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Construction of Fowlpox Virus Vectors with Intergenic Insertions: Expression of the β-Galactosidase Gene and the Measles Virus Fusion Gene

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A DNA fragment from fowlpox virus cloned on a plasmid vector was modified to contain foreign DNA inserts within an intergenic region. In a first step, a 32-base-pair intergenic region from the fowlpox virus genome corresponding to the position of the thymidine kinase locus in the vaccinia virus genome was enlarged to 55 base pairs by site-directed mutagenesis. A unique restriction endonuclease site introduced upstream of the intergenic region was then used to insert various foreign DNA fragments. The *lacZ* gene encoding β-galactosidase and the measles virus gene encoding the fusion protein were positioned downstream of two vaccinia virus p7.5 promoter elements in either a direct repeat or inverted repeat orientation. Foreign DNA inserts contained within the fowlpox virus sequence were transferred to the viral genome by homologous recombination occurring in cells infected with a fowlpox virus temperature-sensitive mutant and transfected with both wild-type viral DNA and plasmid DNA. Recombinant viruses were selected for the expression of β-galactosidase activity by screening for blue plaques in the presence of a chromogenic substrate. Stable recombinants expressing both the *lacZ* gene and the unselected measles gene were obtained when the p7.5 promoter was present as an inverted repeat. However, when the p7.5 promoter was in the direct repeat orientation, viral recombinants which initially expressed both gene inserts readily deleted the *lacZ* gene flanked by the promoter repeat. The methods described enable precise insertion and deletion of foreign genes in the fowlpox virus genome and could be applied to other intergenic regions of the same virus as well as other poxviruses.

Fowlpox virus (FPV), a member of the *Avipoxvirus* genus, has recently been developed as a live virus vector for the delivery of antigens both to its natural avian hosts (6, 30) as well as to mammalian hosts (31). The conservation of many molecular features between FPV and vaccinia virus (VV), an orthopoxvirus, as well as the knowledge acquired in the genetic manipulation of VV (20, 24) have enabled rapid progress in the construction of FPV recombinants. The genetic organization of FPV and VV is similar within defined regions of the genomes with notable exceptions such as the thymidine kinase gene (3, 12). Extensive amino acid homologies between a number of virus-encoded proteins have been found even though the nucleotide sequences of the two viruses diverge considerably (2, 3, 7, 12, 32). Furthermore, initial studies have suggested that the promoter sequences of FPV and VV are functionally equivalent (4, 6, 30). Thus, the techniques used for the insertion and successful expression of foreign genes within the FPV genome have largely been based on methods previously devised for VV. We describe a novel strategy for the construction of FPV recombinants in which foreign genes are inserted and expressed within an intergenic region of the genome. We also developed a method which enables the deliberate deletion of a marker gene initially convenient for the construction of a recombinant virus. These techniques should be valuable for the achievement of the full potential of FPV or other poxviruses as recombinant virus vaccines and expression vectors.

MATERIALS AND METHODS

Virus. The Salsbury vaccine strain and recombinants or mutants derived from it were propagated on primary chick embryo fibroblasts (CEF). The FPV *ts*1 mutant was isolated from a virus stock that had been grown in the presence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10 μg/ml). This mutant gave a 10,000-fold-higher titer at 33°C than at 39.5°C. FPV DNA was prepared from infected cells by the procedure described by Esposito et al. (13). Measles virus F protein was immunoprecipitated as previously described (11).

Cloning procedures and site-directed mutagenesis. Molecular cloning procedures were essentially as described by Maniatis et al. (21). Site-directed mutagenesis (34) was done on an FPV DNA segment cloned as a 1,756-base-pair (bp) *Eco*RI-*Pst*I fragment (Fig. 1) into the single-stranded bacteriophage vector M13TG131. In a first step, a mutation was created by using an oligonucleotide with the sequence AA CAACCTAATATCACTATAGGATCCTTGTTAAAAAG GAAT and then the mutant obtained was further modified by using the oligonucleotide CCTAATATCACTACTGAAGCG CAACTAGGATCCTTGTTAA, where the underlined portions correspond to the desired insertions. The predicted nucleotide sequences of the mutants were checked by the dideoxynucleotide method. The modified FPV fragment was then recovered as a *Bgl*II-*Pst*I fragment and ligated into the small bacterial plasmid pPoly II (19) that had been cut with *Bam*HI and *Pst*I. This procedure generated a plasmid containing an FPV fragment in which a unique *Bam*HI site introduced by site-directed mutagenesis was located between the two open reading frames (ORFs) F7 and F9. The *Bam*HI site was then used for insertion of foreign DNA elements. The VV p7.5 promoter contained between a 5′ *Sal*I site and a 3′ *Sca*I site and encompassing 268 bp (18, 28, 33) was modified to have a *Bgl*II site at its 5′ end and a *Bam*HI site at its 3′ end. These two sites were used to isolate

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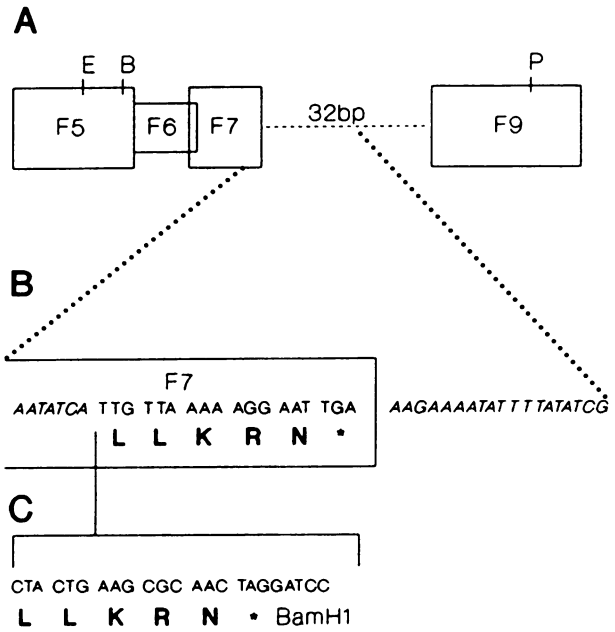

FIG. 1. Modification of the intergenic region separating ORFs F7 and F9. The top part of the figure schematically represents the ORFs designated F5, F6, F7, and F9 on the FPV genome. The expected direction of transcription and translation of each of the ORFs is from left to right. Note that 32 bp separate the F7 and F9 ORFs. The letters E, B, and P (*Eco*RI, *Bgl*II, and *Pst*I, respectively) indicate the positions of unique restriction endonuclease sites used for subcloning. The nucleotide sequence of the 3′ end of the F7 ORF as well as 19 of the 32 nucleotides of the intergenic region that follow it are represented in panel B. Amino acids encoded by the last five codons of the F7 ORF are also indicated in panel B. The nucleotide sequence inserted by site-directed mutagenesis into the F7 ORF as well as the amino acids it encodes and the newly created *Bam*HI site are represented in panel C.

the promoter fragment on an enlarged 297-bp fragment and ligate it into the newly created *Bam*HI site between the F7 and F9 ORFs. A plasmid containing the promoter running in the same direction as the two flanking genes was isolated. The ligation procedure maintained a single *Bam*HI site at the 3′ end of the p7.5 promoter. A *lacZ* fusion gene (3.8 kilobase pairs [kbp]) encoding β-galactosidase activity was isolated from pCH110 (17) with *Hin*dIII at the 5′ end and *Bam*HI at the 3′ end, modified by the addition of a *Bgl*II linker at the 5′ end, and then inserted into the *Bam*HI site downstream and in the same orientation as the p7.5 promoter. This generated a plasmid designated pTG2137 (Fig. 2) which contained a unique *Bam*HI site at the 3′ end of the *lacZ* gene and which was used for further cloning procedures. A second p7.5 promoter isolated as described above as a 297-bp fragment was inserted in either of the two orientations into the *Bam*HI site. Each of the two plasmids generated had a *Bam*HI site at the 3′ end of the second promoter into which the 1.9-kbp measles virus gene encoding the fusion protein (8, 11) was inserted. First, the 5′ end of the gene was inserted as a *Bgl*II-*Bam*HI fragment and then the 3′ end was added as a *Bam*HI fragment. Maps for the two plasmids, which were designated pTG2195 and pTG3113, are depicted in Fig. 2.

Transfection and isolation of recombinants. The procedure used to isolate FPV recombinants was adapted from one previously used for VV (10, 18). CEF in 30-mm petri dishes were infected with the FPV *ts*1 mutant at about 0.2 PFU per cell. After 1 h of adsorption at room temperature, fresh medium containing 5% serum was added and the cells were incubated at 33°C for 2 h. The medium was then removed, and the cells were transfected with 0.25 ml per dish of a calcium phosphate precipitate containing FPV wild-type DNA (about 200 ng) and the appropriate recombinant plasmid (about 50 ng). After 1 h at room temperature, cells were covered with fresh medium and incubated for 4 h at 39.5°C. Cells were then submitted to a 1-min treatment with 10% glycerol in culture medium, washed twice with phosphate-buffered saline, and incubated in fresh medium at 39.5°C for 5 days. Infected cultures were frozen and thawed, and their titers were determined on CEF at 37°C under a liquid overlay. Five days later, the medium was removed and fresh medium containing 1% agar and 300 μg of 5-bromo-4-chloro-3-indoyl-β-galactoside (X-Gal) was added. The following day, blue plaques could be picked and their titers amplified by infection of CEF. Further plaque purifications were performed by adding an agarose overlay immediately after virus adsorption and a second overlay containing 600 μg of X-Gal per ml after 5 days of incubation at 37°C.

DNA hybridization. Total DNA from infected CEF was purified and digested with the appropriate restriction enzyme, and the fragments obtained were separated on 1% agarose gels. DNA fragments were then blotted to nitrocellulose filters and hybridized to DNA probes radioactively labeled with [α-^{32}P]dCTP by random primed synthesis (15).

RESULTS

Plasmid vectors for construction of FPV recombinants. Previous studies have shown that one region of FPV DNA displays a genetic organization similar to the region of VV DNA that surrounds the thymidine kinase (*tk*) locus. However, a *tk* gene is lacking within the corresponding FPV gene cluster (3, 12) and is located in another area of the genome (7). We chose the intergenic region that separates the ORFs designated F7 and F9 of the FPV genome and corresponds to the position of the *tk* locus in the VV genome as a potential site for insertion of foreign genes (Fig. 1A). DNA surrounding this region (1,756 bp) was cloned as an *Eco*RI-*Pst*I fragment into the single-stranded phage vector M13TG131, and the intergenic region was modified in two successive steps by site-directed mutagenesis. The purpose of these steps was to replace the 3′ end of the nucleotide sequence of the F7 ORF by different but equivalent codons, introduce a unique *Bam*HI cloning site after the modified 3′ end, and hence enlarge the intergenic space separating F7 and F9 from 32 to 55 nucleotides (Fig. 1B and C). Enlargement of the intergenic region was intended to prevent a possible overlap between the promoter for the F9 ORF and the 3′ coding sequence of the F7 ORF as often occurs for coding regions in the VV genome, whereas the 3′ codons of the F7 gene were changed to avoid introduction of a sequence repetition. The altered FPV fragment was transferred for further manipulation to a small plasmid vector by using the *Bgl*II and *Pst*I sites. Three different plasmids were then generated to construct FPV recombinants (Fig. 2). In each of them, a DNA segment containing the VV p7.5 promoter (33) was inserted at the newly created *Bam*HI site in the same transcriptional orientation as the surrounding genes. The *lacZ* gene encoding β-galactosidase was considered to be a potentially convenient marker gene from work with VV (9, 23) and was therefore inserted downstream from the VV promoter in all the plasmids. In the plasmids designated pTG2195 and pTG3113, a second VV promoter was inserted

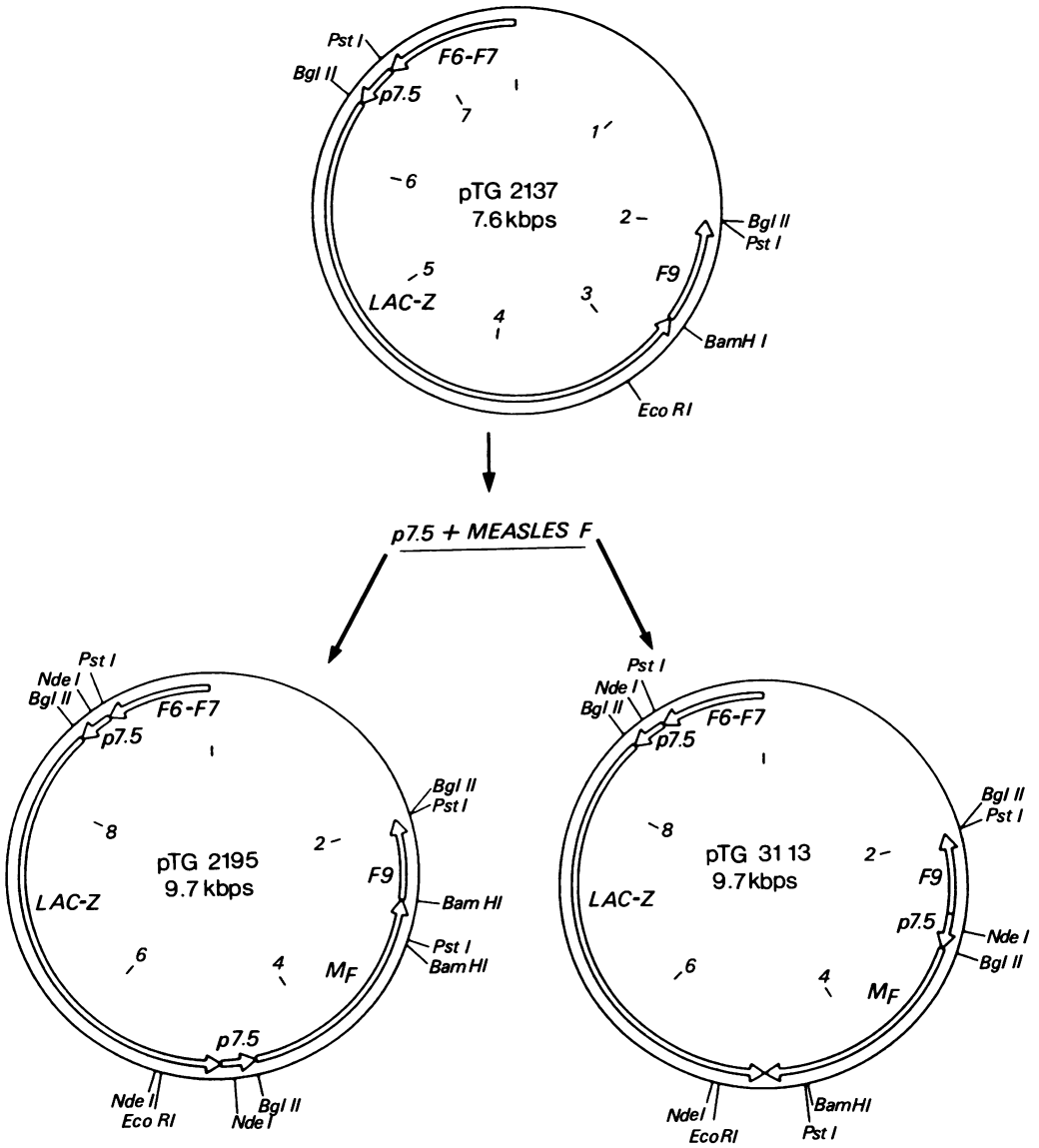

FIG. 2. Plasmid vectors for the generation of FPV recombinants. Each of the plasmids is represented in a circular configuration, with the different genetic elements drawn to scale relative to one another. A scale in kilobase pairs is provided within each circle. The arrows indicate the direction of transcription of the p7.5 promoter elements and the direction of reading of the ORFs. The symbols F6-F7 and F9 refer to the corresponding ORFs on the FPV genome, whereas the symbols *LAC-Z* and M_F refer to the β-galactosidase gene and the measles virus fusion gene, respectively. The positions of several restriction endonuclease sites used for plasmid analysis are also indicated. The plasmid designated pTG2137 was used to derive pTG2195 and pTG3113 by the addition in either of the two possible orientations of a second p7.5 promoter element and the measles virus fusion protein gene.

downstream of the *lacZ* gene in either of the two possible orientations. This created a 297-bp repeat separated by the 3.8-kbp *lacZ* gene. Finally, a foreign gene, the measles virus gene encoding the fusion protein, was positioned downstream of the second VV promoter in the correct orientation with respect to the promoter element.

Isolation of FPV recombinants. CEF were infected with the FPV *ts*1 mutant at a nonpermissive temperature for the mutant (39.5°C) and transfected with wild-type FPV DNA as well as the recombinant plasmids. After 5 days of infection, the virus produced was used to infect fresh monolayers and assayed for the presence of virus plaques expressing β-galactosidase activity. Blue plaques appeared at a low frequency (0.01 to 0.1%) only in cells transfected with plasmids containing the *lacZ* gene. When transfections were performed with plasmids pTG2137 and pTG3113, about three successive plaque purifications were necessary to dilute out contaminant virus that did not display the blue-plaque phenotype. With further passages, the recombinant viruses named FPV 2137 and FPV 3113, according to the plasmids they were derived from, yielded only blue plaques, thus demonstrating their genetic stability. In contrast, virus derived from transfection with pTG2195, designated FPV 2195 blue, displayed an unstable blue-plaque phenotype despite extensive plaque purifications. After the third and fourth plaque purifications, we found that, respectively, 2.5 and 4% of the plaques failed to stain blue. Several of the latter were picked and designated FPV 2195 white. Instability of the

FPV 2195 blue recombinant was predicted from results with similar VV recombinants we had previously constructed which contained a direct repeat of the p7.5 promoter (unpublished data) as well as from extensive studies on recombination in VV (1, 29). The direct repeats of the p7.5 promoter surrounding the *lacZ* gene are expected to participate in intermolecular or intramolecular recombination which would delete out the *lacZ* gene and give rise to recombinants containing only one p7.5 promoter upstream of the unselected foreign gene. Homologous recombination between the indirect repeats in FPV 3113 could generate nonviable genomes with either two left ends or two right ends or viable genomes in which the alignment of the *lacZ* gene and fusion gene inserts would be inverted.

Analysis of DNA from FPV recombinants. To determine the position of the foreign DNA inserts within the recombinant virus genomes, DNA from infected cells was digested with various restriction endonucleases, blotted onto nitrocellulose filters, and hybridized to appropriate radioactive probes (Fig. 3, top). Results from the hybridization experiments were analyzed with respect to the genome structures predicted for each of the recombinant viruses (Fig. 3, bottom). A cloned 3.5-kbp *Eco*RI fragment from the FPV genome was chosen as an FPV-specific probe because it entirely overlapped the 1.5-kbp *Bgl*II-*Pst*I fragment which had been used to construct the recombinant plasmids. This FPV *Eco*RI fragment hybridized to its 3.5-kbp counterpart in *Eco*RI-cut FPV DNA (Fig. 3A, lane 1). In contrast, the same probe hybridized to a higher-molecular-weight fragment in FPV 3113 (Fig. 3A, lane 2) which corresponded closely in size to a doublet expected in the recombinant virus DNA (4.8 and 4.9 kbp), assuming that the foreign DNA had integrated into the FPV genome by homologous recombination on both sides of the insert (see map for FPV 3113 in Fig. 3). Furthermore, the 3.5-kbp probe hybridized to a single 3.1-kbp fragment in FPV wild-type DNA cut with *Bgl*II (Fig. 3B, lane 1), whereas it hybridized to two *Bgl*II fragments (2.4 and 1.2 kbp) in FPV 3113 (Fig. 3B, lane 2) which were also predicted for a genomic structure which results from double homologous recombination. In another experiment in which a measles virus F gene probe was used (Fig. 3C), 2.4- and 4.1-kbp fragments were detected in FPV 3113 DNA, whereas the same probe did not hybridize to wild-type FPV DNA. Longer exposure of the autoradiographs enabled the detection of weakly labeled DNA fragments in FPV 3113 DNA which could correspond to fragments generated by recombination between the two p7.5 promoters as discussed above (data not shown).

Hybridization analysis of *Eco*RI-cut DNA from the FPV 2195 blue recombinant showed, as expected, a pattern similar to FPV 3113 DNA (Fig. 3A, compare lanes 2 and 3). However, FPV 2195 blue DNA was clearly distinct from FPV 3113 when cut with *Bgl*II (Fig. 3B, compare lanes 2 and 3). Two *Bgl*II fragments (4.0 and 1.2 kbp) were revealed by the probe in FPV 2195 blue which again corresponded to the sizes predicted if double homologous recombination had occurred.

The phenotype of FPV 2195 white (negative for β-galactosidase activity) suggested as discussed above that it had lost the *lacZ* gene and one of the p7.5 promoters by homologous recombination between two p7.5 promoters on the same genome or on different genomes. Hybridization of the FPV probe to *Eco*RI-cut DNA from FPV 2195 white revealed a 5.6-kbp fragment (Fig. 3A, lane 4) consistent with the genome structure represented in Fig. 3. Furthermore, hybridization of a measles virus probe to *Pst*I-cut DNA from FPV 2195 white showed that it no longer contained the 5.7-kbp fragment found in FPV 2195 blue but had a new 1.8-kbp fragment (Fig. 3C, lane 4) that could only have arisen through the suggested deletion. Note that a 1.8-kbp fragment was also faintly detectable in FPV 2195 blue, which is in agreement with the proposal that this virus population generates segregants equivalent to FPV 2195 white.

Synthesis of measles virus F protein in cells infected with FPV recombinants. CEF were infected with wild-type FPV or each of the recombinants containing the measles virus F gene and labeled with [^{35}S]methionine from 38 to 42 h postinfection. Cell lysates were then immunoadsorbed to protein A-Sepharose with guinea pig serum raised against measles proteins and analyzed by polyacrylamide gel electrophoresis. All the recombinant viruses, whether they contained the *lacZ* gene or not, induced the same level of synthesis of the measles virus F protein precursor (F^0) as well as its proteolytic cleavage products F1 and F2 (results shown only for FPV 3113 in Fig. 4).

DISCUSSION

The construction of poxvirus recombinants has so far relied on the identification of nonessential viral genes into which foreign DNA segments may be inserted. We demonstrated that it is also feasible to insert foreign DNA between coding sequences while still maintaining virus viability. To achieve this goal, care was taken to ensure the integrity of promoter sequences, which may overlap both coding and noncoding sequences. In the example reported, site-directed mutagenesis was used to enlarge an intergenic region from 32 to 55 bp and to replace the 3′ codons of the upstream ORF by equivalent codons to restore the intact ORF while avoiding the creation of a direct repeat. Although we have no formal proof that the ORFs surrounding the intergenic region are essential for FPV multiplication, indirect evidence that this is the case for the F9 ORF has been obtained. A recombinant FPV constructed by selecting for integration of a p7.5 *lacZ* gene block immediately upstream of the 32 bp preceding the F9 ORF displayed an unstable blue-plaque phenotype and was also shown to have integrated the bacterial plasmid portion of the vector (data not shown). This observation suggested that only single recombination events could occur with the recombinant plasmid containing a 32-bp intergenic region and that instability resulted from the sequence repetition. Analysis of the DNA from the FPV recombinants containing a 55-bp intergenic region reported in this study showed that they arose through homologous recombination on both sides of the foreign DNA and that their *lacZ*-associated phenotype is stable. Furthermore, the measles virus F gene, an unselected foreign gene physically linked to the *lacZ* gene, was also maintained and expressed in cells infected with the recombinant viruses. Moreover, experiments to be presented elsewhere have demonstrated that the F protein is immunologically functional since mice vaccinated with FPV 3113 are protected from a fatal measles encephalitis.

In contrast to previous methods, the insertion of foreign DNA sequences within intergenic regions might be expected to maintain the original phenotypic properties of the parental virus. This would be an asset when the construction of live virus recombinants from a previously accepted vaccine strain is undertaken. FPV vaccines are already attenuated for poultry, and further attenuation by gene disruption could result in an increase in the dose required for vaccination. The possibility of inserting foreign genes into intergenic

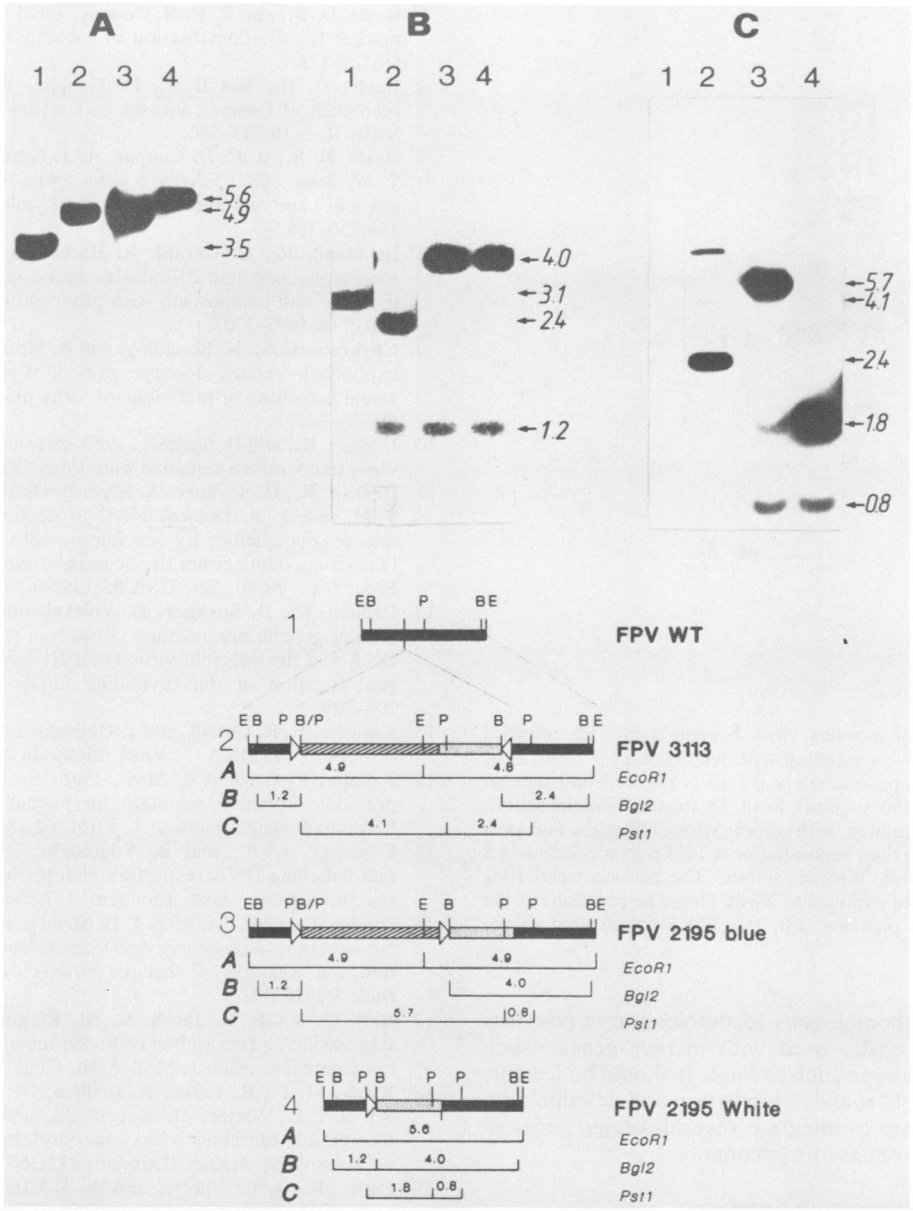

FIG. 3. Hybridization analysis of viral DNA from FPV recombinants. In the top of the figure, DNA from wild-type FPV (lanes 1), FPV 3113 (lanes 2), FPV 2195 blue (lanes 3), or FPV 2195 white (lanes 4), was cut with *Eco*RI (A), *Bgl*II (B), or *Pst*I (C), and the fragments obtained were separated on 1% agarose gels. The DNA fragments were then denatured, transferred to nitrocellulose filters, and hybridized with a radiolabeled *Eco*RI fragment of wild-type FPV (A and B) or a radiolabeled measles virus fusion protein gene (C). The size in kilobase pairs of each of the fragments revealed by hybridization is provided at the right of each of the panels. Note that a fragment not accounted for appears in panel C, lane 2, which is probably derived from incomplete digestion of the sample. The bottom part of the figure schematically represents the genomic structure of the *Eco*RI fragment of wild-type FPV DNA used in the hybridization experiments as well as the predicted structures of each of the recombinant DNA genomes. The letters E, B, and P indicate the positions of the restriction endonuclease sites for *Eco*RI, *Bgl*II, and *Pst*I, respectively. The bold black lines correspond to FPV DNA. A vertical line interrupts FPV wild-type DNA at a position corresponding to the intergenic region where foreign DNA was inserted. The open arrows represent the VV p7.5 promoters with their direction of transcription. The striped rectangles correspond to the *lacZ* gene, whereas the dotted rectangles represent the measles virus fusion protein gene. The calculated size in kilobase pairs is provided for each of the fragments expected to hybridize in the top part of the figure.

regions appropriately modified by site-directed mutagenesis also considerably increases the potential number of insertion sites in poxvirus genomes.

In addition to methods that rely on the use of VV genes as selective markers (20, 25, 27), foreign genes such as the *lacZ* gene (9, 23), the neomycin resistance gene (16, 22), the luciferase gene (26), or the xanthine guanine phosphoribosyl transferase gene (5, 14) have been valuable for the isolation of VV recombinants. However, a particular marker gene can only be used for a single insertion event, and when vaccine application is considered, it may not be acceptable to retain some marker genes. We devised a strategy which allows precise deletion of a marker gene once its selective properties have been taken advantage of. This method, which relies

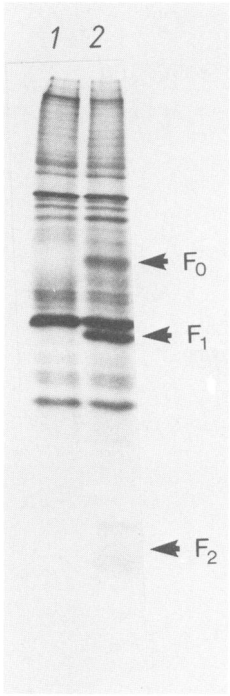

FIG. 4. Synthesis of measles virus F protein in CEF infected with FPV 3113. CEF were infected with wild-type FPV (lane 1) or FPV 3113 (lane 2) at approximately 0.1 PFU per cell and labeled with [^{35}S]methionine (50 μCi/ml) from 38 to 42 h postinfection. Proteins immunoprecipitated with antimeasles antibodies and protein A-Sepharose were then separated on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate. The gel was treated for fluorography, dried, and exposed to X-ray film. The positions of the measles virus-specific proteins (F0, F1, F2) are indicated in the margin.

on the efficiency of homologous recombination in poxvirus infections, is most easily used with marker genes which confer a visual phenotype such as *lacZ*. It should be feasible to go through multiple rounds of insertion and deletion with the same marker gene to integrate several foreign genes in different regions of a poxvirus genome.

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