Selection kinetics during serial cell culture passage of mixtures of wild-type *Autographa californica* nuclear polyhedrosis virus and its recombinant Ac360–β-gal

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Detailed analysis of the selection process in serial co-infections of cell cultures by wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) strain E2 (AcNPV/E2) and Ac360–β-gal, a genetically engineered strain, shows that the unaltered strain was clearly dominant even when it initially constituted the minority component in the inoculum. A method of calculating a selection coefficient that quantifies the relative advantage of one strain of virus over the other under specific culture conditions is described. Calculated selection coefficients were relatively homogeneous and almost exclusively favoured the progenitor. Selection pressure was not influenced by the relative proportions of the two strains in the population. Selection coefficients, as determined in the present study, may be useful for evaluating the effect of a genetic alteration on viral fitness under specified conditions. Unexpected high frequencies of mixed phenotype plaques were observed during infectivity titrations of media from early serial passages of co-infected cultures. Statistical evaluation implicates some non-heritable combinational phenomenon. Virus plated from mixed phenotype plaques show high segregation of phenotypes implying that genetic recombination does not contribute in a major way to the high mixed phenotype frequencies. Electron microscopic examination of virion pellets from infected 72 h cell culture media similarly argue against co-envelopment as a major contributory factor to the high frequency of mixed phenotype plaques. The cause remains undetermined.

Introduction

The potential for development of undesirable phenotypes that have a decided selective advantage over their natural progenitors and sympatric species has been the basis for passionate debate about the hazards of environmental release of genetically engineered organisms (Sharples, 1983; Simberloff & Colwell, 1984; Brill, 1985; Davis, 1987). Recombinant baculoviruses have been experimentally released under carefully controlled conditions in the U.K. (Bishop et al., 1988; Bishop & Possee, 1990) with the intention of eventually using these agents as viral insecticides.

Nuclear polyhedrosis viruses (NPVs), subfamily Eubaculovirinae, have been particularly suitable for biotechnological application due to certain unique features (Webb & Summers, 1990). Among the most important are the following. (i) They produce two types of virions containing identical genomic DNA and capsids but with envelopes of different origin. Extracellular virus obtains its envelope from modified cell surface plasma membrane by budding and functions in the cell-to-cell spread of the virus. It is essential for cell culture propagation of the virus. Polyhedra-derived virus (PDV), in contrast, obtains its *de novo* synthesized envelopes within the nucleus and is normally occluded within large, virus-encoded paracrystalline protein inclusion bodies, the nuclear polyhedra. Nuclear polyhedra are characteristic of Eubaculovirinae infections, function in host-to-host spread of the virus in the natural environment and are dispensable for cell culture propagation of the virus. (ii) The PDV-occluding matrix of nuclear polyhedra is formed by a viral protein, polyhedrin. The polyhedrins of different viruses form a family of highly conserved viral structural proteins (Rohrmann, 1986) which are encoded by hyperexpressed late baculoviral genes (Blissard & Rohrmann, 1990). The strong promoters possessed by these genes and the dispensable nature of polyhedrins for *in vitro* virus replication have made polyhedrin genes favourable sites for insertion and efficient expression of large numbers of foreign genes (Webb & Summers, 1990). Additionally, the absence of polyhedron formation by these expression vectors greatly diminishes their potential environmental
survival (Summers & Smith, 1985; Webb & Summers, 1990). Baculoviral recombinant strains have also been constructed with the intent of improving pesticidal characteristics (Maeda, 1988; Hammock et al., 1990).

The availability of recombinants, some bearing reporter genes, of the M type NPV of Autographa californica (Ac) provided an opportunity to study in detail the selection kinetics in mixed cultures of a recombinant and its natural progenitor during extended serial passage. Described in this report are results of in vitro experiments using an AcNPV recombinant, Ac360-β-gal (Luckow & Summers, 1988), and the genetically homogeneous unaltered progenitor strain, AcNPV/E2 (Smith & Summers, 1979). Detailed statistical analyses of the results were carried out and selection coefficients calculated and compared.

**Methods**

**Viruses and cells.** Viruses and cells were kindly provided by Dr M. Summers (Texas A & M University, College Station, Tex., U.S.A.). Plaque-purified AcNPV/E2 was the unaltered progenitor virus. The plaque phenotype of this virus was identifiable by the presence of polyhedral inclusions in the nuclei of infected cells. Ac360-β-gal, the recombinant, is a construct with the coding sequence for β-galactosidase inserted in-phase plus 36 bp downstream from the polyhedrin gene translation start site (Luckow & Summers, 1988). Ac360-β-gal plaques were identified with the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) at 40 μg/ml (Bethesda Research Laboratories).

Virus stocks were propagated in Spodoptera frugiperda IPLB-Sf21-AE cell (Sf) cultures. Monolayers of Sf cells were also utilized for plaque assays for virus infectivity titrations. The cells were grown in Grace's medium (Gibco) plus 10% foetal bovine serum (FBS; Gibco) and 0.13 g/ml yeastolate (Difco). The cells were maintained in logarithmic growth at 27 °C. Cell viability was tested by the trypan blue exclusion method.

**Preparation of AcNPV/E2 and Ac360-β-gal stocks.** Culture flasks (Costar, 75 cm²) were seeded with Sf cells at a density of 5 x 10⁶ cells per flask. Cells were permitted to attach at 27 °C for 2 h and then 2 ml per flask of diluted, plaque-purified AcNPV/E2 or Ac360-β-gal virus was added at an m.o.i. of 0.01. Each flask was rocked at room temperature for 1 h, an additional 8 ml of Grace's medium with 10% FBS and 0.13 g/ml yeastolate was added and the cultures were incubated at 27 °C for 72 h. After centrifugation of media from infected cultures at 400 g for 20 min to remove cells and large cellular debris, the supernatant, containing the released virus, was stored at 4 °C. Infectious titres of these stocks were determined as p.f.u./ml (Bethesda Research Laboratories).

**Selection experiment: serial passage of AcNPV/E2 - Ac360-β-gal mixtures.** Mixtures of AcNPV/E2:Ac360-β-gal in 10:1, 1:1 and 1:10 ratios were prepared based on the infectious titre (p.f.u./ml) of the stocks. The mixtures were serially passaged in separate cell cultures as described below. Sf cells (2.5 x 10⁶) were seeded into 25 cm² flasks (Costar) and allowed to attach for 2 h at 27 °C. Each culture was then infected with 1 ml of one of the AcNPV/E2:Ac360-β-gal mixtures. The virus was allowed to adsorb for 1 h at room temperature, the inoculum was removed and fresh medium added to each culture. After a 72 h incubation at 27 °C, non-occluded free virus present in culture medium was separated from cell-associated and occluded virus by centrifugation at 400 g for 20 min. Supernatant containing the non-occluded virus was diluted and used as inoculum for the next passage as described below. The remaining supernatant was stored at 4 °C. Pure, unmixed AcNPV/E2 and Ac360-β-gal were passaged in serial cultures under the same conditions as controls concurrently with the mixed cultures.

Cells for the first passage were inoculated at an m.o.i. of 1. In subsequent serial passages, the infectious titres of progeny virus were assumed to reach 6 x 10⁶ p.f.u./ml, a nominal value for AcNPV/E2 infections. Accordingly, the clarified culture media were diluted 1:20 to obtain inocula that would infect cell cultures for the next passage at an m.o.i. of about 1 to prevent defective particle formation. Later titrations showed the actual m.o.i. to be between 0.3 and 0.6.

**Plaque assays.** Cells for plaque assays were obtained from cultures in logarithmic growth and exhibited viabilities of greater than 80% based on the trypan blue exclusion criterion. Plaque assays were conducted essentially as described by Wood (1977) using IPLB-Sf21 cells. The plates were incubated at 27 °C for 4 days. Plaques were then counted with a dissecting microscope. Duplicate plates were run for each sample in all plaque assays.

Selection experiment data were obtained by determining the proportion of AcNPV/E2 to Ac360-β-gal after each serial passage. The number of each of four different plaque phenotypes produced in the assay of the clarified medium from cultures initiated at different progenitor:recombinant ratios were tallied. The plaque phenotypes were (i) blue plaques without inclusions which represent Ac360-β-gal, the recombinant, (ii) colourless, refractile plaques with nuclear polyhedra due to AcNPV/E2, (iii) mixed phenotype plaques that were blue but in which nuclear polyhedra were also present and (iv) "clear" plaques due to foci of cells showing c.p.e. without any nuclear polyhedra or blue colouration. Clear plaques were extremely rare and not included in the calculations. The mixed phenotype and clear plaques were verified by examination with an inverted phase contrast microscope to ensure that they did not represent overlapping AcNPV/E2 and Ac360-β-gal plaques or AcNPV/E2 plaques with few polyhedra. In the enumeration of AcNPV/E2 to Ac360-β-gal plaques for calculation of the ratio between progenitor and recombinant during serial passages, the number of mixed phenotype plaques was added to both the AcNPV/E2 and Ac360-β-gal totals because it was found that the majority of mixed phenotype plaques are due to combinatorial events and would segregate upon replaquing (see Results). Titres of total virus were also calculated from the plaque assay data for all cultures after serial passage.

**Fidelity of mixed phenotypes.** Representative mixed phenotype plaques were picked from early (P5) and later (P15) serial passages, resuspended in medium, diluted and replaqued to determine whether the mixed phenotype represented true recombinants or was due to other combinatorial phenomena such as multiple envelopment or superinfection and would segregate upon replaquing. Passages were chosen to determine whether the origin of the mixed phenotype was different during early serial passage when their frequencies were maximal and later after continued passage. Occasionally, mixed phenotype plaques observed after a single plaque purification were again replaqued to determine the distribution of phenotypes among the progeny.

**Productivity and growth kinetics of AcNPV/E2 and Ac360-β-gal in single passage cultures.** To begin to explain the outcome of the selection experiments, the kinetics of replication of AcNPV/E2 and Ac360-β-gal during single passages in pure and mixed (1:1) cultures were investigated. Culture media were periodically assayed for the release of infectious progeny AcNPV/E2 or Ac360-β-gal into the medium for 98 h post-infection (p.i.) in the following manner. Sf cells (2.5 x 10⁶) in 25 cm² flasks were inoculated with 1 ml of virus (m.o.i. of 1) for 1 h at room temperature. The inoculum was removed, the cells were washed once with medium and after the addition of 5 ml
of fresh medium flasks were incubated for 1, 6, 12, 18, 24, 36, 48, 72 or 96 h. At the end of the period, the culture media were centrifuged at 400 g for 20 min and the supernatant collected and stored at 4 °C. (In the case of the 1 h sample, there was no additional incubation at 27 °C.) The virus titre was determined by plaque assay as described previously except that the X-gal concentration was 60 µg/ml and the overlay contained 100 µg penicillin and 100 units streptomycin/ml.

Data analysis. Replicate plaque assays from each passage were tested for homogeneity by fitting log-linear models to the number of plaques of each phenotype and determining the reduction in 'goodness-of-fit' incurred by pooling terms for replicates. This testing was carried out using Proc Cat model of the SAS statistical analysis system. Homogeneous replicates were pooled for calculating proportions and s.e.m.s and for testing for monotonic trend. Bartholomew's test for increasing proportions (Fleiss, 1981) as implemented in STAT-SAK (Dallal, 1986) was used to test whether significant trends in ratio changes were occurring with serial passage.

A selection coefficient was defined to quantify the selective advantage of AcNPV/E2 over Ac360-β-gal. If the titres of the strains can be thought of as undergoing exponential or logistic growth in titre during a passage, then the relationship between the proportion of AcNPV/E2 and passage number is (Crow & Kimura, 1970):

\[ P_{t+1} = \frac{1 + \frac{P_t e^{-s}}{P_t}}{1 + \frac{1 - P_t}{P_t}} \]

Here, \( P_t \) is the proportion of AcNPV/E2 at the beginning of the time period, \( s \) is the selection coefficient in units of the reciprocal of the generation time (72 h), and \( k \) is the number of passages the system was followed. \( P_t \) and \( P_{t+1} \) were estimated from the data, and used to estimate \( s \) with a formula derived by solving equation 1 for \( s \):

\[ s = \ln \left( \frac{P_{t+1} - 1}{P_t} \right) - \ln \left( \frac{P_t e^{-s}}{P_t} - 1 \right) \]

Estimates of \( s \) were plotted versus \( P_t \) and examined graphically for constancy using the scatterplot smoother LOWESS (Cleveland, 1979). A further test of the constancy of \( s \) was afforded by testing the significance of the coefficient of a term in \( k^2 \) in (1). As the estimates of \( s \) from (2) are not statistically independent, and because the variance of the estimates depend upon the initial proportion of AcNPV/E2, single estimates of \( s \) for each series were calculated using the fact that (1) is the formula for a logistic curve. Generalized linear model methods (McCullagh & Nelder, 1983) were used to fit (1) to the estimates of the proportion of AcNPV/E2 at each passage in a series.

The exponential rate of increase associated with the growth curves relating the titre of virus of pure and mixed cultures of AcNPV/E2 and Ac360-β-gal to time were estimated from the slope of the relationship between log titre and time during the log phase of the appropriate growth curves. The maximum titre in the cultures was estimated as the mean of the titres of the last three time points, during which there was virtually no change in titre. Kinetic parameters for the two viruses were then compared statistically in each experiment by adding the differences between strains at each time point, squared and divided by the estimate of the variance of the difference, together into a chi-squared variable with as many degrees of freedom as time points in the experiment.

Electron microscopy of virus pellets. Pellets of virions from culture media at 24 and 72 h were examined to determine whether virions consisting of co-enveloped nucleocapsids were present. This was important because one possible type of combinatorial phenomenon is co-envelopment of AcNPV/E2 and Ac360-β-gal nucleocapsids to form multinucleocapsid virions (Bilimoria, 1986).

Results

Selection during serial passages of mixed cultures

The results of two separate series of serial passages of mixtures of AcNPV/E2 and Ac360-β-gal initiated at three different ratios (1:10, 1:1 and 10:1) are presented in Table 1, which shows the proportions of AcNPV/E2 over 30 consecutive passages, and the 95% confidence limits. The results and trends of the two series were essentially similar and, for most calculations, both sets of data were used. For the sake of brevity and clarity, however, only data from series 1 are graphically presented in Fig. 1. Table 1 and Fig. 1 illustrate that AcNPV/E2 had a decided selective advantage over Ac360-β-gal regardless of the starting ratio of the two viruses. There is no indication of the development of an equilibrium between recombinant and progenitor virus. The recombinant virus was not detected after passage 20 in cultures initiated at 10:1 AcNPV/E2:Ac360-β-gal and at passage 30 in 1:1 and 1:10 cultures in both series.

The combined total infectious titres in mixed infections of AcNPV/E2:Ac360-β-gal appear to drop over 30 passages (Fig. 2). This was true of infections initiated at all three AcNPV/E2:Ac360-β-gal ratios.

Selection coefficients, \( s \), were calculated to determine the intensity of selection during serial passage of mixed virus cultures initiated at different ratios of AcNPV/E2:Ac360-β-gal. Values of \( s \) were calculated for each consecutive pair of passages and plotted against the proportion of AcNPV/E2 (Fig. 3). The increased scattering of values near unity on the abscissa is because the proportion of Ac360-β-gal appears in the denominator of the function used to estimate \( s \). As this proportion approaches zero, fluctuations in the proportion of AcNPV/E2 are amplified and result in the scattering of values. The curve through the estimates of \( s \) in Fig. 3 is the result of smoothing the estimates of \( s \) with the scatterplot smoother LOWESS (Cleveland, 1979) to approximate the relationship between the selection coefficient for a passage and the initial proportion of AcNPV/E2 for that passage. Most of the estimates of \( s \) are positive, and there is no trend with increasing proportion of AcNPV/E2. The smoothed curve of the estimates of \( s \) is close to a horizontal line with the y
Fig. 1. Proportion of AcNPV/E2, the natural progenitor strain, during serial passage in mixed cultures with Ac360-β-gal, a recombinant (series 1). Curves show the selective advantage of the natural progenitor in IPLB-Sf21 cell cultures initiated at different AcNPV/E2 : Ac360-β-gal initial ratios: ■, 1:10; +, 1:1; □, 10:1.

Fig. 2. Changes in total virus titre during serial passage (series 1). Mixed infections were started at different AcNPV/E2:Ac360-β-gal initial ratios: symbols as described for Fig. 1.

intercept between 0.2 and 0.3. This emphasizes that there is little or no difference in selection intensity at different proportions of AcNPV/E2. A single selection coefficient was then fitted to each series of data. These estimates are listed in Table 2. The selection coefficients in the different experiments are quite similar. The homogeneous values of s and the positive running average indicate that selection was uniform and predominantly in favour of AcNPV/E2.

**Titre changes of AcNPV/E2 and Ac360-β-gal passaged in unmixed serial cultures**

The dominance of AcNPV/E2 in mixed infection may simply be due to its consistently greater productivity (release of infectious particles) during serial passage in culture. To test this possibility, the trends in the productivity of AcNPV/E2 and Ac360-β-gal in serial, pure cultures run concurrently with the mixed virus cultures were examined.

The titres attained by AcNPV/E2 were compared to those of Ac360-β-gal at equivalent serial passages and the results were pooled over the entire experiment. The data (not shown) indicate that the infectious titres attained by AcNPV/E2 were on average about 1.7 times higher than that of Ac360-β-gal at every passage (virus effects were contrasted in an ANOVA of In-transformed
In vitro selection of AcNPV strains

Fig. 3. Estimates of the selection coefficient, $s$, for the natural progenitor virus AcNPV/E2 during serial passages with the recombinant strain Ac360-β-gal. Serial passages were initiated at different AcNPV/E2:Ac360-β-gal initial ratios: □, 1:10, series 1; ■, 1:10, series 2; △, 1:1; series 1; ▲, 1:1; series 2; ○, 10:1, series 1; ●, 10:1, series 2. The relatively homogeneous values of $s$ indicate a fairly constant selection pressure and the positive running average indicated by the line demonstrates that selection predominantly favoured AcNPV/E2.

Fig. 4. Single passage growth kinetics of pure AcNPV/E2 (a), pure Ac360-β-gal (b) and a 1:1 mixed culture of AcNPV/E2 and Ac360-β-gal (a and b) in IPLB-Sf21 cells. Pure cultures were infected at a multiplicity of 1.0. Mixed cultures were infected with an inoculum containing AcNPV/E2 and Ac360-β-gal, each at multiplicities of 0.5. Symbols represent: (a) □, series 1 AcNPV/E2 culture; +, series 2 AcNPV/E2 culture; ○, series 2 mixed culture; (b) ■, series 1 Ac360-β-gal culture, +, series 2 Ac360-β-gal culture; ●, series 2, mixed culture.

Table 2. Estimates of the selection coefficients ($s$), the S.E.M. and approximate 95% confidence limits (CL) from mixed cultures of AcNPV/E2 and Ac360-β-gal passed 30 times

<table>
<thead>
<tr>
<th>Initial ratio</th>
<th>$s$</th>
<th>S.E.M.</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series 1</td>
<td>0.256</td>
<td>0.042</td>
<td>0.173-0.339</td>
</tr>
<tr>
<td>Series 2</td>
<td>0.297</td>
<td>0.247</td>
<td>0.248-0.345</td>
</tr>
<tr>
<td>1:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series 1</td>
<td>0.247</td>
<td>0.013</td>
<td>0.223-0.271</td>
</tr>
<tr>
<td>Series 2</td>
<td>0.129</td>
<td>0.019</td>
<td>0.091-0.167</td>
</tr>
<tr>
<td>10:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series 1</td>
<td>0.238</td>
<td>0.017</td>
<td>0.204-0.272</td>
</tr>
<tr>
<td>Series 2</td>
<td>0.321</td>
<td>0.017</td>
<td>0.287-0.356</td>
</tr>
</tbody>
</table>

*AcNPV/E2:Ac360-β-gal.

whether the dominant strain had a shorter replication cycle or reached higher titres (Fig. 4). In pure culture, the infectious titre of both AcNPV/E2 and Ac360-β-gal started to increase at 12 h and peaked at 72 h p.i. when the inoculum was usually harvested for the next serial passage. Maximum titres of $2.2 \times 10^7$ and $1.8 \times 10^7$ p.f.u./ml were attained by AcNPV/E2 and Ac360-β-gal, respectively. Neither of the two virus strains consistently produced higher infectious titres at 72 h than the other in the two series ($P > 0.5$ for 72 h infectious titre and initial rate of increase). Thus the reason for the selective advantage of AcNPV/E2 remains to be ascertained. Comparison of 72 h titres attained by each strain in the single mixed 1:1 AcNPV/E2:Ac360-β-gal culture showed a trend similar to that of the pure culture results. AcNPV/E2 reached a higher titre than Ac360-β-gal in this specific experiment.

High frequency of mixed phenotype plaques

Compared to the results of earlier studies (Summers et al., 1980), the data also show unexpectedly high
Fig. 5. Relationship of mixed phenotype plaques to the ratio of AcNPV/E2:Ac360-β-gal in the virus population. The figure shows that log₁₀ of the number of mixed phenotype plaques (p.f.u./ml) increases proportionally with the ratio represented numerically on the abscissa as log₁₀ of the arithmetic product of the titre of AcNPV/E2 and the titre of Ac360-β-gal (series 1). Symbols (□, +, △) represent the same initial ratios as indicated for Fig. 1.

Table 3. Proportion of mixed phenotype plaques in serial passages initiated at different ratios (AcNPV/E2 : Ac360-β-gal)

<table>
<thead>
<tr>
<th>Initial ratio</th>
<th>Passage</th>
<th>1:10 Proportion</th>
<th>1:1 Proportion</th>
<th>10:1 Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcNPV/E2</td>
<td>Ac360-β-gal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.036 (111)*</td>
<td>0.086 (163)</td>
<td>0.074 (296)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.053 (94)</td>
<td>0.300 (190)</td>
<td>0.062 (146)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.104 (134)</td>
<td>0.253 (296)</td>
<td>0.095 (211)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.117 (103)</td>
<td>0.242 (240)</td>
<td>0.037 (109)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.122 (123)</td>
<td>0.213 (169)</td>
<td>0.021 (188)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.060 (546)</td>
<td>0.073 (1078)</td>
<td>0.024 (721)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.038 (468)</td>
<td>0.048 (834)</td>
<td>0.023 (814)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.058 (362)</td>
<td>0.031 (450)</td>
<td>0.010 (498)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.077 (208)</td>
<td>0.024 (332)</td>
<td>0.004 (243)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.045 (308)</td>
<td>0.000 (221)</td>
<td>0.000 (201)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.036 (331)</td>
<td>0.008 (395)</td>
<td>0.002 (446)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.016 (426)</td>
<td>0.003 (340)</td>
<td>0.000 (323)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0.000 (492)</td>
<td>0.000 (589)</td>
<td>0.000 (825)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.000 (78)</td>
<td>0.000 (64)</td>
<td>0.000 (144)</td>
<td></td>
</tr>
</tbody>
</table>

* Total number of plaques are in parentheses.

If the high frequency of mixed phenotype plaques is due to a combinational phenomenon the data should then indicate the following. (i) Among cultures with the same titres of infectious particles, the highest proportions of mixed phenotype plaques should occur when the probability of pairing of the two genotypes is maximal, i.e. when the ratio of AcNPV/E2:Ac360-β-gal is near 1:1. Numerically, this implies that the mixed phenotype titre should be proportional to the arithmetic product of the fraction of AcNPV/E2 multiplied by the fraction of Ac360-β-gal. (ii) The ratio between the titre of mixed phenotype plaques and the arithmetic product of the titres of AcNPV/E2 and Ac360-β-gal should remain fairly constant with passage.

Analysis of the data shows both appear to be true. Fig. 5 demonstrates that the titres of mixed phenotype plaques increase as a power function of the product of the titres of the two strains. In fact, the slope of the least-squares line through the data in Fig. 5 is about 0.6, and through the data for series 2 (not shown) is about 0.5, suggesting that the titre of mixed phenotype plaques is proportional to the square root of the product of the titres of the two strains. The ratio of the titre of mixed phenotype plaques to that of AcNPV/E2 multiplied by the titre of Ac360-β-gal does not change systematically with passage (Fig. 6).

Virus derived from both early (fifth) and later (15th) passages of mixed phenotype plaques showed very high rates of segregation of phenotypes when plaqued (Table 4). Similar rates of segregation (>95%, data not shown) were exhibited when the virus from selected progeny plaques was subjected to an additional plaque assay step.
As expected, the environmental persistence of the polyhedrin-deficient constructs was greatly decreased.

Even in cultures where the initial ratios favoured AcNPV/E2 inexorably increased until it predominated.

The relative ‘fitness’ of genetically engineered and natural progenitor strains has been a core issue concerning the hazards posed by the release of genetically altered agents into the environment. The concern is that dominant genotypes with undesirable phenotypic characteristics can be created.

Controlled environmental release of three genetically altered, marked AcNPV strains carried out in the U.K. have compared genetic and biological properties of parental AcNPV, AcNPV with a synthetic marker polynucleotide inserted in the 3’ non-translated sequence of the polyhedrin gene, AcNPV in which the whole polyhedrin gene including its transcriptional promoter was replaced by another oligonucleotide sequence which purposely did not alter expression of other genes, and an AcNPV strain in which β-galactosidase was substituted for the polyhedrin-coding region (Bishop & Possee, 1990). No differences in infectivity, host range or genetic stability were observed. With the exception of loss of polyhedrin predicted by deletion of its gene, viral translational products of all the constructs were similar. As expected, the environmental persistence of the polyhedrin-deficient constructs was greatly decreased.

A quantitative indication of the strength and direction of selection for organisms under defined conditions can be provided by the selection coefficient, s. In contrast to earlier studies, the present experiments quantitatively investigated the selection kinetics during serial passage in mixed cultures of the progenitor AcNPV/E2 and a recombinant Ac360-β-gal. The results of these studies (Table 1, Fig. 1) demonstrate that the proportion of AcNPV/E2 inexorably increased until it predominated even in cultures where the initial ratios favoured Ac360-β-gal. The number of passages required for AcNPV/E2 to become pre-eminent was a function of the starting ratio. The selection coefficients (Fig. 2) confirm these findings. Relatively homogeneous values of s demonstrate that selection pressure was uniform and appeared to be independent of the composition of the mixed viral population. That is, selection pressure did not increase or decrease with the proportion of either strain. Non-parametric estimation of the relationship between s values and AcNPV/E2 proportions resulted in a smooth, positive, horizontal line indicating the progenitor was uniformly favoured in mixed culture. If an equilibrium ratio between the two strains is achieved, it is beyond the resolution of the present experiment to detect.

Although the present results are relevant for assessing the effects of genetic alterations on viral phenotype in vitro, the applicability of these findings to selection in the insect host or environment remains undetermined. However, selective forces that function in these simpler systems may nevertheless occur in more complex situations such as in the larval haemocoel.

Comparison of the productivity of the two strains in pure, concurrent serial cultures demonstrated that AcNPV/E2 attained a roughly 1.7-fold higher average titre than Ac360-β-gal during each passage. Such a difference in mixed cultures could be a major determinant of the eventual predominance of the natural progenitor virus. The apparent selective disadvantage of Ac360-β-gal may be a consequence of the genetic effects of the construct, cumulative reversion to the progenitor phenotype during the experiment, the synthesis or properties of the polyhedrin-β-galactosidase fusion protein, the absence of normal polyhedrin synthesis or possibly a combination of these factors.

An unexpected finding of the serial passage results was the high proportion of mixed phenotype plaques observed during the early passages of mixed cultures. Frequencies as high as 30% far exceeded the 7% observed in earlier studies of genetic recombination at the molecular level (Summers et al., 1980). That the high frequencies of mixed phenotype plaques are due to combinational phenomena and not to genetic recombination is strongly supported by the demonstration that virus from mixed phenotype plaques from either early or late passages exhibited very low phenotype fidelity (Table 4). Over 90% of the progeny segregated into either AcNPV/E2 or Ac360-β-gal phenotypes and additional plaque formation by a limited number of second generation mixed phenotype plaque isolates resulted in similar segregation rates. Taken together, these results strongly indicate that a combinational phenomenon plays a major role in the production of mixed phenotype plaques. However, it is likely that at least a very small number of such plaques are due to genetic recombinants.

### Table 4. Frequency of progeny plaque phenotypes produced by virus from mixed phenotype plaques at early and late passage*

<table>
<thead>
<tr>
<th>Passage</th>
<th>Mixed (%)</th>
<th>Ac360-β-gal (%)</th>
<th>AcNPV/E2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>7.5 ± 6.4 (100)</td>
<td>71.9 ± 20.8 (663)</td>
<td>20.6 ± 15.5 (282)</td>
</tr>
<tr>
<td>15</td>
<td>5.0 ± 3.8 (97)</td>
<td>63.6 ± 29.4 (1082)</td>
<td>31.4 ± 27.0 (639)</td>
</tr>
</tbody>
</table>

* x ± S.D. (no. plaques).
Some of the combinational processes possibly causing mixed phenotype plaques may be coalescence of infection foci due to physical proximity, chance co-infection of cells by individual or physically adherent virions of the two strains, or co-envelopment of AcNPV/E2 and Ac360-β-gal nucleocapsids. Co-envelopment is a contributing factor but not a major one because the highest frequency of co-enveloped nucleocapsids in virions from culture media 72 h p.i. was 14%. The explanation may be more mundane. The observed elevated number of mixed phenotype plaques may be due simply to the use of two strains that produce phenotypically distinguishable plaques near optimal ratios (1:1), revealing the basal rate at which coalescence of infection foci and chance co-infection skew the results of baculovirus titration assays.

In summary, the present study shows that the selection coefficients estimated when a derived strain and the natural progenitor strain are co-cultured can be useful as an indicator of the effect of the genetic alteration on the fitness of the phenotype and its success in replicating in the presence of its progenitor under standard cell culture conditions. The present results demonstrate that the AcNPV construct, Ac360-β-gal, is at a selective disadvantage in mixed cultures with AcNPV/E2. Why a strain that is altered in a gene presumed to be dispensable for \textit{in vitro} replication is at a disadvantage is a puzzle. Perhaps if comparisons similar to those of the present study are made with the AcNPV constructs reported by Bishop & Possee (1990), effects equivalent to those seen here will be demonstrated if the regulatory effect of polyhedrin gene transcription on the 3.2 kb antisense RNA transcript which traverses this region (Ooi & Miller, 1990) is a critical event in the replication process of the virus.

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\section*{References}


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