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Genetic recombination of pseudorabies virus: evidence that homologous recombination between insert sequences is less frequent than between autologous sequences

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Summary. We studied in vivo recombination between a thymidine kinase (TK) negative, glycoprotein E (gE) negative, attenuated strain and a virulent strain of pseudorabies virus (PRV) in pigs. To simplify the detection of recombination we inserted different but overlapping (375 bp) parts of the E1 gene of classical swine fever virus into the gG locus of both virus strains. Recombination between the E1 sequences of these viruses results in reconstitution of the complete E1 coding sequence and expression of the E1 protein. Since E1 is highly immunogenic, we expected to detect in vivo recombination in co-inoculated pigs by the presence of serum antibodies against E1. However, after co-inoculation of pigs with high doses of both virus strains, we were unable to detect antibodies against E1, suggesting that in vivo recombination did not occur or remained below the detection limit. Analysis of individual progeny viruses showed that 13 out of 995 (1.3%) possessed a recombinant TK-negative gE-positive phenotype. In contrast, no E1-positive viruses were detected among 5000 analyzed. This result showed that in vivo recombination between the two virus strains did occur, but was much more frequent between the TK and gE loci than between the E1 sequences. Similar results were obtained in in vitro recombination experiments in which possible growth differences between the various virus strains were excluded. The different recombination frequencies could not be attributed to the difference in distance of the genetic loci since recombination between mutations at a distance of 266 bp in the TK gene occurred as frequent as recombination between the TK and gE genes which are separated by approximately 60 kilobasepairs. These results indicate that some property of the E1 sequence and/or the location of the E1 sequence within the PRV genome affects the frequency of recombination.

Introduction

Pseudorabies virus (PRV) is the causative agent of Aujeszky's disease in pigs (reviewed in [22]). The disease is distributed world-wide and has a large impact on the pig industry. To control or eradicate the disease, pigs are vaccinated with conventionally attenuated or genetically engineered PRV strains. One example of an engineered PRV vaccine is strain 783 which is thymidine kinase (TK) and glycoprotein E (gE) deficient (TK^-gE^-) [18]. This strain has been extensively examined for its safety and efficacy [25, 26]. Recently, van Zijl et al. [28] used this strain to express the surface protein E1 of classical swine fever virus. Pigs that were immunized with the carrier vaccine were protected against both classical swine fever and Aujeszky's disease.

Although vaccination with carrier vaccines is a promising approach, possible risks might be connected with the use of such viruses [16]. One of the potential risks of the use of carrier vaccines is genetic recombination between the attenuated vector virus and a virulent field virus. Recombination might result in transfer of the inserted heterologous gene(s) from the safe attenuated vector strain to a virulent field strain. In vivo recombination between attenuated strains of herpesviruses has been demonstrated by several investigators. Lethal recombinants of herpes simplex virus type-1 (HSV-1) were recovered from the brains of mice that were co-inoculated with the weakly neuroinvasive ANG and KOS strains [13]. Restriction enzyme pattern analysis of virus isolated from the brains of mice that were co-inoculated with a TK-strain of HSV-2 and the non-neuroinvasive (syn) strain of HSV-1 showed that even distantly related viruses are able to recombine [20]. In sheep, recombination between attenuated strains of PRV has been demonstrated [9,15]. In pigs, recombination between attenuated strains of PRV has been shown by PCR analysis [4,10]. Furthermore, analysis of field isolates indicated that recombination between genomic variants is not uncommon [3].

Previously we examined recombination of non-lethal PRV strains in mice [8]. Our results indicated that in vivo recombination occurred only when high doses of two mutant strains were administered at the same location. Recombination between two ribonucleotide reductase (RR) negative mutants occurred less frequently than recombination between a TK^- and a RR^- mutant or between two TK^- mutants, suggesting that the probability of in vivo recombination depended on the genotypes of the virus strains used. Furthermore, recombination between genetic loci that were separated by 266 bp was as efficient as recombination between loci that were separated by 10 kbp, suggesting that the distance between the loci did not affect recombination. In general, this study implied that the probability of recombination between attenuated vaccine strains [4, 9, 10, 15] is different from that between a vaccine strain and a wild-type strain.

This report describes recombination between a virulent PRV strain and an attenuated TK^-gE^- vaccine strain in its natural host, the pig. By using these strains as carriers for foreign sequences (derived from the E1 gene of classical swine fever virus) we show that homologous recombination between these

foreign sequences is much less frequent than between autologous PRV sequences.

Materials and methods

Cells and viruses

The highly virulent wild-type PRV strain NIA-3 [1] was the parent strain of all strains used in this study (Table 1). Strain M205 is a derivative of the TK^-gE^- vaccine strain 783 with the E1 gene of classical swine fever virus inserted in the gG locus [28]. The TK^- strain TK1 contains a 19 bp deletion in the TK gene. This deletion is identical to that of vaccine strain 783 [18]. Strain Begonia, hereafter named TK2, is a vaccine strain that contains a small deletion in the TK gene and a deletion of 2055 bp, encompassing the gE gene, in the unique short region of the genome [29]. The construction of strains NIA.E1, NIAΔpE1 and 783.E1ΔA is described below. All viruses were propagated on porcine kidney (SK6) cells [14]. TK-negative mouse L–M cells (ATCC CCL 1.3) were used to determine the TK-phenotype of progeny virus (described below). All cells were cultured in DMEM supplemented with 5% fetal calf serum.

Construction of mutant viruses

DNA manipulations were performed essentially as described by Sambrook et al. [23]. Plasmid pMZ67 [28] contains the *Hin*dIII-B fragment of strain 783 with the E1 gene of classical swine fever virus inserted in the gG locus. A 2.5 kb *Xho*I-*Kpn*I fragment of pMZ67, containing the E1 gene and adjacent PRV sequences was cloned in pSP73 (Promega). The resulting plasmid was designated p.E1. The gG promoter which drives expression of the E1 gene was removed from plasmid p.E1 by deleting a 34 bp *Fsp*I-*Bam*HI fragment, resulting in pΔpE1. Plasmid pPEh18 has been used to map epitopes of E1 [27]. The truncated E1 protein encoded by this plasmid lacks antigenic domain A. The *Nhe*I-*Sma*I fragment of p.E1 was replaced by the *Nhe*I-*Bgl*II fragment of plasmid pPEh18 resulting in plasmid p. E1ΔA that lacks domain A of protein E1.

The *Xho*I-*Kpn*I fragment of plasmid p.E1 and DNA of strain NIA-3 were co-transfected in SK6 cells construct strain NIA.E1. $E1^+$ plaques (approximately 1%) were identified immunohistochemically by using monoclonal antibodies against E1 [30]. This mutant virus has the "worst case" recombinant phenotype $TK^+gE^+E1^+gG^-$ and may arise by recombination between the two $E1^-$ mutant strains described below. The *Xho*I-*Kpn*I fragment of plasmid p.ΔpE1 and *Eco*RI-digested DNA of strain NIA.E1 were co-transfected in SK6 cells to construct strain NIA.ΔpE1. Similarly, the *Xho*I-*Kpn*I fragment of plasmid p.E1ΔA and *Eco*RI-digested DNA of vaccine strain M205 were co-transfected in SK6 cells

Table 1. Names and genotypes of PRV strains

Virus	Genotype	Reference
NIA-3	wild-type	[1]
M205	$TK^-gE^-gG^-E1^+$	[28]
NIA.E1	gG^-E1^+	this paper
NIA.ΔpE1	gG^-E1^-	this paper
783.E1ΔA	$TK^-gE^-gG^-E1^-$	this paper
TK1	TK^-	for TK deletion see [18]
TK2	TK^-gE^-	[29]

to construct strain 783.E1ΔA. *Eco*RI cleaves the viral genomes of NIA.E1 and M205 at a unique site in the E1 sequence. By using *Eco*RI-digested viral DNA instead of intact viral DNA in co-transfection experiments, the percentage of recombinants increased to approximately 50%. All viruses were plaque purified 3 times and viral DNA was isolated and analyzed by Southern blot analysis as described [6]. Names and genotypes of all viruses used in this paper are summarized in Table 1.

Animals

Dutch Landrace pigs of twenty-weeks-old were obtained from the specific pathogen free herd of the Institute for Animal Science and Health (ID-DLO). Pigs were randomly assigned to experimental groups. During the experiment, each group was housed in a separate isolation room.

Experimental design

Ten 20-weeks-old pigs were co-infected intranasally with a mixture of 10^5 PFU of NIA.ΔpE1 and 10^7 PFU of strain 783.E1ΔA. The pigs were inoculated with 0.5 ml of the virus suspension which was slowly administered into each nostril during inspiration. As a control, five pigs were inoculated with 10^5 PFU of NIA.ΔpE1, and five pigs were inoculated with 10^7 PFU of strain 783.E1ΔA. To determine the amount of E1-expressing virus that is required to induce the production of antibodies against E1, two groups of three pigs were inoculated intranasally with 10^2 and 10^3 PFU, respectively, of strain NIA.E1.

Collection of samples

Swab specimens of oropharyngeal fluid were collected daily from the day before inoculation till 10 days after inoculation. Swabs were extracted with 4 ml of DMEM supplemented with 5% fetal calf serum and antibiotics. To determine the virus content per gram of oropharyngeal fluid, the weight of the collected fluid was measured after centrifugation of the swabs.

Serological detection of antibodies against E1

Blood samples were collected on the day before inoculation and once every week after inoculation of the pigs. The presence of antibodies against E1 was examined by using an E1-specific complex-trapping-blocking assay as described [31].

In vitro recombination

Monolayers of SK6 cells were co-infected with NIA.ΔpE1 and 783.E1ΔA with an m.o.i. of 10, 1 or 0.1. Progeny virus was harvested 24 h after infection and examined for their TK and gE phenotypes as described below. The same progeny virus was also examined for the expression of domain A of the E1 protein (see below). To determine the frequency of in vitro recombination within the TK gene, monolayers of SK6 cells were co-infected with strains TK1 and TK2 with an m.o.i of 10, 1 or 0.1. The TK phenotype of progeny virus from the latter experiment was examined as described below.

Competition experiment

To examine whether growth differences between $E1^-$ and $E1^+$ mutants might influence in vitro recombination experiments, we mixed NIA.ΔpE1 and NIA.E1 in a ratio of 100:1.

Monolayers of SK6 cells were inoculated with the virus mixture (m.o.i. = 1) and after 24 h progeny virus was harvested by two freeze-thaw cycles followed by low speed centrifugation. The original inoculum and the progeny were titrated on SK6 cells and the ratio of $E1^-$ versus $E1^+$ plaques was determined immunohistochemically (see below).

Analysis of progeny virus

Virus titers in oropharyngeal fluid samples of all inoculated pigs were determined by titration on SK6 cells. Progeny virus obtained from co-inoculated pigs was examined immunohistochemically for the expression of E1 by using monoclonal antibodies specific for domain A [30]. The TK and gE phenotypes of progeny virus from co-inoculated pigs were determined after titrating swab suspensions on TK-negative mouse L–M cells. Monolayers that contained 10 to 200 plaques were examined by a TK-assay [24] and immunohistochemically by using a monoclonal antibody specific for gE. After plaques had developed, growth medium was removed, and replaced by medium containing 1:100 diluted rabbit anti-PRV antiserum to prevent reinfection. After incubation for 1 h at 37 °C, 50 uCi of ^{3}H-thymidine was added to the wells and the incubation was continued for 4 to 6 h at 37 °C. The monolayers were washed 4 times with PBS, dried and frozen. At this stage, gE^+ plaques were identified. After the assay, the wells were washed with PBS, dried, and incubated with En^{3}Hance (Amersham). Then the wells were dried again and exposed to X-ray film. An autoradiogram of a TK^+ plaque shows a dark rim indicative of ^{3}H-thymidine incorporation (see Fig. 4).

Results

Construction of virus strains

A problem in examining in vivo recombination is to detect small amounts of recombinant virus among large amounts of the parental viruses. In a previous study in which we examined in vivo recombination of different PRV strains in mice, we made use of the fact that recombination between attenuated PRV strains can yield wild-type PRV which is highly lethal for mice. Thus, we were able to monitor recombination by examining survival of co-inoculated mice [8]. Pigs are relatively resistant against PRV, therefore this system could not be used to examine recombination in pigs. Furthermore, we wanted to study recombination between a virulent wild-type strain and an attenuated strain. One of the results of the experiments in mice was that recombination between loci which were separated by only 266 bp was as efficient as recombination between loci that were separated by 10 kbp [8]. In an attempt to simplify the detection of recombination in pigs, we constructed two virus strains that contained different but overlapping parts (375 bp) of the E1 sequence of classical swine fever virus. Recombination between the E1 sequences of these strains will yield a virus that expresses domain A of glycoprotein E1. After co-inoculation of the parent strains in pigs, the presence of serum antibodies against the highly immunogenic E1 protein should reveal that recombination has occurred.

The strains that were used to study in vivo recombination are shown in Fig. 1. Strain NIA.ΔpE1 contains the E1 coding sequence inserted into the gG locus of wild-type strain NIA-3. The E1 gene of this strain lacks a functional promoter and therefore it does not express E1. Strain 783.E1ΔA contains the first 375 bp

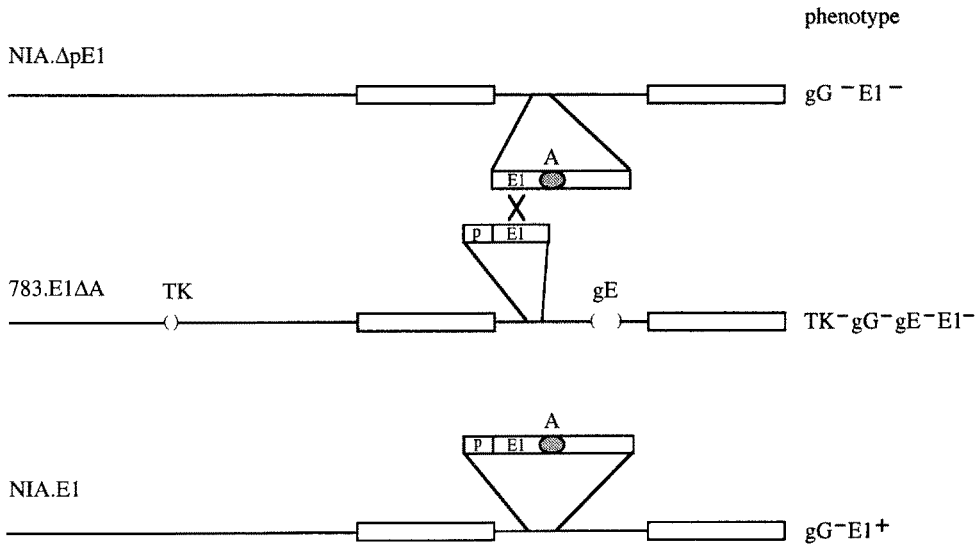

Fig. 1. Schematic map of the genomes of PRV strains NIA. ΔpE1, 783.E1ΔA and NIA.E1. Strain NIA.E1 contains the E1 gene of classical swine fever virus inserted behind the gG promoter in the unique short region of PRV. Strain NIA.ΔpE1 contains the same E1 gene but lacks a function gG promoter. Strain 783.E1ΔA lacks domain A and the putative transmembrane region of E1. Homologous recombination between the E1 sequences of NIA.ΔpE1 and 783.E1ΔA results in an $E1^+$ phenotype. Recombination between the TK and gE loci of NIA.ΔpE1 and 783.E1ΔA may result in a TK^-gE^+ phenotype. The filled circle represents domain *A* of the E1 protein; *p* represents the gG promoter that drives expression of the E1 gene. Note: only the prototype orientation of the PRV genome is shown; the unique short region is normally present in two orientations

of the E1 gene inserted behind the gG promoter in the gG locus. The 3′-terminal part of the E1 gene including the region encoding domain A of E1 and the transmembrane region are absent. The virus furthermore contains a deletion in the TK gene and a deletion encompassing the gE gene in the unique short region. Due to these deletions this strain is highly attenuated [28]. Strain NIA.E1 contains the complete E1 gene inserted behind the gG promoter in the gG locus. This virus expresses the E1 gene and can be generated by recombination of the two virus strains described above.

Figure 2 shows a Southern blot analysis of NIA.ΔpE1 and 783.E1ΔA. To demonstrate that the promoter region of gG is absent in NIA.ΔpE1, DNA of NIA.ΔpE1 and wild-type strain NIA3 was digested with *Bam*HI. The nick-translated plasmid pE1 (see Materials and methods) was used as a probe. *Bam*HI fragments 7 and 10 are fused in NIA.ΔpE1 as a result of the deletion of the gG promoter sequence resulting in a fragment of approximately 10 kb (lane 2). To demonstrate that the C-terminal part of the E1 gene is absent in strain 783.E1ΔA, DNA of 783.E1ΔA and plasmid pE1ΔA was digested with *Xho*I and *Kpn*I. Both strain 783.E1ΔA (lane 3) and plasmid pE1ΔA (lane 4) contain the same *Xho*I-*Kpn*I fragment of 1.4 kb. The 2.5 kb fragment in lane 4 represents the pSP73 vector.

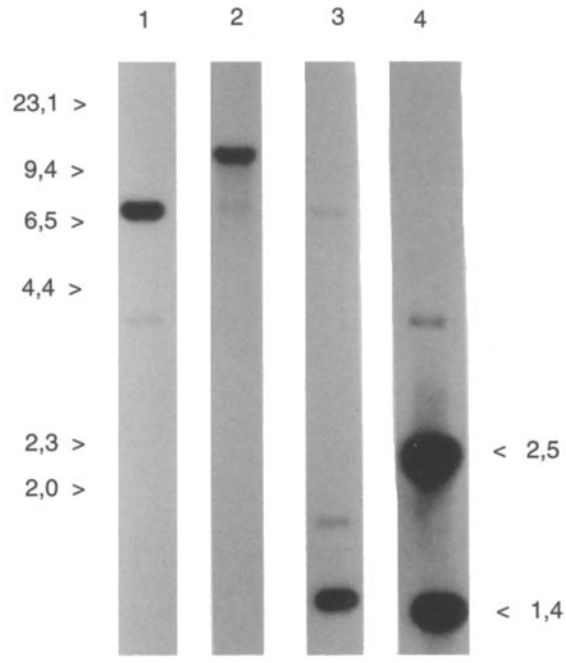

Fig. 2. Southern blot analysis of strains NIA.E1, NIA.ΔpE1 and 783.E1ΔA (cf. Fig. 1). Viral DNA of NIA.E1 and NIA.ΔpE1 was digested with *Bam*HI (*1* and *2*). DNA of 783.E1ΔA and plasmid pE1ΔA was digested with *Xho*I and *Kpn*I (*3* and *4*). Plasmid pE1 was used as a probe. *1* shows the position of the *Bam*HI-7 fragment that contains the E1 gene. As a result of the deletion of the gG promoter in NIA.ΔpE1 the *Bam*HI site between fragments 7 and 10 is absent, resulting in a fragment of approximately 10 kb (*2*). The 1.4 kb *Xho*I-*Kpn*I fragment of 783.E1ΔA (*3*) has the same size as the *Xho*I-*Kpn*I fragment of plasmid pE1ΔA (*4*). The 2.5 kb band in *4* represents plasmid sequences which hybridize with the labeled pE1 probe. Minor bands are the result of partial digestion. The positions and sizes of the marker fragments (*Hind*III digest of lambda DNA) are shown on the left

Both mutant strains NIA.ΔpE1 and 783.E1ΔA are unable to express the E1 gene as judged by immunohistochemistry (data not shown). In vitro recombination experiments with NIA.ΔpE1 and 783.E1ΔA showed that $E1^+$ recombinants could be generated (see below).

Infection of pigs

In order to examine in vivo recombination, 10 pigs were co-inoculated intranasally with 10^5 PFU of NIA.ΔpE1 and 10^7 PFU of 783.E1ΔA. As a control, we inoculated both strains separately. To determine the virus titer that is required to elicit an antibody response against E1, 2 groups of 3 pigs were inoculated with 10^2 and 10^3 PFU respectively of the $E1^+$ virus NIA.E1. All pigs that were co-inoculated with NIA.ΔpE1 and 783.E1ΔA, and all pigs inoculated with NIA.ΔpE1 showed severe signs of Aujeszky's disease but none of the pigs died during the experiment. Clinical symptoms were not observed in animals infected with 10^7 PFU of 783.E1ΔA, or 10^2 or 10^3 PFU of NIA.E1.

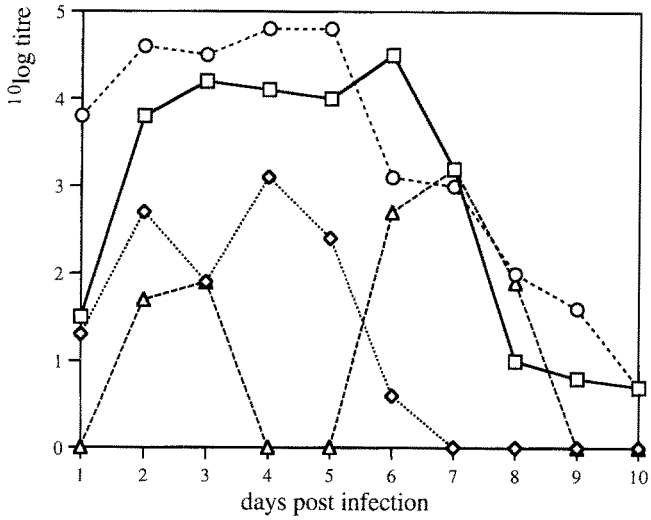

Fig. 3. Virus contents (^{10}log) in the oropharyngeal fluid of pigs inoculated with 10^3 PFU of NIA.E1 (△), 10^5 PFU of NIA.ΔpE1 (□), 10^7 PFU of 783.E1ΔA (◇), or co-inoculated with 10^5 PFU of NIA.ΔpE1 and 10^7 PFU of 783.E1ΔA (○)

Progeny virus recovered from the oropharyngeal fluid of the infected pigs was titrated on SK6 cells (Fig. 3). Pigs co-inoculated with NIA.ΔpE1 and 783.E1ΔA excreted virus in the oropharyngeal fluid from day 1 to day 10 after inoculation. When the virus strains were inoculated separately, pigs inoculated with 10^5 PFU of NIA.ΔpE1 excreted considerably more virus, and for a longer period of time, than pigs inoculated with 10^7 PFU of 783.E1ΔA. In contrast, no virus was detected in the oropharyngeal fluid of pigs inoculated with 10^2 PFU of NIA.E1, whereas only one pig that was inoculated with 10^3 PFU of NIA.E1 excreted virus. The latter finding indicates that a dose of 10^3 PFU is near the lower limit for successful intranasal infection of 20-week old pigs.

In vivo recombination between the E1 sequences

In order to examine whether recombination occurred between the E1 sequences of strains NIA.ΔpE1 and 783.E1ΔA, we collected serum of the co-inoculated pigs at days 7, 14, 21, and 28 after inoculation. The presence of antibodies against domain A of the E1 protein was examined by using an E1-specific complex-trapping-blocking assay [31]. No antibodies directed against the E1 protein were detected in sera of these pigs. Of the control animals inoculated with 10^2 or 10^3 PFU of NIA.E1, the one pig that excreted virus developed serum antibodies against E1 at week 3 after inoculation, indicating that the minimal dose required for successful infection of the nasal epithelium was also sufficient for the induction of a detectable level of serum antibodies against E1. Therefore, the absence in co-inoculated pigs of antibodies against E1 suggested that recombination within the E1 sequences of NIA.ΔpE1 and 783.E1ΔA did not occur or was below the detection limit.

Because this result was unexpected, approximately 5000 progeny plaques from swab specimens taken at days 5, 6, and 7 after co-inoculation, were examined for the expression of E1 by means of immunohistochemistry [30]. No $E1^+$ plaques were detected suggesting that in vivo recombination occurred infrequently, if at all.

In vivo recombination between the TK and the gE loci

We subsequently examined whether in vivo recombination could be demonstrated by analyzing progeny viruses on their TK and gE phenotypes. Because strain NIA.ΔpE1 is TK^+gE^+, and strain 783.E1ΔA is TK^-gE^-, progeny viruses that possess TK^+gE^- or TK^-gE^+ phenotype are the result of in vivo recombination. Of each pig, we selected three swab suspensions with the highest virus titer (mainly detected at day 5, 6 and 7 after inoculation) and titrated these on TK-negative mouse L–M cells. In total, 995 individual progeny plaques were examined with regard to their ability to incorporate ^{3}H-thymidine (indicating a TK^+ phenotype) and to express the gE protein (Table 2). The results demonstrated that 1.3% of the progeny viruses possessed the TK^-gE^+ recombinant phenotype (example shown in Fig. 4). Recombinants were found in 6 out of 10 co-inoculated pigs. These findings demonstrated that in vivo recombination between a virulent PRV strain and an attenuated strain, under the conditions used here, was not a rare event. Therefore, the difference between the results of both analyses (E1-phenotype vs TK^-gE^+ phenotype) suggested that the frequency of recombination between the E1 sequences was much lower than between sequences located between the TK and the gE loci.

In vitro recombination

In order to examine whether the observed differences were due to differences in the frequency of the actual molecular recombination event or due to differences in in vivo growth properties, we determined the in vitro recombination

Table 2. Number of gE^+TK^- recombinant viruses recovered from the oropharyngeal fluid of 10 pigs at days 4, 5, 6, and 7 after intranasal co-inoculation of NIA.ΔpE1 and 783.E1ΔA

Swab day	No. of gE^+TK^- plaques	No. of viruses examined	% of recombinant plaques
4	4	268	1.5
5	7	501	1.4
6	2	168	1.2
7	0	58	<1.7
Total	13	995	1.3

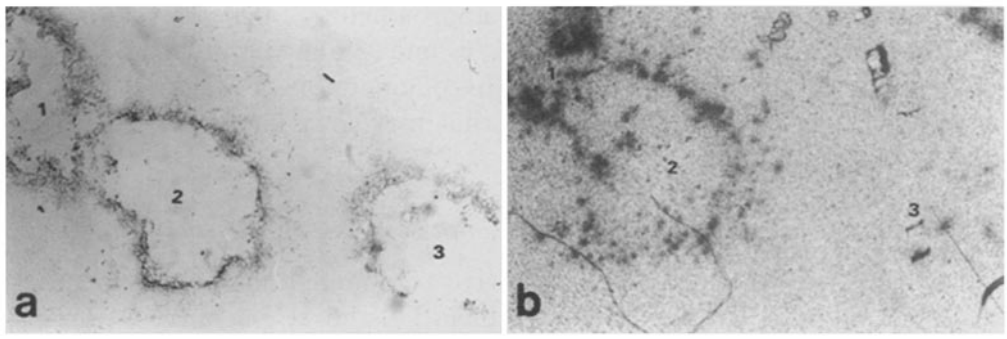

Fig. 4. Progeny virus of co-inoculated pigs examined for their gE and TK phenotypes. **a** Three progeny plaques (*1–3*) were stained with an anti-gE monoclonal antibody. **b** Autoradiogram of the same plaques analyzed by the TK-assay. Plaque *3* was identified as a gE^+TK^- recombinant

frequencies. Monolayers of SK6 cells were co-infected with NIA.ΔpE1 and 783.E1ΔA with an m.o.i. of 10, 1 or 0.1. Infection at high m.o.i. results in cells that are co-infected by both viruses. Therefore, all generated genotypes are complemented by the same set of wild-type viral gene products, thus excluding possible growth differences. Twenty-four hours after infection, progeny virus was harvested and titrated, and the percentage of recombinants was determined by using a TK-assay and gE immunohistochemistry. The percentage of recombinants with a TK^-gE^+ phenotype was 2.5, 3.5, and 0.7 after an infection with an m.o.i. of 10, 1, and 0.1, respectively (Table 3). Analysis of the frequency of $E1^+$ recombinants revealed that the percentage of $E1^+$ recombinants was 0.20, 0.06 and 0.03 respectively. These results clearly indicated that the frequency of in vitro recombination between the E1 sequences was much lower than between sequences located between the TK and gE loci.

The possibility that expression of E1 influenced the in vitro replication of PRV was examined by a competition experiment. A mixture of $E1^+$ and $E1^-$ mutants (ratio 1:100) was titrated before and after a passage on SK6 cells. Because the ratio ($E1^+/E1^-$) remained unchanged (data not shown) we concluded that expression of E1 did not influence replication of PRV, which is in agreement with previous results [19, 24].

To examine whether the low frequency of recombination between the E1 sequences was due to the relatively short distance (375 bp) of the overlap, we also co-infected monolayers of SK6 cells with two TK^- strains. The positions of the mutations (small deletions) within the TK genes of these strains are separated by only 266 bp. Analysis of progeny virus showed that the percentage of TK^+ recombinants was comparable with the percentage of TK^-gE^+ recombinants (Table 3). These results indicated that a relatively short distance between genetic loci does not necessarily reduce the frequency of recombination. The possibility that the presence of a functional TK protein influenced the results of the recombination experiment is highly unlikely since Jamieson et al. [12] demonstrated that herpesviruses with or without a functional TK gene replicate

Table 3. Percentage of in vitro recombinants after co-infection of SK6 cells with NIA.ΔpE1 and 783.E1ΔA, or TK1 and TK2

Inoculated virus strains	Phenotype of recombinants	% of recombinants m.o.i.		
		10	1	0.1
NIA.ΔpE1 × 783.E1ΔA	gE^+TK^- [a]	2.5	3.5	0.7
NIA.ΔpE1 × 783.E1ΔA	$E1^+$ [b]	0.20	0.06	0.03
TK1 × TK2	TK^+ [c]	5.8	8.3	1.8

[a] Analyzed by combined TK-assay and gE immunohistochemistry
[b] Analyzed by E1 immunohistochemistry (mean of 2 independent experiments)
[c] Analyzed by TK-assay

equally well in exponentially growing cells that provide cellular TK activity. Furthermore, we previously examined replication of PRV strain NIA-3, vaccine strain 783 (TK^-gE^-), and mutant strain 783H$^+$ (TK^-gE^-). Comparison of the growth rate by single step kinetics showed that all three viruses replicated equally well [7].

Discussion

Expression of heterologous genes by a carrier virus is an attractive approach to induce a protective immune response against two or more pathogens [2, 11, 17, 28]. However, a possible risk of the use of such carrier viruses is in vivo recombination between the carrier vaccine and a wild type virus. Recombination may result in the transfer of the inserted heterologous DNA from the attenuated vaccine strain to a wild type virus. The biological consequences of gene transfer will depend on the site of insertion and the function of the inserted gene. The site of insertion is important because homologous recombination results in exchange of DNA between identical loci. Transfer of heterologous DNA that is inserted in an important virulence gene or in an essential gene will therefore knock out the virulence gene or the essential gene of the recipient field virus [21]. The function of the inserted gene is important because the gene product may increase the virulence or the tropism of the recipient virus.

Previously we examined the circumstances that affected efficient recombination of mutants of PRV in mice and found that recombination was dose dependent [8]. Furthermore, we found that the virus dose required to detect recombination depended on the virus strains used and is probably affected by the replicative capacity of the parent viruses in the host. Therefore, it likely that the frequency of in vivo recombination between two attenuated strains differs from the frequency of recombination between a wild-type virus and an attenuated strain.

Despite intensive vaccination, wild-type PRV is still present in the pig population in many countries and thus recombination between a vaccine strain

and wild-type PRV can occur. This report is the first study dealing with recombination between a virulent (TK^+gE^+) PRV strain and an attenuated (TK^-gE^-) PRV strain in pigs. Recombination between these strains can be determined by screening progeny plaques for their TK and gE phenotypes. However, the frequency of recombination might be low, making the screening for recombinants laborious and time consuming. Therefore, an in vivo detection method that would detect small amounts of recombinant virus among a large amount of parental viruses would be advantageous. To detect in vivo recombinants we tried to make use of the immune system of the host. Two viruses, NIA.ΔpE1 and 783.E1ΔA, were constructed that only after genetic recombination could generate a virus that expressed domain A of the E1 protein of classical swine fever virus. After co-inoculation of pigs we were unable to detect serum antibodies against domain A of E1. Because an antibody response against E1 was detectable in a control pig, these results suggested that in vivo recombination did not occur or remained below the detection limit. Since recombination has been demonstrated after co-inoculation of pigs [3, 4, 10] progeny viruses were also analyzed on their TK and gE phenotypes. We found that 1.3% of the viruses possessed the recombinant TK^-gE^+ phenotype, indicating that in vivo recombination occurred relatively frequently. Recombinants were found in 6 out of 10 co-inoculated pigs and since the number of progeny plaques examined was low (between 0 and 267 per pig) the actual number of pigs in which recombination occurred was probably even higher. In vitro experiments were performed under conditions that excluded differences in growth, to determine the recombination frequencies between the different genetic loci. The results confirmed that recombination occurred much more frequently between the TK and gE loci than between the E1 sequences. The most plausible explanation for the observed difference would be that the recombination frequency is dependent on the distance between genetic loci. However, we found that the size of the overlap between the E1 sequences (375 bp) was not a limiting factor, since recombination between mutations that were separated by only 266 bp within TK gene was as frequent as recombination between the TK and gE loci which are separated by approximately 60 kb (Table 3). Our previous recombination experiments in mice [8] also suggested that recombination frequencies were independent of the distance between the genetic markers. Moreover, Fathi et al. [5] recently described that the frequency of recombination between mutants of vaccinia virus are largely independent of the distance between the mutations. These authors attributed this lack of length dependency to a high rate of viral DNA interchange. Our result seems to be in agreement with this interpretation.

We conclude that the relatively low frequency of recombination between the E1 sequences is either caused by some property of the E1 sequence itself or by the location of the E1 sequence within the viral genome. A possible explanation for the influence of the E1 sequence itself might be the GC content. The genome of PRV has a relatively high GC content of 73%, whereas the E1 sequence has a GC content of approximately 50%. The viral DNA replication/recombination

machinery may have adapted to the high GC content of the PRV genome and may therefore be less efficient on the E1 sequence. We do not know whether the genomic location affects the frequency of recombination in PRV. During the construction of various recombinant viruses we have never observed significant differences in the percentage of recombinants that was obtained when fragments were used that originated either from the Ul region or the Us region of the PRV genome. This observation would argue against an effect of the genomic location on the frequency of recombination.

In vivo homologous recombination between different PRV strains is possible and can theoretically result in recombinants with undesirable properties. However, the result of homologous recombination between a carrier vaccine and a wild-type strain can be predicted. By using known virulence genes as insertion sites, potential risks connected with the transfer of genetic material between a carrier vaccine and a wild-type strain may be kept to a minimum. In the long run, the use of phenotypically complemented mutants that carry a foreign gene in an essential PRV gene may be the best strategy for designing safe, self-restricted carrier vaccines [21].

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