pMH9	38 ± 10
293/neo	pHSRV2	13 ± 2.8
	pcHSRV2	$9.2 \times 10^3 \pm 1.6 \times 10^3$
293/Bet1	pHSRV2	28 ± 2.1
	pcHSRV2	$4.5 \times 10^3 \pm 1.2 \times 10^3$
293/Bet2	pHSRV2	15 ± 1.2
	pcHSRV2	$8.2 \times 10^3 \pm 1.9 \times 10^3$
293/Bet4	pHSRV2	18 ± 1.8
	pcHSRV2	$1.5 \times 10^4 \pm 7.0 \times 10^2$
293/Bet6	pHSRV2	79 ± 0.9
	pcHSRV2	$9.1 \times 10^2 \pm 18$

FIG. 5. Release of cell-free virus after transfection of Bet^- and Bet^+ cells with infectious HFV genomes. The cells were transfected with the depicted infectious genomes. In contrast to pHSRV2 and pcHSRV2, pMH9 can only perform one round of replication due to a large deletion in the U3 region of the 3′LTR. The U3-GFP cassette in pMH9 is irrelevant for this experiment. At 48 h after transfection, the virus in the cell-free supernatant was titered on BHK/LTR(HFV)lacZ indicator cells. The results from three independent experiments are shown. Transient transfection of 293 cells with pHSRV2 yielded only very low amounts of virus. These cells also fail to support productive HFV replication. However, virus yields after transfection of these cells with pcHSRV2 were in the same range obtained by transfection of 293T cells with this plasmid (Lindemann and Rethwilm, 1998; Moebes *et al.*, 1997). (IP) Internal promoter.

1986) were cultivated in DMEM containing 10% FCS and HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) in the case of PA317 cells. HFV plasmid-derived viruses were generated by $CaPO_4$-mediated transfection of BHK-21 or 293T cells (Ausubel *et al.*, 1987) and were abbreviated with the plasmid name lacking the

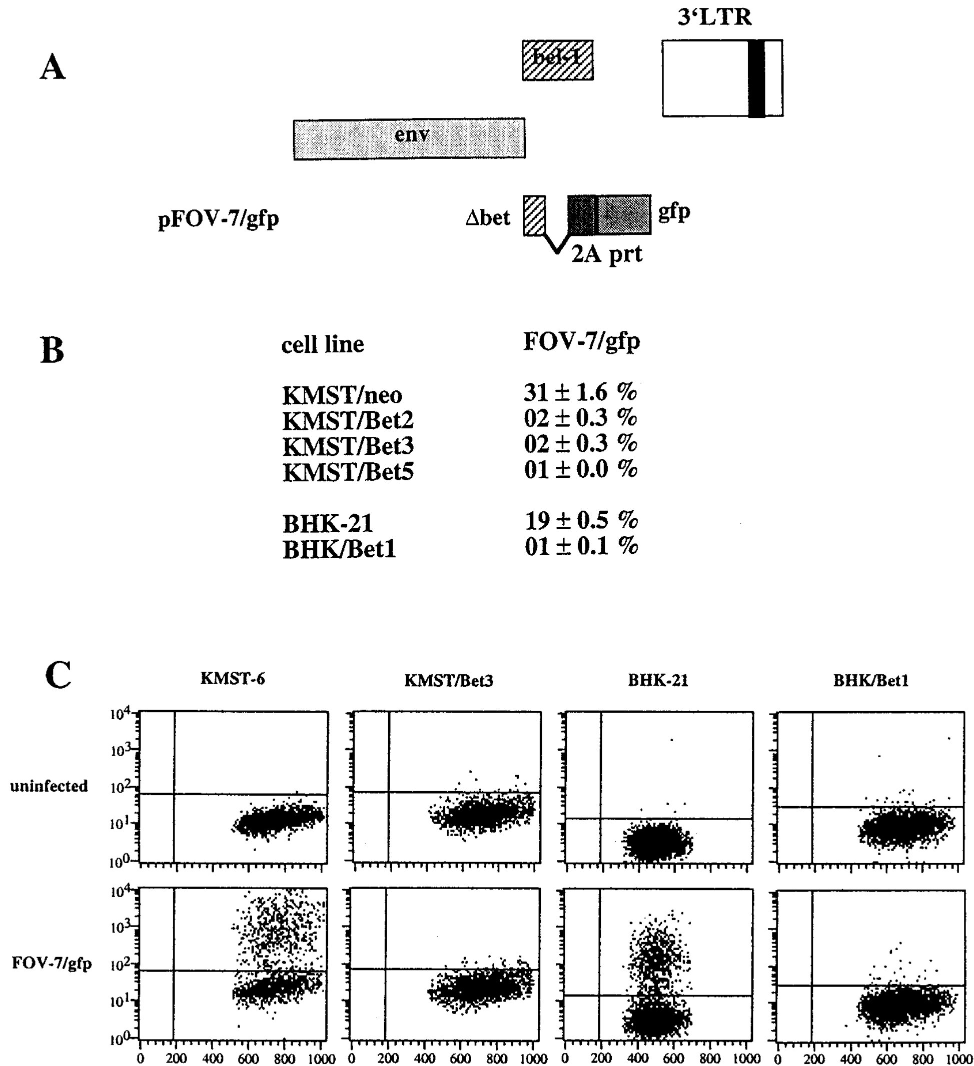


FIG. 6. Transduction efficiencies of Bet^- and Bet^+ cells infected with GFP-expressing HFV vector. (A) A 3′region of the pFOV-7/gfp vector. GFP is initially expressed as a fusion protein to a truncated Bet protein. The fusion partners are cleaved by the *cis*-encoded 2A protease derived from foot-and-mouth-disease virus (Schmidt and Rethwilm, 1995). (B) 2×10^4 Bet^+ and Bet^- cells were infected with cell-free vector virus at an m.o.i. of 1.5, and the GFP expression levels were determined 72 h later by FACScan analysis. The results of three independent experiments are presented. (C) Examples of the FACS images demonstrating the lack of activity of FOV-7/gfp in expressing the indicator gene in Bet^+ cells. The forward scatter is shown on the x axis, and the fluorescence intensity is shown on the *y* axis.

"p." The uncloned FFV isolate (Chiswell and Pringle, 1978) was grown on CRFK cells. Cell-free (0.45-μm filtrate) virus preparations were stored at −70°C for use. HFV titrations were performed on BHK/LTR(HFV)lacZ cells as described previously (Schmidt and Rethwilm, 1995). FK/LTR(FFV)lacZ cells are CRFK cells with an integrated lacZ gene under control of the FFV U3 region. The generation of this cell line is described below. It was used to titrate FFV in a similar way to the titration of HFV on BHK/LTR(HFV)lacZ cells (Schmidt and Rethwilm, 1995), except that 8×10^4 FK/LTR(FFV)lacZ cells per well were seeded onto 12-well plates (Nunc) the day before inoculation with virus instead of 2.5×10^4 BHK/LTR-(HFV)lacZ cells.

Generation of Bet protein-expressing cells

After the insertion of *Mun*I linkers upstream and downstream of the repaired bet insert of the HFV cDNA clone pA65 (Hahn *et al.*, 1994), the complete bet coding sequence (EMBL accession number X79000) was inserted as a 1.56-kb *Mun*I fragment into the *Eco*RI cloning site of the retroviral vector pLEN (Adam and Miller, 1991). pLEN-bet(HFV) was transfected into the ecotropic packaging cell line GP+E-86 using $CaPO_4$ (Ausubel *et al.*, 1987). The supernatant of the transfected cells was used to transduce the amphotropic packaging cell line PA317, which was selected in 0.5 mg/ml G418. The supernatant of the pooled G418-resistant PA317 packaging cells was

used to generate Bet protein expressing BHK-21, Vero A, KMST-6, and 293 cells. The cells were selected in 0.5 mg/ml G418, and single-cell clones were established by limiting dilution.

The expression of the Bet protein in the packaging cells and in the transduced target cells was monitored by indirect immunofluorescence using a rabbit antiserum generated against recombinant ORF-1 protein as described (Baunach *et al.*, 1993). In addition, Bet expression in the stable transduced single cell clones was analyzed by immunoblot using the same antiserum (Hahn *et al.*, 1994).

Recombinant DNA

All recombinant DNA work was done by established techniques (Ausubel *et al.*, 1987). The vector pFOV-7/gfp, which expresses GFP (S65T) (Chalfie *et al.*, 1994; Lindemann *et al.*, 1997) under control of the HFV LTR and IP, has been described recently (Nestler *et al.*, 1997). pMH9 is a derivative of pcHSRV2 (Lindemann and Rethwilm, 1998; Moebes *et al.*, 1997), which harbors the internal spleen focus forming virus (SFFV) U3/gfp expression cassette (Baum *et al.*, 1997; Fischer *et al.*, 1998; Heinkelein *et al.*, 1998) and has a deletion of a 0.56-kb *Bst*EII–*Xba*I fragment from the 3′LTR U3 region.

The FFV U3-R fragment from −1070 to +48 was PCR amplified from DNA of FFV-infected CRFK cells using primers derived from the published FFV LTR sequence (Bodem *et al.*, 1996). A *Bgl*II site (underlined) was attached to the 5′ primer (5′-GC<u>AGATCT</u>TGTCATGGGC-CAAAGAGAATTC), and a *Kpn*I site (underlined) was attached to the 3′ primer (5′-GC<u>GGTACC</u>AGAGTCT-CAATCTCACCCTGG). The *lacZ* gene from pCMVβ (Clonetech) was excised as a 3.53-kb *Not*I fragment and inserted into the *Not*I site of a pcDNA vector harboring the zeocine resistance cassette (InVitrogen). The CMV enhancer–promoter was excised from this plasmid as a 0.9-kb *Bgl*II–*Kpn*I fragment, and the 1.12-kb *Bgl*II–*Kpn*I-digested amplicon containing the FFV U3 region was inserted. The resulting plasmid was linearized with *Bgl*II and used to generate a stable transfected CRFK cell clone by limiting dilution on selection in 400 μg/ml zeocine (Eurogentec), similar to the previously described BHK/LTR(HFV)lacZ cells (Schmidt and Rethwilm, 1995).

Infection experiments

The cells were infected with replication competent cell-free virus at the m.o.i. indicated in the figure legends. Virus replication was monitored by transferring cell-free supernatant (0.45-μm filtrate) to the indicator cell lines. The MLV-derived retroviral vector pSFG.GFPS65T (Lindemann *et al.*, 1997) was packaged using the pHIT transfection system (Soneoka *et al.*, 1995) and was pseudotyped with the VSV G protein using pHIT.VSV-G or with the chimeric HFV env construct pCenv.HFVΔ2MuLV (Lindemann *et al.*, 1997). The virus-containing supernatant was divided into equal parts and used to infect an equal number (2×10^4) of Bet$^+$ and Bet$^-$ cells. The recipient GFP-expressing cells were identified 72 h after transduction or infection by fluorescence-activated cell sorting on a FACScan. The number of positive cells was quantified by using the Lysis II and CellQuest software packages (Becton Dickinson).

Transient transfection experiments

Next, 2×10^5 BHK-21 or 8×10^4 293 cells, which were seeded onto six-well plates on the day before transfection, were transfected with 3 μg of HFV DNA, either pHSRV2 (Schmidt and Rethwilm, 1995), pcHSRV2 (Lindemann *et al.*, 1997; Moebes *et al.*, 1997), or pMH9 (Fig. 4), together with 2 μg of pCH110 (Hall *et al.*, 1983) by $CaPO_4$ cotransfection (Ausubel *et al.*, 1987). Cell-free viral titers (0.45-μm pore size filtrate) were determined 48 h after transfection on BHK/LTR(HFV)lacZ cells. For CAT assays, 3×10^5 BHK-21, KMST-6, and Vero A cells were transfected with 2 μg pf pHSRV2, 2 μg of p5′cat(−777/+4) (Erlwein and Rethwilm, 1993), and 1 μg of pCH110 using Lipofectin (GIBCO BRL). The CAT activity was determined in cellular lysates as described previously (Erlwein and Rethwilm, 1993). Cotransfection of the *lacZ*-encoding plasmid pCH110 was done to account for different transfection efficiencies. The results were normalized for similar β-galactosidase expression levels, which were measured as reported previously (Norton and Coffin, 1985). Results were obtained from three to eight independent experiments and are presented in the figures as mean values with standard error.

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