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Expression of porcine pseudorabies virus genes by a bovine herpesvirus-1 (infectious bovine rhinotracheitis virus) vector

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Summary. Recombinant DNA techniques were used to insert foreign genes into bovine herpesvirus-1 [infectious bovine rhinotracheitis virus (IBRV)] vectors which were attenuated by deletion and/or insertion mutations in the IBRV thymidine kinase (tk) gene. In one recombinant, the regulatory and coding sequences of the late pseudorabies virus (PRV) glycoprotein gIII gene, were inserted into the early IBRV tk gene. This recombinant efficiently expressed the PRV gIII gene indicating that immediate early IBRV proteins were competent to transactivate the late PRV gIII gene. IBRV vector viruses were also prepared in which the coding sequences of the early PRV tk gene, the late PRV gIII gene, and the *E. coli* β-galactosidase gene were ligated to the late IBRV gIII promoter. Genotypes and phenotypes of the recombinant viruses were verified by restriction endonuclease and molecular hybridization experiments, thymidine plaque autoradiography, β-gal plaque assays, and by immunoprecipitation experiments on extracts from ^{3}H-mannose-labelled cells. The recombinant IBRV expressing β-gal from the IBRV gIII promoter has been useful as an intermediate in the construction of IBRV vectors harboring foreign DNA sequences. The infectivity of the IBRV recombinant that expressed PRV gIII from the IBRV gIII promoter, was neutralized by polyclonal PRV antisera and by monoclonal antibodies to PRV gIII. The PRV gIII glycoprotein synthesized by the preceding recombinant has been used to coat microtiter test plate wells in a PRV gIII differential diagnostic test kit.

Introduction

Modified-live pseudorabies virus (PRV) and bovine herpesvirus-1 [infectious bovine rhinotracheitis virus (IBRV)] mutants irreversibly attenuated by deletions in the thymidine kinase (tk) gene and with marker deletions in the homologous PRV gIII and IBRV gIII genes (which are also homologous to the

herpes simplex virus gC gene) provide safe, efficacious, and rationally designed vaccines for the control of economically important porcine and bovine herpesvirus diseases [12, 14, 16–18], and in conjunction with sensitive and specific differential blocking ELISA diagnostic tests enable veterinarians to distinguish vaccinated animals from those infected with field strains [9, 16]. Furthermore, these attenuated herpesvirus vaccines are useful as vectors for the expression of foreign proteins which can be used as antigens in diagnostic kits and as immunogens to prevent other diseases.

Previously, we described recombinant IBRV vectors which expressed foot-and-mouth disease virus (FMDV) capsid protein (VP 1) epitopes as parts of fusion proteins with the major IBRV envelope glycoprotein gIII [10, 13]. The nucleotide sequences encoding the FMDV VP 1 epitopes were positioned at the aminoterminal end of the coding region of the IBRV gIII gene from which 63 to 114 nucleotides had been deleted. We now describe recombinant IBRV vectors which efficiently express a "late" glycoprotein and an "early" enzyme protein of the porcine PRV, as well as *E. coli* beta-galactosidase [2]. DNAs encoding these proteins were inserted at two different nonessential sites on the IBRV genome. The data to be presented show that the expression of these porcine virus and bacterial genes can be driven by a "late" IBRV promoter, and also, that the porcine virus glycoprotein gIII gene inserted in the recombinant can be expressed from the native "late" PRV gIII promoter. The PRV gIII protein produced by the cells infected with the IBRV/PRV gIII recombinant virus has been employed as the antigen to coat microtiter test plates in a blocking ELISA PRV gIII differential test kit (Diasystems Celisa Omnimark PRV, Tech America, Kansas City, MO) [9].

The efficient expression of the PRV and bacterial genes inserted in the IBRV gIII gene is noteworthy, because, contrary to preceding findings, the IBRV gIII gene and other foreign genes inserted in the PRV gIII gene of a PRV vector have been expressed poorly [32, 33; M. Kit and S. Kit, unpubl. experiments].

Materials and methods

Cells and viruses

Virus strains were propagated in rabbit skin [Rab 9, Rab(Bu)] or bovine kidney (MDBK) cells as described [17]. The parental IBRV used to prepare vaccine and vector virus strains was the tk^+ Los Angeles (LA) strain of IBRV (American Type Culture Collection (ATCC) VR-188). The tk^- IBRV(NG) dltk vaccine strain was constructed from IBRV(LA) by genetically engineering a 343 bp (Bgl II to Sac I) deletion from the IBRV tk gene and by inserting in place of the deleted sequences a synthetic oligonucleotide sequence, designated NG (codons for NovaGene), which contains stop signals in all 3 reading frames. IBRV(NG) dltkdlgIII is a marker vaccine with deletions in the IBRV tk and gIII genes [16]. PRV(BUK-5) is a tk^+ PRV plaque purified from the attenuated Bucharest (BUK) strain of PRV. PRV(BUK-d13) is a tk^- deletion mutant derived from PRV(BUK-5) [15].

Autoradiography

Thymidine plaque autoradiography experiments were carried out, as described [15, 17, 18] to analyze the tk^+ or the tk^- phenotypes of IBRV recombinants.

Synthesis of oligodeoxyribonucleotides

Oligodeoxyribonucleotide sequences and adaptors were synthesized on a Systec 1450 automated DNA synthesizer (Minneapolis, MN) using cyanoethyl phosphoramidite chemistry [6].

Virus neutralization assay

Confluent cultures of MDBK cells in 6-well tissue culture trays (Falcon Tissue Culture Products, Becton Dickinson Labware, Oxnard, CA) were infected with wild-type IBRV(LA) or recombinant IBRV(NG) dltk dlgIII (PRV g92). Normal pig sera, pig PRV antisera (VN = 1 : 64), mouse monoclonal antibodies (mAb) to PRV gIII, and bovine IBRV antisera (VN = 1 : 64) were diluted 1 : 4, 1 : 4, 1 : 8, and 1 : 20, respectively, in phosphate buffered saline solution, mixed with an equal volume of virus, and incubated for 1 h at 37 °C. Then, 0.1 ml per well of the virus plus serum mixture was added to the cell monolayers and adsorbed for 1 h at 34.5 °C. The infected cell monolayers were overlaid with 1% methylcellulose in growth medium. After incubation for 4 days at 34.5 °C, the media were aspirated and the cells were rinsed with saline solution, fixed with ethanol for 5 min at room temperature and stained with 0.1% crystal violet for 5 min. The wells were rinsed with tap water, dried, and the plaques were counted.

Analyses of viral DNAs

IBRV DNAs were prepared as described, digested with restriction endonucleases, the fragments were separated by agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide, visualized with a long-range UV-illuminator and photographed [15]. The restriction nuclease fragments were transferred to nitrocellulose sheets by Southern blotting procedures and hybridized to ^{32}P-labeled DNA probes. Nicktranslated, ^{32}P-labeled DNA probes with specific activities greather than 2×10^8 cpm/μg DNA were prepared as described [15].

To identify recombinant IBRV with PRV (gIII) gene insertions, a nick-translated probe, designated pSal was used. The PRV fragment of the pSal probe was obtained from plasmid pBUK : Stu 12/Pst I by subcloning the 1.1 kb Sal I fragment of pBUK : Stu 12/Pst I into the Sal I site of pBR 322 [18]. To identify recombinant IBRV with PRV tk gene insertions, a nick-translated probe was prepared from tk coding sequences of pSP 64/PRV tk [11, 18, 27].

Immunoprecipitation of ^{3}H-mannose-labeled glycoproteins synthesized in virus-infected cells

To demonstrate the expression of PRV gIII by IBRV-recombinant viruses, cells infected with parental IBRV and PRV and with candidate recombinant viruses harboring PRV gIII DNA inserts were labeled with ^{3}H-mannose as described [18]. Nonionic detergent extracts of the ^{3}H-mannose-labeled, virus-infected cells were prepared, incubated at 4° C for 8 h with antisera, immunoprecipitated with pansorbin (formalin-fixed *Staphylococcus aureus*), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography [18].

Results

Isolation of recombinant IBRV expressing PRV gIII glycoprotein from native PRV gIII promoter

A recombinant IBRV was constructed with the entire PRV gIII gene, including its 5′ promoter and 3′ polyadenylation signals inserted into the nonessential tk

gene of IBRV. Following infection of susceptible cells with this recombinant, the late PRV gIII gene [previously designated PRV g92 or PRV g92 (gIII)] [15, 16] was expressed from its native PRV promoter. Irreversible attenuation of the IBRV recombinant was achieved by inactivating the tk gene through the insertion of the foreign DNA sequence into the tk gene coding sequences and through the deletion of a 0.46 kb Bgl II to Mlu I nucleotide sequence from the IBRV tk gene. Isolation of the IBRV recombinant was facilitated by selection techniques which differentiated tk^+ parental IBRV from recombinant tk^- IBRV [8, 15].

A plasmid, designated pSP 65/IBRV tk/PRV g92, was constructed by inserting a 3.5 kb Mlu I to Nru I fragment from pBUK: Stu 12/Pst I into the Bgl II to Mlu I site of pSP 65 (Exo 36) (Fig. 1). pBUK: Stu 12/Pst I was constructed by cloning the 4.0 kb Pst I fragment of PRV(BUK-5) DNA, which contains the PRV g92 (gIII) gene plus its 5′ and 3′ flanking sequences, at the Pst I site of pBR 322 [11]. pSP 65 (Exo 36) was obtained by cloning the IBRV tk gene plus flanking sequences into pSP 65 (Promega Biotech, Madison WI) [8, 27]. pSP 65/IBRV tk/PRV g92 was then constructed by cleaving pBUK: Stu 12/Pst I with Mlu I plus Nru I, and cleaving pSP 65 (Exo 36) with Mlu I plus Bgl II. The

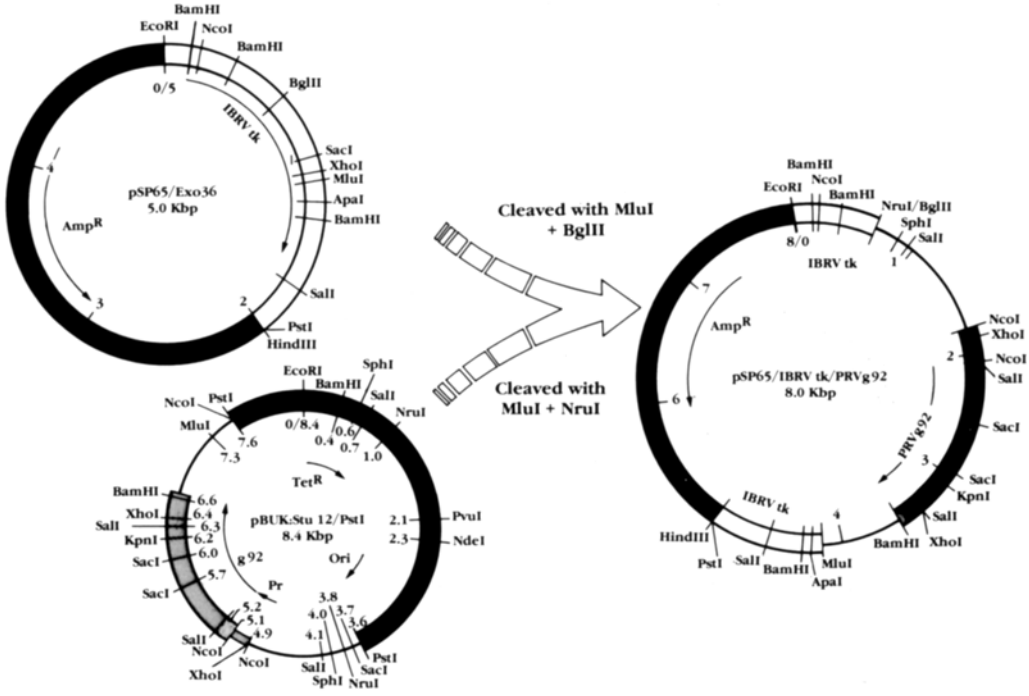

Fig. 1. Restriction endonuclease maps of plasmids used to construct pSP 65/IBRV tk/PRV g92, which contains the PRV g92 (gIII) gene inserted in the IBRV tk gene in place of the Bgl II to Mlu I nucleotide sequence of the IBRV tk gene. Black bars represent pSP 65 and pBR 322 DNA sequences, and the white bars represent IBRV DNA sequences. The stippled bars represent the PRV g92(gIII) gene within the 4 kb Pst I fragment of PRV DNA. The smaller stippled bars represent the PRV g92(gIII) promoter

restriction nuclease digested fragments were treated with mung bean nuclease to produce blunt ends, the fragments were mixed, ligated with phage T4 ligase and used to transform *E. coli* K12 RR1. Restriction nuclease mapping of ampicillin resistant colonies served to identify the 0.8 kb plasmid designated pSP 65/IBRV tk/PRV g92.

A tk^- deletion/insertion mutant of IBRV was obtained by homologous recombination as depicted in Fig. 2. Rab 9 cells were transfected with infectious DNA from tk^+ IBRV (LA) plus pSP 65/IBRV tk/PRV g92 which had been predigested with Eco RI and Pst I. The virus harvest from the transfection was enriched for tk^- IBRV deletion/insertion mutants by passaging the harvest in tk^- Rab(BU) cells in selective media containing 50 μg/ml bromodeoxyuridine (BrdUrd). The harvest from this first selection was titrated and a second selection step was carried out. The harvest from the second selection was titrated in Rab 9 cells, canditate tk^- recombinants were picked at random from plaques, virus pools were prepared and 96 tk^- IBRV deletion/insertion mutant candidates were obtained. To identify the desired recombinant containing PRV g92 (gIII) DNA sequences, molecular hybridization experiments were carried out using a ^{32}P-labelled probe (pSal) containing PRV gIII sequences [27]. Two of the 96 candidate IBRV recombinants hybridized to the pSal probe, indicating that these clones, designated IBRV dltk (PRV g92) clones 6-2 and 7-30, had an insertion of the PRV g92 (gIII) gene in the IBRV tk gene.

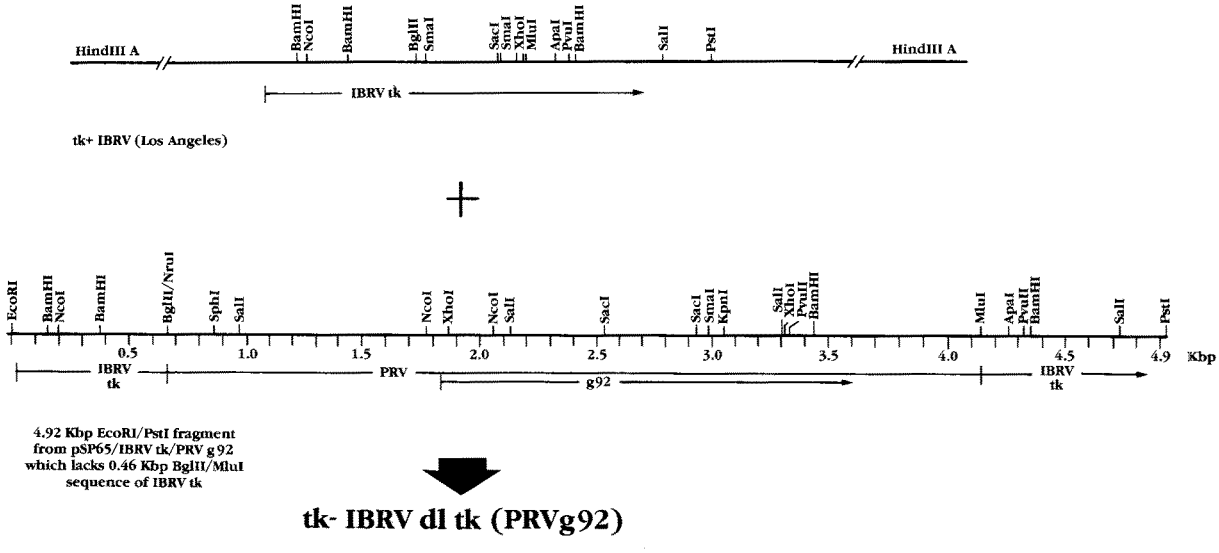

Fig. 2. Hind III-A restriction fragment of the parental tk^+ IBRV(LA) which was co-infected with the 4.92 kb Eco RI to Pst I fragment from pSP 65/IBRV tk/PRV g92 (Fig. 1). This Eco RI to Pst I fragment lacks the 0.46 kb Bgl II to Mlu I sequence of the coding region of the IBRV tk gene and contains, instead, a PRV DNA insert capable of expressing the PRV g92(gIII) gene, to give rise to the tk^- IBRV mutant, IBRV dltk (PRV g92), by homologous recombination. The IBRV mutant expresses the PRV g92(gIII) gene from the PRV g92 (gIII) promoter

Southern blotting experiments on Pst I, Kpn I, Hind III, and Bam HI digests of viral DNAs verified the structure of IBRV dltk (PRV g92) clone 6-2. These experiments showed that the 6.7 kb Kpn I fragment of IBRV(LA), which contains the IBRV tk gene, was absent from IBRV dltk (PRV g92) and that two new fragments of about 5.9 kb and 4.2 kb appeared among the restriction fragments of IBRV dltk (PRV g92) clone 6-2, consistent with the insertion of the 3.5 kb Nru I and Mlu I fragment of the PRV g92 gene into the IBRV tk gene at the Bgl II and Mlu I sites. Also, the Hind III-A fragment of IBRV-dltk (PRV g92), which contains the IBRV tk gene, was larger than that of the wild-type IBRV(LA) strain, as predicted. None of the wild-type IBRV(LA) restriction fragments hybridized with the pSal probe. However, Bam HI, Hind III, and Pst I fragments of 3.0 kb, 24 kb, and 6.5 kb, respectively, of IBRV dltk (PRV g92) hybridized to the pSal probe, as did two 5.9 kb and 4.2 kb Kpn I fragments. These experiments demonstrated that IBRV dltk (PRV g92) clone 6-2 contains PRV g92 (gIII) sequences inserted in the IBRV tk gene.

Construction of a gIII$^-$ IBRV-based viral vector with the PRV tk coding sequences inserted adjacent to the IBRV gIII promoter

In the preceding example, the "early" IBRV tk gene was the site of insertion of foreign PRV g92 (gIII) sequences. Figure 3 summarizes the strategy used to construct a recombinant IBRV with an insertion of the coding region of the early PRV tk gene plus its downstream translational stop and polyadenylation signals into the "late" IBRV gIII gene. The PRV tk gene promoter sequences were not included so that the expression of the PRV tk gene was driven by the "late" IBRV gIII promoter. In addition, the PRV tk gene sequences replaced the coding sequences of the IBRV gIII gene. Furthermore, essential coding sequences of the IBRV tk gene were deleted, so that in the viral recombinant, the PRV tk gene would be the only source of viral tk enzyme expression.

pSP 64/PRV tk, which contains the coding region of the PRV tk gene ligated to the SP6 promoter in pSP 64 (Promega Biotech, Madison, WI), was prepared by cloning the coding region of the PRV tk gene and sequences 3′ to the PRV tk gene, i.e., the 2.2 kb Bam HI to Pvu II fragment shown in Fig. 3, into the unique Bam HI and Sma I site of the polyclonal cassette of pSP 64 [15, 18]. Figure 3 also shows the restriction endonuclease map of the Kpn I to Hind III fragment of plasmid pLAHK, which contains the IBRV gIII gene [10]. pLAHK dlApa I was obtained by deletion of Apa I sequences from pLAHK so as to remove the coding region of the IBRV gIII gene. The coding region of the PRV tk gene and the PRV sequences 3′ to the PRV tk gene were inserted adjacent to the IBRV gIII promoter in pLAHK dlApa I to produce pLAHK dlApa I/PRV tk by: (1) cleaving pLAHK dlApa I with Bam HI so as to separate the IBRV gIII promoter from the IBRV gIII coding sequences; (2) cleaving pSP 64/PRV tk with Bam HI and Kpn I to produce a 1.6 kb Bam HI/Kpn I fragment; (3) ligating the Bam HI termini of the cleaved plasmids so as to place the PRV

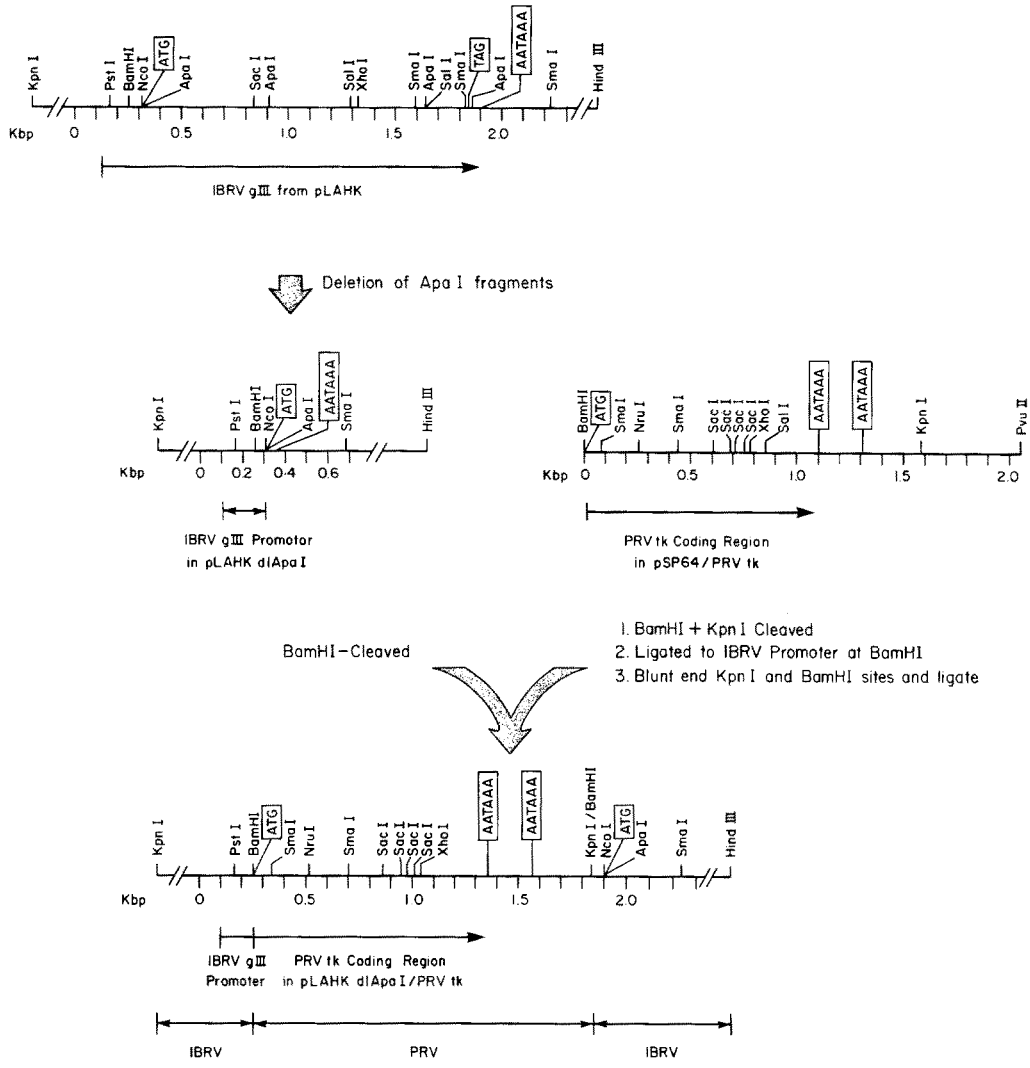

Fig. 3. Summary of the derivation of pLAHK dlApa I/PRV tk. The restriction endonuclease map of plasmid pLAHK shows the IBRV gIII promoter region, the translational start (ATG) and stop (TAG) signals, and the polyadenylation (AATAAA) signal. The restriction endonuclease map of the Bam HI Pvu II fragment from pSP 64/PRV tk which contains the coding region of the PRV tk gene is also shown. Homologous recombination between pLAHK dlApa I/PRV tk and infectious DNA from IBRV(NG) dltk dlgIII gave rise to the IBRV recombinant which expresses the PRV tk gene from the IBRV gIII promoter

tk gene coding region adjacent to the IBRV gIII promoter; (4) treating the overhanging Kpn I and Bam HI termini of the resulting reaction mixture with mung bean nuclease to produce blunt ends; and (5) ligating the blunt ends to produce pLAHK dlApa I/PRV tk.

To obtain by homologous recombination a gIII$^-$ deletion mutant in which the PRV tk gene replaces the coding region of the IBRV gIII gene, the hybrid plasmid pLAHK dlApa I/PRV tk was mixed with infectious DNA from

IBRV(NG) dltk dl gIII, in which a part of the IBRV tk gene and the entire coding region of the IBRV gIII gene was deleted [11, 13, 16], and the DNA mixture was transfected into MDBK cells [15]. The progeny virus obtained following this cross was enriched for IBRV recombinants expressing the tk enzyme, by passing the progeny virus in tk^- Rab (BU) cells in HATG medium [15]. Virus harvests were then titrated in MDBK cells, diluted to about 100 PFU/ml, and about 20 PFU of HATG-selected virus were plaque purified in MDBK cells. Viruses isolated from individual plaques were then used to infect MDBK cells which had been seeded in 96-well tissue culture plates. In this manner 192 clones of HATG-selected viruses were prepared from individual plaques and served as master plates.

To identify HATG-resistant IBRV recombinants with PRV tk gene inserts, a nick-translated ^{32}P-pSP 64/PRV tk probe was prepared as described [15], and hybridized to Pvu II, Pst I, and Hind III fragments from candidate IBRV(NG) dltk dl gIII (PRV tk) recombinants and from parental IBRV(NG) dltkdl gIII. The 10.5 kb Hind III, 1.8 kb Pst I, and 3.3 kb Pvu II DNA fragments, which contain the promoter region of the IBRV gIII gene, were present in the restriction nuclease fragments of parental tk^- IBRV(NG) dltk, but absent from the DNA fragments of $gIII^-$ deletion/insertion mutant IBRV(NG) dltk-dlgIII (PRV tk) clone 14. The ^{32}p-labelled pSP 64/PRV tk probe did not hybridize to any of the parental virus DNA fragments, but did hybridize to 12.1 kb Hind III, 3.4 kb Pst I, and 5.2 kb Pvu II fragments of recombinant IBRV(NG)-dltk dlgIII (PRV tk), clone 14, as expected from the insertion of a 1.6 kb Bam HI to Kpn I fragment of PRV DNA at the Bam HI cleavage site of the IBRV gIII promoter (Fig. 3). The results demonstrate that the HATG-resistant recombinant between IBRV(NG) dltk dlgIII (PRV tk) clone 14 is a homologous recombinant between IBRV(NG) dltk dlgIII and pLAHK dlApa I/PRV tk, and that, in the recombinant virus, the PRV tk coding region is placed 3′ to the IBRV gIII gene promoter.

Construction of a tk^- $gIII^-$ IBRV-based virus vector expressing the PRV g92 (gIII) gene driven by the IBRV gIII promoter

Plasmid pLAHK dlApa I/PRV g92 was obtained by inserting a 2.4 kb Nco I fragment derived from pBUK: Stu 12/Pst I (see Fig. 1), which contains the coding region of PRV g92 (gIII) plus PRV translational stop and polyadenylation signals, into the Nco I site of pLAHK dlApa I (Fig. 4). Thus, plasmid pLAHK dlApa I/PRV g92 contains the coding sequences of the PRV g92 (gIII) gene immediately downstream from the promoter regions of IBRV gIII (see Fig. 3). To obtain, by homologous recombination, a tk^- $gIII^-$ IBRV deletion/insertion mutant in which the PRV g92 (gIII) gene replaces the coding region of the IBRV gIII gene so that the PRV g92 (gIII) gene is expressed under the control of the late IBRV gIII promoter, infectious DNA from IBRV(NG) dltk dlgIII, in which a part of the IBRV tk gene and the entire coding region of the IBRV gIII gene were deleted, was mixed with hybrid plasmid pLAHK dlApa I/PRV

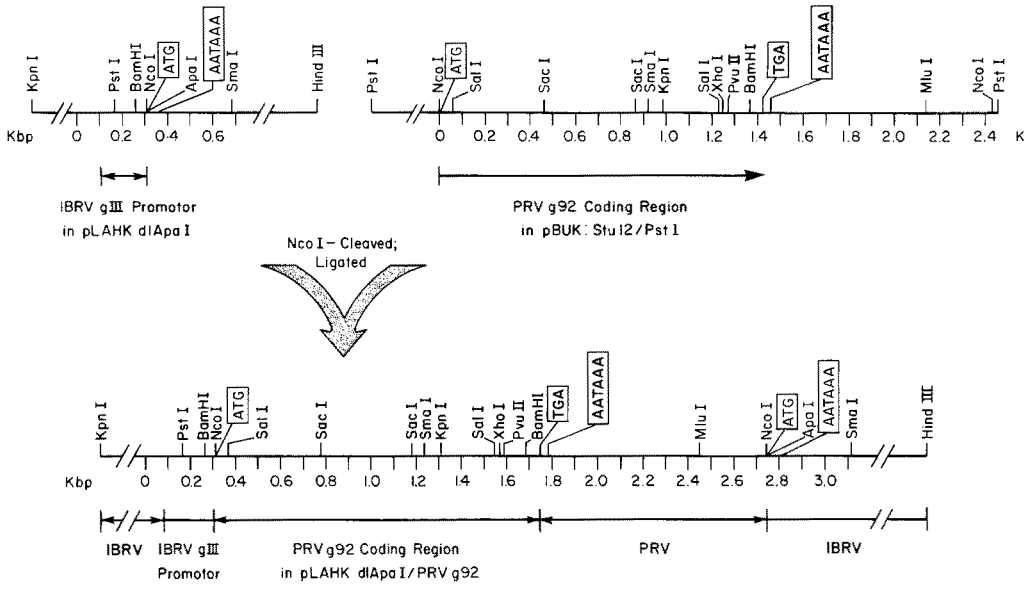

Fig. 4. Restriction endonuclease map of the IBRV gIII promoter in pLAHK dlApa I (see Fig. 3) and of the coding region of the PRV g92(gIII) gene in pBUK : Stu 12/Pst I (see Fig. 1). In order to insert the coding region of the PRV g92(gIII) gene and its 3′ flanking sequences adjacent to the IBRV gIII promoter, pLAHK dlApa I and pBUK : Stu 12/Pst I were cleaved with Nco I and the resulting DNA fragments were religated to produce pLAHK dlApa I/PRV g92. Homologous recombination between pLAHK dlApa I/PRV g92 and infectious DNA from IBRV(NG) dltk dlgIII gave rise to the mutant IBRV(NG) dltk-dlgIII (PRV g92) which expresses the PRV g92 gene from the IBRV gIII promoter

g92 and transfected into MDBK cells. The progeny were cloned, and the clones were screened for IBRV recombinants harboring PRV g92 (gIII) nucleotide sequences, using a nick-translated ^{32}P-labeled pSal probe, as described previously.

Molecular hybridization experiments demonstrated that 3 out of 192 clones of candidate recombinants hybridized to the pSal probe. Two of the isolated homologous recombinants were designated IBRV(NG) dltk dlgIII (PRV g92) clone 7 and clone 9 and were saved for further analyses. Viral DNAs were prepared from these two recombinants and from parental IBRV(NG) dltk dlgIII, the DNAs were cleaved with restriction endonucleases Hind III, Kpn I, and Pst I, and the DNA fragments were separated by electrophoresis on agarose gels, and analyzed as described (Fig. 5). The restriction endonuclease analyses showed that the Hind III restriction profiles of the recombinants and of the parental IBRV(NG) dltk dlg92 DNA were similar, except that the 10.5 kb fragment observed in the parental virus DNA was absent from the recombinant DNAs and a new 12.9 kb fragment was present in the recombinant virus DNAs, but not in the parental virus DNA (Fig. 5A). The pSal probe hybridized to the 12.9 kb Hind III fragment of the recombinant DNAs, but did not hybridize with any of the Hind III fragments of parental DNA

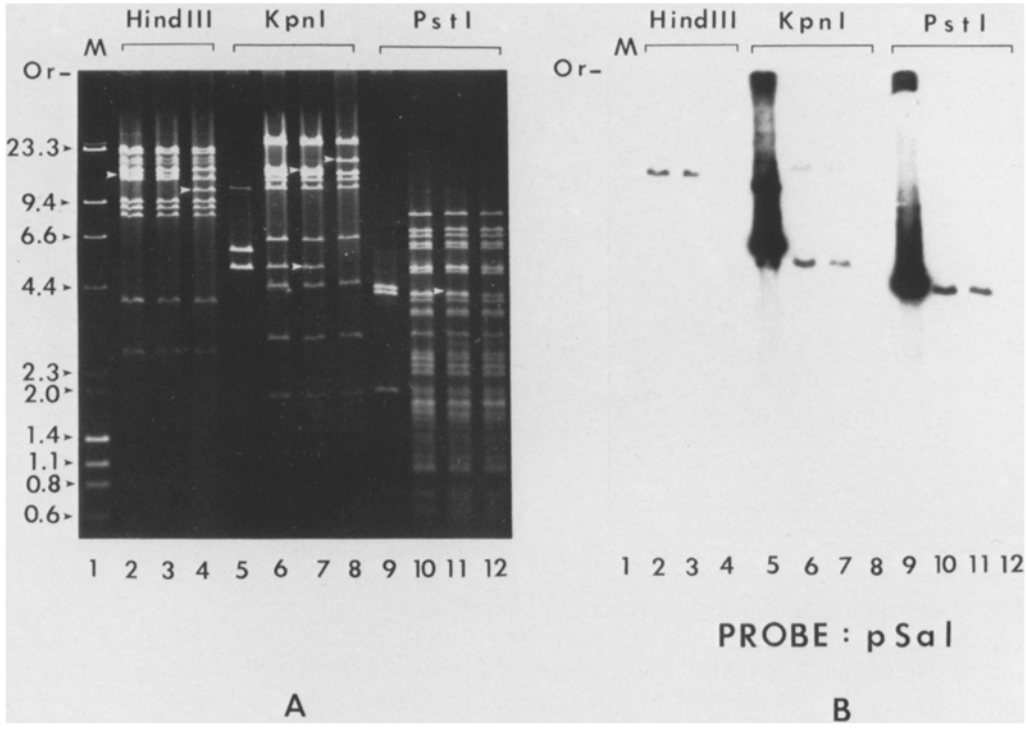

Fig. 5. A Ethidium bromide-stained agarose gel fragments of Hind III-, Kpn I- and Pst I-digested DNA from the parental tk$^-$ IBRV(NG) dltk dlgIII strain (*4, 8,* and *12*) and from the recombinants IBRV(NG) dltk dlgIII (PRV g92) clone 7 (*3, 7,* and *11*) and IBRV(NG) dltk dlgIII (PRV g92) clone 9 (*2, 6,* and *10*) which express the PRV g92 (gIII) gene. *5* and *9*, respectively, show Kpn I and Pst I fragments obtained by restriction endonuclease digestion of pLAHK dlApa I/PRV g92. *1* shows the size in kb of marker fragments from Hind III-digested phage lambda DNA and Hae III-digested phage phi X174 DNA. The arrows point to DNA fragments which differ in size in the parental and recombinant viruses. **B** Autoradiographs demonstrating molecular hybridization of the ^{32}P-labeled pSal probe to DNA fragments from IBRV(NG) dltk dlgIII (PRV g92) clones 7 and 9, and to pLAHK dlApa I/PRV g92, all of which contain PRV DNA inserts, but not to DNA fragments from the parental tk$^-$ IBRV(NG) dltk dlgIII strain

(Fig. 5B). The 10.5 kb Hind III fragment of IBRV(NG) dltk dlgIII contains the promoter region of IBRV gIII. The results demonstrated that the 2.4 kb DNA fragment from pLAHK dlApa I/PRV g92, which contains the coding region of the PRV g92 (gIII) gene, was incorporated into the IBRV genome proximal to the IBRV gIII gene promoter. Further restriction endonuclease analyses with Kpn I and Pst I confirmed this conclusion. The parental IBRV and the recombinant viruses differed in their Kpn I restriction profiles in that a 17 kb fragment was present in the parental virus but absent from those of the recombinant viruses, and that 14 kb and 5.3 kb fragments were present in the profiles of the recombinant viruses but absent from those of the parental virus. The 5.3 kb fragment of the recombinant virus hybridized with the pSal probe, but the 17 kb fragment of the parental virus did not. It may be noted that the 5.3 kb fragment

is also present in the Kpn I digest of pLAHK dlApa I/PRV g92 (Fig. 5B). Digestion with Pst I yielded 4.3 kb fragments from the recombinant viruses and from pLAHK dlApa I/PRV g92, which also hybridized with the pSal probe. Thus, the above results confirm that IBRV(NG) dltk dlgIII (PRV g92) clones 7 and 9 contain the IBRV gIII gene promoter sequence and that the PRV g92(gIII) coding region replaced the IBRV gIII coding region.

tk Enzyme phenotypes of IBRV recombinants

Thymidine plaque autoradiographic analyses were carried out in tk^- Rab(BU) cells to ascertain the tk phenotypes of the IBRV recombinants harboring inserts of foreign genes. These experiments demonstrated that monolayers of mock-infected tk^- Rab(BU) cells did not have virus plaques and did not incorporate ^{14}C-thymidine into cellular DNA (data not shown). However, tk^- Rab(BU) cell monolayers infected with wild-type tk^+ IBRV(LA) or with recombinant viruses IBRV(NG) dltk dlgIII, IBRV dltk (PRV g92) clone 6-2, IBRV(NG)-dltk dlgIII (PRV tk) clone 14, and IBRV(NG) dltk dlgIII (PRV g92) clone 9 had many plaques. Plaques made by the wild-type IBRV(LA) were labeled with ^{14}C-thymidine, as expected, but plaques made by IBRV(NG) dltk dlgIII, IBRV dltk (PRV g92) or IBRV(NG) dltk dlgIII (PRV g92) were not labeled, because these viruses had deletions in the IBRV tk gene and they were not capable of inducing a functional tk activity. In contrast, plaques made by IBRV(NG) dltk dlgIII (PRV tk) were heavily labeled with ^{14}C-thymidine, because the PRV tk gene had been inserted adjacent to the IBRV promoter and was efficiently expressed under the control of the IBRV gIII promoter. The results demonstrate that the insertion mutants, IBRV(NG) dltk dlgIII (PRV tk) clone 14 had a tk^+ phenotype and that insertion mutants IBRV dltk (PRV g92) clone 6-2 and IBRV(NG) dltkdl gIII (PRV g92) clone 9 had tk^- phenotypes.

Detection of PRV g92 (gIII) following infection of Rab9 cells with recombinants IBRV dltk (PRV g92) and IBRV dltk dlgIII (PRV g92)

To verify that PRV g92 (gIII) was expressed in cells infected with IBRV recombinants containing PRV g92 (gIII) inserts, Rab9 cells were infected with PRV(BUK-5) and IBRV(LA) as positive and negative controls, respectively, or with IBRV dltk (PRV g92) clones 6-2 and 7-30, and labeled with D-(2,6-^{3}H) mannose, as described [18]. Labeled extracts from virus-infected cells were prepared, mixed with PRV antisera, immunoprecipitated and analyzed [18].

Figure 6 demonstrates that PRV antisera immunoprecipitated mannose-labeled glycoproteins with molecular weights of about 115–122 kDa, 92–87 kDa, 72–78 kDa, and 55–62 kDa from extracts of PRV(BUK-5)-infected cells. The labeled 92–97 kDa and 72–78 kDa bands contain PRV g92(gIII) and a PRV g92 (gIII) precursor, respectively [18]. Figure 6 also shows that PRV antisera did not immunoprecipitate any labeled glycoprotein from the wild-type

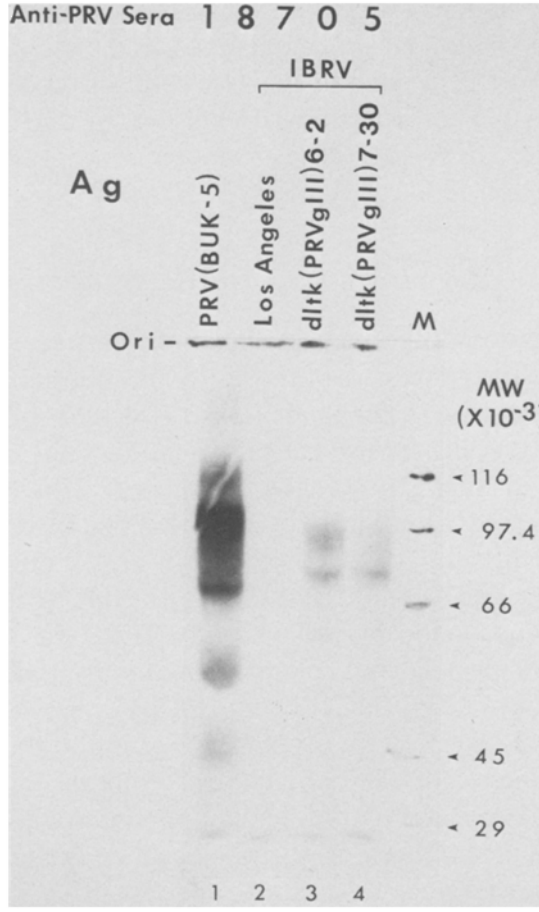

Fig. 6. Immunoprecipitation experiments using PRV antisera to detect the PRV g92(gIII) antigen in detergent extracts of ^{3}H-mannose-labeled cells infected with PRV(BUK-5), with IBRV(LA) and with recombinants, IBRV dltk (PRV g92) clone 6-2 and clone 7-30. Recombinant IBR viruses produced ^{3}H-mannose-labeled proteins of about 92,000 to 97,000 daltons and about 72,000 daltons [PRV g92(gIII) and a partially glycosylated precursor] which reacted with the PRV-specific antisera, but the ^{3}H-mannose-labeled extracts from parental tk^{+} IBRV(LA) did not react with the PRV antisera. Molecular weight markers are shown at the right

IBRV(LA)-infected cells, but did immunoprecipitate labeled glycoproteins of 92–98 kDa and 72–78 kDa from extracts of recombinant IBRV dltk (PRV g92)-infected cells. Additional control experiments demonstrated that labeled glycoproteins from mock-infected cells were not immunoprecipitated by PRV antisera nor by polyclonal IBRV antisera. Likewise, polyclonal IBRV antisera did not immunoprecipitate labeled glycoproteins from PRV-infected cells, but, as expected, did precipitate six or more IBRV-specific glycoproteins with molecular weights ranging from about 55 kDa to 180 kDa from extracts of cells infected with IBRV(LA) and IBRV dltk (PRV g92). These results demonstrate that both the PRV g92(gIII) gene, which had been inserted into the IBRV tk

gene of IBRV(LA), and IBRV-specific-glycoprotein genes were expressed in the recombinant IBRV dltk (PRV g92).

In a second experiment, ^{3}H-mannose-labeled extracts from mock-infected and virus-infected-cells were immunoprecipitated with mAb to PRV g92(gIII) and analyzed as described [18] (data not shown). The results showed that the 92–97 kDa PRV g92(gIII) was precipitated from extracts of cells infected with IBRV dltk (PRV g92) clone 6-2, IBRV(NG) dltk dlgIII (PRV g92) clone 9, and from PRV gIII-positive PRV(BUK-d13) cells, but not from extracts of mock-infected cells. Hence, the results demonstrate that the insertion mutants, IBRV(NG) dltk dlgIII (PRV g92) and IBRV dltk (PRV g92) were expressing the PRV g92(gIII) glycoprotein.

Virus neutralization

Polyclonal bovine IBRV antisera inhibited plaque formation by wild-type IBRV(LA) and by the recombinant IBRV(NG) dltk dlgIII (PRV g92) by 95–97%, as expected (Table 1). To learn whether antibodies against PRV could also inhibit plaque formation by the recombinant harboring a PRV g92 (gIII) insert, IBRV(NG) dltk dlgIII (PRV g92) and IBRV(LA) were treated with polyclonal PRV antisera and with mAb to PRV gIII. The polyclonal PRV antisera and the mAb to PRV gIII had little or no inhibiting effect on plaque formation by IBRV(LA), but, in contrast, both inhibited IBRV(NG) dltk dlgIII (PRV g92) plaque formation by 65% and 47%, respectively (Table 1).

Construction of IBRV expressing E. coli β-gal gene from the IBRV gIII locus

An IBRV insertion mutant in the gIII gene expressing the *E. coli* β-gal gene from the IBRV gIII promoter was obtained by transfection of Rab9 cells with plasmid pLA (PrGIII-β-gal) : 31 (Fig. 7) and infectious IBRV(NG) dltk DNA. The derivation of plasmid pLA (PrGIII-β-gal) : 31 from plasmids pMC 1871, which contains the *E. coli* β-gal gene [2], and a plasmid, designated pLA (GIII): 31 dlHind III/Hpa I dlNS, is shown in Fig. 7. This plasmid was constructed from cloning vector pIBI 31 (International Biotechnologies, Inc., New Haven,

Table 1. Virus neutralization of wild-type and recombinant IBRV strains

Description of sera	IBRV (Los Angeles)		IBRV(NG) dltk dlgIII (PRV g92)	
	PFU	%inhibition	PFU	%inhibition
Normal pig sera (control)	74	0	172	0
Pig PRV antisera	80	0	60	65
Mouse mAb to PRV gIII	60	19	92	47
Bovine IBRV antisera	4	95	6	97

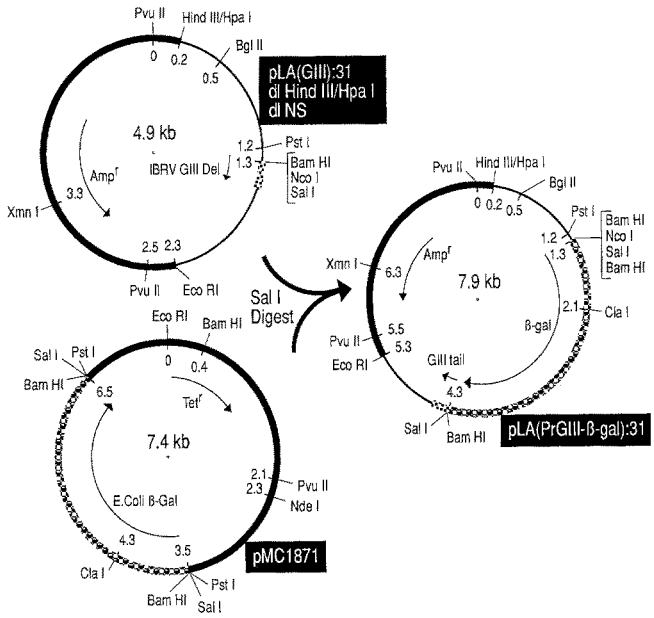

Fig. 7. Restriction endonuclease maps of plasmids used to construct pLA (PrGIII-β-gal) : 31 which contains the *E. coli* β-gal gene adjacent to the IBRV gIII promoter

CT) by inserting the IBRV gIII gene plus flanking sequences in pIBI 31 to obtain plasmid pLA (GIII): 31 dlHind III/Hpa I, and then modifying the latter plasmid to provide convenient insertion sites and an in-phase reading frame for the β-gal gene. This was accomplished by deleting a 1.4 kb Nco I to Sal I fragment containing the gIII coding sequences from the IBRV gIII gene in pLA (GIII) : 31 dlHind III/Hpa I (see Fig. 3) and then ligating the synthetic oligodeoxyribonucleotide adaptor,

5′-CATGGCAGAATCCT
CGTCTTAGGACAGCT-5′,

to the Nco I/Sal I digest of pLA (GIII) : 31 dlHind III/Hpa I.

In order to verify that the oligodeoxyribonucleotide adaptor had placed the *E. coli* β-gal gene in an proper translation phase with the ATG GCA GAA TCC TGT CGA codons of the adaptor, Bam HI fragments of 25 bp and 88 bp from the DNA region at the start of the β-gal gene were cloned into phage M13mp19 and sequenced, as described [24]. The sequences confirmed that the β-gal gene had been inserted in phase with the ATG translational start codon of the adaptor. The progeny IBRV from the transfection of IBRV(NG) dltk DNA and pLA (PrGIII-β-gal) : 31 were screened for recombinants expressing β-gal by colorimetric plaque assay [2, 4]. Candidate recombinants identified by the conversion of the colorless X-gal substrate to an insoluble colored product were plaque purified twice and virus and viral DNA pools were prepared. To verify the identity of IBRV recombinants harboring *E. coli* β-gal inserts, the

DNA from one of the candidate recombinants, designated IBRV (PrGIII-β-gal), was digested with Hind III, Bam HI, Kpn I and Sal I, the restriction fragments were separated by gel electrophoresis, stained with ethidium bromide and photographed. The restriction patterns of the parental IBRV(NG) dltk for Hind III and Sal I were not significantly distinct from that of IBRV (PrgIII-β-gal). However, Bam HI cleavage of IBRV (PrGIII-β-gal) revealed the appearance of a new 3 kb fragment and the approximately 20 kb Bam HI fragment of IBRV(NG) dltk was replaced by a fragment of about 18 kb. Furthermore, Kpn I cleavage of IBRV (PrGIII-β-gal) revealed that a fragment of about 21 kb had replaced the 18 kb Kpn I fragment of IBRV(NG) dltk. These results are consistent with the expected homologous recombination between IBRV(NG) dltk and pLA (PrGIII-β-gal), to produce a virus that expresses *E. coli* β-gal.

Discussion

The preceding experiments describe recombinant IBRV vectors that efficiently express the foreign PRV gIII (late; gamma 2) gene [5, 18, 25] inserted into the nonessential IBRV tk (early) gene [34], and recombinant IBRV vectors that efficiently express the early PRV tk gene, the late PRV gIII gene, and the *E. coli* β-galactosidase gene [2] inserted into another nonessential IBRV gene, that is, the late IBRV gIII gene [3, 23]. By analogy with herpes simplex virus and PRV [20, 22], additional nonessential genes probably exist in the IBRV genome, as for example in the unique short (Us) region of the IBRV genome, and could also be used as insertion sites for foreign genes.

The expression of late (gamma 2) viral genes by cells infected with IBRV and other alphaherpesviruses requires prior viral DNA synthesis and the function of proteins encoded by immediate-early viral genes [3, 5, 20, 21, 23]. In the case of recombinant virus, IBRV dltk (PRV g92), the entire PRVgIII gene, including the native PRV gIII promoter, was inserted into the IBRV tk gene. Hence, the expression of this late PRV gIII gene in the recombinant virus denotes that immediate early proteins of IBRV, presumably in conjunction with host transcription factor(s) [19, 20], can recognize and transactivate the heterologous, native late promoter sequences of the PRV gIII gene. For recombinants, IBRV(NG) dltk dlgIII (PRV tk), IBRV(NG) dltk dlgIII (PRV g92), and IBRV (PrGIII-β-gal), the coding sequences of the early PRV tk, the late PRV g92 (gIII), and the *E. coli* β-galactosidase genes replaced the coding sequences of the IBRV gIII gene and were ligated to the IBRV gIII promoter sequence. Thus, the expression of these foreign genes was, in these cases, driven by the homologous late (gamma 2) IBRV gIII gene promoter, which was, likewise, transactivated by immediate early IBRV proteins.

The IBRV (PrGIII-β-gal) recombinant can be recognized by the colored plaques generated after infected monolayers are overlaid with X-gal [2, 4] and has been useful as an intermediate in the construction of IBRV recombinants harboring other foreign DNA sequences ligated to the IBRV gIII promoter

sequence. For example, by transfecting susceptible MDBK cells with DNA from IBRV (PrGIII-β-gal) plus plasmids containing foot and mouth disease virus VP1 epitope sequences, and selecting for recombinant viruses which failed to express the *E. coli* β-gal gene in the X-gal plaque assay, we were able to isolate recombinant IBRV-FMDV viruses, which expressed FMDV VP1 epitope sequences as part of a fusion protein with IBRV gIII [10, 13].

The high levels of expression of PRV gIII by IBRV(NG) dltk dlgIII-(PRV g92) enabled us to utilize the PRV gIII produced by this recombinant as the antigen to plate microtiter test wells in a sensitive and specific PRV gIII differential blocking ELISA test [9]. This recombinant may also have the potential for affording cattle protection from fatal PRV infection.

Plaques produced by the IBRV(NG) dltk dlgIII (PRV g92) recombinant were stained by mAb to PRV gIII. In addition, as shown in Table 1, this recombinant IBRV was neutralized by polyclonal PRV antisera and by mAB to PRV gIII, thereby providing functional evidence that the PRV g92 (gIII) glycoprotein was presented on the surface of IBRV(NG) dltk dlgIII (PRV g92) virus particles. Previously, plaque immunoassays, virus neutralization, and immunoelectron microscopy analyses had demonstrated that the FMDV-IBRV gIII fusion proteins produced by the IBRV-FMDV (monomer) and IBRV-FMDV (dimer) recombinants were expressed on the surface of virus particles and on the surface of virus-infected cells [10, 13].

The efficient expression of PRV and *E. coli* genes ligated to the IBRV gIII promoter in recombinant IBRV-infected cells contrasts with analogous experiments on the expression of foreign genes inserted in the PRV gIII gene, which is homologous to the IBRV gIII and the HSV gC genes. Studies have shown that the expression of foreign genes inserted into the PRV gIII gene is comparatively poor. For example, in our laboratory, it was found that the insertion of the entire IBRV gIII gene including the regulatory plus coding sequences at the Nco I site of the PRV gIII gene resulted in very low levels of synthesis of IBRV gIII protein (unpubl. experiments). Formation of IBRV gIII protein was increased by deleting the promoter sequences of the IBRV gIII gene and ligating the coding sequences of the IBRV gIII gene directly to the PRV gIII promoter. However, comparisons of the color produced by coating microtiter wells of ELISA test plates with the PRV gIII and IBRV gIII antigens made in recombinant IBRV and PRV, respectively, indicated that only one tenth as much IBRV gIII antigen was produced by the PRV (IBRV gIII) recombinant-infected cells as PRV gIII antigen made by IBRV(NG) dltk dlgIII (PRV g92)-infected cells. Likewise, we observed that when *E. coli* β-gal coding sequences were ligated to the PRV gIII promoter sequences, the recombinant PRV (PrGIII-β-gal) expressed β-gal activity very poorly, unlike the recombinant IBRV (PrGIII-β-gal), which expressed much higher levels of β-gal.

Whealy et al. [33] inserted the entire HSV-1 gC gene including regulatory sequences in place of its homolog, the PRV gIII gene, in the genome of a PRV recombinant. The HSV-1 gC promoter functioned in the PRV genome and

authentic HSV-1 gC protein was produced, albeit at a low level, in infected cells. The HSV-1 gC protein was present at the cell surface but was not detected in the PRV envelope. These investigators [32] have likewise reported that PRV gIII-*E. coli* lac Z hybrid viruses poorly express the bacterial gene. Whealy et al. [32] also constructed infectious PRV mutants expressing chimeric viral membrane proteins comprised of portions of the PRV envelope glycoprotein gIII and the human HIV-1 envelope glycoproteins gp 120 and gp 41. All the chimeric genes contained the transcription control sequences and the first 157 codons of the PRV gIII gene fused to different regions of the HIV-1 envelope. Although the mutant viruses expressed novel glycosylated fusion proteins, the levels of expression were lower than expected due primarily to instability or altered processing of the hybrid mRNA. The hybrid protein was poorly detected on the cell surface and was not detected in the PRV envelope.

The experiments described herein illustrate intriguing differences in the expression of foreign genes driven by homologous gIII promoters of closely related alphaherpesviruses. The reasons for these differences require further study. IBRV occupies an intermediate position in the evolution of alphaherpesviruses with regard to the number and layout of immediate early genes [19, 31, 35]. Whereas PRV and equine herpesvirus 1 produce a single immediate early transcript encoding different related species of immediate early proteins, the human herpesviruses, varicella-zoster, and HSV-1, encode four or five immediate early proteins from different immediate early transcripts arranged similarly to the arrangement of IBRV, which generates three major immediate-early transcripts from two divergent and spliced transcription units. In the case of HSV-1, three of the five immediate early gene products (ICP0, ICP4, and ICP27) can transactivate expression of the beta or gamma genes during viral infection or in transient expression systems. ICP27 can inhibit or augment the individual capabilities of ICP4 and ICP0 to stimulate the expression of chimeric genes containing viral gene promoters [28] and ICP0 can play a role in the de novo synthesis of infectious virus during the transfection of viral DNA [1]. The IBRV IER 4.2 transcript appears to be homologous to the HSV-1 ICP4 transcript and to the PRV transcript. These transcripts encode the major transactivating immediate early proteins, that is, VP 175 of HSV-1 and the 180 kDa proteins of IBRV and PRV [35]. The IBRV IER 2.9 and IER 1.7 transcripts may be homologous to the HSV-1 ICP0 and ICP22 transcripts, respectively. It remains to be determined, however, whether the unavailability of the IBRV IER 2.9, IER 1.7, and IER 4.2 transcripts and their putative translation products in PRV recombinants partly accounts for the poor expression of the IBRV gIII gene inserts in PRV.

Differential regulation by viral transactivating genes has previously been observed by Inchauspe and Ostrove [7], who found that varicella-zoster gene products homologous to HSV-1 ICP4 and ICP27 stimulate a variety of viral and cellular gene promoters, including the HSV-1 tk promoter, but that the expression of a recombinant vector containing the varicella-zoster tk promoter

could not be stimulated by HSV-1 infection or by the HSV-1 ICP4 or ICP0 proteins expressed during cotransfection experiments.

Enquist and coworkers [5] found that the nucleotides encoding the signal sequence of PRV gIII were critical for RNA accumulation and protein localization. They constructed a PRV mutant with a 63 bp deletion of the sequences encoding amino acids 2 to 22 of the PRV gIII gene. The gIII polypeptide made by this signal sequence mutant was not detected in the viral envelope, nor was it aberrantly localized to the culture medium. In addition, the mutant gIII allele was expressed at lower levels than was the wild-type gene. Enquist et al. [5] pointed out that several inverted repeats lie in the DNA sequences that correspond to the 5′ noncoding and coding regions of PRV gIII mRNA, that these inverted repeats might lead to DNA-mediated control of gene expression, perhaps by acting as binding sites for regulatory proteins, or as a consequence of mRNA secondary structure. The 5′ sequence of gIII mRNA has the potential to fold into two stemloop structures, which might have a role in the stable formation and efficient expression of PRV gIII. By contrast, our computer analyses indicate that similar inverted repeats are not found in the promoter-signal sequence regions of IBRV gIII.

Robbins et al. [26] found that a single amino acid change in the PRV gIII signal sequence of leucine to proline was responsible for greatly reduced expression of PRV gIII in the Bartha vaccine strain. In this case, the reduced amount of gIII was not mediated at the level of transcription but rather reflected defects in gIII maturation and localization. The observations on the Bartha strain of PRV suggest that the expression of the foreign IBRV gIII gene in PRV recombinants might be increased by deleting the IBRV gIII signal sequence and inserting the truncated IBRV gIII gene in phase with the PRV gIII promoter plus PRV signal sequence. Experiments to test this hypothesis are in progress. It should be noted, however, that, in the HIV-1 recombinants constructed by Whealy et al. [32], even though the PRV gIII promoter and signal sequences were intact, and the HIV sequences were inserted 157 codons downstream from the PRV gIII translational start site, the level of expression of the PRV-HIV-1 fusion proteins was low.

Finally, it may be noted that the poor expression of foreign gene inserts in the PRV gIII gene described here may be idiosyncratic for the late PRV gIII gene. Several recent reports have described the expression of foreign genes ligated to the PRV gX promoter in PRV recombinants [22, 25, 29, 30]. Nevertheless, by development of recombinants which more efficiently express foreign gene inserts driven by the PRV gIII promoter, our understanding of gamma 2 herpesvirus control mechanisms can be improved.

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