JOURNAL OF VIROLOGY, Oct. 1990, p. 4691–4696
0022-538X/90/104691-06$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 64, No. 10

Linker Insertion Mutagenesis of Herpesviruses: Inactivation of Single Genes within the Us Region of Pseudorabies Virus

NIELS DE WIND,[1] ALICE ZIJDERVELD,[1] KOEN GLAZENBURG,[2] ARNO GIELKENS,[2] AND ANTON BERNS[1]*

Division of Molecular Genetics of The Netherlands Cancer Institute and Department of Biochemistry, University of Amsterdam, Antoni van Leeuwenhoek Huis, Plesmanlaan 121, 1066 CX Amsterdam,[1] and Department of Virology, Central Veterinary Institute, 8221 RA Lelystad,[2] The Netherlands

Received 12 March 1990/Accepted 16 June 1990

We describe a technique for the systematic inactivation of nonessential genes within the genome of a herpesvirus without the requirement for phenotypic selection. This technique is based on the insertion of an oligonucleotide containing translational stop codons at a random site within a large cloned viral DNA fragment. Mutant virus is then reconstituted by cotransfection with overlapping viral clones, together comprising the entire viral genome, as described previously (M. van Zijl, W. Quint, J. Briaire, T. de Rover, A. Gielkens, and A. Berns, J. Virol. 62:2191–2195, 1988). This technique was used to construct, in a single experiment, a set of 13 viable pseudorabies virus strains with oligonucleotide insertions within all known genes of the Us region except for the gp50 gene, which proved essential for virus growth in cell culture. The growth rate in porcine kidney cells of mutants of all nonessential Us genes was similar to that of the parental virus, with the exception of a mutant of the recently identified protein kinase gene.

Like other herpesviruses, pseudorabies virus (PRV) has a complex linear DNA genome, with a size of about 150 kilobase pairs (kbp) encoding at least 50 proteins (see Fig. 1A). The majority of the PRV genes has not been mapped on the viral genome, and no function has been assigned to most of the viral gene products. Several techniques for the mutagenesis of herpesvirus genomes have been described. Most are based on introduction of a mutation within a cloned viral fragment and successive recombination in tissue culture of the mutant fragment into the viral genome (7, 33, 35, 42). Alternative methods for the generation of mutant virus use selective growth conditions in vitro (9, 18, 19, 41). In this report, we describe an approach for linker insertion mutagenesis of large segments of the PRV genome in a single experiment. The technique takes advantage of the observation that digestion in the presence of ethidium bromide of covalently closed DNA molecules by restriction enzymes with multiple recognition sites within the fragment preferentially yields full-length linear molecules (25). We applied this technique to a 27-kbp clone containing the Us region of PRV, using restriction enzymes with 4-bp recognition sequences. At the site of linearization, an oligonucleotide containing both translational stop codons in all reading frames and an *Eco*RI site was inserted. Similar approaches, used for the construction of linker insertion mutants within a small viral clone, mostly a single cloned gene, have been described (3, 4, 38). A set of mutant PRV strains was generated by overlap recombination after cotransfection of the oligonucleotide-containing Us clones with overlapping clones, together comprising the entire viral genome, as described previously (13, 40).

This method enabled us to construct a series of 13 viable PRV mutants in a single experiment. Individual genes were inactivated as a result of premature translational termination without any previous knowledge of gene organization and without the requirement for phenotypic selection of the mutants. The reliability of this technique was validated by (i) determination of the sequence flanking a series of oligonucleotide insertion sites, (ii) identification of transcripts containing an inserted oligonucleotide, and (iii) characterization of protein products, synthesized in vitro and in vivo from a gene with the oligonucleotide inserted at different positions within the protein-coding domain.

MATERIALS AND METHODS

Cells and virus. Cell line PK15 and the PRV isolate NIA-3 have been described previously (5). Virus was routinely propagated in SK6 cells, a porcine kidney cell line. Both cell lines were cultured in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum. Growth of virus strains in SK6 cells was determined essentially as described previously (15).

DNA and RNA techniques. Total DNA was extracted from infected cells as described previously (6). Total RNA was isolated 7 h after infection of a monolayer of SK6 cells with 10 PFU of virus per cell, as described previously (37). Common DNA and RNA techniques were performed essentially as described elsewhere (17). In vitro transcription of cloned genes was performed by using the vector pSP64. In vitro translation of RNA was done in the presence of [^{35}S]methionine. Kits for transcription and translation were supplied by Promega Biotec and used according to protocols provided by the supplier. DNA sequence analysis was performed according to the dideoxy-chain termination method (36), using deaza-dGTP instead of dGTP to eliminate compressions.

Western immunoblotting. Monolayers of SK6 cells were infected with virus at 10 PFU per cell in the presence of tunicamycin (1 μg/ml) to inhibit N-linked glycosylation. At 16 h after infection, cells were lysed with phosphate-buffered saline containing 1% (vol/vol) Triton X-100, 0.5% (wt/vol) deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted to nitrocellulose. The blot was incubated with anti-gI monoclonal antibody 3/6 (kindly provided by H.-J. Rziha), washed, and incubated with goat anti-mouse antibodies conjugated to alkaline phos-

* Corresponding author.

phatase. Glycoprotein gI was visualized with a standard alkaline phosphatase staining.

Linker-scanning mutagenesis of the PRV Us region. A palindromic 20-mer oligonucleotide, 5'-TAGGCTAGAAT TCTAGCCTA-3', was used for insertional inactivation of PRV genes. The oligonucleotide has the following characteristics: (i) TAG translational stop codons in all reading frames and (ii) an *Eco*RI restriction site that is absent from the genome of PRV NIA-3. Insertion of this oligonucleotide in any protein-encoding gene, in any orientation, and in any reading frame should result in premature translational termination. Furthermore, the presence of the *Eco*RI site in the oligonucleotide greatly facilitates mapping of the site of insertion of the oligonucleotide. The 27-kbp *Hin*dIII-B fragment of PRV NIA-3, containing the Us region (Fig. 1A), was cloned in the *Hin*dIII site of a pBR322 derivative in which the *Eco*RI site had been deleted by using the Klenow fragment of *E. coli* DNA polymerase I. Three parallel 25-μg portions of covalently closed plasmid DNA were digested in a volume of 125 μl for 15 min at 37°C with either one of the restriction endonucleases *Fnu*DII (2 U), *Hae*III (1 U), and *Rsa*I (0.5 U). Reactions were performed in the following buffer solutions: *Fnu*DII, 20 mM Tris hydrochloride (pH 7.5)–8 mM $MgCl_2$–50 μg of ethidium bromide per ml; *Hae*III, 20 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–8 mM $MgCl_2$–5 μg of ethidium bromide per ml; *Rsa*I, 20 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–8 mM $MgCl_2$–0.5 μg of ethidium bromide per ml. Under these conditions, approximately 30% of the DNA was cleaved once to yield full-length linear molecules, as judged by agarose gel electrophoresis. Since each of these restriction enzymes has a 4-bp recognition specificity, the DNA is considered to be linearized at quasi-random sites. The full-length linear DNA was purified by CsCl-ethidium bromide density gradient centrifugation, followed by preparative agarose gel electrophoresis and electroelution. The purified linear DNA fragments were ligated with a 50-fold molar excess of the kinase-treated oligonucleotide. Concatemers of ligated fragments and oligonucleotide were cleaved by *Eco*RI. Unit-length fragments were purified by preparative gel electrophoresis and electroelution. Of this DNA, 0.5 μg was recircularized by ligation in a volume of 400 μl. DNA was precipitated from the ligation mixture, dissolved in 10 μl of TE, and used to transform *Escherichia coli* K-12 DH5 by the method of Hanahan (8). The recombinant clones were examined for integrity by digestion with *Bam*HI, followed by agarose gel electrophoresis. The site of insertion of the oligonucleotide was determined by double digestions with the restriction enzymes *Bam*HI-*Eco*RI and *Bgl*II-*Eco*RI, respectively, followed by agarose gel electrophoresis.

Reconstitution of mutant PRV. PRV with the oligonucleotide inserted at the desired position within the *Hin*dIII-B fragment was reconstituted by cotransfection of cell line PK15 with the mutant *Hin*dIII-B fragment and the overlapping, purified viral insert fragments from cosmids c-179, c-27, and either c-443 or c-447 as described previously (40). Cosmid c-447 was derived from cosmid c-443 (40) by deleting 8 kbp of DNA from the right end (starting at an *Xba*I site within the *Bam*HI-5 fragment, 3 kbp to the right of the *Bam*HI-8–*Bam*HI-5 junction; Fig. 1A), thereby reducing the overlap with the mutant *Hin*dIII-B fragment. Mutant virus obtained in this way was plaque purified three times on SK6 cells. Integrity and identity of the mutant viral DNA were verified by restriction enzyme digestions of total DNA from infected cells.

RESULTS

Viability of PRV mutants. Insertion of the oligonucleotide at quasi-random unique sites within a clone of the Us region of PRV was used to generate 14 mutant Us clones (Fig. 1A). An additional Us mutant, named RI, was obtained by inserting the oligonucleotide in a unique *Bgl*II site within the gp50 gene (Fig. 1A). This set of 15 mutagenized Us clones was chosen for the reconstruction of mutant viruses. For that purpose, purified mutant insert fragments were cotransfected with purified overlapping PRV fragments, derived from cosmid clones, together comprising the complete genomic information of PRV. Only insertions RI and 322, located within the gp50 gene (28), did not yield mutant PRV progeny, indicating that the gp50 protein is essential for viability of the virus. All other oligonucleotide insertions in the Us region yielded infectious mutant PRV. Mutant 169, carrying an oligonucleotide in the internal repeat region in the *Bam*HI-10 fragment, yielded, besides wild-type (wt) virus, a PRV mutant with an insertion in both IR and TR (*Bam*HI-10 and *Bam*HI-12 fragments). A *Bam*HI-*Eco*RI double digest of wt NIA-3 DNA and of the mutant PRV strains is depicted in Fig. 1B.

Growth of Us mutants in tissue culture cells. Mutant strains 549, 247, 149, 522, and 361 were chosen for the determination of viral growth in SK6 (porcine kidney) cells. These mutants represent 5' insertions in the recently mapped (M. van Zijl, H. van der Gulden, N. de Wind, A. Gielkens, and A. Berns, J. Gen. Virol., in press) protein kinase (549), gX (247), gp63 (149), gI (522), and 11K (361) genes (Fig. 1A). As no 5' insertion of the recently discovered (van Zijl et al., in press) 28K gene was obtained, we constructed a Δ28K mutant virus by exchanging the 5.2-kbp *Bgl*II-*Eco*RI fragment of mutant clone 357 (Fig. 1A) for the 4.6-kbp homologous fragment of clone 351 (Fig. 1A) and cotransfecting this clone with overlapping viral fragments as described, resulting in mutant virus Δ351-357. All mutants except 549 showed no consistent differences in growth titers and identical end titers as compared with wt NIA-3 (Fig. 2). Strain 549, having an insertion of the oligonucleotide in the protein kinase gene, between 133 and 134 bp 3' of the major start codon for translation (van Zijl et al., in press), displayed a lower growth rate than all other PRV strains tested and also a lower virus end titer (Fig. 2).

Nucleotide sequences adjacent to insertion sites. To determine whether alterations had occurred in PRV sequences bordering the insertion sites, *Sau*3A-*Eco*RI fragments of both sides of the inserted oligonucleotide of seven mutant *Hin*dIII-B fragment clones (as indicated in Fig. 1A) were cloned in M13mp11 and sequenced. In none of the cases was a deletion or alteration in the sequence found in the PRV DNA flanking the oligonucleotides.

Transcription of gI and 11K mutants. We examined whether insertion of the oligonucleotide had affected the size or relative level of the corresponding transcript. Total RNA was isolated from PK15 cells 7 h after infection with wt NIA-3 virus with mutants 522, 326, and 324 (all with an insertion in the gI gene) or with mutant 361 (having an insertion in the 11K gene, which is cotranscribed with the gI gene). After separation on 1% agarose-formaldehyde gels, RNA was blotted to nitrocellulose and hybridized with a gI probe (a 1,052-bp *Bst*EII-*Sph*I fragment) and with gX probe pMZ14 (a 591-bp *Bam*HI-*Bst*EII fragment) as an internal control (see Fig. 1A for the map positions of the probes). In all cases, both the levels and the sizes of the transcripts were

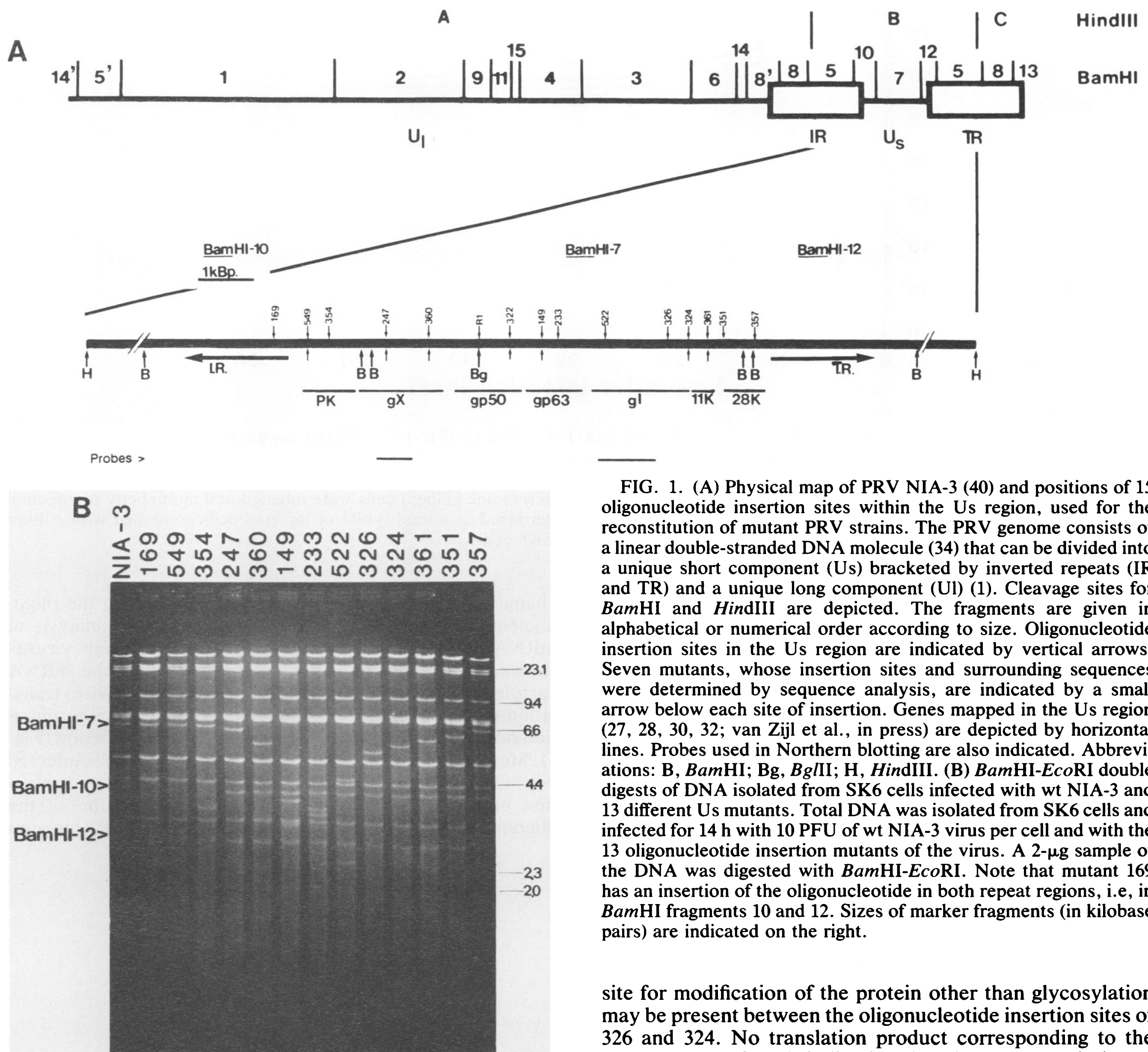

FIG. 1. (A) Physical map of PRV NIA-3 (40) and positions of 15 oligonucleotide insertion sites within the Us region, used for the reconstitution of mutant PRV strains. The PRV genome consists of a linear double-stranded DNA molecule (34) that can be divided into a unique short component (Us) bracketed by inverted repeats (IR and TR) and a unique long component (Ul) (1). Cleavage sites for *Bam*HI and *Hin*dIII are depicted. The fragments are given in alphabetical or numerical order according to size. Oligonucleotide insertion sites in the Us region are indicated by vertical arrows. Seven mutants, whose insertion sites and surrounding sequences were determined by sequence analysis, are indicated by a small arrow below each site of insertion. Genes mapped in the Us region (27, 28, 30, 32; van Zijl et al., in press) are depicted by horizontal lines. Probes used in Northern blotting are also indicated. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII. (B) *Bam*HI-*Eco*RI double digests of DNA isolated from SK6 cells infected with wt NIA-3 and 13 different Us mutants. Total DNA was isolated from SK6 cells and infected for 14 h with 10 PFU of wt NIA-3 virus per cell and with the 13 oligonucleotide insertion mutants of the virus. A 2-μg sample of the DNA was digested with *Bam*HI-*Eco*RI. Note that mutant 169 has an insertion of the oligonucleotide in both repeat regions, i.e, in *Bam*HI fragments 10 and 12. Sizes of marker fragments (in kilobase pairs) are indicated on the right.

indistinguishable from those seen in the control NIA-3 (data not shown).

Translational termination caused by the oligonucleotide in vitro. *Dra*I-*Bam*HI fragments, containing a PRV fragment starting 46 bp 5′ from the start codon of the gI gene and including the genes for gI and 11K of wt NIA-3 virus and of mutants 522, 326, 324, and 361 (Fig. 1A), were subcloned in *Hin*dII-*Bam*HI-digested pSP64. RNA was synthesized in vitro and translated in a rabbit reticulocyte lysate. The synthesized proteins were analyzed by SDS-PAGE and autoradiography. In all cases, the mutant gI proteins, synthesized in vitro, showed a size as predicted from the site of insertion of the oligonucleotide in the gI gene (Fig. 3). This proves that the oligonucleotide indeed causes efficient translation termination in vitro. As previously described for gI proteins synthesized in vitro from some PRV strains (20, 21), gI migrates as a doublet except for mutants 522 and 326, which displayed a single band, suggesting that some signal or site for modification of the protein other than glycosylation may be present between the oligonucleotide insertion sites of 326 and 324. No translation product corresponding to the 11K gene was found, indicating that the 11K protein is not cotranslated with the gI protein.

Translational termination caused by the oligonucleotide in vivo. SK6 cells were infected with control NIA-3 and with mutant viruses 522, 326, 324, and 361 (Fig. 1A). Total protein was separated by SDS-PAGE and electroblotted to nitrocellulose, and gI proteins were visualized by consecutive incubation with a gI-specific monoclonal antibody and a goat anti-mouse antibody conjugated with alkaline phosphatase, followed by a standard alkaline phosphatase staining. In all mutants except 522, the gI-specific bands showed the predicted size (Fig. 4). Mutant 522 gave no visible gI product, possibly because of instability of the polypeptide.

DISCUSSION

In this report, we describe the rapid generation of PRV mutants by the insertion of a 20-mer oligonucleotide at quasi-random sites within a large cloned subgenomic fragment. Cotransfection of each of the mutant fragments with

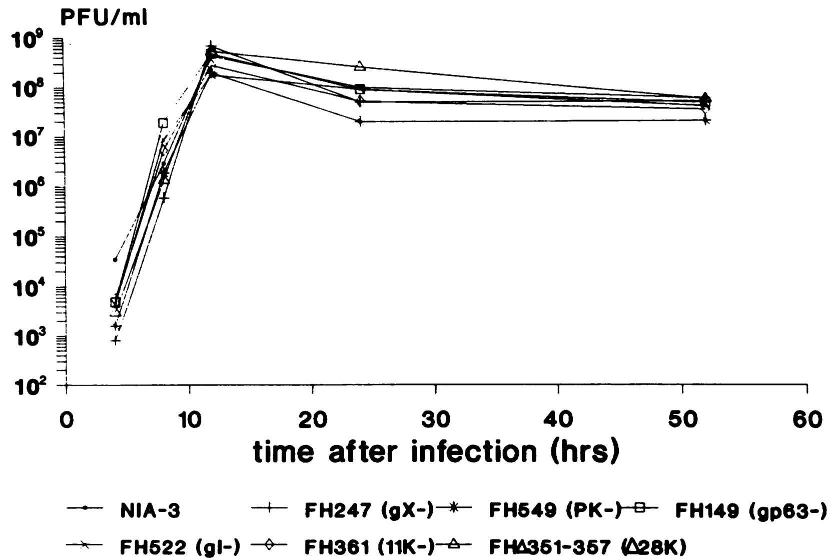

FIG. 2. Growth curves of mutants of every nonessential Us gene. SK6 (porcine kidney) cells were infected at a multiplicity of infection of 5 by six Us mutants and by wt NIA-3. Virus yield (in PFU) was determined in a total lysate of infected cells combined with culture supernatant at various time points after infection. Virus was titrated on SK6 cells.

overlapping PRV fragments, derived from cosmid clones and containing the complete genomic information of the virus, yields a PRV strain with an oligonucleotide inserted at a unique site, provided that the mutation is not lethal for the virus. By this strategy, the expression of a single gene can be abolished without requiring a phenotypic selection protocol. We show by a number of analyses that this method is highly reliable. Firstly, sequence analysis of the flanking DNA of seven randomly chosen oligonucleotide insertion sites showed that in all cases the oligonucleotide was inserted in a cleavage site of one of the three enzymes that were used to digest the *Hin*dIII-B clone (data not shown). No deletions or changes had occurred in the viral DNA flanking the oligonucleotide. Furthermore, Northern (RNA) blot analysis of mRNA from cells infected with viable mutant viruses showed no alterations in the relative levels of the mRNA carrying the oligonucleotide (data not shown). in vitro translation of mRNA showed that efficient translational termination occurred at the site of the oligonucleotide insertion (Fig. 3). Moreover, Western blotting of protein from cells infected with virus mutants demonstrated that, also in vivo, termination of translation occurs at the site of insertion of the oligonucleotide (Fig. 4). The presence of an *Eco*RI recogni-

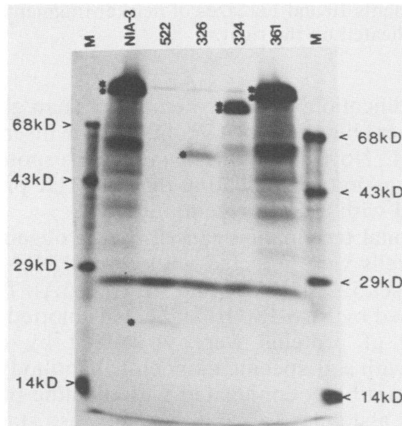

FIG. 3. Glycoprotein gI, synthesized in vitro by wt NIA-3 and by mutants 522, 326, 324, and 361. Glycoprotein gI genes of wt NIA-3, of gI oligonucleotide insertion mutants 522, 326, and 324, and of 11K insertion mutant 361 were cloned into pSP64. Glycoprotein gI mRNA was synthesized in vitro and translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Protein was separated on a denaturing SDS-polyacrylamide gel, and glycoprotein gI was visualized by autoradiography. Asterisks indicate the truncated gI proteins synthesized. Lane M, molecular weight markers; sizes are indicated in kilodaltons (kD).

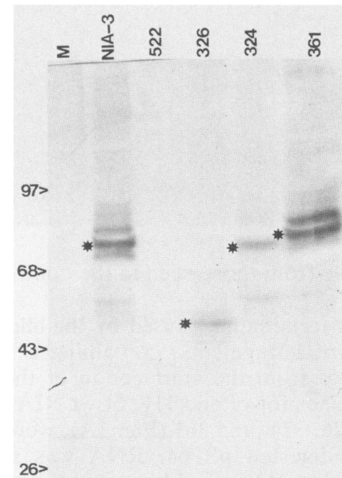

FIG. 4. Western blot of gI proteins, synthesized in vivo by NIA-3 and mutants 522, 326, 324, and 361. SK6 (porcine kidney) cells were infected with wt NIA-3, with gI oligonucleotide insertion mutants 522, 326, and 324, and with 11K insertion mutant 361. Cells were lysed at 16 h after infection, total protein was separated on a denaturing polyacrylamide gel and blotted, and glycoprotein gI was visualized by using a monoclonal antibody, followed by a standard alkaline phosphatase staining. Asterisks indicate the gI proteins synthesized. Sizes of molecular weight markers (in kilodaltons) are indicated. Lane M, mock-infected SK6 cells.

tion site within the oligonucleotide enables rapid mapping of the site of insertion, both within the mutagenized cloned fragment and within the reconstituted mutant virus. The unique *Eco*RI recognition site also facilitates further manipulation of the clone and thereby of the mutant PRV virus, as was demonstrated with the construction of a mutant with a deletion of the 28K gene. Finally, this approach enables the construction of multiple oligonucleotide insertion mutants within a single gene, facilitating the assignment of functional sites within the protein. The latter is exemplified by the analysis of insertion mutants within the gI gene, in which we mapped a putative signal or site for modification of the gI protein between the oligonucleotide insertion sites of mutants 326 and 324 by an in vitro study (Fig. 3).

In the related herpes simplex virus type 1, most of the genes in the Us region are dispensable for growth in tissue culture cells (16, 42). The same applies for PRV; mutants of the gX (39), gp63 (23, 24, 43), and gI (22, 24, 43) genes were previously constructed. Large deletions of the gp63, gI, 11K, and 28K genes are present in attenuated PRV strains such as vaccine strains Bartha and Norden (19, 29). The Us deletions in these strains probably have emerged during passage of the virus on chicken embryo fibroblasts (19). These mutants displayed a normal growth rate in porcine kidney cells, as was reported for the Bartha mutant (2, 15) and the Norden mutant (19). A gX mutant has not previously been tested for growth in porcine kidney cells; however, a PRV mutant carrying a deletion of the gene was demonstrated to be as virulent for mice as was the corresponding wild-type virus (39). This report is the first to describe a PRV mutant with a defective protein kinase gene, as was demonstrated by sequence analysis (this report) and Western blotting experiments (van Zijl et al., in press). Deletion mutants of the homologous herpes simplex type 1 protein kinase gene have been described (5, 16, 31). The PRV protein kinase mutant clearly has the lowest growth rate and the lowest end titer in porcine kidney cells of all mutant strains constructed and tested in this study. The inability to obtain oligonucleotide insertion mutants of the gp50 gene strongly suggests that this gene is the only Us gene indispensable for replication of PRV in cultured cells. The latter view was supported by the observation that viable virus containing oligonucleotide insertions RI and 322 in the gp50 gene (Fig. 1A) can be generated by overlap recombination in gp50-producing SK6 cells (B. Peeters et al., unpublished data). The herpes simplex virus gD gene, which is homologous with gp50 at the sequence level (28) and seems to be involved in the same path of entry of virus into cells (26), has been demonstrated to be essential for virus replication, playing an important role in the penetration of the virus into the cell (10–12, 14).

By using the technique described here, saturating oligonucleotide mutagenesis of the PRV genome can easily be achieved. Mutants viable in tissue culture may have deficiencies in genes required for modulation of viral or host gene expression, host range, or the establishment of latency. Therefore, analysis of the phenotype of these mutant PRV strains in cultured cells and in the pig, the natural host for PRV, can provide insight in PRV gene function and in virus-host interactions.

ACKNOWLEDGMENTS

We thank Maddy van Zijl and Hanneke van der Gulden for support and for making available many of the clones used in this study.

This work was supported by the Netherlands Technology Foundation, Duphar (a member of the Solvay Animal Health Group), and Intervet International BV (subsidiary of AKZO).

LITERATURE CITED

1. **Ben-Porat, T., A. Deatly, R. A. Veach, and M. L. Blankenship.** 1979. Analysis of the structure of the genome of pseudorabies virus. Virology **95**:285–294.
2. **Ben-Porat, T., J. DeMarchi, J. Pendrys, R. Veach, and A. S. Kaplan.** 1986. Proteins specified by the short unique region of the genome of pseudorabies virus play a role in the release of virions from certain cells. J. Virol. **57**:191–196.
3. **Cai, W., S. Person, C. DebRoy, and B. Gu.** 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1: an analysis of linker insertion mutants. J. Mol. Biol. **201**:575–588.
4. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex type 1 (HSV-1) ICP4 genes specifying nonsense peptides. Nucleic Acids Res. **15**:4491–4511.
5. **Frame, M. C., F. C. Purves, D. J. McGeoch, H. S. Marsden, and D. P. Leader.** 1987. Identification of the herpes simplex virus protein kinase as the product of viral gene US3. J. Gen. Virol. **68**:2699–2704.
6. **Gielkens, A. L. J., J. T. van Oirschot, and A. J. M. Berns.** 1985. Genome differences among field isolates and vaccine strains of pseudorabies virus. J. Gen. Virol. **66**:69–82.
7. **Goldstein, D. J., and S. K. Weller.** 1988. An ICP6::*lacZ* insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J. Virol. **62**:2970–2977.
8. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning, a practical approach, vol. 1. IRL Press, Ltd., Oxford.
9. **Harland, J., and S. M. Brown.** 1988. Generation of a herpes simplex virus type 2 variant devoid of *Xba*I sites: removal of the 0.91 map coordinate site results in impaired synthesis of glycoprotein g-2. J. Gen. Virol. **69**:113–124.
10. **Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso.** 1987. Neutralizing antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61**:3356–3364.
11. **Johnson, D. C., and M. W. Ligas.** 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. **62**:4605–4612.
12. **Johnson, R. M., and P. G. Spear.** 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex infection. J. Virol. **63**:819–827.
13. **Kapoor, Q. S., and G. Chinnadurai.** 1981. Method for introducing site-specific mutations into adenovirus 2 genome: construction of a small deletion mutant in VA-RNAI gene. Proc. Natl. Acad. Sci. USA **78**:2184–2188.
14. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
15. **Lomniczi, B., A. S. Kaplan, and T. Ben-Porat.** 1987. Multiple defects in the genome of PRV can affect virulence without detectably affecting replication in cell culture. Virology **161**:181–189.
16. **Longnecker, R., and B. Roizman.** 1987. Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science **236**:573–576.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **McGregor, S., B. C. Easterday, A. S. Kaplan, and T. Ben-Porat.** 1985. Vaccination of swine with thymidine kinase-deficient mutants of pseudorabies virus. Am. J. Vet. Res. **46**:1494–1497.
19. **Mettenleiter, T. C., B. Lomniczi, N. Sugg, C. Schreurs, and T. Ben-Porat.** 1988. Host-cell specific growth advantage of pseudorabies virus with a deletion in the genome sequences encoding a structural glycoprotein. J. Virol. **62**:12–19.
20. **Mettenleiter, T. C., N. Lukacs, and H.-J. Rziha.** 1985. Mapping

of the structural gene of pseudorabies virus glycoprotein A and identification of two non-glycosylated precursor polypeptides. J. Virol. **53**:52–57.

21. **Mettenleiter, T. C., C. Schreurs, H.-J. Thiel, and H.-J. Rziha.** 1987. Variability of pseudorabies virus glycoprotein 1 expression. Virology **158**:141–146.
22. **Mettenleiter, T. C., C. Schreurs, F. Zuckermann, and T. Ben-Porat.** 1987. Role of pseudorabies virus glycoprotein gI in virus release from infected cells. J. Virol. **61**:2764–2769.
23. **Mettenleiter, T. C., C. Schreurs, F. Zuckermann, T. Ben-Porat, and A. S. Kaplan.** 1988. Role of glycoprotein gIII of pseudorabies virus in virulence. J. Virol. **62**:2712–2717.
24. **Mettenleiter, T. C., L. Zsak, A. S. Kaplan, T. Ben-Porat, and B. Lomniczi.** 1987. Role of a structural glycoprotein of pseudorabies virus in virulence. J. Virol. **61**:4030–4032.
25. **Parker, R. C.** 1980. Conversion of circular DNA to linear strands for mapping. Methods Enzymol. **65**:415–426.
26. **Petrovskis, E. A., A. L. Meyer, and L. E. Post.** 1988. Reduced yield of infectious pseudorabies virus and herpes simplex virus from cell lines producing viral glycoprotein gp50. J. Virol. **62**:2196–2199.
27. **Petrovskis, E. A., and L. E. Post.** 1987. A small open reading frame in pseudorabies virus and implications for evolutionary relationships between herpesvirus. Virology **159**:193–195.
28. **Petrovskis, E. A., J. G. Timmins, M. A. Armentrout, C. C. Marchioli, R. J. Yancey, Jr., and L. E. Post.** 1986. DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J. Virol. **59**:216–233.
29. **Petrovskis, E. A., J. G. Timmins, T. M. Gierman, and L. E. Post.** 1986. Deletions in vaccine strains of pseudorabies virus and their effect on synthesis of glycoprotein gp63. J. Virol. **60**:1166–1169.
30. **Petrovskis, E. A., J. G. Timmins, and L. E. Post.** 1986. Use of λgt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella-zoster virus glycoproteins. J. Virol. **60**:185–193.
31. **Purves, F. C., R. M. Longnecker, D. P. Leader, and B. Roizman.** 1987. Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. J. Virol. **61**:2896–2901.
32. **Rea, T. J., J. G. Timmins, G. W. Long, and L. E. Post.** 1985. Mapping and sequence of the gene for the pseudorabies glycoprotein which accumulates in the medium of infected cells. J. Virol. **54**:21–29.
33. **Roizman, B., and F. J. Jenkins.** 1985. Genetic engineering of novel genomes of large DNA viruses. Science **229**:1208–1214.
34. **Rubenstein, A. S., and A. S. Kaplan.** 1975. Electron microscopic studies of the DNA of defective and standard pseudorabies virions. Virology **66**:385–392.
35. **Ryan, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist.** 1987. Analysis of pseudorabies virus glycoprotein gIII localization and modification by using novel infectious viral mutants carrying unique *Eco*RI sites. J. Virol. **61**:2962–2972.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
37. **Selten, G., H. T. Cuypers, and A. Berns.** 1985. Proviral activation of the putative oncogene pim-1 in MuLV-induced T-cell lymphomas. EMBO J. **4**:1793–1798.
38. **Stone, J. C., T. Atkinson, M. Smith, and T. Pawson.** 1984. Identification of functional regions in the transforming protein of fujinama sarcoma virus by in-phase insertion mutagenesis. Cell **37**:549–558.
39. **Thomsen, D. R., C. C. Marchioli, R. J. Yancey Jr., and L. E. Post.** 1987. Replication and virulence of pseudorabies virus mutants lacking glycoprotein gX. J. Virol. **61**:229–232.
40. **van Zijl, M., W. Quint, J. Briaire, T. de Rover, A. Gielkens, and A. Berns.** 1988. Regeneration of herpesviruses from molecularly cloned subgenomic fragments. J. Virol. **62**:2191–2195.
41. **Wathen, M. W., and L. M. K. Wathen.** 1984. Isolation, characterization and physical mapping of a pseudorabies virus mutant containing antigenically altered gp50. J. Virol. **51**:57–62.
42. **Weber, P. C., M. Levine, and J. C. Glorioso.** 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science **236**:576–579.
43. **Zuckermann, F. A., T. C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat.** 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. **62**:4622–4626.