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Regulated Expression of the Feline Panleukopenia Virus P38 Promoter on Extrachromosomal FPV/EBV Chimeric Plasmids

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Feline panleukopenia virus/Epstein-Barr virus (FPV/EBV) chimeric expression plasmids were constructed to study regulation of the structural protein gene of the parvovirus, FPV, in a homologous cell culture system. Detection and quantitation of activity from the native FPV promoter, P38, was facilitated by fusing the *Escherichia coli lacZ* gene with the FPV structural protein gene. Feline cell lines which stably maintained these plasmids extrachromosomally were established. Constitutive β-galactosidase activity was low but increased up to 40-fold after infection with FPV. Expression of β-galactosidase was only detected when the FPV/*lacZ* gene was oriented in the same transcriptional direction as the Epstein-Barr virus gene coding for EBNA-1. When a small open reading frame upstream of the FPV/*lacZ* initiation codon was deleted, β-galactosidase expression increased another 4.7- to 26-fold. These changes in β-galactosidase activity indicate that expression of the FPV structural protein gene is regulated both transcriptionally and posttranscriptionally.

Feline panleukopenia virus (FPV) is an autonomous parvovirus which causes enteritis, leukopenia, and developmental abnormalities in cats. Parvoviruses are small, nonenveloped DNA viruses which contain a linear, single-stranded genome approximately 5,000 bases in length. A number of these viruses have been either partially or completely sequenced (1–3, 5, 6, 28, 33, 35, 38, 41). Analysis of these sequences reveals that the genomes of these viruses contain two large open reading frames, each encompassing approximately half of the genome. The left-hand open reading frame is transcribed from a promoter, P4, located at map position 4 and encodes the nonstructural proteins NS1 and NS2. The two nonstructural proteins have a common amino terminus but through differential mRNA splicing differ in their carboxy termini (7, 16). The right-hand open reading frame encodes the structural proteins VP1 and VP2 which in most parvoviruses are expressed from a promoter, P38, located at map position 38. Although the entire sequence of VP2 is contained within VP1, these two proteins are translated from slightly different transcripts which differ in splice donor usage (16, 24).

In studies involving regulation in both autonomous and helper-dependent parvoviruses, NS1 has been shown to be multifunctional. NS1 is required for replication of the viral genome (13, 30, 43) and has also been shown to be involved in the regulation of structural protein expression. In transient expression experiments using the autonomous parvovirus H-1, NS1 has been shown to be required for efficient expression of genes transcribed from P38 (32). This increased expression was shown to be mediated through a *cis*-acting region (TAR) 137 to 116 nucleotides upstream from P38 (34). Temperature-sensitive mutants in NS1 have been shown to be incapable of synthesizing structural proteins in the minute virus of mice (MVM) (45). Using point mutations that only affected the NS1 coding region, Doerig et al. (9) have shown that NS1 is sufficient to *trans* activate P39 in MVM(i). Similar positive regulation of the structural gene promoter has been reported in adeno-associated virus (21, 44). Conversely, expression of the structural proteins of MVM from a bovine papillomavirus vector demonstrated no requirement for NS1 (26, 29).

To quantitatively study gene expression from the FPV promoter P38, the *Escherichia coli lacZ* gene was fused with the FPV structural protein gene. The FPV/*lacZ* gene was used in the construction of FPV/Epstein-Barr virus (FPV/EBV) chimeric expression plasmids. FPV/EBV chimeric plasmids were introduced into Crandell feline kidney (CRFK) cells (8), and stably transfected cell lines were established. These expression plasmids were able to replicate extrachromosomally in CRFK cells.

The use of stable transfectants allows one to work with a uniform population of cells and eliminates potential problems resulting from variation in the efficiency of transfection. Since the FPV/*lacZ* gene is maintained on a stably replicating multiple-copy plasmid, it is not subject to disruption or rearrangements during integration and expression is not influenced by neighboring cellular genes or chromosomal structures near the site of integration. By using β-galactosidase (β-Gal) activity as a quantitative indicator of P38-mediated gene expression, we have demonstrated that expression of the FPV structural proteins is positively regulated at the transcriptional level and negatively regulated after transcription.

MATERIALS AND METHODS

Cells, virus, and transfections. CRFK cells (8) were grown in RPMI 1640 with L-glutamine supplemented with 5% fetal bovine serum and penicillin-streptomycin (complete medium). Viral infection was performed by removing the growth medium and replacing it with the FPV suspension. Virus was adsorbed to the cells for 1 h at room temperature, followed by addition of the appropriate growth medium and incubation at 37°C.

Transfections were performed in 60-mm dishes. Each dish was seeded at 5×10^5 cells and incubated overnight at 37°C. Cells were transfected with 1 μg of DNA in the presence of 30 μg of Polybrene (17) per ml, followed by a 4-min shock with 22.2% dimethylsulfoxide after a 6-h incubation at 37°C. The cells were incubated overnight at 37°C in complete medium prior to selection. Selection was performed by replacement of complete medium with selective medium

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(complete medium containing 200 μg of hygromycin B per ml). The selective medium was changed twice weekly until small colonies were observed (10 to 14 days). The colonies were isolated and expanded into cell lines. The transfected cells were maintained in selective medium throughout all experiments.

Plasmid constructions. (i) *lacZ* fusion. The plasmid pFPML2dlacZ, containing the FPV/*lacZ* gene, was produced by inserting the *E. coli lacZ* gene into the unique *Bgl*II site of the FPV capsid protein gene (5), thus positioning the *lacZ* gene in a location that would include it in both the VP1 and VP2 transcripts. To ensure fusion in the proper translational reading frame, the *Sma*I-*Sac*I fragment of pLG400 (11), containing the 5′ portion of *lacZ*, was inserted into *Sma*I-*Sac*I-digested pMC1871 (39), creating pMC400. This positioned a *Bam*HI site in the desired translational reading frame in *lacZ*. The *lacZ*-containing *Bam*HI fragment of pMC400 was then inserted into the unique *Bgl*II site of pFPML2d, creating pFPML2dlacZ. pFPML2d had previously been constructed by inserting the FPV-containing *Eco*RI fragment of pEH20 (5) into the *Eco*RI site of pML2d (37).

(ii) Expression vectors. pDClacZ was constructed by directionally cloning the large *Xba*I-*Bam*HI fragment of pFPML2dlacZ into the *Xba*I-*Bam*HI sites of the polylinker in p220.2, a derivative of pHEBo1 (42). The transcriptional orientation of the FPV/*lacZ* gene is opposite that of the EBNA-1 gene in pDClacZ.

pDClacZb was constructed by directionally cloning the large *Xba*I-*Sal*I fragment of pFPML2dlacZ into the *Xba*I-*Sal*I sites of the polylinker in p220.2. The transcriptional orientation of the FPV/*lacZ* gene is the same as that of the EBNA-1 gene.

pDClacZbΔMC was constructed by isolating the 1.1-kilobase *Eco*RI-*Sca*I and the 5.7-kilobase *Xmn*I-*Bam*HI fragments from pFPML2dlacZ. These two fragments were simultaneously cloned into the *Eco*RI-*Bam*HI sites of pML2d, creating pFPML2dlacZΔMC. This removed the FPV fragment from the *Sca*I site at nucleotide 1014 to the *Xmn*I site at nucleotide 1159 (the FPV base designation from Carlson et al. [5] is used throughout this report). pDClacZbΔMC was created by directionally cloning the large *Xba*I-*Sal*I fragment of pFPML2dlacZΔMC into the *Xba*I-*Sal*I sites of the polylinker of p220.2. The transcriptional orientation of the FPV/*lacZ* gene is the same as that of EBNA-1.

β-Gal histochemistry. Growth medium was removed, and the cells were rinsed in 136 mM NaCl–26 mM KCl–10 mM $NaHPO_4$–14 mM KH_2PO_4 (PBS). The cells were fixed for 5 min at 4°C in 2% formaldehyde–0.2% glutaraldehyde diluted in PBS. The fixative was discarded, and cells were again rinsed in PBS. The histochemical reaction was performed by overlaying the cells with a solution containing 1 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) per ml, 5 mM $K_4Fe(CN)_8 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS and incubating at 37°C overnight (36). The X-Gal solution was diluted from a dimethyl sulfoxide stock solution of 40 mg/ml. The histochemical reaction was stopped by repeated washing in PBS.

β-Gal quantitation. Growth medium was removed, and the cells were rinsed in PBS. The cells were removed from the flask with a rubber policeman, suspended in 5 ml of PBS, pelleted (5 min, 750 × *g*), and suspended in 100 ml of 250 mM sucrose–10 mM Tris hydrochloride (pH 7.5)–10 mM EDTA. Cell extracts were prepared by three freeze-thaw (−80°C, 37°C) cycles and clarified by centrifugation for 10 min in a microcentrifuge (40). Cell extract (50 μl) was added to 350 μl of Z buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM 2-mercaptoethanol [pH 7.0]) and 80 μl (4 mg/ml) of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) in 0.1 M PO_4 buffer (23). The reaction was incubated at 30°C until a faint yellow color developed (10 to 60 min). The reaction was stopped by the addition of 200 μl of 1 M Na_2CO_3 at pH 11. β-Galactosidase activity was determined spectrophotometrically at 420 nm and expressed as nanomoles of ONPG cleaved per minute per milligram of protein. The protein concentration of each extract was determined spectrophotometrically at 595 nm by using the Bradford method (4). Bradford reagent was mixed with 10 μl of cell extract, and the A_{595} was compared with a bovine serum albumin standard curve.

DNA isolation. Total cellular DNA was isolated by lysing cells in 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–10 mM EDTA–0.5% sodium dodecyl sulfate–200 μg of protease K per ml and incubating at 37°C overnight. The cell lysate was extracted three times with phenol and three times with chloroform. DNA was precipitated by addition of 3 volumes of ice-cold ethanol, collected with a glass rod, and suspended in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. The concentration was determined spectrophotometrically at 260 nm. Low-molecular-weight DNA was isolated by the method of Hirt (14).

Plasmid rescue. Low-molecular-weight DNA isolated by the method of Hirt was used to transform *E. coli* HB101. Plasmid DNA from transformed bacteria was isolated by alkaline lysis (15) and analysed by restriction mapping.

Slot blot analysis. (i) DNA. The number of plasmid molecules per cell was determined by slot blot analysis. Various concentrations of pDClacZb as a control and of total cellular DNA were denatured by adding one-third volume of 1 N NaOH and heating at 65°C for 1 h. This was followed by the addition of 0.1 volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before the mixture was applied to a GeneScreen Plus membrane by vacuum pressure in a slot blot apparatus (Schleicher & Schuell, Inc.). Each slot was washed three times with 10× SSC, and the membrane was dried at room temperature prior to prehybridization in 50% deionized formamide–10% dextran sulfate–1% sodium dodecyl sulfate–1 M NaCl–50 mM Tris hydrochloride (pH 7.5) for 4 h at 42°C. Hybridization was performed by adding 1 ml of denatured hybridization solution containing 10^7 cpm of ^{32}P-labeled pDClacZb and 1 mg of herring sperm DNA and incubating at 42°C for 16 h. The membrane was removed from the hybridization solution and washed twice in 2× SSC for 5 min at room temperature, twice in 2× SSC–1% sodium dodecyl sulfate for 30 min at 65°C, and twice in 0.1× SSC for 30 min at room temperature. The membrane was dried and exposed to X-ray film until an appropriate exposure was achieved. The intensity of the exposure on the X-ray film was determined by using a model 1650 Transmittance/Reflectance scanning densitometer (Bio-Rad Laboratories) coupled to an Hewlett-Packard 3392A integrator. By using the known concentration of pDClacZb as a reference, the total number of plasmid molecules present in a given sample of total cellular DNA was determined. By using 7.2×10^{-6} μg as an estimate of the amount of DNA in a mammalian cell, the number of cells represented by a given concentration was calculated. The number of plasmids per cell was determined by dividing the total number of plasmid molecules by the total number of cells represented.

(ii) RNA. Total RNA was isolated as described below. RNA was quantitated spectrophotometrically and diluted to

the appropriate concentration in 7× SSC–2.2 M formaldehyde. Samples were denatured by heating to 65°C for 15 min before being applied to a GeneScreen Plus membrane as described above. Each slot was washed three times with 10× SSC; the membrane was dried at room temperature before being baked at 80°C for 2 h. The membrane was then treated as described above.

Isolation of RNA and Northern (RNA) blot analysis. Total RNA was isolated by lysing the cells in the presence of 4 M guanidine thiocyanate and centrifugation through a 5.7 M CsCl cushion. The RNA was quantitated spectrophotometrically, separated under denaturing conditions in 1% agarose gels containing 2.2 M formaldehyde, and blotted overnight onto nitrocellulose filters. Following blotting, the filters were baked at 80°C for 2 h. Prior to hybridization, the filters were rehydrated in 0.1× SSC. Prehybridization was performed at 37°C for a minimum of 6 h in 50% formamide–5× Denhardt solution–10 mM EDTA–500 μg of denatured carrier DNA per ml–50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0)–0.1% sarcosyl–1 M NaCl. Prehybridized filters were transferred to fresh prehybridization solution containing the ^{32}P-labeled probe (10^6 cpm/ml) and incubated at 37°C overnight. Following hybridization, the filters were washed briefly in 2× SSC–0.1% sarcosyl at room temperature, twice in 2× SSC–0.1% sarcosyl at 37°C for 30 min, and twice in 2× SSC–0.1% sarcosyl at 50°C for 30 min. The filters were then exposed to X-ray film until an appropriate exposure was achieved.

RESULTS

Stable extrachromosomal maintenance of FPV/EBV plasmids in feline cells. EBV replicons consisting of the replication origin (*oriP*) and the gene encoding the Epstein-Barr nuclear antigen (EBNA-1), the product of which binds (27) and activates *oriP*, are stably maintained extrachromosomally in a variety of mammalian cells (46). Plasmid p220.2, a derivative of the shuttle plasmid pHEBo1 (42), contains these EBV elements and a 43-base-pair (*Sma*I-*Hae*III) polylinker from pUC12, inserted at the *Nar*I site. This plasmid also contains the bacterial hygromycin B phosphotransferase gene transcribed from the herpes simplex virus thymidine kinase gene promoter, which functions as a dominant selectable marker in eucaryotic cells.

To facilitate the study of FPV structural protein gene expression, plasmids were constructed containing the reporter gene, *lacZ*, from *E. coli*, which encodes β-Gal. The *lacZ* gene lacking a translational initiation codon was put under the control of the FPV promoter P38 by inserting it into the unique *Bgl*II site of the FPV capsid gene in such a way as to maintain the proper translational reading frame. The *lacZ* sequence was fused to the capsid gene downstream of the intervening sequences and therefore, both VP1/β-Gal and VP2/β-Gal fusion proteins could be made. FPV/EBV expression plasmids pDClacZ (Fig. 1A) and pDClacZb (Fig. 1B) were constructed by inserting the FPV/*lacZ* gene into p220.2. The FPV/*lacZ* gene was transcribed in the opposite direction from the EBNA-1 gene in pDClacZ and in the same direction as the EBNA-1 gene in pDClacZb. Both of these plasmids were introduced into CRFK cells by Polybrene transfection (17). Hygromycin-resistant colonies were isolated and expanded into cell lines. Cell lines containing pDClacZ were designated CZ cells; cell lines containing pDClacZb were designated CZb cells.

To determine whether the EBV-based plasmids that had been constructed were maintained extrachromosomally in CRFK cells, low-molecular-weight DNA was selectively isolated by Hirt extraction (14) from cell lines CZb5 and CZ21, which had been transfected with pDClacZb and pDClacZ, respectively. These DNA samples were used to transform competent *E. coli* cells. The plasmid DNA from the resulting transformants was isolated, purified, and analyzed by digestion with restriction endonucleases. Fractionation of these digests by agarose gel electrophoresis showed no apparent changes in the plasmids as a result of their growth in mammalian cells (data not shown).

The average number of plasmid copies in stably transfected cell lines was determined by slot blot hybridization and densitometry. Slots representing 10 μg of total DNA from three cell lines and 1 ng of pDClacZb DNA were scanned and integrated. The areas of the peaks for the cell line DNA preparations were divided by the area of the plasmid DNA peak, giving ratios of 1.56, 1.96, and 0.74 for cell lines CZb5, CZb1, and CZ21, respectively. By assuming a haploid genome size of 3.3×10^9 base pairs and a plasmid size of 1.58×10^4 base pairs, the average plasmid copy number was calculated to be 65 copies per cell for CZb5 cells, 82 copies per cell for CZb1 cells, and 31 copies per cell for CZ21 cells.

Expression of β-Gal in CRFK cells. Stably transfected cell lines were analyzed for β-Gal activity by histochemical staining with X-Gal. The cleavage of X-Gal by β-Gal forms an insoluble blue precipitate, which is easily visible microscopically within positive cells. Histochemical analysis revealed that a very low percentage of cells (<1%) constitutively expressed β-Gal (Fig. 2A).

Previously, it had been reported that the P38 promoters of the autonomous parvoviruses H-1 and MVM exhibit low basal activity but can be *trans* activated by the parvovirus nonstructural protein NS1 (9, 32). To investigate whether the P38 of FPV could be similarly induced, CZb cells were infected with FPV. Histochemical analysis at 2 days postinfection revealed that approximately 10% of the cells stained positive for β-Gal (Fig. 2B). When an identical experiment using CZ cells was performed, no positive cells were detected in either infected or uninfected cultures. These data strongly suggested that a viral gene product was responsible for the activation of P38. The results also indicate that P38 was only inducible when aligned in the same transcriptional direction as the EBNA-1 gene. Recently, an identical dependence on transcriptional orientation was reported when p220.2 was used to express human gamma interferon in CV-1 and 293 cells (47).

To quantitate the induction of β-Gal activity following infection with FPV, cell extracts from stably transfected cell lines were analyzed spectrophotometrically for the ability to cleave ONPG. The data presented in Table 1 indicate that FPV infection of CZb cells increased β-Gal activity 7.9- to 17.6-fold over uninfected cells. Induction experiments were repeated many times, and although the absolute nanomoles of ONPG cleaved per minute per milligram of protein varied from experiment to experiment, FPV-infected CZb cells consistently showed increases in β-Gal activity 10- to 40-fold over uninfected cells. Throughout these experiments, none of the CZ cells were induced.

The effect of FPV inoculum upon β-Gal expression was examined in an attempt to explain the variation observed in the levels of induction and to strengthen the hypothesis that the induction of β-Gal activity was mediated by a viral product. Identical CZb5 cultures were inoculated with 5×10^4, 1×10^5, and 2×10^5 50% tissue culture infective doses ($TCID_{50}$) of FPV and assayed for β-Gal activity at 3 days

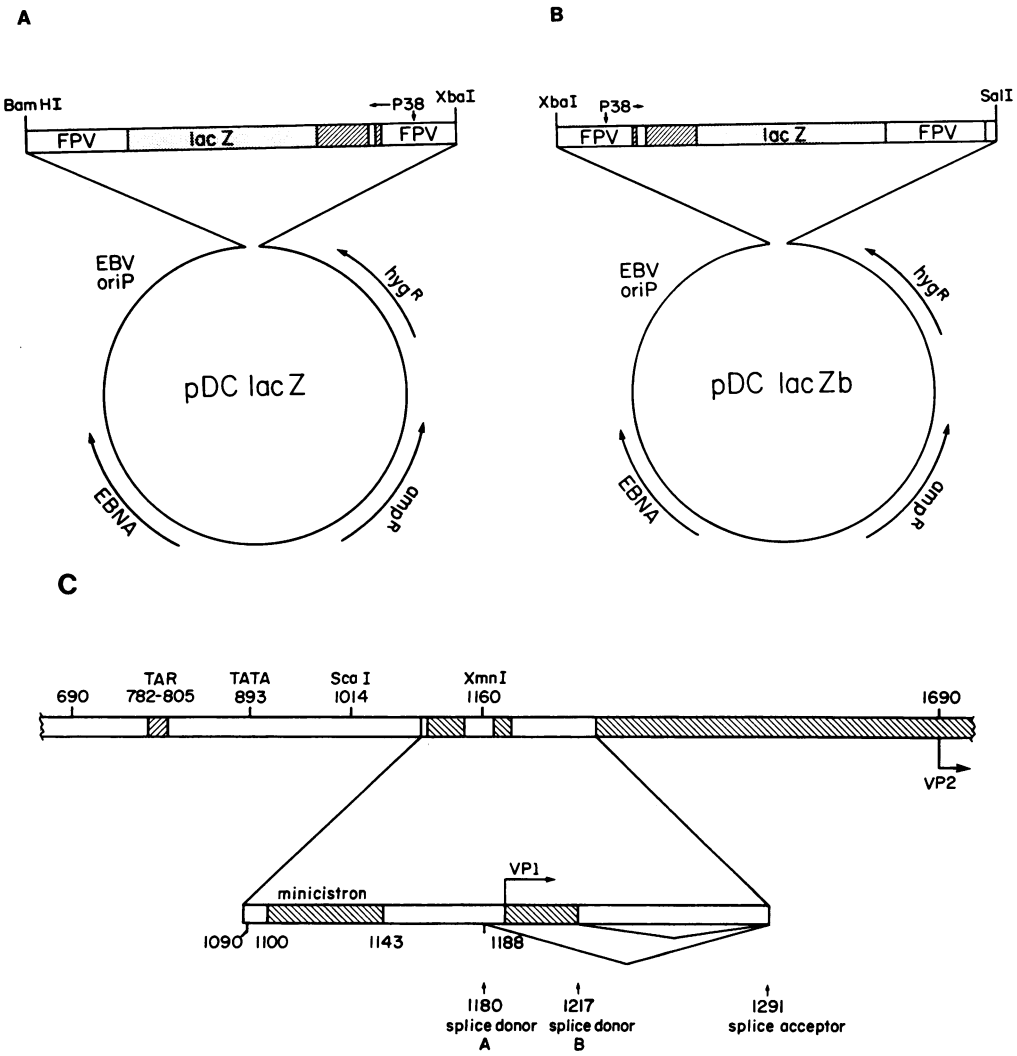


FIG. 1. FPV/EBV chimeric expression vectors containing the FPV/*lacZ* gene. Diagrams of the basic plasmids used in this study. ▨, FPV portion of the FPV/lacZ protein; □, *lacZ* portion of the FPV/lacZ protein, which was inserted at nucleotide 2260 of the FPV sequence (5). Also shown are the locations of P38 and the restriction sites used in the construction of these two plasmids. (A) pDClacZ. The entire FPV/*lacZ* gene, including the regulatory region gene, was inserted into the *Xba*I-*Bam*HI sites of p220.2. The direction of transcription by P38 is opposite that of the EBNA-1 gene. (B) pDClacZb. The entire FPV/*lacZ* gene, including the regulatory region, was inserted into the *Xba*I-*Sal*I sites of p220.2. The direction of transcription by P38 is the same as that of the EBNA-1 gene. (C) Diagram of the genomic region (nucleotides 690 to 1690) responsible for control of P38-mediated expression. The putative control regions, including the TAR sequence, the TATA box, the minicistron, splice donors, and the acceptor, and the initiation sites of VP1 and VP2 are shown. ▧, Portion of the genome coding for the viral proteins. Also shown are the locations of the restriction sites used to delete the minicistron.

postinfection. The culture inoculated with 5×10^4 $TCID_{50}$ cleaved 127 nmol of ONPG per min per mg, the culture inoculated with 1×10^5 $TCID_{50}$ cleaved 207 nmol of ONPG per min per mg, and the culture inoculated with 2×10^5 $TCID_{50}$ cleaved 300 nmol of ONPG per min per mg. The data from this experiment indicate that expression of β-Gal increased in proportion to the FPV inoculum.

The temporal appearance of β-Gal following infection was next examined. Identical CZb5 cultures were infected with 10^5 $TCID_{50}$ of FPV and lysed at 0, 8, 16, 24, 32, 48, and 72 h postinfection. Quantitation of β-Gal activity in these samples (Fig. 3) revealed that little β-Gal was detected until 24 h postinfection. From 24 to 72 h postinfection, the expression of β-Gal in FPV-infected CZb5 cultures increased at a near-linear rate. This lag period suggests that viral gene expression was required for induction of β-Gal expression.

Northern blot analysis was performed with a β-Gal-specific probe to determine whether this induction was due to an increase in the level of mRNA transcribed from P38. An mRNA species of 5.7 kilobases was predicted from the FPV/*lacZ* fusion gene. Comparison of lanes 3 and 4 in Fig. 4 shows a dramatic increase in the amount of the expected β-Gal-specific message in the infected samples, indicating that the induction was at the transcriptional level. It is interesting that although no increase in β-Gal expression was observed in CZ cells, there was an increase in the level of P38 transcription in these cells following infection (Fig. 4, lanes 7 and 8).

All of these data indicate that the P38 of FPV is transcrip-

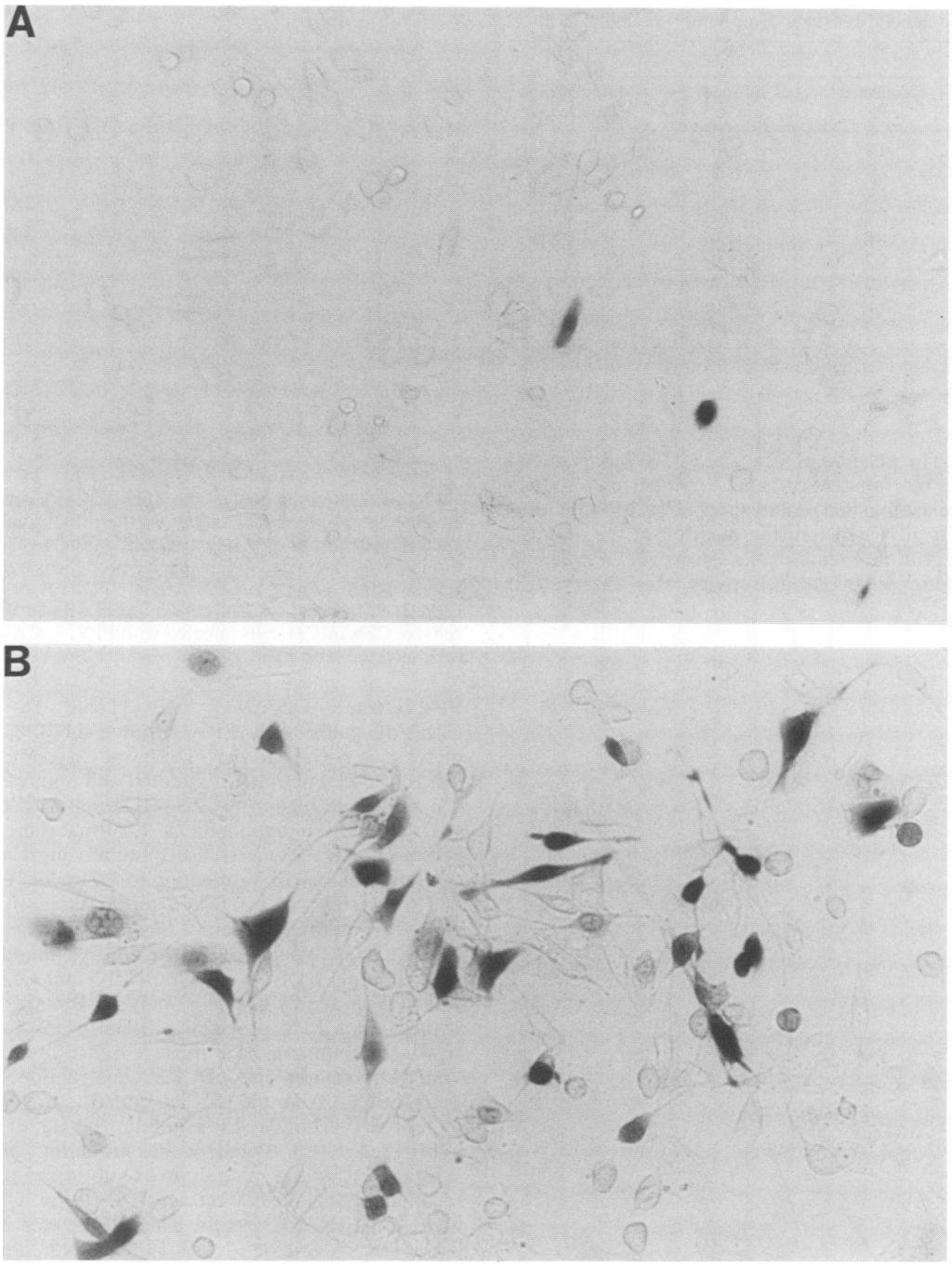


FIG. 2. Histochemical staining of CZb cells. CZb cells were histochemically stained with the indicator substrate, X-Gal, which forms a blue precipitate when cleaved by β-Gal. (A) uninfected CZb cells. (B) CZb cells infected with FPV.

tionally activated by a viral product. These data are consistent with previous observations (9, 21, 32, 44, 45) and suggest that *trans* activation of P38 by NS1 is a highly conserved function in parvoviruses.

Deletion of the 5′-proximal minicistron. The genome of FPV contains a short open reading frame (minicistron) capable of encoding a 14-amino-acid peptide (Fig. 1C). This small open reading frame, from bases 1100 to 1143, is located directly upstream of the putative initiation sites for VP1 and VP2 (5). The sequence surrounding the initiation codon fits the consensus initiation sequence (19) much better than do the putative VP1 and VP2 initiation sites. Therefore, the FPV minicistron could act as a posttranscriptional regulatory element in much the same way as the minicistron upstream of the small t antigen of simian virus 40 (SV40) (18).

The regulatory function of the FPV minicistron was investigated by constructing pDClacZbΔMC, a plasmid identical to pDClacZb (Fig. 1B) but with a 145-base-pair deletion in the FPV sequence (nucleotides 1014 to 1159), which deletes

TABLE 1. Influence of FPV infection on β-Gal activity in stably transfected cell lines

Cell line	Sp act[a]		
	Uninfected cells	Infected cells	Infected cells/ uninfected cells
CRFK	4.8	11.0	2.7
CZ42	2.7	1.9	0.7
CZ43	1.9	1.4	0.7
CZ45	1.7	1.5	0.9
CZb1	11.8	94.0	7.9
CZb5	6.7	118.0	17.6
CZbΔMC14	1.3	271.0	209.0
CZbΔMC23	5.8	478.0	82.5
CZbΔMC31	2.7	397.0	147.0

[a] Activity is expressed in nanomoles of ONPG cleaved per minute per milligram of protein.

the minicistron. The plasmid pDClacZbΔMC was introduced into CRFK cells by transfection, and a number of cell lines (CZbΔMC) were established. Analysis of CZbΔMC cell lines for β-Gal activity revealed that the deletion of the minicistron had no effect in uninfected cells. However, a dramatic effect in the induction of β-Gal activity was observed in FPV-infected CZbΔMC cells (Table 1). Infection of CZbΔMC cells with FPV resulted in a 83- to 209-fold increase in β-Gal activity over basal CZbΔMC activity. The ratios of β-Gal activity in infected and uninfected cells were 4.7- to 26-fold higher in CZbΔMC than in CZb cells.

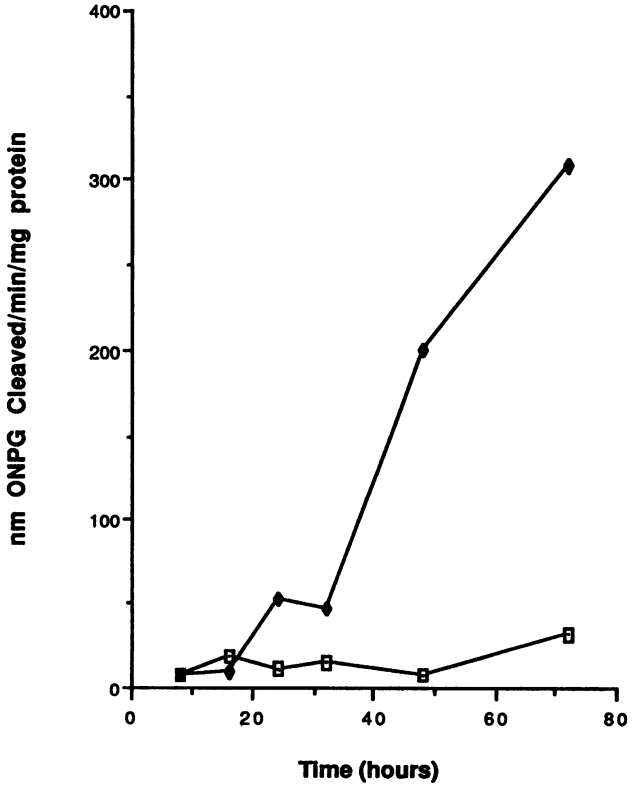


FIG. 3. Spectrophotometric analysis of β-Gal activity in infected CZb5 cells. Cell extracts were prepared by freeze-thawing and clarified by centrifugation. Extracts were incubated at 30°C with the colorless substrate ONPG, which produces a yellow product when cleaved by β-Gal. Color intensity was determined spectrophotometrically at 420 nm. The protein concentration of each extract was also determined. β-Gal-specific activity was defined as nanomoles of ONPG cleaved per minute per milligram of protein. β-Gal activity was determined at the indicated time points following infection with 10^5 $TCID_{50}$ of FPV. ⊡, Uninfected cells; ◆, infected cells.

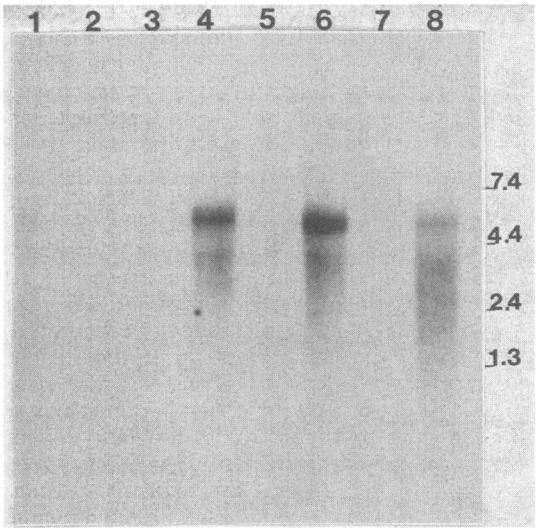


FIG. 4. Determination of the effect of FPV infection on P38-mediated transcription by using Northern blot analysis. To determine the effect that FPV infection had on the level of transcription from P38, a ^{32}P-labeled β-Gal-specific probe was hybridized to total cellular RNA. Lanes: 1, CRFK cells; 2, CRFK cells infected with FPV; 3, CZb5 cells; 4, CZb5 cells infected with FPV; 5, CZbΔMC31 cells; 6, CZbΔMC31 cells infected with FPV; 7, CZ55 cells; 8, CZ55 cells infected with FPV.

Two possible explanations for this increase in β-Gal activity are (i) P38 was transcriptionally more active because of more efficient FPV infection in CZbΔMC cells or (ii) the P38 transcript was translated more efficiently in CZbΔMC cells. To determine which of these mechanisms was causing the increased β-Gal activity, the relative abundance of β-Gal and FPV mRNA from infected CZb5 and CZbΔMC31 cells was determined by slot blot analysis. Blots were hybridized with either a FPV probe or a β-Gal probe to compare the relative amounts of FPV mRNA transcribed from both the viral genome and the pDClac construction and from the β-Gal mRNA from the pDClac constructions only. Densitometry of the bands representing 100 ng of each sample hybridized with a β-Gal-specific probe and of bands representing 10 ng of the same samples hybridized to a FPV probe resulted in β-Gal/FPV ratios of 0.356 for CZb5 cells and 0.162 for CZbΔMC31 cells. Thus, there was 2.19-fold more β-Gal mRNA compared with FPV mRNA in the CZb5 cells than in the CZbΔMC31. Therefore, an increase in efficiency of FPV infection could not account for the increase in β-Gal activity observed in CZbΔMC cells. The fact that there is over twice as much β-Gal-specific transcript with respect to FPV transcript in the infected CZb5 cells and less β-Gal activity suggests that the transcripts deficient in the minicistron (CZbΔMC31 cells) are translated more efficiently, indicating that the 5′-proximal minicistron has a posttranscriptional regulatory role in the synthesis of FPV capsid proteins.

DISCUSSION

The experiments presented here made use of shuttle vectors based on EBV. These vectors have been shown to

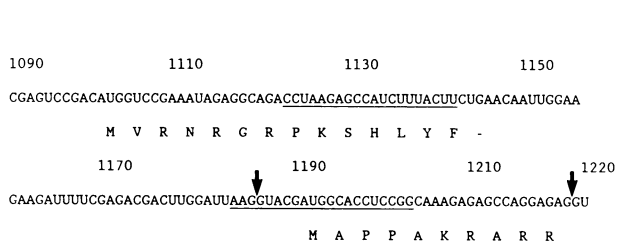
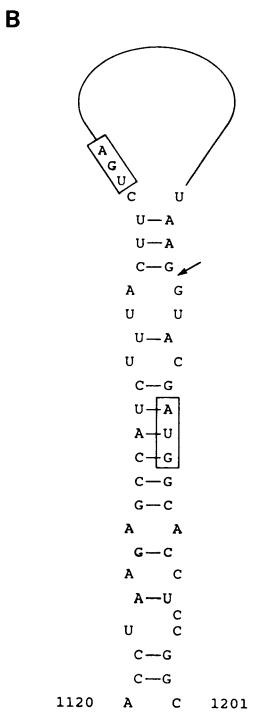


FIG. 5. Minicistron and potential secondary structure in the 5′ region of the FPV structural protein mRNA. (A) The mRNA of the FPV structural proteins contains a small open reading frame (minicistron) from bases 1100 to 1142 which could posttranscriptionally regulate P38-mediated expression. The underlined sequences indicate the inverted repeats that potentially form the stem of the stem-loop structure shown in Fig. 5B. (B) A potential secondary structure which could influence splicing or initiation, thereby influencing the ratio of VP1 transcript to VP2 transcript. The arrows indicate the putative splice donors for VP1 and VP2; note that the VP2 donor is within the stem of the secondary structure. Also shown are the initiation sites for the minicistron (1100) and VP1 (1198).



be stably maintained in an extrachromosomal state in a number of mammalian cell types, including human, monkey, canine, and, in this work, feline cells. These shuttle plasmids allow stable cell lines carrying multiple copies of the expression plasmid to be constructed without the risk of rearrangement, insertional mutagenesis, or variation in expression because of insertion site. Furthermore, the expression construction can easily be reisolated from the cells by plasmid rescue from extrachromosomal DNA prepared by the Hirt (14) extraction procedure.

Expression of parvovirus genes was once thought to be relatively unregulated. However, these studies, along with other recent work (9, 32), indicate that control of capsid gene expression from the P38 promoter is highly dependent on expression of other viral gene products. In these studies, the *E. coli* reporter gene *lacZ* was fused with the FPV structural protein gene, allowing the level of parvoviral gene expression from the P38 promoter to be determined quantitatively. Analysis of β-Gal expression in CRFK cells that had been stably transfected with pDClacZb indicated that constitutive activity of P38 was very low. The expression of β-Gal in these cells could be induced up to 40-fold following infection with FPV. This observation, coupled with the observations that β-Gal expression increased as a function of time following FPV infection and that gene expression was proportional to the viral inoculum, clearly indicated that expression from FPV P38 was *trans* activated, either directly or indirectly, by a FPV-specified product. Northern blot analysis of these cells confirmed that the increased expression was due to transcriptional *trans* activation. Although it has not been directly shown, it is likely that this *trans* activation was mediated by the nonstructural protein NS1.

Data were also presented which indicate that the expression of the structural proteins of FPV is regulated posttranscriptionally. The two capsid proteins of FPV, VP1 and VP2, are encoded by the right-hand open reading frame. The entire amino acid sequence of VP2 is contained in VP1. VP1 contains approximately 140 additional amino acids at the amino terminus. It is thought that in FPV, as in MVM and H-1, VP1 and VP2 are synthesized from different mRNA species generated by alternative splicing of the P38 transcript. In FPV, the mRNA for VP2 is thought to be generated by joining the 5′ splice junction at nucleotide 1182 to the 3′ splice junction at 1291, while the mRNA for VP1 results from the joining of the 5′ splice junction at 1218 to the 3′ splice junction at 1291 (Fig. 1C).

Examination of the nucleotide sequence near these splice junctions reveals several features that could be involved in the posttranscriptional control of the expression of VP1 and VP2. There is a pair of inverted repeat sequences at nucleotides 1121 to 1140 and 1180 to 1200 (Fig. 5A) which are potentially able to form a stem-loop secondary structure which has a calculated ΔG of −3.6 kcal/mol (1 kcal = 4,184 J) (Fig. 5B) (10). This putative secondary structure could influence both the splicing of the primary transcript and translation of VP1. The 5′ splice donor for the VP2 mRNA at nucleotide 1182 is in the stem of the secondary structure. This could influence the choice of 5′ splice junctions, increasing the possibility of the VP1 transcript being produced. On the translational level, the initiation codon for VP1 at 1188 is also contained in the stem of the secondary structure and thus would be relatively unavailable for binding of ribosomes to initiate synthesis of VP1.

In addition to the postulated secondary structure, the primary transcript contains a minicistron of 14 codons extending from the AUG at nucleotide 1100 to the UGA terminator at 1142. This minicistron would be present in both VP1 and VP2 mRNAs. The sequence around the minicistron initiator codon conforms to the consensus sequence (19) better than do the putative initiation codons for VP1 and VP2. Although the presence of minicistrons upstream from eucaryotic genes generally has an inhibitory effect on the downstream gene, translation of the minicistron in the VP1 mRNA would disrupt the secondary structure and could make the VP1 initiation codon more available for binding ribosomes.

The significance of these potential control elements for splicing is far from obvious, and their relative importance in the expression of proteins from the P38 promoter is difficult

to predict. However, there was an increase in β-Gal activity when they were deleted. CZbΔMC cells containing the deletion construction, pDClacZbΔMC, which removed both the minicistron and the leftward inverted repeat, routinely expressed more β-Gal activity than did CZb cells. Unfortunately, immunoblot analysis indicated that the expected VP1/β-Gal and VP2/β-Gal fusion proteins are apparently quickly processed to a 112-kilodalton form, and it was not possible to determine whether the increase was due to enhanced translation from VP1 or VP2 initiator codons or both.

If the minicistron was translated, the resulting peptide would contain three arginines and a lysine. A basic peptide such as this might have a role in binding nucleic acids. A comparison of the FPV sequence with the sequences of two strains of canine parvovirus a host range variant of FPV, shows that canine parvovirus strain B (31) could code for the peptide although one lysine codon would be changed to glutamic acid, reducing the net positive charge. Surprisingly, the canine parvovirus strain N sequence (28) shows a change in the initiator codon from AUG to GUG. The sequences of MVM and H-1 do not contain either the minicistron or the inverted repeat sequences. Short upstream minicistrons encoding small peptides that potentially regulate viral gene expression have been described in SV40 (18) and avian leukosis virus (12). In the case of SV40, the two upstream AUGs in the leader of the early mRNA dramatically decrease expression at the small t antigen in vitro. It is evident that initiation at internal AUGs occurs in mammalian cells (20, 22, 25). We believe that the expression of the structural proteins of FPV provides yet another example of this phenomenon. It appears that these small DNA viruses which were once considered simple are quite complex and exquisitely regulated.

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