Genetic recombination between two strains of Aujeszky's disease virus at reduced multiplicity of infection

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The potential for in vivo genetic recombination has been suggested for Aujeszky's disease virus (ADV) stemming from the use of modified-live vaccines carrying vaccine-specific deletion mutations. This scenario serves as a model for the study of biological factors that affect in vivo herpesviral recombination and, ultimately, the natural evolution of herpesviruses. This report describes experiments evaluating the efficiency of ADV recombination under in vitro conditions of low multiplicity infection which were felt to represent in vivo conditions more closely than previous in vitro measurements of ADV recombination. A series of experiments were performed to measure in vitro recovery of recombinant viral progeny following co-infection of cell monolayers with non-saturating concentrations of two parental strains of ADV. A simple statistical model was generated to estimate the rate of cellular co-infection. After a single replicative cycle infectious viral progeny were recovered and their genotypes determined at two alleles by characterization of two gene loci using a battery of PCR assays. Recovery rates of parental and recombinant progeny genotypes were calculated from the PCR data. The observed frequency of recovery of recombinant viral progeny closely approximated the values projected by the model calculations. The data from our system suggest that at low multiplicity of infection, the rate of genetic recombination is a function of the probability of cellular co-infection.

Introduction

The potential for in vivo genetic recombination has been suggested recently for Aujeszky's disease virus (ADV) (Katz et al., 1990a, b; Henderson et al., 1990, 1991; Dangler et al., 1993). The development of vaccine-derived recombinants has been proposed as a conceivable, although unproven, outcome of the practice of using modified-live vaccines on infected herds of swine. Recombination might occur as the result of co-infection of animals by both vaccine and wild-type strains. Experiments to test this hypothesis have resulted in the isolation of recombinant ADV genotypes from swine which had been simultaneously co-inoculated with two distinct vaccine strains of ADV (Henderson et al., 1991; Dangler et al., 1993).

Herpesviral recombination has been studied routinely in vitro using a high m.o.i., e.g. 5 to 10 p.f.u./cell, to obtain synchronous co-infection of target cells and one-step growth cycles. Using this approach, previous studies have reported the relationship of recombination frequencies and map distances between marker loci, and potential mechanisms of herpesviral recombination (Ben-Porat et al., 1982; Ihara et al., 1982; Brown et al., 1992). It seems unlikely that natural recombination would occur in vivo under conditions of high m.o.i. with synchronous infection of all available target cells by both parental virus strains. Furthermore, because of the saturating concentration of infectious virus applied to the cell monolayers in these earlier reports, the relationship between estimated cellular co-infection rates and the observed frequency of viral recombination could not be described. At a low m.o.i. the frequency of both in vitro and in vivo genetic recombination should become a function of the number of virus-cell interactions that lead to co-infection of individual cells. In this report we describe an analysis of in vitro recombination frequency under conditions of comparatively low m.o.i. and felt to represent in vivo conditions more closely than previous in vitro measurements of ADV recombination.

Methods

Statistical prediction of cell infection and maximal viral recombination.

A simple model was developed to estimate a threshold value for the frequency of recombinant genotype recovery from a population of $N_0$ cells co-inoculated with two distinct parental genotypes W and V at m.o.i. W = X and m.o.i. V = Y. The model was intended to estimate the effects of infectious virus concentrations when the number of infectious
viral particles is less than the number of cells in the target population. Under the condition of low infectious virus concentration, the infected cell fraction was estimated using the Poisson distribution (Dulbecco, 1980). The hypothetical maximum calculated by the model is proposed to serve only as a point of reference for comparing observed frequencies of recombination. Any negative effects due to factors that interfere with the molecular interactions required for recombination or subsequent replication of recombinant progeny were expected to be reflected in a reduction in the observed frequency of recovery of recombinant viral progeny.

Based on the model, the proportion of cells that become infected by strain W, P(W), at the time of inoculation is $1 - e^{-x}$. Similarly, the proportion of cells that become infected by strain V, P(V), equals $1 - e^{-y}$. To simplify the model under conditions of low m.o.i., the contribution of the relatively small number of cells multiply infected with particles of the same strain are considered to be equivalent to single-infected cells. The estimated proportion of cells which become infected by both strains, $P(\text{WV})$, is $P(W)P(V)$. Consequently, in a population of $N_o$ target cells, $P(\text{WV})N_o$ of the cells would be co-infected. Of the remaining cells, $[P(W) - P(\text{WV})]N_o$ would be solely infected with parental strain W, and $[P(V) - P(\text{WV})]N_o$ would be solely infected with strain Y.

Liberal assumptions were incorporated to generate a hypothetical maximum value for the frequency of recovery of recombinant progeny from the model. An assumption was made that recombination events involve only parental genome molecules during the first replicative cycle. This is supported by the observation that progeny genomes do not appear to interact significantly with parental genome molecules during recombination (Ben-Porat et al., 1982). A second assumption, that each co-infection of a cell consistently resulted in recombination and yielded only recombinant progeny, was used to incorporate a maximal level of efficiency in the steps leading to recombination and generation of infectious progeny. This assumption is supported in part by the observation that recombination precedes DNA replication (Ben-Porat et al., 1982). This condition suggests that progeny virus would not arise prior to recombination and, after recombination, would arise following the generation of recombinant templates, thereby maximizing the proportion of recombinant progeny virions generated. For the purposes of the calculations as presented here, a third condition that each parental or recombinant genomic template ultimately generated $K$ progeny following a single generation of viral replication was used to assume no loss or increase in replication or packaging efficiency between parental or recombinant progeny genomes. Applying these assumptions, the cells solely infected with strain W would each yield $P(W) - P(\text{WV})KN_o$ of W parental-type progeny, and the co-infected cells containing two recombinant genome templates would yield $2[P(\text{WV})]KN_o$ progeny. The hypothetical maximal frequency of recombinant progeny as a proportion of the total number of progeny virus recovered after a single generation of viral replication would be $2[P(\text{WV})]/[P(W) + P(V)]$. Accordingly, if strains W and V were inoculated onto a cell monolayer, each at an m.o.i. of 0.1, the maximal frequency of recombinant progeny virions after a single generation of viral replication would be 9.5%.

**Viruses.** Parental strain W was the Shope strain of ADV which has wild-type sequences in both the thymidine kinase (TK) and glycoprotein X (gX) gene loci. Parental strain V was a commercial modified-live PRV vaccine strain which has a 285 bp deletion in the TK gene and has a lacZ gene inserted into the site of its deleted gX gene. The genotypes of these two strains will be referred to as TK + gX + and TK − gX −, respectively. Recombination between these two parental strains will be based on the identification of TK + gX + and TK − gX + isolates. Virus-infected cell cultures were maintained in medium 199 with penicillin/streptomycin and 5% fetal bovine serum at 37°C. 5% CO₂. Virus stocks were divided into aliquots and frozen at −80°C. Aliquots of virus were titrated for p.f.u. concentration prior to co-infected monolayers.

**Co-infection of cell cultures.** A series of five independent experiments were performed to measure the frequency of recovery of recombinants from cell cultures after inoculation at an m.o.i. of 0.1 for each parental strain. In experiment 1, both parental strains were inoculated simultaneously onto a Vero cell monolayer at a calculated m.o.i. of 0.1. Following a 1 h adsorption period, the monolayer was washed with fresh medium. The cells were incubated at 37°C, 5% CO₂ and harvested 6 h following the adsorption period. Because of the low m.o.i. used to infect the cell cultures, eclipse periods were used to establish the lengths of incubation to preclude the contribution to the progeny virus pool by released progeny virus and subsequent rounds of cell infection. In experiment 1 the 6 h incubation period corresponded to the eclipse period of the vaccine parental strain and was 2 h in excess of the eclipse period for the wild-type parental strain. Because the 6 h interval was less than double the eclipse period of the wild-type strain (i.e. fewer than two viral generations), interactions between parental and progeny genomes from subsequent cycles of cellular infection were believed to be minimized.

Experiment 2 was conducted as described for experiment 1, with the exception that the infected cells were harvested at 4 h post-adsorption. This incubation period corresponded to the eclipse period of the wild-type parental strain and was approximately 2 h shorter than the eclipse period of the vaccine parental strain. Experiments 3 and 4 were executed as described for experiment 1 and served as independent replicate experiments.

A final experiment was carried out to test the model calculation when disparate concentrations of virus were used to co-inoculate the cell monolayers. In experiment 5, three Vero cell monolayers were co-inoculated and harvested at 6 h post-adsorption. All three monolayers were inoculated with the wild-type parental strain at a calculated m.o.i. of 0.1. The first monolayer was simultaneously inoculated with the vaccine parental strain at a calculated m.o.i. of 0.1. An aliquot of the working stock of vaccine parental strain, prepared for the first monolayer, was serially diluted to provide a calculated m.o.i. of 0.05 and 0.025 for simultaneous inoculation of the second and third Vero cell monolayers, respectively.

In each experiment, after the appropriate incubation period the infected cell cultures were scraped to form a suspension in the culture medium. The cell suspension was sonicated, clarified by low-speed centrifugation, and stored in aliquots at −80°C. Aliquots of the infected medium were serially diluted to identify the appropriate dilution for individual plaque isolation. Using this dilution samples of the medium were inoculated onto Vero cell monolayers in Petri dishes and covered by an agar overlay. After neutral red staining of the infected monolayer, individual plaques were picked and propagated by inoculation onto fresh monolayers in 24-well plates. The plaque clone-infected cells were harvested after the c.p.e. attained > 80%.

**Analysis of progeny genotypes.** The progeny genotypes were analysed using a previously described approach for sample preparation and PCR amplification of gene sequences from the TK and gX gene loci (Dangler et al., 1992, 1993). The technique was used previously to identify recombinant ADV genotypes recovered from experimentally co-infected swine.

(i) **Sample preparation.** Infected cell pellets were lysed in a medium consisting of reaction buffer (50 mm-KCl, 10 mm-Tris-HCl pH 9.0, 0.01% gelatin, 0.1% Triton X-100) containing 0.45% NP40, 0.45% Tween 20, and 60 µg of proteinase K/ml (Higuchi, 1989). The cell suspension was incubated at 37°C for 1 h and heated to 95°C for 10 min.

(ii) **PCR.** The PCR assay for the ADV TK gene was performed as described (Dangler et al., 1992). A second PCR was performed on each sample to assess the presence of the wild-type gX gene or the lacZ gene.
Table 1. Observed distribution of ADV genotypes recovered following in vitro co-infection of Vero cell monolayers

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation period* (h)</th>
<th>Calculated m.o.i.†</th>
<th>N</th>
<th>Parental genotype</th>
<th>Recombinant genotype</th>
<th>Frequency of recovery of recombinant genotype [%]</th>
<th>Ratio of parental genotype recovery§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>0.1:0.1</td>
<td>251</td>
<td>TK + gX +</td>
<td>TK - gX -</td>
<td>TK + gX + TK - gX + 10.0% [6.9-13.1%]</td>
<td>1:2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.1:0.1</td>
<td>221</td>
<td>TK + gX +</td>
<td>TK - gX -</td>
<td>TK + gX + TK - gX + 2.7% [0.9-4.5%]</td>
<td>4:1</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.1:0.1</td>
<td>245</td>
<td>TK + gX +</td>
<td>TK - gX -</td>
<td>TK + gX + TK - gX + 6.9% [4.2-9.6%]</td>
<td>2:1</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.1:0.1</td>
<td>237</td>
<td>TK + gX +</td>
<td>TK - gX -</td>
<td>TK + gX + TK - gX + 7.2% [4.4-10.0%]</td>
<td>2:3</td>
</tr>
<tr>
<td>5A</td>
<td>6</td>
<td>0.1:0.1</td>
<td>240</td>
<td>TK + gX +</td>
<td>TK - gX -</td>
<td>TK + gX + TK - gX + 7.9% [5.0-10.8%]</td>
<td>3:2</td>
</tr>
<tr>
<td>5B</td>
<td>6</td>
<td>0.1:0.05</td>
<td>339</td>
<td>TK + gX +</td>
<td>TK - gX -</td>
<td>TK + gX + TK - gX + 3.8% [2.1-5.5%]</td>
<td>8:3</td>
</tr>
<tr>
<td>5C</td>
<td>6</td>
<td>0.1:0.025</td>
<td>365</td>
<td>TK + gX +</td>
<td>TK - gX -</td>
<td>TK + gX + TK - gX + 4.1% [2.4-5.8%]</td>
<td>20:9</td>
</tr>
</tbody>
</table>

* Time post-adsorption.
† TK + gX + : TK - gX -.
‡ C.I., confidence interval.
§ TK + gX + / TK - gX -.

Results and Discussion

Distribution of recovered progeny genotypes

The PCR battery distinguished between the four projected combinations of the TK and gX alleles. The results of experiments 1 to 4 are described in Table 1. As a general feature, in each of the first four experiments the recovery of the wild-type parental genotype (TK + gX +) exceeded the recovery of the other three genotypes, including the vaccine parental genotype, TK - gX -. Even though the cells in these experiments were exposed to each parental strain at equal m.o.i. The differences in the frequency of recovered parental genotypes was thereby considered to be an indication of disparity in the extent of cellular infection and replication by the two parental strains. The ratio of parental genotype recovery was calculated as an index of this disparity (Table 1). Because the two parental strains had similar single-step growth curves (data not shown), with the exception of an approximately 2 h lag in the eclipse period of strain V, the unequal production and recovery of parental genotypes was attributed to asynchrony in the early viral infection processes. This point is illustrated in experiment 2, in which a 4 h incubation period was used, thereby truncating the time for replication of strain V progeny virions. The disparity between the frequency of recovered parental genotypes is greater than seen in experiments 1, 3 and 4, in which 6 h incubation periods were used, thereby permitting both parental strains to span their respective eclipse periods.

Despite the asynchrony in the parental strain replication, the recovery of recombinant progeny genotypes in experiments 1 to 4 ranged from 2.7% to 10% of all progeny genotypes characterized. In comparison, as described earlier, a projected maximal frequency of recombinant genotype recovery of 9.5% was calculated from a simplified model describing cellular co-infection rates. Although the observed values seem to be good approximations of the projected value, the model also predicts equal frequencies for recovering the parental genotypes, a feature not emulated by the observed data. Furthermore, an inverse relationship exists in experiments 1 to 4, between the observed frequency of recombinant genotype recovery and the ratio of parental genotype recovery. In order to characterize this relationship more clearly, the model equation was manipulated to present a scenario in which disparate frequencies of parental genotype recovery would be projected.

Relationship between recovery of parental genotypes and recovery of recombinant genotypes

A curve was generated using the described model conditions with the exception of altering the input parental strain V concentration (m.o.i.), as a means of varying the amount of strain V genome molecules available to the recombination process. The curve describes a projected relationship between frequency of recombinant genotype recovery and recovery of parental genotypes at dissimilar frequencies. In Fig. 1, the
observed data points from experiments 1 to 4 are plotted with 90% confidence intervals, and compared with the curve describing the projected relationship between the frequency of recombinant genotype recovery and ratio of parental genotype recovery. The observed data points show a close relationship with the calculated projection over a short, relatively linear span of the curve. However, the observed values from experiments 1 to 4 lack sufficient range to support or refute the asymptotic relationship projected by the model.

Experiment 5 was performed to determine the extent to which the observed recovery of recombinants would follow the projected curve as illustrated in Fig. 1. To minimize the effects of sampling variation when preparing viral inocula and infecting the monolayers, serial dilutions of a single vaccine strain aliquot were used to inoculate parallel cell monolayers. The observations are described in Table 1. Once again, the frequency of wild-type genotype recovery predominated. Dilution of parental strain V had a clear effect on the recovery rate of the TK−gX− parental genotype, and the ratio of parental genotype (TK+gX+/TK−gX−) recovery was increased in proportion to the dilution factor. Reduction of the calculated m.o.i. to 0.05 for strain V was associated with a predicted decrease in recombinant genotype recovery. An additional twofold reduction of vaccine parental strain (i.e. m.o.i. of 0.025) did not result in further reduction of the observed frequency of recombinant genotype recovery. Whereas the observed result was higher than the projected values, this final observation was suggestive of the asymptotic curve projected by the statistical model (Fig. 2).

Sources of error

Deviations within the series of replicate experiments, in which the parental strains were inoculated at equivalent m.o.i. of 0.1 and, perhaps, the deviations in the magnitude of observed values might be attributed to several factors. Sampling errors in the measurement of virus stock concentrations and the counting of target cells in monolayers, when incorporated into the described calculations, can lead to substantial deviations in projected values, and presumably would result in deviations in observed values. Differences in parental or progeny virus characteristics affecting the multiple steps of virus replication may alter the frequency of recovered recombinant progeny, as illustrated by the disparity between the eclipse periods of the two parental strains used in this study. However, apart from this deviation between the parental strains, the one-step growth curves of the two strains appeared similar, achieving similar plateau concentrations of virus after 24 h.
Relationship between estimated rate of cellular co-infection and recovery of recombinant genotypes

In a general sense, the described experimental results confirm the efficiency of herpesviral recombination in vitro. The close parallel between the observed frequency values and the maximal values projected by our simplified model of co-infection adds a quantitative sense to the degree of efficiency of the recombination mechanism. However, it is opportune at this time to note that the model is not a mathematical representation of ADV recombination. The model is in essence an equation for estimating the rate of cellular co-infection. The failure of assumptions used in this model are obvious in the case of cellular co-infection at high m.o.i. Under this circumstance, the generous assumptions applied in this study would project an improbable frequency of recombinant recovery approaching 100%. The model could be refined significantly by the incorporation of a series of variables signifying different mechanistic processes contributing to recombination, differences in parental strain characteristics that affect replication, and differences in map distances between viral gene loci. Fortunately, even in its simplified form the projection illustrates a close relationship between target cell co-infection rate and ADV recombination, and suggests that the rate of in vitro genetic recombination can be expressed as a function of the number of virus-cell interactions. This finding suggests that the rate of generation of recombinant ADV genotypes in swine infected by multiple ADV strains would be modulated by factors that affect the distribution of different virus strains to common target cells, thereby limiting or enhancing the likelihood of cellular co-infection. If a similar close relationship between the estimated co-infection rates and the generation of recombinant progeny occurs in vivo, measurements of absolute and relative infectious virus concentrations at the level of target cell populations could be used as indices for the likelihood of in vivo recombination.

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References


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