Host Cell-Specific Growth Advantage of Pseudorabies Virus with a Deletion in the Genome Sequences Encoding a Structural Glycoprotein

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Several attenuated strains of pseudorabies virus contain genomes that carry a deletion in their short unique (Us) component. The sizes of the deletions are different in the various attenuated strains; the deletions may include part of one of the inverted repeats as well as part of the Us region of the genome. In most cases, the deletion includes the gene encoding the glycoprotein gI. The attenuated strains with a deletion in their S component have a common history of having been cultivated in chicken embryo fibroblasts (CEF). We show here that passage of wild-type virus in CEF promotes the emergence of populations of virions with a deletion in their S component. The emergence of these mutants is the result of their growth advantage over the wild type and is related to the lack of expression of gI, as shown by the following. (i) The Norden strain (which has a deletion in the Us) was marker rescued to restore an intact Us. The nonrescued Norden strain had a growth advantage over the rescued Norden strain in CEF. (ii) Passage of wild-type (gI+) virus in CEF but not in rabbit kidney or pig kidney cells resulted invariably in the emergence of virions whose genomes had a deletion in the S component. (iii) Passage of a gI− mutant in CEF did not result in the emergence of such virions. The emergence of virions with a deletion in their S component thus appears to be linked to gI expression. We conclude that gI is deleterious to the growth of pseudorabies virus in CEF and that this effect is cell type specific.

The restriction endonuclease digestion patterns of the genomes of different isolates of pseudorabies virus (PrV) vary greatly (1, 10, 11, 21, 26). Many of the changes in the migration rates of specific restriction fragments of PrV DNA are the result of insertions and deletions (6; unpublished results). The biological significance of these changes is largely unknown. In principle, deletions or insertions in sequences in a variant viral genome may, under certain growth conditions, confer upon the virus useful biological functions, leading to the dominance in the replication of the variant virus over the parental virus. Indeed, we have shown previously that some modifications of the standard structure of the PrV genome may provide the virus with an improved ability to perpetuate itself under certain environmental conditions (2, 6, 18, 40).

Deletions in the short unique (Us) component of the genome are present in the genomes of several viable variants of PrV (7, 17, 22, 27). Interestingly, several attenuated strains of PrV consist of homogeneous populations of virions with genomes that carry such deletions. The experiments described in this report were designed to uncover the basis for the evolution of the virion populations carrying these deletions and to investigate the possibility that, under certain conditions, those deletions confer a growth advantage upon the virus.

Our results show the following. (i) Genomes with a deletion in the S component accumulate readily upon passage of wild-type virus strains in cultures of chicken embryo fibroblasts (CEF) but more rarely in other cell lines. (ii) Virions carrying this deletion have a growth advantage over the nondeleted virions in CEF cells. (iii) The growth advantage of virions with genomes that carry a deletion in the S component is related to the elimination of the gene encoding the glycoprotein gI; genomes with a deletion in the S component are enriched for during growth in CEF because the expression of gI appears to interfere with optimum growth of the virus in these cells.

MATERIALS AND METHODS

Virus strains and cell cultures. PrV(Ka) is a strain that has been carried in our laboratory for more than 25 years; its origin is uncertain (15). PrV90 is a recent field isolate. The Norden and Bartha avirulent vaccine strains were received from P. S. Paul; the origins of these strains have been described previously (29). The Tatarov (MK25) strain was obtained from A. L. J. Gielkens. The attenuated derivatives of the Buk strains were independently isolated after parallel passage of the Buk 120 strain in CEF (34-36, 41-45). These strains were gifts from R. Skoda and A. Zuffa. Buk Z300 is an avirulent strain isolated by A. Zuffa from a virus population of Buk strain after it was passaged 300 times in CEF. Buk Z900 is a virus strain that lost its virulence for swine after 900 passages in CEF. Buk Z920 is a plaque isolate derived from Z900; in contrast to Z900, it has lost its virulence for rabbits. Buk SK624 is a virus strain isolated in the laboratory of R. Skoda that lost its virulence for piglets after 624 passages in CEF. The SK900 and Norden strains were derived by further independent passage of SK624 in CEF. Both were further attenuated.

Rabbit kidney (RK) cells, pig kidney (PK) cells, and CEF were cultivated in Eagle synthetic medium supplemented with 3% dialyzed bovine serum. Virus was titrated by plaque assay in RK or PK cells.

Isolation of deletion-M13 insertion mutants in glycoprotein gI. The detailed method used to construct mutants with deletions and insertions in the gI-coding sequences will be...
described elsewhere (Mettenleiter et al., manuscript in preparation). In brief, fragment BamHISalI 7A of the PrV genome, which encompasses the gene encoding gl (22, 28), was cloned into pBR325. The plasmid was cleaved at a unique BstEII site present approximately 350 nucleotides downstream from the 5' end of the gl-coding sequences, and approximately 100 nucleotides from each end were removed by digestion of the linearized plasmid with the exonuclease Bal 31. The DNA was dephosphorylated with bacterial alkaline phosphatase, and a HaeIII fragment of M13 which includes the multiple cloning site was introduced.

Radiochemicals and enzymes. [α-32P]dCTP was purchased from New England Nuclear Corp. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. Purification of virions and extraction of DNA. Virions were purified as described previously (5). Sodium sarkosinate (final concentration, 2%) was added to the samples, which were heated (60°C for 15 min) and digested with nuclelease-free pronase (1 mg/ml) for 2 h. The DNA was then extracted four times with phenol-chloroform-isoamyl alcohol (50:48:2) and either dialyzed against buffer (0.01 M Tris, 0.001 M EDTA, pH 7.6) or alcohol precipitated.

Restriction enzyme digestion and gel electrophoresis of DNA fragments. Digestion and agarose gel electrophoresis of viral DNA were carried out as described previously (4). Filter strips to which restriction fragments of PrV DNA were fixed were prepared by the method of Southern (37).

Nick translation of cloned PrV DNA restriction fragments. PrV DNA restriction fragments, cloned in pBR325 as described previously (16), were nick translated by the method of Rigby et al. (31).

Immunoprecipitation. Immunoprecipitation was performed as described previously (13).

RESULTS

Mapping deletions in the S component of attenuated strains. During the course of studies designed to elucidate the genetic basis for the virulence of PrV, we examined the characteristics of several independently isolated vaccine strains of PrV and mapped their genomes by the Southern technique (37), as has been described previously for the Bartha and Norden vaccine strains (17). Various changes were observed in the structure of the genomes of the vaccine strains. Of particular interest was the finding that a majority had a deletion in the S component of their genomes similar to the deletion previously observed in the Norden and Bartha vaccine strains (12, 17).

Figure 1 shows the KpnI digestion patterns of the DNA of the various PrV strains. KpnI fragment I, which includes part of the U8 (Fig. 2), is larger in the genomes of wild-type virions (Fig. 1, lanes 7 and 8) than of the attenuated strains (KpnI fragment I is indicated in each case [O]). Five of the seven attenuated strains analyzed (lanes 1, 3, 4, 5, and 11) have relatively large deletions in KpnI fragment I; the two other attenuated strains (lanes 2 and 6) have smaller deletions in KpnI fragment I. In all of these strains, the normalized KpnI fragment I disappeared and a smaller fragment appeared. The smaller fragment was indeed a deleted form of KpnI fragment I was shown conclusively by hybridization with cloned KpnI fragment I of wild-type virus (data not shown).

Of the more than 200 plaque isolates of PrV(Ka) that were analyzed during the course of other experiments, 2 plaque isolates also had a large deletion in KpnI fragment I. One of these plaque isolates was obtained from a virus stock that had been passaged several times in CEF (Fig. 1, lane 10); the other was obtained from a population containing defective interfering particles that had been passaged at high multiplicity in RK cells (lane 9). Some restriction enzyme polymorphism involving KpnI fragment I was also sometimes observed in primary isolates of PrV. However, large deletions in this fragment, similar to those observed in some of the attenuated strains, were not a characteristic of primary isolates of PrV (data not shown).

Most of the U8 region has been sequenced, and detailed restriction maps of that region of the genome are available (27, 28). The availability of these restriction maps allowed us to map the deletions in some of the vaccine strains in detail. The results (Fig. 2) revealed the following. (i) The sizes of the deletions, and consequently probably the sequences that border the deletions, were different in the different virus variants, indicating that the deletions did not result from specific recombinational events. (ii) In most cases, the deletions included the sequences encoding gI. Indeed, gI was not synthesized by cells infected with any of the variant strains carrying the large deletion in the S component (Table 1).

The deletion in strain Buk Z920 exhibits some interesting features. In this strain, part of one of the inverted repeats bracketing the U8 and part of the U8 including the gene encoding gl have been deleted. Some of the sequences that are normally part of the inverted repeats were thereby converted to unique sequences. Furthermore, reiterations of some sequences of the U8 were also observed in the genome of this strain.

The mapping of the various virus variants (Fig. 2) revealed that a large part of the U8 of PrV was nonessential for growth in vitro. In addition, part of the sequences of the inverted repeats did not need to be diploid to allow virus growth in vitro. These findings are similar to those previously reported for herpes simplex virus; mutants of both herpes simplex virus types 1 and 2 have been described in which parts of the U8 and of one of the inverted repeats have been deleted (8, 14, 20, 30, 39).

Repeated passage of PrV in CEF results in the emergence of populations of virions with a deletion in the S component. The
vaccine strains that have a deletion in their S component have a common history of being either repeatedly passaged in CEF (to attenuate them) or grown in CEF. We therefore considered the possibility that growth in CEF might promote the emergence of virions with genomes carrying a deletion in the S component. To determine whether this was the case, four different isolates of wild-type PrV were passaged repeatedly at low multiplicity (0.01 PFU per cell) in either CEF, PK, or RK cells. The virion populations produced by these cells after different passage levels were isolated; the DNA was purified and digested with restriction enzymes and analyzed by the Southern technique (37). Figure 3 shows the Kpnl digestion patterns of the DNA obtained from one PrV strain [PrV(Ka)] passaged in CEF. As the virus was pas-

TABLE 1. Reactivity of monoclonal antibodies against viral glycoproteins with extracts of cells infected with different virus variantsa

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>gII cpm (× 10^6) precipitated with the indicated monoclonal antibody</th>
<th>gl</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>M5</td>
<td>M3</td>
</tr>
<tr>
<td>Buk Z300</td>
<td>151 (+)</td>
<td>128 (+)</td>
</tr>
<tr>
<td>Buk Z900</td>
<td>162 (+)</td>
<td>163 (+)</td>
</tr>
<tr>
<td>Buk SK900</td>
<td>155 (+)</td>
<td>155 (+)</td>
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<tr>
<td>Buk Sk900</td>
<td>165 (+)</td>
<td>145 (+)</td>
</tr>
<tr>
<td>Buk SK900</td>
<td>156 (+)</td>
<td>136 (+)</td>
</tr>
<tr>
<td>Norden</td>
<td>162 (+)</td>
<td>121 (+)</td>
</tr>
<tr>
<td>Norden rescued</td>
<td>162 (+)</td>
<td>121 (+)</td>
</tr>
<tr>
<td>PrV(Ka)</td>
<td>190 (+)</td>
<td>166 (+)</td>
</tr>
<tr>
<td>PrV(Ka)</td>
<td>165 (+)</td>
<td>153 (+)</td>
</tr>
<tr>
<td>PrBpS</td>
<td>195 (+)</td>
<td>201 (+)</td>
</tr>
<tr>
<td>Pr16T</td>
<td>175 (+)</td>
<td>183 (+)</td>
</tr>
<tr>
<td>Bartha</td>
<td>227 (+)</td>
<td>241 (+)</td>
</tr>
<tr>
<td>PrV(Ka)gI−(M13)</td>
<td>197 (+)</td>
<td>229 (+)</td>
</tr>
</tbody>
</table>

a [3H]glucosamine-labeled cell extracts obtained from cultures infected with the different virus strains were immunoprecipitated by the indicated monoclonal antibodies as described in Materials and Methods. In all cases, the background (<10^6 cpm) was subtracted. The amount of radioactivity precipitated was determined.

b +, Reactivity of the monoclonal antibody; −, lack of reactivity.

c ND, Not determined.
also resulted in deletion of these deleted sequences. However, following further passage, KpnI fragments, deleted forms of enzymes (data not shown). On the other hand, after 50 passages of the four virus strains in RK cells, the emergence of virus populations with a deletion in the S component was observed in only one of the strains, and, of the four virus strains that were passaged 50 times in PK cells, none became enriched for genomes with a deletion in the US. Thus, whereas passage in CEF appears to result invariably in the appearance of virions with genomes that have a deletion in the S component, passage in RK or PK cells does so far more rarely.

**Growth advantage of virions with a deletion in the S component.** The following interpretation is compatible with the results described above: genomes with a deletion in the S component arise spontaneously, and virions with genomes that carry this deletion have a growth advantage in CEF.

To assess whether this interpretation is correct and whether the deletion in the S component contributes to a growth advantage of PrV in CEF, we determined whether restoration of an intact S component to the Norden vaccine strain (which has a deletion in the S component; Fig. 2) would change its ability to replicate in CEF. A Norden strain variant with an intact S component was obtained by cotransfecting cells with the DNA of the Norden strain and with PstI fragment I of the PrV(Ka) genome, a fragment that encompasses the region deleted from the Norden genome (17, 19). The progeny was plaque assayed, and plaques were analyzed by hybridization and Southern analysis for the presence of sequences in the S component normally deleted from the Norden strain. An isolate (rescued Norden) with an intact S component was identified (Fig. 5). (The Norden strain has a deletion in the S component which includes parts of BamHI fragment 7 and fragment 12 including the BamHI site between them. As a consequence, a fusion fragment 7 + 12 composed of parts of each of these fragments is formed.) As expected, in contrast to the parental Norden strain, the rescued Norden isolate expressed glycoprotein gl (Table 1).

PK cells and CEF were infected with a 10:1 mixture of rescued Norden and parental Norden virus. This mixed virus

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**FIG. 3.** KpnI restriction patterns of the DNA of virions obtained after different numbers of passages in CEF cells. Strain PrV(Ka) was passaged repeatedly at low multiplicity (0.01 PFU per cell) in CEF cells. After the indicated number of passages, virions were purified, and the DNA was extracted and digested with KpnI. The digests were electrophoresed and stained with ethidium bromide.

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**FIG. 4.** Hybridization patterns of KpnI restriction digests of DNA obtained after different numbers of passages in CEF cells. Restriction digests similar to those described in the legend to Fig. 3 were electrophoresed, transferred to nitrocellulose filters, and hybridized to nick-translated BamHI fragment 7 (see map in Fig. 2). p, Passage.
FIG. 5. Restriction digests of Norden DNA and rescued Norden DNA. Norden virus was marker rescued to restore to it an intact U₈, as described in the text. DNA preparations of PrV(Ka) (lane A), Norden (lane B), and rescued Norden (lane C) were digested with BamHI or KpnI, electrophoresed, and stained with ethidium bromide. The positions of BamHI fragments 7, 7 + 12, and 12 and KpnI fragments I and I* are indicated (O).

The population was passaged at low multiplicity nine times in either CEF or PK cells. Because the cells were infected at low multiplicity (0.01 PFU per cell), much of the virus yielded by the cultures was produced by cells in which complementation between the two virus variants had not taken place. Figure 6 shows the hybridization patterns of BamHI digests of the DNA obtained from these virus populations to nick-translated BamHI fragment 7. In the original virus mixture, the fragment characteristic of the Norden rescued strain (BamHI fragment 7) was, as expected, approximately 10 times more abundant than that of the fragment characteristic of the Norden strain (fragment 7 + 12). The relative abundance of the two fragments did not change detectably after passage in PK cells. However, after passage in CEF, the fragment characteristic of the Norden strain (BamHI fragment 7 + 12) increased in abundance relative to that of the fragment characteristic of the rescued Norden strain (BamHI fragment 7), and the two fragments were present in almost equal amounts. Thus, the parental Norden strain (with a deletion in its S component) had become enriched for in the virion population after passage in CEF. Therefore, it appears that the deletion of the sequences in the S component confer upon the virus a growth advantage in CEF, i.e., the presence of an intact S component is deleterious to virus growth. This effect was not detectable in PK cells.

Although the presence of some sequences in the S component of the genome appears to be deleterious to virus growth in CEF, this effect is subtle. No difference in the final yield of infectious virus or in the kinetics of replication of the virus was observed when the Norden strain and the Norden rescued variants were grown individually in either CEF or PK cells (data not shown). The selective growth advantage of the Norden strain over the rescued Norden variant in CEF was detected only after passage at low multiplicity of a mixture of the two virus variants in cultures of CEF.

Passage in CEF of virions that do not express gI does not result in the emergence of populations of virions with a deletion in the S component of their genomes. The results described above show that virions with a deletion in the S component appear to have a growth advantage in CEF over virions with an intact S component. Furthermore, although several different populations of virions that have been passaged in CEF may have different deletions in their S component, they all lack the sequences encoding gI and, consequently, fail to synthesize gI (Fig. 2 and Table 1). We considered the possibility that the expression of gI may be deleterious to growth of PrV in CEF.

To determine whether this is indeed the case, we ascertained whether populations of mutants of PrV(Ka), which do not express gI, would become enriched for virions with a deleted S component after passage in CEF as is the case for wild-type virus. To this end, the parental virus and a gI- mutant [PrV(Ka)gI- (M13)] in which sequences of phage M13 had been inserted near the 5’ end of the gI gene and which does not express gI (Table 1) were passaged in CEF. After 19 passages, the virions produced by the cells were purified, and the DNA was extracted and analyzed for deletions in the S component. In two independently passaged populations of wild-type virus, deletions in the S component of the genome were observed (Fig. 7). In the DNA of one population (wild type, passage 19a), KpnI fragment I had become undetectable and a smaller fragment, KpnI fragment I*, appeared. Fragment I* hybridized only weakly to the sequences of BamHI fragment 7 that were used as a probe because a large part of the sequences of KpnI fragment I that normally hybridize to BamHI fragment 7 had been deleted. The other population of PrV(Ka) passaged in CEF (Fig. 7; wild type, passage 19b) contained a mixture of genomes; some had retained an intact KpnI fragment I, whereas others had a deletion in KpnI fragment I. The deletion in KpnI fragment I was smaller in the genomes of the passage 19b population than it was in that of the passage 19a population, but in both cases it included the sequences encoding gI. This was determined by hybridization of the Southern blots to sequences derived specifically from the gI gene (data not shown). Interestingly, each population was relatively homogeneous with respect to the type of deletion in the S component it had acquired.

FIG. 6. Hybridization pattern of restriction digests of the DNA of mixtures of Norden and rescued Norden after passage in CEF or PK cells. Rescued Norden and Norden virions were mixed at a ratio of 10:1 and were then passaged at low multiplicity (0.01 PFU per cell) nine times in either CEF or PK cells. Virions were purified; their DNA was extracted, digested with BamHI, electrophoresed, transferred to nitrocellulose filters, and probed with nick-translated BamHI fragment 7 (see map in Fig. 2).
that virus in digested with genomes with deletion in a virus of deletion. We conclude that the virus of wild-type PrV in CEF (Fig. 3, 4, and 7) but occurs less readily in RK or PK cells. The emergence of populations of virions with the deletion in the S component appears therefore to depend upon the cell type in which the virus is passaged. A host cell-specific accumulation of a deletion mutant of HSV-1 has also been reported (33).

It is likely that the deletions in the S component of the genome upon passage of PrV in CEF arise because of instabilities in the virus genome, i.e., are the result of recombinational events. The recombinational events generating the deletions in the S component do not appear to be sequence specific because several deleted forms of the genome appeared initially upon passage of the virus in CEF, one becoming predominant (probably because it has some selective advantage) upon additional passages (Fig. 4). This can also be deduced from the finding that different attenuated strains and different wild-type strains passaged in CEF acquire different size deletions in their S component (Fig. 1 and 2; data not shown), indicating that the recombinational events leading to these deletions can occur at several different loci of the viral genome.

PrV grows poorly in CEF and becomes adapted only after repeated passage in these cells. Several changes in the wild-type virus genome are observed after this adaptation process has taken place. (i) Sequences normally present at the left end of the L component become translocated next to the internal inverted repeat, and consequently the L component becomes invertible (18). (ii) In many cases some of the sequences of the U, normally adjacent to the internal inverted repeat are deleted (18). (iii) Sequences from the S component of the genome, including the sequence encoding gI, are deleted (this paper). Each of the three modifications mentioned above can occur independently of one another, and virions that have experienced only one of these changes can be isolated at early levels of passage in CEF (unpublished results).

Although populations of wild-type virions acquired a deletion in their S component upon passage in CEF, a characteristic that confers upon them a growth advantage in those cells, a population of gI- mutants did not become enriched for genomes with such a deletion after being passaged similarly in CEF. Because the gI- mutant possessed all of the sequences that border the deletions that normally appear after passage of wild-type virus in CEF, all the recombinational events leading to the emergence of the deleted genomes were possible. The lack of enrichment for genomes with a deletion in the S component, after passage of gI- mutants in CEF, indicates therefore that deletion of part of the S component does not have the same selective advantage in CEF for gI- virions as it does for gI+ virions. We conclude that the selective advantage of virions with genomes carrying a deletion in their S component resides in the lack of synthesis of gI by cells infected with these virions and that gI is deleterious to growth of PrV in CEF.

**DISCUSSION**

The studies described in this report were based on the observation that some independently isolated vaccine strains of PrV have a deletion in the S component of their genomes. Because all of these strains had been passaged (or grown) in CEF, we considered the possibility that growth of the virus in CEF may select for virions with genomes carrying this deletion. Our results indicate that, indeed, selection for mutants with a deletion in the S component occurs invariably during growth of the wild-type PrV in CEF (Fig. 3, 4, and 7) but occurs less readily in RK or PK cells. The emergence of populations of virions with the deletion in the S component appears therefore to depend upon the cell type in which the virus is passaged. A host cell-specific accumulation of a deletion mutant of HSV-1 has also been reported (33).

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The PrV genome encodes at least eight different glycoproteins; four of these, including gI, are nonessential for growth in vitro (22, 27, 32, 38). We have studied in some detail the function of gI. This glycoprotein exerts a subtle effect on virus growth in cell culture. Inactivation of gI, in conjunction with a defect in at least one other viral function, inhibits the
release of the virus from RK cells but not from PK cells (3, 24). We show here that the expression of gl is, on the other hand, deleterious to growth of wild-type PrV in CEF. It appears, therefore, that the effect of gl on virus growth in cell culture is cell type specific and is modulated by the expression of other viral functions. The cell type-specific effect exhibited by the gl glycoprotein indicates that it may play an important role in cell tropism and in the pathogenesis of the virus. Indeed, gl mutants have been shown to have reduced levels of virulence for both chickens and swine (25).

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LITERATURE CITED


