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The *bet* Gene of Feline Foamy Virus Is Required for Virus Replication

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Foamy viruses (FV) are complex retroviruses with additional *bel* genes located between *env* and the 3′ long-terminal repeat. The functions of the *bel 2* and *bet* genes are unknown and both are dispensable for replication of the prototypic human foamy virus in cell cultures. We examined the function(s) of *bel 2* and *bet* of the distantly related feline foamy virus (FFV) in the proviral context. Mutagenesis was used to alter the Bel 2 and Bet or to abrogate their expression. The Bel 2/Bet mutants showed a 1000-fold reduced viral titer in feline kidney cells; in human 293T cells, viral titer was only about 10-fold reduced compared to wild-type FFV. In both cell types, the Bel 2/Bet mutations resulted in a reduced release of FFV particles. The results indicate that FFV Bet is required for efficient virus replication. The functions of the Bel 2 and Bet proteins are discussed.

Key Words: spumaretrovirus; feline foamy virus; accessory *bel 2* and *bet* genes.

INTRODUCTION

Spumaretroviruses or foamy viruses (FV) are retroviruses with a complex genome organization (Cullen, 1991; Linial, 1999; Löchelt and Flügel, 1995; Rethwilm, 1995; Lecellier and Saib, 2000). Important features of their replication strategy and molecular biology appear to be unique among the known retroviruses. This is best illustrated by the expression of Pol proteins from a spliced transcript, the mode of Gag–Env interaction during viral morphogenesis and budding, proteolytic processing of Gag and Pol proteins, and defined aspects of reverse transcription, genome integration, and gene expression (Baldwin and Linial, 1998; Bodem *et al.,* 1996; Enssle *et al.,* 1996; He *et al.,* 1996; Heinkelein *et al.,* 2000; Löchelt, 2001; Löchelt and Flügel, 1996; Pfrepper and Flügel, 2001; Pietschmann *et al.,* 1999; Wilk *et al.,* 2001, Yu *et al.,* 1996).

A hallmark of FVs is the efficient utilization of an internal promoter (IP) to direct expression of the regulatory and accessory *bel* genes located between *env* and the 3′ long-terminal repeat (LTR) (Löchelt *et al.,* 1993, Löchelt, 2001). The Bel 1 protein, also designated Tas for transactivator of spuma virus, is the viral transcriptional transactivator for LTR- and IP-derived gene expression (Keller *et al.,* 1991; Löchelt *et al.,* 1993; Mergia *et al.,* 1990; Rethwilm *et al.,* 1991; Zou and Luciw, 1996). Human foamy virus (HFV) Bel 1 is essential for viral replication and has the ability to specifically activate expression of defined human host cell genes (Baunach *et al.,* 1993; Löchelt *et al.,* 1991; Wagner *et al.,* 2000; Yu and Linial, 1993). Sparse data on the biological function of Bel 2 and Bet are available. Deletion of HFV Bel 2 and Bet have been reported to have either no effect or only to reduce the viral titer marginally, especially when cell-free HFV transmission was assayed (Adachi *et al.,* 1995; Baunach *et al.,* 1993; Lee *et al.,* 1994; Yu and Linial, 1993). However, cells expressing the HFV Bet protein were shown to be resistant to HFV superinfection and it is assumed that an early event during infection was blocked by Bet (Bock *et al.,* 1998).

Expression of HFV Bel 2 in infected cells is low and no data exist on its subcellular localization (Löchelt *et al.,* 1991). In contrast, Bet is highly expressed in infected cells and predominantly localized in the cytoplasm of infected or transfected cells (Löchelt *et al.,* 1991). Bet is generated by a splice event fusing the N-terminal shared domain of Bel 1 to the coding sequence of Bel 2 (Bodem *et al.,* 1998; Muranyi and Flügel, 1991). Since the Bel 2 start codon is downstream of the Bet splice acceptor, Bel 2 is fully contained in Bet. Bet is expressed in naturally infected cats and primates since antibodies against Bet are consistently detectable and are considered to be of diagnostic value (Alke *et al.,* 2000; Hahn *et al.,* 1994). The mode of Bel 2/Bet expression and their genomic localization is conserved. The degree of sequence homology of Bel 2/Bet proteins is, however, low among known FVs (Renshaw and Casey, 1994; Tobaly-Tapiero *et al.,* 2000). Genomic RNA that lacks the *bet* intron is reverse transcribed in FV-infected cells and this DNA has been implicated in establishing a persistent infection (Saib *et al.,* 1993).

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                                        ΔSD
                                        Δ Bet-SD
Bet   MASKYPEEGP ITEGVEEDFN SHSTSGLDLT S..... ..... ..... ..... .....    31
Bel1  MASKYPEEGP ITEGVEEDFN SHSTSGLDLT SGNKEEPLIS LALLSMHTSK IVVWIRDHFF VKILSFGGKQ KLYYICNQCH    80
                                        A

Bet   ..... ..... ..... ..... .....VGKNP EHPRRILLVL QHLIAYAEAT IKKQDVPGPL    66
Bel1  KGIPESGYIT LNTKYYLYEK GPTETGTKGL TLMRRHVQNS PCFLNSRKES GTPKTDPTRP ATSYSLCRSD YQEAGCSRPT   160

            ΔB2                                     MCS:  ASVRRGP
              T                                     BBtr: ASRYQE*
Bet   LPILSPYVMA WDNPQNVVTR LVNLGESWKK YLLSPGWKDC GERDLTMLTR ELL    VPG IGLVQIAATL TKTYVLMCNG   142
Bel1  PSNSESVCNG LGQPSERGHT SGESGGIMEE VSFISWLEGL WGEGFDYAN*                                    209

Bet   RCITGSRTDP DCDPLFCKLL CWKQNIQDPR ECNLEEWCLY SLDPEHDPLW DPKMIVRRHR NLLPYCMRPF LIWMNYISHN   222

Bet   PLTQQCIMMK TLNMLWRAQA DDPSDVASLY PRVKVFKASH FDIFGSASGN SEERVSWAKE NSHRGEYSLL PSSDDEEEEM   302

Bet   SEREELLCHI NQCQQKLFYP GGTTDVLGME SNVWLTKFVN IKFPKGTKVI RLPDGKFIAC DPELKPLLQE LKFLDRATSE   382

Bet   SSDSE*                                                                                    387
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FIG. 1. Schematic presentation of the FFV Bel 2/Bet mutations introduced into the proviral FFV genome. The FFV Bet (top line) and Bel 1 (bottom line) sequences are shown in the single-letter code. Mutated residues and the alterations affecting Bet and Bel 2 are shown above the sequence in boldface letters; the single mutation of Bel 1 is shown below the sequences. Asterisks mark translational stop codons. Dots represent the Bet intron sequences. An additional gap in Bel 2 and Bet is due to the mutations in clones pFeFV-MCS and pFeFV-BBtr. The Bel 2 sequence is marked by underlining. Abbreviations used for the different proviral constructs: ΔSD, pFeFV-ΔSD; ΔB2, pFeFV-ΔB2; MCS, pFeFV-MCS; BBtr, pFeFV-BBtr. Mutant pFeFV-ΔBB contains the mutations of clones pFeFV-ΔSD and pFeFV-ΔB2.

To investigate the role of the feline foamy virus (FFV) *bel 2/bet* genes, specific mutations were introduced into both genes in the context of the proviral genome. The mutant phenotypes were analyzed in comparison to the wild-type (wt) genome and Bet was shown to be essential for infectivity. Bet appears to be required for the efficient release of viral particles. These studies are essential to understand the biology of FVs in more detail and to exploit FVs as novel vectors for gene therapy applications (Hill *et al.,* 1999).

RESULTS

Construction of proviral FFV Bel 2/Bet mutants

As described under Materials and Methods and summarized in Fig. 1, defined mutations were introduced into the FFV proviral DNA clone pFeFV-7 (Zemba *et al.,* 2000) to inhibit expression of Bet (mutant pFeFV-ΔSD) or Bel 2 (mutant pFeFV-ΔB2). Additional mutants served to shorten Bel 2 and Bet (mutant pFeFV-BBtr) and to slightly alter the Bel 2 and Bet proteins (mutant pFeFV-MCS). The mutations were inserted in a way which did not or only slightly affect the overlapping *bel 1* gene (Fig. 1). To exclude detrimental effects of the Bel 1 Gly32Ala substitution in clones pFeFV-ΔSD and pFeFV-ΔBB, the FFV LTR luciferase (luc) reporter construct (Winkler *et al.,* 1997) was cotransfected with the different FFV *bel 2/bet* mutants as source of the Bel 1 transactivator into CRANDELL feline kidney (CRFK) cells. The Bel 1-mediated transactivation of luc expression gene was comparable in wt and mutant proviruses 1 day after transfection (p.t.) (data not shown).

Titer of proviral FFV Bel 2/Bet mutants

The phenotypes of the different FFV *bel 2/bet* mutants were analyzed in comparison to the parental clone pFeFV-7 by transfection into feline CRFK cells and human 293T cells. The viral titers were always determined from cell-free culture supernatants using the FFV-FAB indicator cells for titration (Zemba *et al.,* 2000). Especially in feline CRFK cells, the FFV *bel 2/bet* mutants showed a severely reduced viral infectivity in comparison to the wt proviral genome pFeFV-7. Mean average viral titers were determined in at least three independent transfection experiments into CRFK and 293T cells and are shown in Figs. 2A and 2B, respectively. One day p.t., the mutant proviruses showed about 10-fold reduced titers in CRFK and 293T cells. During further incubation, the viral titer increased in wt pFeFV-7-transfected CRFK (A) and 293T cells (B). In contrast, the Bel 2/Bet mutants displayed 72 h p.t. into CRFK cells at least 1000-fold decreased titer than with wt FFV. In 293T cells, infectivity of the mutants increased by time but remained about 10-fold reduced compared with the wild-type provirus. Serial passages of the Bel 2/Bet mutants in CRFK and 293T cells resulted in the complete loss of infectivity, pointing to an important role of these proteins in FFV replication.

Bel 1 expression and Bel 1-mediated transactivation was not impaired in any of the mutants as discussed above since the intracellular level of the Bel 1-induced β-galactosidase expression in individual FFV-FAB reporter cells was comparable in mutant and wt FFV-infected cells. Thus, the reduced titer can be attributed to the Bel 2/Bet mutations introduced.

Gene expression of proviral FFV Bel 2/Bet mutants in 293T cells

293T cells were transfected with the wt provirus and the Bel 2/Bet deletion mutants and harvested 1 and 3 days p.t. Cell lysates were used for immunoblotting to

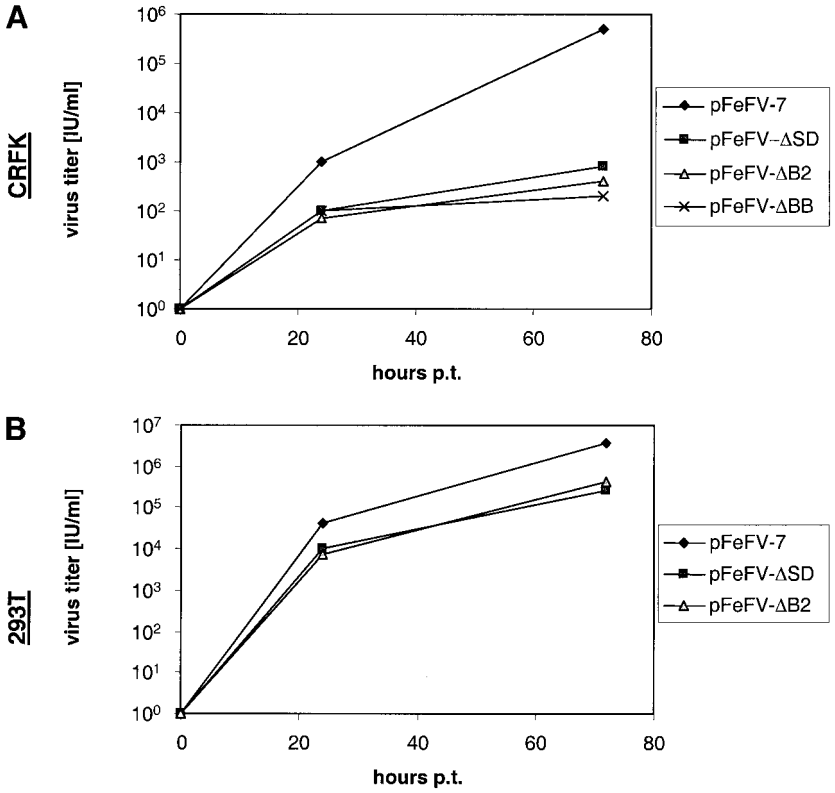

FIG. 2. Growth kinetics of wild-type and mutant FFV. Feline CRFK cells (A) and human 293T cells (B) were transfected with pFeFV-7 (wt) DNA and the mutants pFeFV-ΔSD, pFeFV-ΔB2, and pFeFV-ΔBB. At 24 and 72 h p.t., cell-free supernatants were collected from each culture and virus titers were determined by the FAB assay. The data shown reflect the average of four (A) and six (B) independent experiments, respectively.

determine the expression of viral proteins. To confirm the phenotype of the mutants, FFV gene expression was analyzed with an antiserum directed against Bel 2 and Bet. In 293T cells, Bel 2 was unambiguously detectable 1 and 3 days p.t. of pFeFV-ΔSD DNA (Fig. 3A). It was, however, not expressed by any of the other FFV DNA clones and was also absent in FFV-infected CRFK cells (data not shown). Bet was present in wt pFeFV-7 and pFeFV-ΔB2-transfected 293T cells at comparable levels (Fig. 3A), whereas it was absent in pFeFV-ΔSD-transfected cells, as expected. The expression of FFV Bel 1 was analyzed in parallel and found to be identical in wt- and pFeFV-ΔB2-transfected cells (Fig. 3B). Remarkably, Bel 1 was strongly overexpressed in pFeFV-ΔSD-transfected cells. Due to the inactivation of the Bet SD in mutant pFeFV-ΔSD, Bel 1 (Fig. 3B) and Bel 2 protein (Fig. 3A) expression of this clone is likely increased at the expense of Bet expression.

The expression of Gag and Pol as measured by immunoblotting was almost identical 3 days p.t. but reduced early after transfection of 293T cells with Bel 2/Bet-deleted proviruses compared with wt FFV DNA (data not shown, see also Fig. 6A).

To study whether the relative ratios of the different structural proteins of FFV were changed by the mutations, radioimmunoprecipitation (RIPA) experiments of transfected 293T cells were performed. Cells were labeled 30–48 h after transfection with pUC18, pFeFV-7, pFeFV-ΔSD, and pFeFV-ΔB2 DNAs. The corresponding extracts were precipitated with the FFV-specific cat serum 8014 (Alke *et al.,* 2000) and analyzed by PAGE and autoradiography (Fig. 3C). For the different FFV genomes used, the relative ratios of the FFV Env and Pol precursors and the p52 and p48 Gag proteins were roughly identical, although some differences in overall FFV protein expression were detectable. In summary, overall FFV gene expression in 293T cells appeared to be almost not affected by the Bel 2/Bet mutations except for the alterations in Bel 2 and Bet.

Gene expression of proviral FFV Bel 2/Bet mutants in CRFK cells

Feline CRFK cells were transfected with the wt provirus and the Bel 2/Bet deletion mutants. Cell lysates were analyzed for FFV gene expression by immunoblotting with serum of the experimentally FFV-infected cat 8014 (Alke *et al.,* 2000). Gag and Pol protein expression of clones pFeFV-ΔSD, pFeFV-ΔB2, and pFeFV-ΔBB was slightly reduced early after transfection compared with wt pFeFV-7-transfected CRFK cells (Fig. 4A, left-hand lanes). Structural gene expression of clone pFeFV-7 was greatly increased 3 days p.t. The Bel 2/Bet mutants showed reduced Gag expression compared with the wt

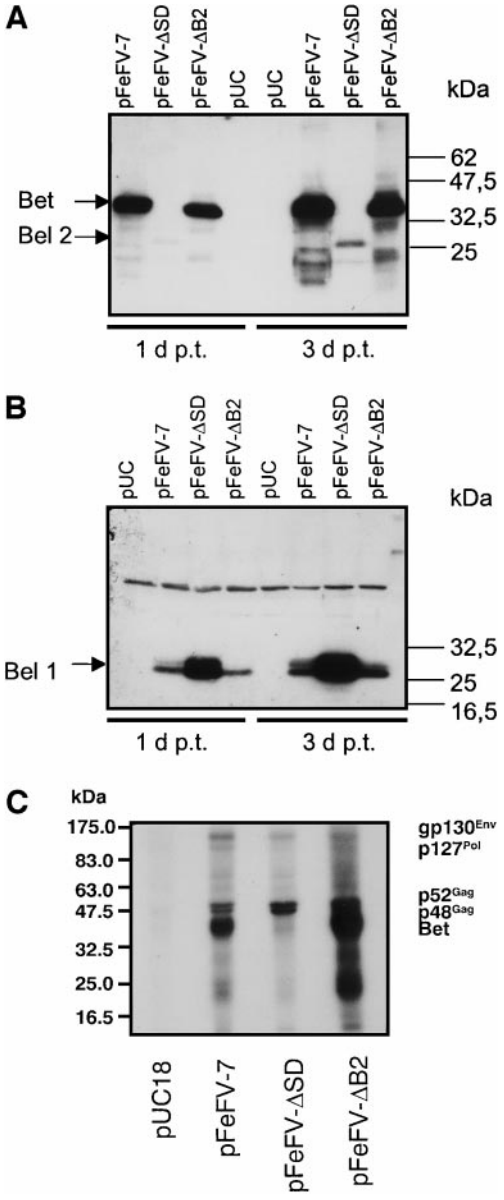

FIG. 3. FFV protein expression in transfected 293T cells. Immunoblot analysis of FFV Bel 2/Bet (A) and Bel 1 (B) protein expression in 293T cells transfected with plasmids pUC18, pFeFV-7 (wt), pFeFV-ΔSD, and pFeFV-ΔB2. Transfected 293T cells were lysed 1 and 3 days p.t. and cellular extracts were analyzed by immunoblotting with hyperimmune sera directed against bacterially expressed Bel 2/Bet (A) and Bel 1 (B, see Materials and Methods). The positions of molecular mass markers are shown in kDa on the right-hand margin, and arrows on the left indicate the detected FFV proteins. (C) RIPA analysis of 293T cells transfected with plasmids pUC18, wt pFeFV-7, pFeFV-ΔSD, and pFeFV-ΔB2. Transfected cells were radioactively labeled 30–48 h p.t. The extracts were precipitated with the FFV cat serum 8014 (Alke *et al.,* 2000) and analyzed by autoradiography. The positions of molecular mass markers and specifically precipitated FFV proteins are indicated.

clone and the level of Pol proteins was close to or even below the detection limit in these cells (Fig. 4A, right-hand lanes, see also Fig. 6B).

RIPA experiments using transfected CRFK cells did not yield specific FFV protein bands due to the low transfec-

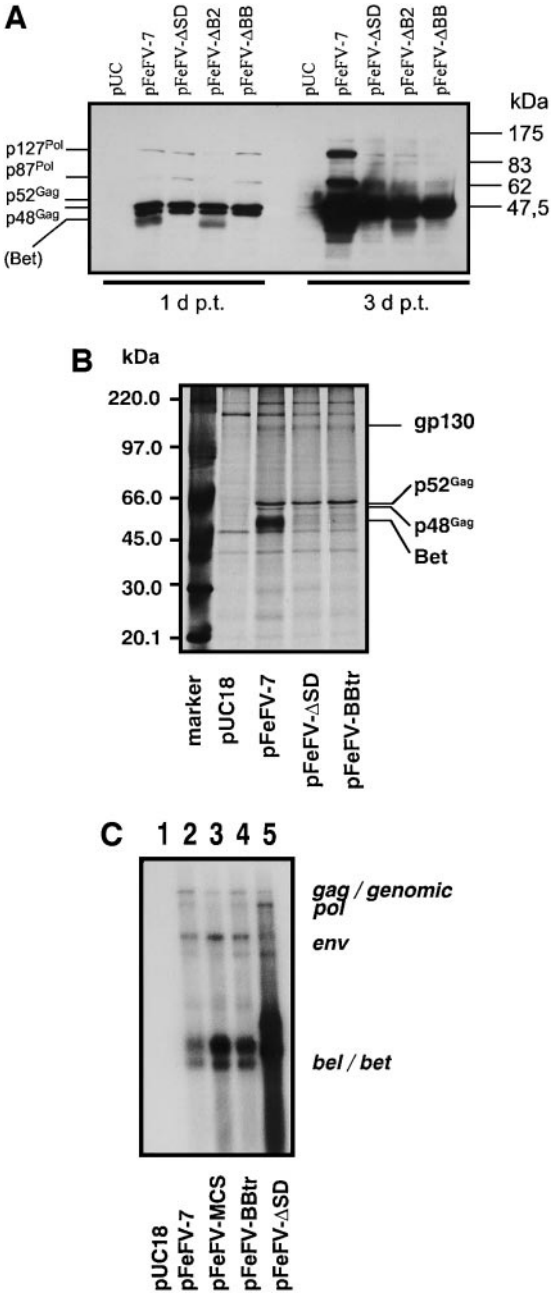

FIG. 4. FFV gene expression in wt- and mutant-transfected and infected CRFK cells. (A) Western blot analysis of FeFV protein expression in CRFK cells transfected with pUC18, pFeFV-7, pFeFV-ΔSD, pFeFV-ΔB2, and pFeFV-ΔBB at 1 and 3 days p.t. Cell lysates were collected from each culture and analyzed by immunoblotting with the FFV-specific cat serum 8014 (Alke *et al.,* 2000). Molecular mass markers are shown in kDa in the right-hand margin. On the left-hand side, arrows indicate the detected FFV proteins. (B) In parallel, CRFK cells were infected with cell-free supernatants of 293T cells transfected with pUC18 DNA or with each 10^5 FFU of wt pFeFV-7-, mutant pFeFV-ΔSD-, and pFeFV-BBtr-transfected 293T cells. Infected CRFK cells were labeled from 30 to 50 h p.i.; protein extracts were harvested and precipitated with cat serum 8014. The position of marker proteins (Rainbow marker, high molecular range, Amersham) and specifically detected FFV proteins are indicated. (C), Northern blot analysis of FeFV mRNA expression in CRFK cells transfected with pUC18 (lane 1), pFeFV-7 (lane 2), pFeFV-MCS (lane 3), pFeFV-BBtr (lane 4), and pFeFV-ΔSD (lane 5) 1 day p.t. Enriched mRNA samples were analyzed by Northern blotting using a probe located in the *bet* intron. The position of defined FFV transcripts is indicated at the right-hand margin.

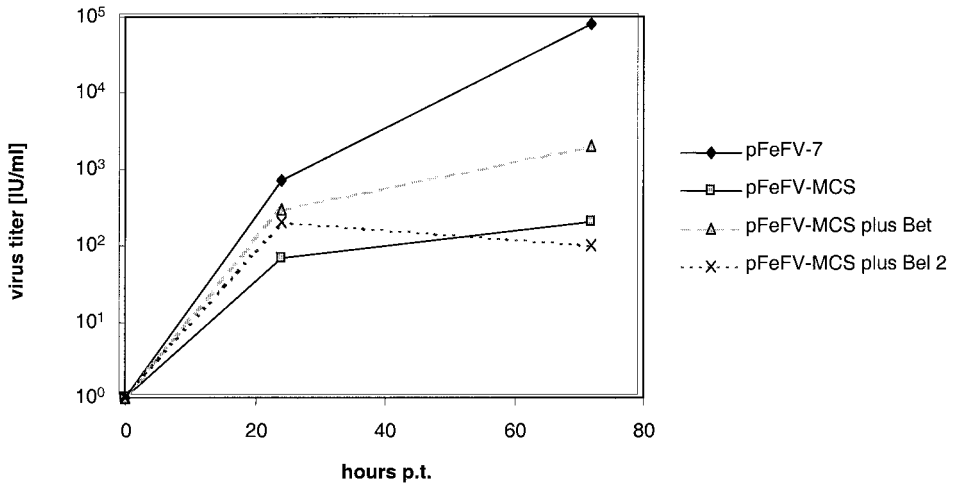

FIG. 5. Transcomplementation analysis of the Bel 2/Bet mutant FeFV-MCS by FFV Bet and Bel 2. The deletion mutant was cotransfected into CRFK cells together with the Bet expression vector pFFVBet and Bel 2 expression vector pFFVBel 2. As control, the wt-clone pFeFV-7 was transfected in parallel. Cell-free supernatants of each culture were collected 1 and 3 days p.t. and the virus titers were measured by the FAB assay. The data shown were taken from one of three independent experiments that showed similar transcomplementation values.

tion efficiency obtained with CRFK cells. To circumvent this problem, the recombinant plasmids were first transfected into 293T cells. Cell-free supernatants were harvested 3 days p.t.; FFV viral titers were determined and each 10^5 FFU was used to infect CRFK cells. The infected CRFK cells were labeled from 30 to 50 h p.i. Protein extracts were harvested and subjected to RIPA using the FFV-specific cat serum 8014 (Fig. 4B). Gag and Env protein expression was almost identical in wt- and mutant-infected cells using autoradiography (Fig. 4B) or phosphorimaging (data not shown). As expected, Bet was only expressed by wt FFV.

To further investigate gene expression of wt and mutant genomes, transfected CRFK cells were harvested 1 day p.t. and poly(A)$^+$ mRNAs were extracted and subjected to Northern blotting using an FFV DNA probe located within the Bet intron (Fig. 4C). All distinct classes of FFV transcripts were expressed; only plasmid pFeFV-ΔSD yielded increased amounts and additional mRNA bands in the *bel/bet* RNA range, which may be the result of alternative RNA processing due to the mutagenesis of the *bet* SD sequence. Whereas the relative expression of the different RNA species was invariant in wt pFeFV-7 and mutants pFeFV-MCS and pFeFV-BBtr, *pol* mRNA appeared to be overexpressed in clone pFeFV-ΔSD. In summary, gene expression of the FFV Bel 2/Bet mutants was reduced in CRFK cells.

Transcomplementation of proviral FFV Bel 2/Bet mutants

To confirm the specificity of the effects on viral infectivity, the Bel 2/Bet mutants were cotransfected into CRFK cells together with the Bet and Bel 2 expression vectors pFFVBet and pFFVBel2. Viral titers were analyzed 1 and 3 days p.t. Representative data for mutant pFeFV-MCS (Fig. 5) showed that part of the viral titer was restored by providing Bet *in trans*. In contrast, coexpression of Bel 2 did not show a significant enhancement of infectivity. The other FFV Bel 2/Bet mutants showed a similar transcomplementation of infectivity by Bet (Table 1; data not shown).

Since coexpression of FFV Bet but not Bel 2 allowed partial reconstitution of viral titers, we addressed the question whether the mutations in Bet introduced in some of the proviral clones (Fig. 1) were primarily responsible for the reduced viral titer observed. Thus, the Bet Met75Thr substitution of clone pFeFV-ΔB2 and the

TABLE 1

Transcomplementation of wt and *bet/bel* 2-Deleted FFV Proviruses by wt and Mutant FFV Bet in CRFK Cells[a]

DNAs cotransfected	1 day after transfection	3 days after transfection
pFeFV-7		
+ pUC18	6×10^2	3×10^5
+ pFFVBet	6×10^3	1×10^5
+ pFFVBet-M/T	9×10^2	3×10^5
+ pFFVBet-MCS	2×10^3	4×10^6
pFeFV-ΔBel2		
+ pUC18	8×10^1	3×10^2
+ pFFVBet	5×10^2	1×10^4
+ pFFVBet-M/T	2×10^1	5×10^2
+ pFFVBet-MCS	7×10^1	3×10^2
pFeFV-ΔSD		
+ pUC18	4×10^0	1×10^1
+ pFFVBet	5×10^2	4×10^3
+ pFFVBet-M/T	3×10^1	5×10^1
+ pFFVBet-MCS	6×10^1	3×10^1

[a] Titers of transcomplementations were determined using FAB cells and are expressed as blue cell units/ml cell culture supernatant.

MCS alterations of clone pFeFV-MCS were introduced by PCR into the wt FFV Bet expression plasmid pFFVBet. The resulting clones pFFVBet-M/T and pFFVBet-MCS and wt clone pFFVBet were used for transcomplementation of wt and Bel 2/Bet mutant proviruses (Table 1). Neither wt Bet nor mutant Bet reproducibly altered infectivity of the wt FFV provirus 1 or 3 days p.t.; the slight increase seen with pFFVBet-MCS DNA was not detectable in additional experiments. This indicates that the mutant Bet molecules used did not display a transdominant negative effect on wt Bet from pFeFV-7. As described above, wt Bet consistently rescued part of the titer of mutants pFeFV-ΔB2 and pFeFV-ΔSD. In contrast, none of the mutant Bet proteins was able to transcomplement the Bel 2 deletion clone pFeFV-ΔB2. The Bet deletion clone pFeFV-ΔSD was strongly transcomplemented by wt Bet and the titer of this clone was slightly increased even by both mutant Bet proteins.

In conclusion, the data suggest that the alterations introduced into Bet and expressed from plasmids pFeFV-ΔB2 and pFeFV-MCS significantly contribute to the defective phenotype of these mutants. Taking into account that coexpression of Bel 2 did not show a significant effect on the viral titer (Fig. 5), the phenotype of the Bel 2/Bet mutants seems to be predominantly, if not exclusively, determined by mutations in *bet*.

FFV Bet does not alter LTR- and IP-directed gene expression

The FFV LTR promoter luc expression construct (Winkler *et al.,* 1997) and a corresponding reporter plasmid of the IP were assayed for effects of Bet on the basal and Bel 1-activated FFV gene expression. Both FFV luc reporter plasmids were cotransfected with wt and mutant FFV Bet expression plasmids pFFVBet, pFFVBet-MCS, and pFFVBet-M/T into 293T cells either in the presence or in the absence of an FFV Bel 1 expression plasmid (Winkler *et al.,* 1997). Coexpression of wt and mutant FFV Bet proteins did not show any significant effect on the basal and Bel 1-transactivated reporter gene expression driven by either the LTR or the internal promoter (data not shown). Similarly, Gag expression was not affected if virions derived from Bel 2 and Bet deletion clone pFeFV-ΔB2 were used to infect CRFK cells transiently expressing wt and mutant Bet proteins (data not shown).

Release of viral particles from proviral FFV Bel 2/Bet mutants

293T cells were transfected with pFeFV-7, pFeFV-ΔSD, and pFeFV-ΔB2 DNA. Three days p.t., aliquots of the cell culture supernatants were used to infect CRFK cells (see below) and the remainder was used to purify released viral particles by centrifugation through a sucrose cushion. The release of FFV particles and FFV gene expression of the transfected 293T cells were analyzed by immunoblotting using cat antiserum 8014. FFV antigen expression by plasmids pFeFV-ΔSD and pFeFV-ΔB2 was slightly reduced compared to the wt FFV provirus (Fig. 6A, left-hand lanes). In contrast, the release of FFV particles from pFeFV-ΔSD- and pFeFV-ΔB2-transfected 293T cells was reduced by a factor of 2 and 4 compared with wt-transfected cells (right-hand lanes). This indicates that antigen expression of the mutants is only slightly decreased, whereas release of mutant particles is about two to four times lower.

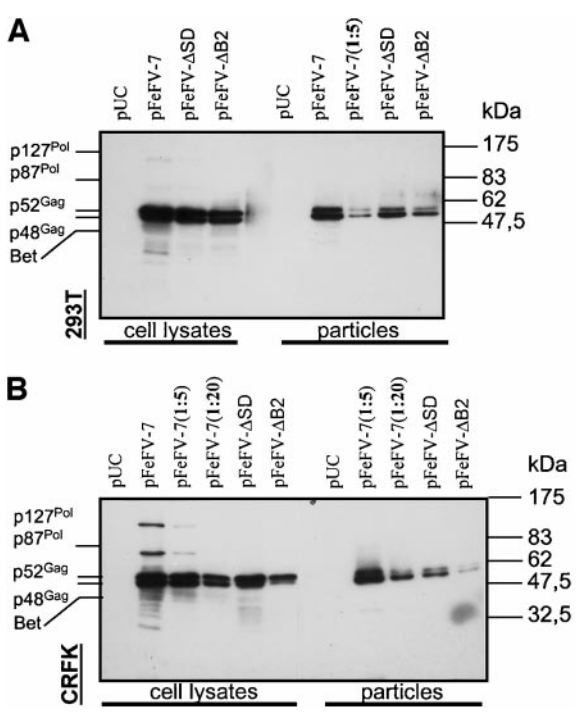

FIG. 6. Analysis of particle release from transfected 293T cells (A) and infected CRFK cells (B). In (A), cells were harvested 3 days p.t. and regular aliquots were subjected to immunoblotting with FFV cat serum 8014 (left-hand lanes). Part of the cell-free culture supernatant was used to purify the released viral particles, lysed and analyzed by immunoblotting in parallel (right-hand lanes). In (B), aliquots of the cell-free supernatants of 293T cells transfected with pFeFV-7, pFeFV-ΔSD, and pFeFV-ΔB2 used in (A) were inoculated on CRFK cells. Cellular antigen and released viral particles were harvested 3 days p.i., as described above and analyzed by immunoblotting with serum of FFV-infected cat 8014. To facilitate the comparison of protein amounts, extracts from plasmid pFeFV-7 were diluted 1:5 and 1:20, as indicated above the blots. Molecular mass markers are shown in kDa on the right-hand margin. On the left-hand side, the detected FFV proteins are indicated.

Equal aliquots of the cell culture supernatants of the transfected 293T cells described above were used to infect CRFK cells. Three days p.i., cell-associated antigen and released virus particles were analyzed as above. Gene expression of the mutants as measured by analyzing cell-associated antigen was reduced 10-fold for pFeFV-ΔSD-infected and more than 20-fold for pFeFV-ΔB2-infected CRFK cells in comparison to the wt-infected cells (Fig. 6B, left-hand lanes). In contrast, the release of

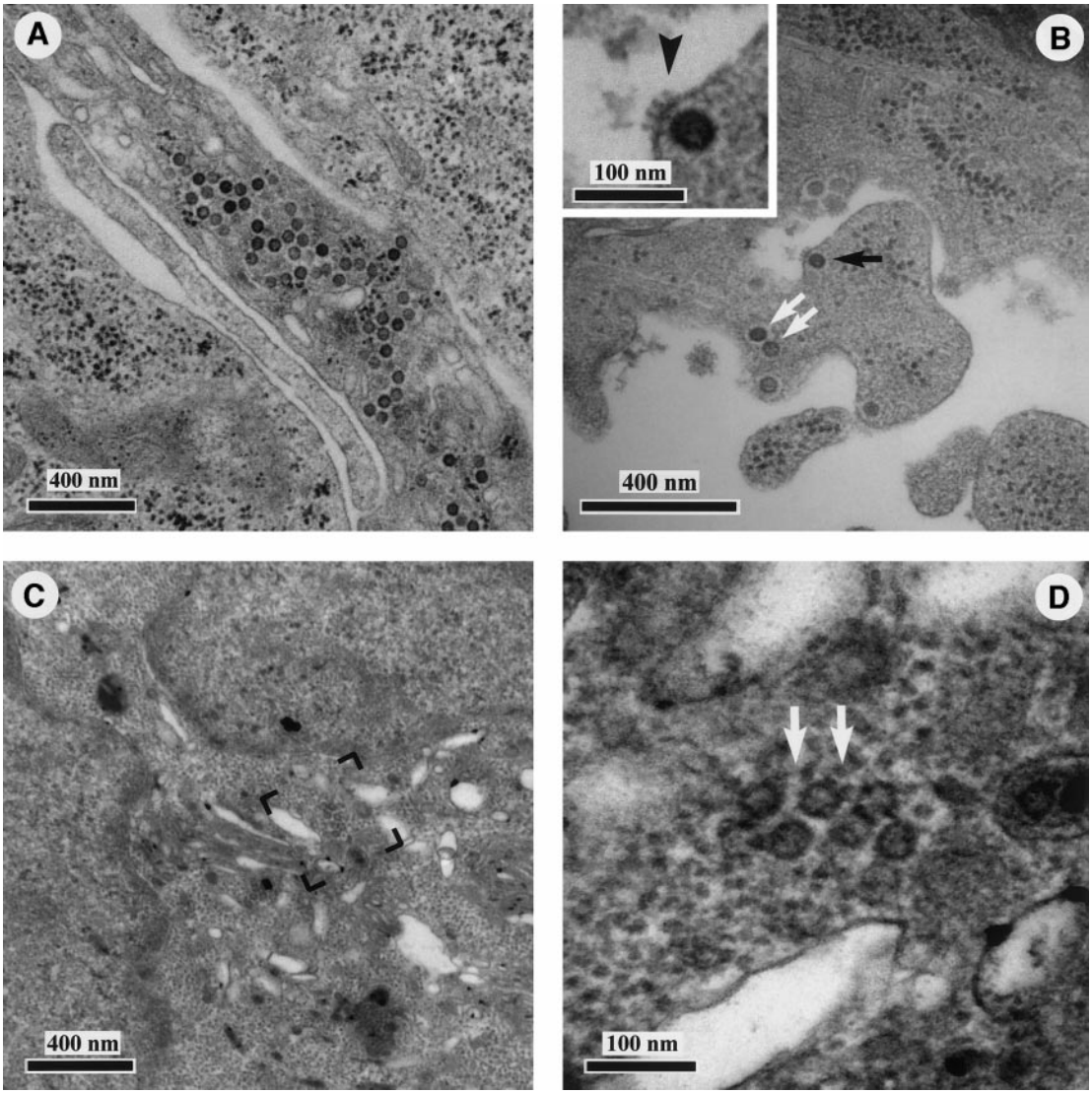

FIG. 7. Electron micrographs of thin sections of 293T cells transfected with wt pFeFV-7 (A and B) and pFeFV-ΔSD (C and D) 2 days p.t. (A) Accumulated preassembled capsids in pFeFV-7 wt transfected cells. (B) Budding of capsids (black arrow) into the extracellular space (bottom) at the cell membrane containing viral spike proteins (arrowhead in the inset) was only observed in cells transfected with pFeFV-7 DNA. The white arrows mark preassembled capsids. In pFeFV-ΔSD-transfected cells (C), only preassembled capsids (boxed space), here in the vicinity of a Golgi-stack, were detected; budding particles were not detectable. (D) Magnification of the boxed space in (C) with preassembled capsids (white arrows). The bars represent 400 and 100 nm, respectively.

particles from pFeFV-ΔSD-infected CRFK cells was more than 20-fold reduced and the release of virions from pFeFV-ΔB2-infected CRFK cells was at least 50-fold reduced in comparison to the release of wt FFV particles. When the release of particles is normalized to the different levels of intracellular FFV antigen expression, the amount of Bel 2/Bet mutant particles in the supernatant shows an about two- to fourfold decrease when compared with the wt FFV genome. When virions released from wt and mutant FFV-infected CRFK cells described in Fig. 4B were subjected to RIPA assays 2 days p.i., Gag specific bands were only detectable in wt and not in mutant-infected cells, although the initial titer and the intracellular gene expression were comparable. In summary, the data demonstrate that the release of Bel 2/Bet mutant FFV particles was reproducibly decreased about two- to fourfold compared to wt particles.

Electron microscopy of Bet/Bel 2-deleted FFV

To explore whether Bet has any effect on particle morphology and assembly, 293T cells were transfected with wt pFeFV-7 DNA and the mutant pFeFV-ΔSD. Two days p.t., cells were washed, fixed, and processed for thin-section electron microscopy. 293T cells transfected with pFeFV-7 DNA contained arrays of preassembled capsids in the cytoplasm in the vicinity of the Golgi apparatus, as known for FVs including FFV (Fig. 7A;

Zemba *et al.,* 1998). Budding of these particles consistently occurred at the plasma membrane in areas with finger-like membrane extrusions (Fig. 7B). Budding into intracellular membrane compartments was almost not detectable. Preassembled capsids were also detectable in mutants pFeFV-Δ SD (Figs. 7C and 7D); however, budding of these particles through intra- or extracellular membranes was not observed. In 293T cells transfected with pFeFV-ΔB2 DNA, budding of preassembled capsids was detectable (data not shown).

Whereas FFV particles were reproducibly detected in pFeFV-7-transfected CRFK cells, virus particles were almost undetectable in CRFK cells transfected with *bet/bel 2* mutant proviruses.

DISCUSSION

The severe, about 1000-fold reduction in titer of FFV genomes containing mutations in the *bel 2/bet* genes in permissive CRFK cells indicates that at least one of these genes is required for productive replication. This is in contrast to HFV, where different Bel 2/Bet mutations did not show a defined phenotype (Adachi *et al.,* 1995; Baunach *et al.,* 1993; Lee *et al.,* 1994) or resulted in less than 10-fold reduced HFV titer detectable only late after infection (Yu and Linial, 1993). Since the authentic target cells of FVs are not known at present, it is possible that the HFV *bel 2/bet* gene may be required for replication in other cell types or cells of other origin. The specificity of the decrease in titer of our FFV mutants was confirmed by rescue of a substantial part of the infectivity of the Bel 2/Bet mutants by coexpression of wt FFV Bet. Surprisingly, Bel 2 did not significantly transcomplement *bel 2* deleted pFeFV-ΔB2, whereas this clone was partially rescued by coexpression of wt Bet. Therefore, we assume that the Bet Met75Thr substitution and not the inactivation of Bel 2 expression in clone pFeFV-ΔB2 was responsible for the reduced infectivity of this clone. Consistent with this suggestion, mutant Bet Met75Thr was not capable of transcomplementing pFeFV-ΔB2 and the remaining mutants. Taken together with the observation that Bel 2 has not been unambiguously identified in FFV-infected CRFK cells except when the Bet splice donor had been deleted (Fig. 3A), it is likely that the modification and/or deletion of Bet was primarily responsible for the phenotypes observed.

Taking into account that mutagenesis of Bet was responsible for the reduced infectivity, it was surprising that not only the complete abrogation of Bet expression (pFeFV-ΔSD), but also truncation of Bet (pFeFV-BBtr), and even subtle modification of its primary sequence (pFeFV-ΔB2, pFeFV-MCS) displayed similar phenotypes as judged by viral titer and transcomplementation. For clones pFeFV-ΔB2 and pFeFV-MCS that contain Bet mutations at residues 75 and 117–119, it is reasonable to conclude that the integrity of this region of Bet may be required for proper folding and secondary or even tertiary structure. Using the DSC secondary structure prediction program (King *et al.,* 1997), the Met75Thr substitution completely abolished an extended β-sheet in FFV Bet that appears to be also present in the equine and bovine FV Bet proteins (data not shown).

The different titers and kinetics of FFV antigen expression of wt pFeFV-7 and the Bel 2/Bet mutants in CRFK and 293T cells may be due to the full permissiveness of CRFK cells for FFV. In contrast, infection of 293T cells with FFV particles is inefficient (unpublished observations), whereas transfection of 293T cells is much more efficient than that of CRFK cells. Thus, 293T cells may reflect the infectivity and gene expression of the input proviral DNA even late after transfection. CRFK cells infected with identical titers of wt and mutant particles showed a similar level of gene expression 2 days p.i., whereas the differences in viral titer of transfected proviruses had been further multiplied by additional rounds of replication 3 days p.t. However, this interpretation does not rule out cell type specific differences of Bel 2/Bet proteins for FFV replication.

The reduced titer is a consistent trait of the Bel 2/Bet mutants described here. The question remains as to which viral function(s) had been affected by the mutations. The data on the release of FFV particles and those obtained by thin-section EM suggest that budding and release of particles are affected (Figs. 6 and 7). Whereas assembly of cytosolic capsid appeared not to be altered, budding of FFV particles through cellular membranes was not detectable in the Bet deletion mutant pFeFV-ΔSD. In contrast, wt FFV budded primarily at the plasma membrane. However, particle release was only reduced in the Bet mutant since FFV antigens were released into the cell culture supernatants and FFV infectivity was detectable. This implies that alternative pathways for virus release may have been used by the Bet mutant or that "typical" budding intermediates were no longer detectable. In clone pFeFV-ΔB2 encoding the Bet Met75Thr mutation, typical budding structures were observed. However, it is unlikely that the about two- to fourfold reduced release of particles solely accounts for the strong differences in titer detected in CRFK cells. It is thus also likely that the infectivity of these mutant particles is compromised, which may be related to budding-related defects in assembly and morphogenesis.

A reduced release of HFV Bel 2/Bet mutant particles very late after infection of human cells has been described by Yu and Linial (1993) and is fully consistent with the data reported here. The observation of Bock *et al.* (1998) that HFV Bet-expressing cells are resistant to HFV superinfection also does not contradict our observations. It is conceivable that Bet that is involved in the release of particles may also interfere with the uptake of virus particles since release and uptake may share common components.

We cannot rule out the possibility that Bel 2 and/or Bet are additionally involved in other steps of FV replication. For instance, Bel 2 or Bet may modulate viral transcription under certain circumstances and/or the utilization of defined transcripts for FV gene expression (Linial, 2000). However, data supporting such an additional function have not been obtained in this study. Further studies are required to fully determine the functions of the FFV Bel 2/Bet proteins in cell cultures and infected cats and to establish why these proteins are apparently almost dispensable in HFV.

MATERIALS AND METHODS

Virus and cells

FFV strain FUV[7] has been described previously and was propagated in CRFK cells (Flower *et al.,* 1985; Winkler *et al.,* 1997). FFV infections were always performed with released, cell-free virus. FFV-FAB titration cells were selected and maintained in CRFK cell medium supplemented with 500 μg/ml G418 (Roche, Mannheim, Germany). FFV titer was assayed using FFV-FAB cells grown in 24-well plates (Zemba *et al.,* 2000). DNA transfection into CRFK cells was performed by electroporation and for 293T cells; the calcium coprecipitation method was used as described previously (Wagner *et al.,* 2000; Zemba *et al.,* 2000).

Construction of proviral FFV *bel 2/bet* mutants

The different FFV Bel 2/Bet mutants are schematically presented in Fig. 1.

pFeFV-MCS. To change defined amino acid residues in Bel 2/Bet and to introduce unique restriction enzyme sites into the infectious FFV clone pFeFV-7 (Zemba *et al.,* 2000), the *bel 2* gene was amplified by PCR with primers located at nt positions 9029 (5′-TTCACGAAGGAGACTATCCAGAGT-3′) and 9809 (5′-CC*GGTACCGGTCCGCGGCGTACGCTAGCTCTAGTTAGC*ATAGTCAAATCCC-3′) using *Pfu* DNA polymerase as recommended by the supplier (Stratagene, Heidelberg, Germany). The underlined sequence of the antisense primer contains recognition sites for *Nhe*I, *Bsi*WI, *Sac*II, *Age*I, and *Kpn*I. The Bel 2 sequence Glu43-Leu44-Leu44 was altered to AlaSerValArgArgGlyPro, corresponding to Bet residues 117–119. The resulting amplicon of about 800 bp was subcloned into pCR2.1TOPO (Topo Cloning Kit, Invitrogen). The FFV insert was excised with *Bsp*EI and *Kpn*I and cloned into the infectious FFV clone pFeFV-7 in a three-component ligation. Recombinant clone pFeFV-MCS and all other mutants described below were thoroughly analyzed by restriction-enzyme digestion and the inserted alterations were confirmed by DNA sequencing.

pFeFV-BBtr. The *bel 2/bet* genes of clone pFeFV-MCS were truncated directly downstream of the *bel 1* gene. To this end, pFeFV-MCS was digested with *Nhe*I and *Age*I, which both cut in the mutated sequence. The ends of the linearized vector were blunt-ended with Klenow DNA polymerase and religated. This resulted in a frameshift (+1) at nt position 9876 fusing the heterologous residues AlaSerArgTyrGlnGlu to the N-terminal 42 residues of Bel 2 and to the N-terminal 116 residues of Bet.

pFeFV-ΔSD. To specifically abolish Bet expression, the SD in *bel 1* required to generate Bet-encoding transcripts by splicing (Bodem *et al.,* 1998) was specifically destroyed by ligation PCR. Two independent PCRs were performed with primers located at nt positions 7163 (5′-CCAATTGGACAAGAGTAGAATCCTATGG-3′) and 9291 (5′-ATT**CG**CT*GA**C**GT**C***AGGTCCAA**T**CCAGAAGTGGAATGGAGT-3′) and with primers at positions 9672 (5′-GCTTCTTGATAGTCGCTTCGG-3′) and 9310 (5′-G**A**TTGGACCT***G**AC**G**TC*AG**CG**AATAAAGAAGAACCTTTGAT-3′); the mutations introduced are in boldface letters. Plasmid pFeFV-7 (Zemba *et al.,* 2000) served as template as defined above. Subsequently, the two amplicons served as template using the external primers at positions 7163 and 9672, leading to a fused PCR product of 2.5 kb, which contained the mutation from GT to CG in the *bet* SD and an additional inserted *Aat*II site (underlined). In the overlapping *bel 1* reading frame, the SD mutation led to a Gly32Ala substitution, whereas the other mutations were silent. The 2.5-kb PCR product was digested with *Pml*I and *Bsp*EI and inserted into the correspondingly digested plasmid pFeFV-7, resulting in DNA clone pFeFV-ΔSD.

pFeFV-ΔB2. As described for mutant pFeFV-ΔSD, the Bel 2 AUG start codon in plasmid pFeFV-7 was specifically deleted. PCR primers located at nt positions 8490 (5′-GAATGCAGGATATTTATCTAAGG-3′) and 9724 (5′-GC**C**GTTACATACG-GA**T**TCA-3′) and located at nt positions 9983 (5′-GCATCACAATCTGGGTCGG-3′) and 9724 (5′-CTGA**A**TCCGTATGTAA**C**GGC-3′) were used. The altered residues are shown in boldface letters. In the resulting PCR product of about 1.5 kb, the *bel 2* ATG start codon was changed to ACG. The mutation did not affect Bel 1 but led to the Met75Thr substitution in Bet. The PCR product was digested with *Bsa*AI and *Kpn*I and inserted into the corresponding sites of pFeFV-7, resulting in clone pFeFV-ΔB2.

pFeFV-ΔBB. The mutant pFeFV-ΔB2, which has the ΔSD and the ΔB2 phenotype, was constructed in analogy to plasmid pFeFV-ΔB2 using pFeFV-ΔSD DNA as template for PCR amplification.

Construction of eukaryotic Bel 2 expression plasmid

The coding sequences of the FFV *bel 2* gene was amplified by PCR as described above using pFeFV-7 template DNA and primers at nt positions 9716 (5′-CAG-T*AAGCTT*-CCAATTCTGAGTCCGT-3′) and 10,686 (5′-CATAATCCAGGTAAATTCAG-3′). A *Hin*dIII site (underlined) was included in the sense primer. The PCR product was

digested with *Hin*dIII and inserted into the *Hin*dIII- and *Sma*I-digested pBC12CMV DNA (Sambrook *et al.,* 1989). The corresponding expression plasmid was designated pFFVBel2 and directed the expression of the 33-kDa Bel 2 protein. The corresponding FFV Bet expression plasmid has been described previously (Alke *et al.,* 2000).

Construction of mutant FFV Bet expression plasmids

The alterations of Bet in proviral clones pFeFV-MCS and pFeFV-ΔB2 were introduced into the FFV Bet expression plasmid pFFVBet (Alke *et al.,* 2000). The *Bsp*EI–*Xba*I fragment of plasmid pFFVBet was replaced by the corresponding DNA fragments of the clones pFeFV-MCS and pFeFV-ΔB2. The identities of the clones pFFVBet-MCS and pFFVBet-M/T were analyzed by restriction-enzyme digestion and Bet protein expression was confirmed by transfection into eukaryotic cells and immunoblotting.

Immunoblotting and RIPA

Cells were harvested 2 days after transfection by lysis in 1% SDS and the protein concentration was determined using the DC protein assay (Bio-Rad, Munich, Germany). Identical amounts of proteins were separated by SDS–PAGE, blotted, reacted with different FFV-specific sera directed against recombinant proteins or a cat hyperimmune serum, and detected as previously described (Alke *et al.,* 2000). Labeling of cells and radioimmunoprecipitation were done as described (Alke *et al.,* 2000).

Northern blotting

Extraction of RNA from transfected CRFK cells, poly(A)$^+$ selection, and Northern blotting were done as described (Wagner *et al.,* 2000). A PCR-amplified FFV DNA fragment located in the *bet* intron (FFV nt 9323–9534) was used as described (Bodem *et al.,* 1998).

Luc expression assays

The FFV LTR luc reporter plasmid has been described previously (Winkler *et al.,* 1997) and a corresponding FFV IP-based construct will be described elsewhere. Luc reporter assays were performed and quantified as described. Each 2 μg of the reporter plasmids and each 2 μg of a Bel 1/Bet expression plasmid or the wt and mutant FFV proviruses were transfected (Winkler *et al.,* 1997). Luc expression was measured using a Luminoskan TL Plus luminometer (Labsystems, Frankfurt, Germany). Luc values were normalized according to coexpressed β-gal activity, which was comparable in the different samples. As controls, the pGL2-promoter vector backbone was analyzed and found to be slightly activated by Bel 1. These values were routinely subtracted from those derived from FFV LTR- and IP luc reporter plasmids.

Transmission electron microscopy

Electron microscopy was done with minor modifications as described (Zemba *et al.,* 1998). In brief, transfected 293T cells grown on glass coverslips were gently washed with PBS 2 days p.t. and fixed for 60 min with 4% formaldehyde and 0.25% glutaraldehyde in PBS. Subsequently, the cells were washed with PBS and postfixed with 1% osmium tetroxide in water for 60 min. After washing two times, the specimens were dehydrated in a graded ethanol series. The 70% step was supplemented with 2% uranyl acetate and cells were stained for 60 min in the dark. Dehydrated cells were then embedded in Epon 812. After polymerization, the glass coverslips were removed in 20% hydrogen fluoric acid for ~60 min. Ultrathin sections were made perpendicular to the cell layer with a diamond knife on a Leica Ultracut UCT, doubly stained with uranyl acetate and lead citrate, and analyzed using a Philips BioTwin CM120 electron microscope.

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