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# Nuclear Localization of the Functional Bel 1 Transactivator but Not of the Gag Proteins of the Feline Foamy Virus

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Interactions between host cells and foamy or spumaretroviruses are different from those of other known retroviruses. Previous work has suggested that the Gag and high-affinity DNA-binding Bel 1 transactivator of human foamy virus are localized in the nuclei of infected cells. Using two independent detection methods, we show here that the functionally active Bel 1 transactivator protein of feline foamy virus is of nuclear localization. In contrast to that reported for the human foamy virus Gag protein, the cat foamy virus Gag proteins exclusively localized in the cytoplasm close to perinuclear regions.


## INTRODUCTION

Spumaviruses, or foamy viruses (FVs), belonging to the retroviruses encode not only the structural proteins Gag, Pol, and Env but also a strong transcriptional transactivator Bel 1 (Tas) that is required for virus replication and that directly binds to DNA target sequences (He *et al.,* 1996; Kang *et al.,* 1998; Zou and Luciw, 1996). Characteristic features of FVs not shared by other complex retroviruses include the presence of a potent internal promoter besides the 5′ long terminal repeat (LTR) promoter (Bodem *et al.,* 1998; Löchelt *et al.,* 1993; Mergia, 1994). In addition, the FV *pol* genes are expressed from a spliced *pol* transcript (Bodem *et al.,* 1996; Yu *et al.,* 1996a).

Recently the nuclear import and export pathways for various proteins and RNAs were studied extensively (Görlich and Mattaj, 1996; Nigg, 1996). The functional properties and cellular localizations of retroviral proteins such as HIV-1 Tat and Rev have been analyzed in detail (Cullen and Malim, 1991), and unique nuclear structures have been reported for the HTLV-I Tax transactivator (Bex *et al.,* 1997), The subcellular localizations of spumaretroviral proteins have not been extensively characterized (Löchelt and Flügel, 1995; Mergia and Luciw, 1991; Rethwilm, 1995).

The feline FV (FeFV) transactivator Bel 1 and Gag proteins are shorter in length than the corresponding human FV (HFV) proteins. Surprisingly, both reveal little, if any, homology on the protein sequence level compared with the corresponding gene products of primate FVs and HFV. The FeFV Bel 1 is expressed exclusively from the internal promoter (Bodem *et al.,* 1998), which is in contrast to that of HFV, for which *bel 1* transcripts were observed that started at the 5′ LTR promoter (Löchelt and Flügel, 1995). The FeFV Bel 1 does not contain the classic basic nuclear localization signal (NLS) but likely contains an unusual bipartite signal motif (Winkler *et al.,* 1997). The HFV Bel 1 has been reported to contain a functional NLS that contains the conserved classic motif (Chang *et al.,* 1995).

The FeFV Gag lacks the Arg-Gly-rich motifs characteristic of the primate FV Gag COOH-terminal domains (Winkler *et al.,* 1997). The HFV Arg-Gly-rich boxes were reported to be responsible for nuclear localization by genetic analysis (Schliephake and Rethwilm, 1994). However, a more recent report clarified that this subcellular localization was not required for infectivity (Yu *et al.,* 1996b).

Through the use of different methods and cell types, the study goal was to determine where the Bel 1 and Gag proteins of FeFV are localized.

## RESULTS AND DISCUSSION

### Localization of the Bel 1 transactivator protein

To determine the subcellular localization of the FeFV Bel 1 transactivator protein, cloning was carried out that resulted in the expression of a GFP-Bel 1 fusion protein.

The plasmid contained *gfp* 5′ of the *bel 1* gene. The variant GFP used had been adapted for mammalian cells with a optimal fluorescence shifted into the red and an activity that is 35 times higher than that of wild-type GFP (Zolotukhin *et al.,* 1996). The expression plasmid pBK-CMV-*gfpbel1* was transfected into either HeLa or Crandell feline kidney (CRFK) cells. Two days after transfec-

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tion, the localization was analyzed with fluorescence microscopy (Fig. 1). No fixation was necessary; thus the resulting image was not affected by different fixation techniques. Examination of the transfected HeLa cells (Figs. 1A and 1B) clearly revealed nuclear localization of the GFP-Bel 1 protein. The same result was obtained when CRFK cells were examined with UV light (Figs. 1C and 1D). To determine whether the recombinant GFP-Bel 1 protein was still biologically active and whether the transactivator activity was comparable with that of wild-type Bel 1, transient expression assays were performed with a cotransfected 5′ LTR-based luciferase plasmid. The data obtained showed that the Bel 1 protein was active and functioned as transcriptional transactivator (Table 1). This subcellular localization was confirmed through direct microinjections of recombinant MBP-Bel 1 protein (data not shown). This result is in agreement with the known capability of Bel 1 to bind DNA with high affinity and of serving as transactivator for transcription.

It is unknown what mechanisms are responsible for exporting spumaviral transcripts from the nuclei into the cytoplasm of infected cells. To examine whether Bel 1 is capable of shuttling between the cytoplasm and cell nuclei, thereby exporting cargo, an MBP-*bel 1* recombinant plasmid was constructed, and Bel 1 was expressed as MBP-Bel 1 fusion protein. The MBP-Bel 1 protein was labeled with FLUOS and microinjected into one of several polynucleated nuclei of HeLa cells grown onto coverslips. After incubation for 30 min, the cells were fixed and examined with fluorescence microscopy. The result shown in Fig. 2 clearly identified Bel 1 as nuclear protein that remained in the nuclei of transfected cells, indicating that FeFV Bel 1 does not shuttle between the cytoplasm and the nucleus under the conditions used. As control, MBP-Bel 1 protein was microinjected into the cytoplasm of HeLa cells. It was observed that the fusion protein was transported into the nucleus but did not shuttle between the two cellular compartments. Inspection of the FeFV Bel 1 sequence showed that a conserved basic NLS motif is not present, which is in contrast to those of the primate FV Bel 1 (Chang *et al.,* 1995). We assume that FeFV Bel 1 contains an unorthodox nonconserved NLS motif, as suggested previously (Winkler *et al.,* 1997).

### Subcellular localization of the GFP-Gag fusion protein

To determine the subcellular localization of the FeFV Gag structural protein, an approach was chosen analogous to that described above for the Bel 1 protein. To determine the subcellular localization of the viral protein Gag, cloning was carried out that resulted in the expression of a Gag-GFP fusion protein. Again, the *gfp* gene was cloned upstream of the viral *gag* gene. Plasmid DNAs (pBK-CMV-*gfpgag*; for details, see Materials and Methods) from two independently prepared *gfp-gag* clones were separately transfected into BHK, CRFK, and COS cells. Examination by UV light microscopy 2 days after transfection showed that the GFP-Gag protein exclusively localized in the cytoplasm in distinct structures close to perinuclear regions. (Figs. 3A–3C). Neither a nuclear localization nor a random cellular distribution of GFP-Gag was found. The narrow point-like clustering of the GFP-Gag fluorescent protein on the outside of nuclei indicates that these regions presumably represent cytoplasmic preassembly sites of FeFV capsids and might be explained by the presence of a morphogenetic signal motif identified by Rhee and Hunter (1990) in type D retroviruses. A closely related signal sequence motif also occurs in the matrix domain of FeFV Gag at a genetically similar position as in that of Mason–Pfizer monkey virus (Winkler *et al.,* 1997). In addition, distinct morphological features adjacent to the nucleus that are reminiscent of those found here were also reported for HFV Gag (Saib *et al.,* 1997).

Additional immunofluorescence experiments with antiserum directed against the matrix domain of FeFV Gag revealed that in CRFK cells, Gag concentrated in perinuclear regions (Fig. 4A). Again, nuclei of infected CRFK cells were not stained with a matrix domain-specific antiserum but rather were stained with DAPI (Fig. 4B). Taken together, our data show FeFV does not localize to the cell nuclei of infected or transfected cells; instead, it localized in perinuclear regions of both BHK and CRFK cells. This might be due to the sequence differences present in the COOH-terminal (NC like) regions of FeFV Gag (Winkler *et al.,* 1997), although alternative explanations cannot be ruled out.

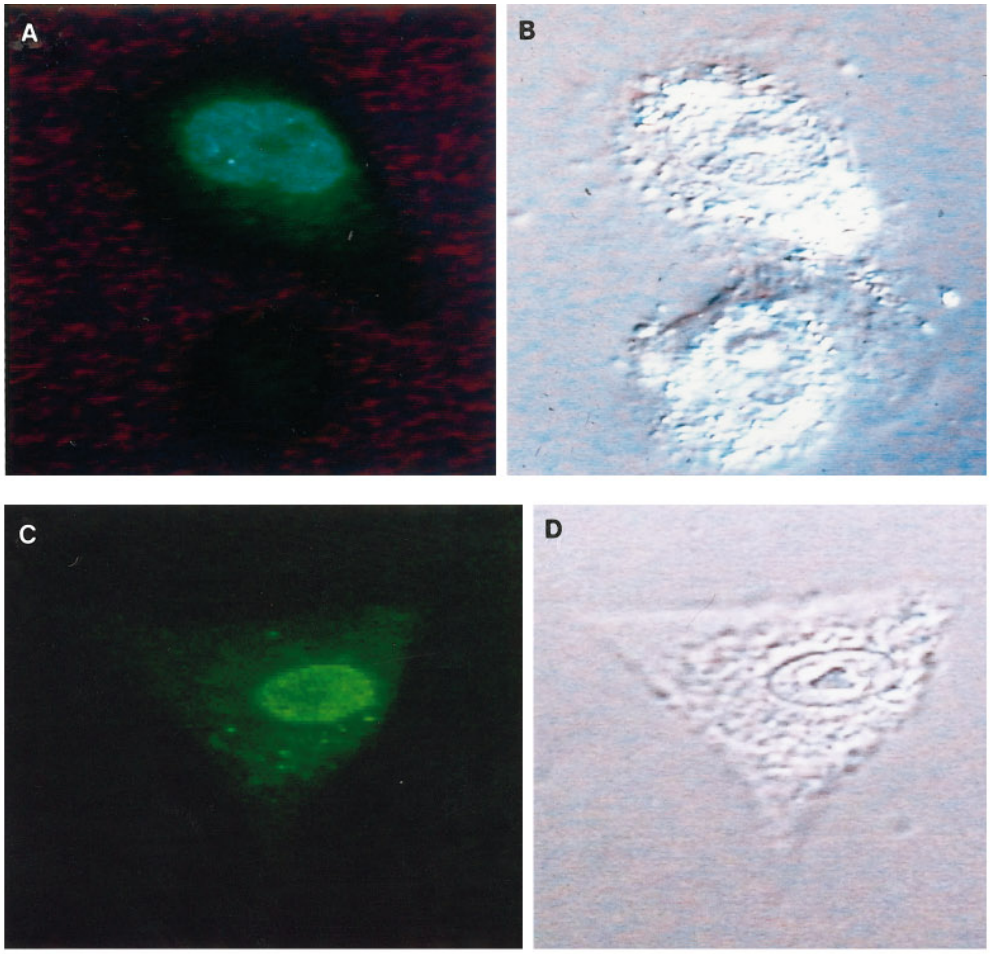
## MATERIALS AND METHODS

### Expression cloning

Two primers were used to amplify *gfp* (green fluorescent protein) from plasmid vector pTR-UF5 (Zolotukhin *et al.,* 1996) with *Pfu* DNA polymerase and cloned into the pBK-CMV plasmid that had been modified by adding *Pst*I and *Spe*I sites (Stratagene, Heidelberg, Germany). The FeFV *gag* gene was amplified with *Pfu* DNA polymerase and two primers (5′-GGACTAGTATGGCTCGAGAATTAA-

FIG. 1. Distribution of the FeFV GFP-Bel 1 fusion protein. pBK-CMV-*gfpbel1* plasmid DNA (20 $\mu$g) was transfected into HeLa cells and visualized 2 days after transfection with fluorescence microscopy (A) and phase contrast (B). (C and D) Analogous experiments with CRFK cells 3 and 4 days after transfection.

FIG. 2. Microinjection of MBP-Bel 1 fusion protein (3 mg/ml) labeled with either rhodamine-labeled IgG (A) or FLUOS (B) into a HeLa cell nucleus. After incubation for 30 min, photographs were taken under UV light (B) and by phase contrast (C).

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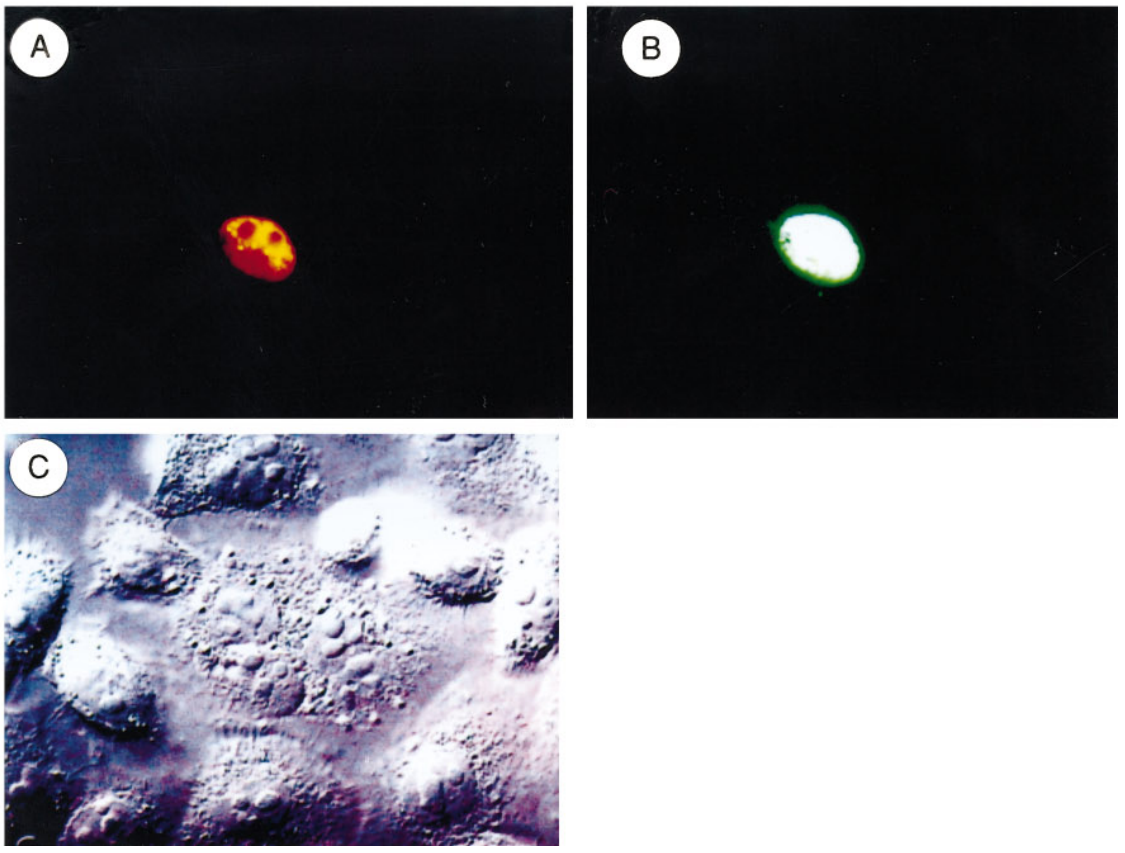


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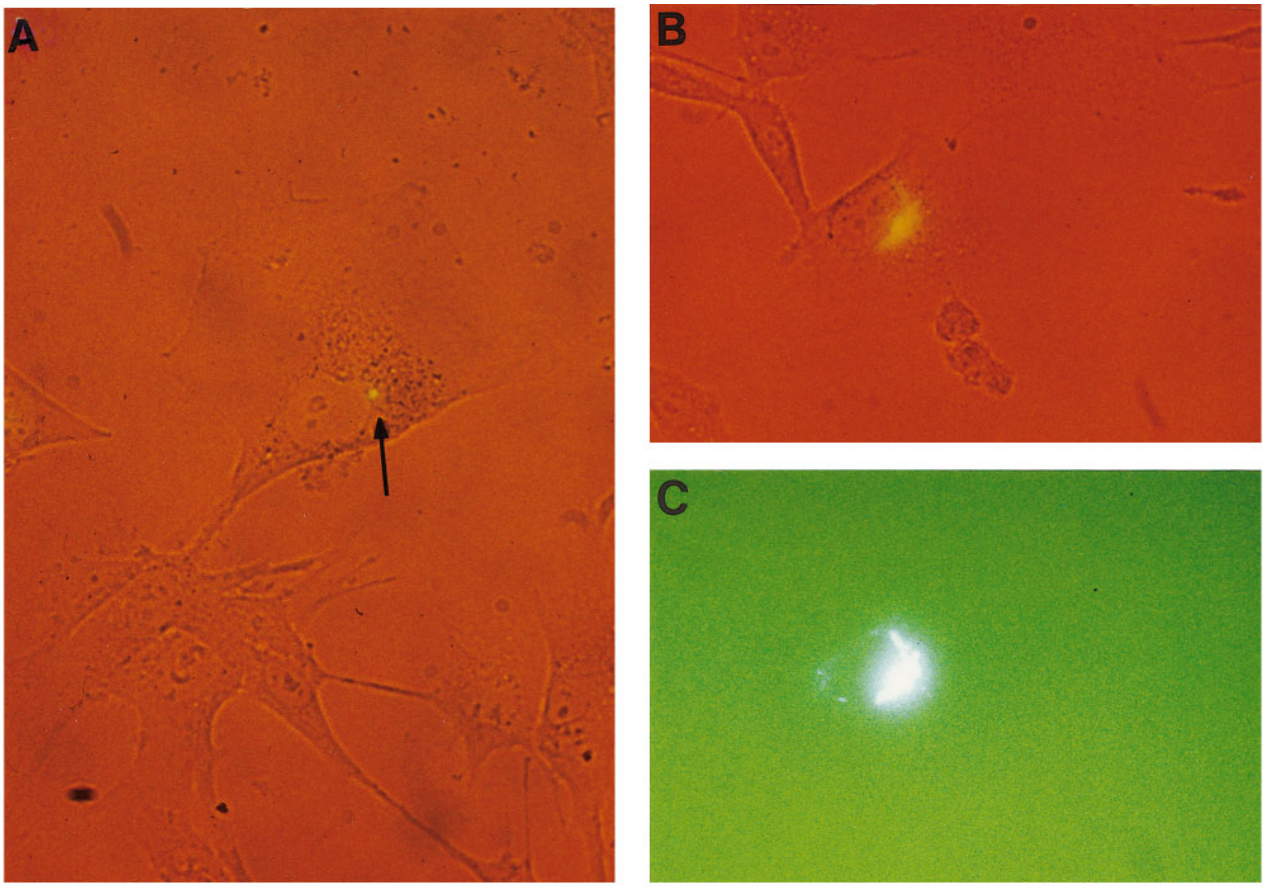


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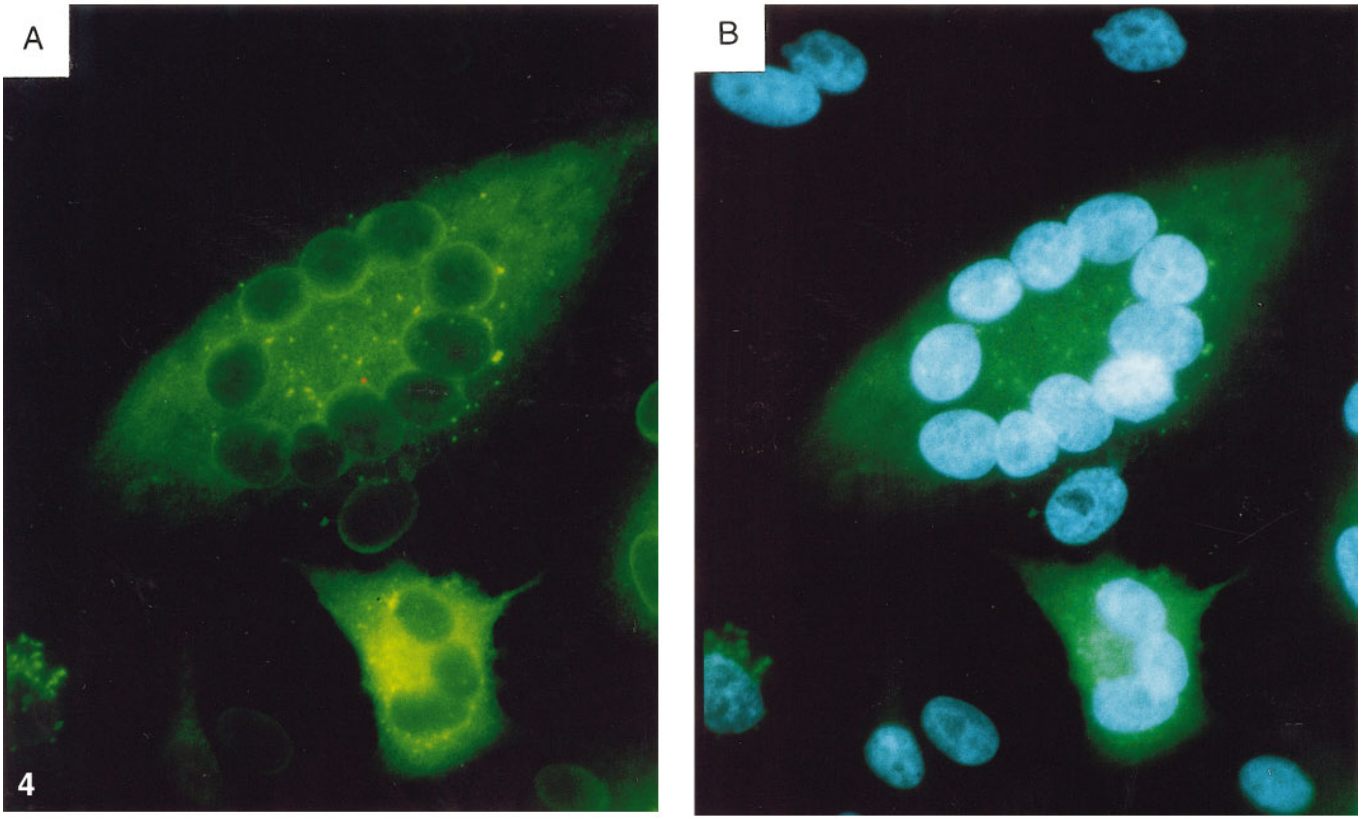


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TABLE 1

Transactivation of FeFV LTR Promoter-Based Luciferase Reporter Plasmids by Cotransfection of FeFV Bel 1 Expression Plasmids in CRFK Cells

| | Luciferase activity[a] | | | |
|---|---|---|---|---|
| Plasmid | pBK-CMV | pBKCMV-Bel1 | pBK-CMV-GFPBel1 | Fold increase |
| pGL2-basic | 9 | 23 | 27 | 2.5–3 |
| pFeFV-LTR | 25 | 214, 148 | 202, 871 | 8355 |

[a] Data are expressed as relative luciferase units normalized to cotransfected $\beta$-Gal activity.

ATCCTCTCC-3′ and 5′-GGATCGATAACCTTTAATCTTTA-CCCCCTTTCTTTCC-3′), cut with *Spe*I, and ligated into the vector pBK-CMV-GFP predigested with *Spe*I plus *Sma*I (rapid ligation kit; Boehringer-Mannheim, Mannheim, Germany). FeFV *gag* was cloned directly downstream of the *gfp* gene of the pBK-CMV-*gfp* plasmid into the same reading frame. Restriction enzyme analysis confirmed the correct orientation and size of the inserts. On expression and subsequent gel electrophoresis under denaturing conditions, a polypeptide of the expected molecular size for the GFP-Gag fusion protein was detectable. To obtain antiserum against the MA domain of FeFV Gag, polymerase chain reaction (PCR) products of FeFV DNA from coordinates 1205s and 2171a as primers were used and cloned into pET16b (Winkler *et al.,* 1997). Bacterially expressed recombinant proteins were purified and used to immunize rabbits.

The plasmid pBK-CMV-*gfp-bel1* was constructed by using PCR-amplified *bel 1* sequences with primers that contained a *Spe*I site at the 5′ end and a *Cla*I site at the 3′ end. The sense primer had the authentic ATG codon directly downstream of the *Spe*I site; the antisense primer had the authentic *bel 1* stop codon. The resulting recombinant plasmid was ligated into plasmid pBK-CMV-*gfp* that had been digested with *Spe*I and *Sma*I. Restriction enzyme analysis and *in vitro* translation was used to verify the correct size and orientation of the inserts.

## Construction of the maltose binding protein-*bel1* plasmid (MBP-*bel1*)

*Bel 1* sequences were obtained through PCR with the sense primer bgl-bel1s 5′-CCACTAGTATGGCTCAAAATA-CCCGGAAGAAGG-3′ from the 3′ LTR plasmid (Bodem *et al.,* 1996) and the antisense primer Fbela 5′-GCCATCG-ATTTGTACCAGGCCTATTCCTGG-3′ (Winkler *et al.,* 1997) with *Pfu* DNA polymerase. The plasmid *pMalbel1* was cut by *Bam*HI and *Sal*I and blunt-end ligated into the pMal-c2 vector (New England Biolabs) digested with *Bgl*II. *Escherichia coli* DH5$\alpha$ cells (GIBCO, Karlsruhe, Germany) were transformed according to standard procedures. The vector-insert borders of all plasmids were confirmed by nucleotide sequencing.

## Cell culture, transfection, and transient expression assays

BHK, CRFK, and HeLa cells were grown as described previously (Bodem *et al.,* 1996). Transfections were done according to Winkler *et al.* (1997). Luciferase assays were carried out with the pFeFV-LTR plasmid as reporter for the determination of transactivation activities of the different *bel 1*-containing constructs under conditions described previously (Winkler *et al.,* 1997; Zolotukhin *et al.,* 1996).

## Immunofluorescence

MBP-Bel 1 fusion protein was labeled with the fluorescein derivative FLUOS (Boehringer) as described previously (Truant *et al.,* 1998). The cellular localization of the injected protein was determined by fluorescence at a magnification of 100×. The rhodamine-conjugated goat anti-mouse IgG antibody was used (Cappel). Photographs were taken after FITC staining at 495 nm and after staining with DAPI (Sigma) at 365 nm.

## Shuttle assay and microinjections

The assays for probing shuttling of the Bel 1 between cytoplasm and nucleus were carried out as described recently (Fridell *et al.,* 1997). Briefly, HeLa cells were seeded onto coverslips and grown in serum-free medium for 12 h. During this period, ~20% of the cells contained polynuclei. Labeled MBP-Bel1 protein was microinjected into one nucleus of the cells that contained polynuclei. After 30 min at 37°C, the microinjected HeLa cells were examined under UV light.

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FIG. 3. Distribution and fluorescence of the GFP-FeFV Gag protein in BHK cells. (A) Plasmid *gfp-gag* (20 $\mu$g of DNA) was transfected into BHK cells and examined by phase contrast and UV light 2 days after transfection at 200× magnification. Arrow indicates GFP-Gag localized in the cytoplasm close to perinuclear regions (B) Photograph taken 4 days after transfection at 400× magnification. (C) Same as in B but in UV light only.

FIG. 4. (A) Distribution and fluorescence of the FeFV Gag protein in FeFV-infected CRFK cells fixed with paraformaldehyde 3 days p.i. Cells reacted with rabbit antiserum raised against the MA domain of FeFV Gag at 1:200 dilution. (B) Nuclear staining of the same area with DAPI showing syncytial CPE.

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