Complementation between Phosphonoacetic Acid-resistant and -sensitive Variants of Herpes Simplex Viruses: Evidence for an Oligomeric Protein with Restricted Intracellular Diffusion as the Determinant of Resistance and Sensitivity

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SUMMARY

Complementation between phosphonoacetic acid (PAA)-resistant ($P^r$) and -sensitive ($P^s$) variants of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) was studied to provide information on the function of the virus-coded DNA polymerase. Complementation within and between serotypes was demonstrated, with the growth of the $P^s$ partner in mixed infections becoming relatively more resistant and the $P^r$ partner relatively more sensitive to PAA than in the corresponding single infections. However, the relative contribution of the $P^s$ partner to the mixed infection had a disproportionately large effect on the resultant sensitivity of the mixed infection which was incompatible with non-interactive (e.g. monomeric) polymerase molecules as determinants of PAA sensitivity and resistance. Although a number of solutions gave equally good fits to the available data, the simplest was obtained by assuming that the functional DNA polymerase was a trimer and that only the $(P^r)^3$ homotrimer was active in the presence of the drug. In addition, yields from mixed infections in the presence of PAA were enriched for the resistant partner relative to yields in the absence of the drug. These latter results suggested that the intracellular distribution of resistant DNA polymerase oligomers was non-random with respect to resistant and sensitive template genomes and that these resistant polymerase molecules were more likely to encounter and replicate resistant than sensitive genomes. Such an explanation seems to require vectorial nuclear-cytoplasm-nucleus translocation and restricted diffusion of transcript and gene products determining resistance.

INTRODUCTION

Phosphonoacetic acid (PAA) and related compounds (e.g. phosphonoformic acid) are effective inhibitors of a number of herpesviruses, including herpes simplex virus types 1 and 2 (HSV-1, HSV-2; see e.g. Overby et al., 1974; Herrin et al., 1977; Eriksson et al., 1980). Results from biochemical (e.g. Leinbach et al., 1976; Reno et al., 1978) and genetic (Hones & Watson, 1977; Purifoy & Powell, 1977; Chartrand et al., 1979, 1980) investigations are consistent with inhibition of the activity of the virus-coded DNA polymerase as the primary site of action of PAA on HSV replication. Phosphonoacetic acid-resistant ($P^r$) variants of HSV are readily selected during virus growth in the presence of the drug and $P^r$ determinants have been linked by both conventional genetic (Hones & Watson, 1977; Purifoy & Powell, 1977) and physical mapping techniques to the DNA polymerase locus of HSV. The $P^r$ markers segregate as single determinants amongst recombinant progeny of conventional
crosses (Honess & Watson, 1977; Honess et al., 1980) and have been located completely within regions of less than 1.3 (HSV-2) or 2.6 (HSV-1) kilobase pairs (kbp) at about 40.2 to 41.0 map units of the HSV genome (Chartrand et al., 1979, 1980; Crumpacker et al., 1980). Estimates of the size of the virus DNA polymerase which is active in \textit{in vitro} assays range from 130000 to about 180000 and purified preparations of this activity contain a major polypeptide in this size range (Weissbach et al., 1973; Powell & Purifoy, 1977; Knopf, 1979; Ostrander & Cheng, 1980). Thus, the coding region for the determinants of PAA resistance (1.3 to 2.6 kbp) can be accommodated entirely within the approx. 4.6 kbp required to encode the major polypeptide of the virus DNA polymerase. The \textit{P} marker therefore seems to represent a useful marker for both biochemical and genetic studies of the herpesvirus DNA polymerase.

We previously reported that \textit{P} variants of HSV would complement the growth of \textit{P}s viruses of homologous and heterologous serotype (Honess & Watson, 1977). In this paper we present a detailed examination of the stoichiometry of this interaction between resistant and sensitive viruses. We consider the interpretation of these experiments on the basis of some simple rules which appear to describe quantitative aspects of the expression of herpesvirus gene products in single and mixed infections. These analyses suggest the functional virus-specified DNA polymerase consists of an oligomer with at least three subunits and that only the trimer with three resistant subunits is active in the presence of the drug. The data further suggests that the mixing of \textit{P} and \textit{P}s subunits is incomplete in mixed infections.

\textbf{METHODS}

\textit{Viruses.} The viruses used were: (i) HSV-1 [HFEM], a syncytial plaque-forming (\textit{syn}), PAA-sensitive (\textit{P}s) strain; (ii) HSV-1 [HFEM] P18cl, a PAA-resistant (\textit{P}) variant of the latter virus whose isolation and properties have been described (Honess & Watson, 1977; Honess et al., 1980); (iii) HSV-1 [B2006], a non-syncytial (\textit{syn+}), \textit{P}s strain which is TK- (Dubbs & Kit, 1964); (iv) HSV-1 [\textit{syn+}.\textit{P}4], a \textit{syn+}, \textit{P}, TK- recombinant selected from a cross between viruses (ii) and (iii) above; (v) HSV-2 [HG52], the Glasgow prototype HSV-2 strain (Timbury & Calder, 1976) which is \textit{syn+}, and \textit{P}s; (vi) HSV-2 [HG52] P7cl, a \textit{P} derivative of the latter virus, cloned after seven consecutive passages in from 50 to 250 \textmu g/ml PAA. All sensitive viruses gave less than 0.1\% of the control plaque counts when assayed in the presence of 125 \textmu g/ml PAA whereas resistant derivatives gave efficiencies of plaque formation which did not differ significantly (i.e. \pm 10\%) from 100\% (see e.g. Honess et al., 1980) in the presence of this concentration of the drug.

\textit{Cells and media.} The BHK-21/C13 line of baby hamster kidney cells was used throughout the virus growth, infectivity titrations (Russell, 1962) and high multiplicity infections. Infected and uninfected cells were grown or maintained at 37 °C using the Glasgow modification of Eagle's medium supplemented with 10\% tryptose phosphate broth and 10\% (uninfected cells) or 2 to 5\% (infected cells) calf serum.

\textit{High multiplicity infections.} Cells were infected as semiconfluent monolayers (2 \times 10^6 to 4 \times 10^6 cells per 5 cm diam. plastic Petri dish or 25 cm$^2$ tissue culture flask) or as suspensions of 0.5 \times 10^7 to 1 \times 10^7 cells/ml, with from 5 to 300 p.f.u./cell of appropriate viruses singly or combined in various proportions (see Results) in a total vol. of 1 ml. After an adsorption period of 1 to 2 h at 37 °C with continuous gentle agitation, cells and residual inocula were separated by decantation (cell monolayers) or low-speed sedimentation (1500 rev/min for 10 min, cell suspensions), washed with 2 \times 5 ml of prewarmed growth medium and overlaid or resuspended in 5 ml of growth medium with no inhibitor or containing 300 \textmu g/ml neutralized PAA (ICN Pharmaceuticals). Infected cultures were removed after 18 to 30 h incubation at 37 °C and the cells resuspended in the culture fluid and disrupted by ultrasonic oscillation. These homogenates were assayed for their content of infectious virus by plaque
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Titration in BHK-21 cells using an overlay containing 0.5% carboxymethylcellulose. Assay plates were fixed with 10% formal saline and stained with crystal violet after 2 to 3 days incubation at 37 °C. Differential counts of syn and syn+ plaques were performed with the aid of a dissecting microscope.

Measurements of infectious centres. Infected monolayer cultures were rinsed with phosphate-buffered saline (PBS) and then removed by trypsin/versene treatment. Aliquots of cells infected in suspension were withdrawn into PBS, pelleted and treated with trypsin in suspension. Trypsin-treated cell suspensions were then resuspended in growth medium, counted in a haemocytometer, and dilutions containing 10, 10² and 10³ infected cells were added to duplicate monolayers of BHK-21 cells and incubated for 2 to 3 days at 37 °C with an overlay medium containing 0-5% carboxymethylcellulose. Plaques initiated by infected cells were counted as above.

Replicates and errors in infectivity titrations. In each experiment examining the effects of PAA, at least four separate cultures were infected under each set of conditions, and pairs of these cultures were combined to give duplicate samples. These duplicate PAA-treated cultures and their corresponding controls from each experiment were titrated in parallel on at least two separate occasions. Acceptable assays consisted of at least duplicate 5 cm plates of each of three 10-fold dilutions where one dilution showed > 50 and < 200 plaques/plate.

RESULTS

Properties of mixed infections with non-defective isolates of HSV-1

From the results of this paper we will deduce certain properties of interactions between P⁰ and P⁸ HSV DNA polymerase molecules. The results we show are from studies of complementation between P⁰ and P⁸ viruses to produce infectious progeny in mixed infections in the presence of PAA. The relationship between these measurements of the yield of progeny and the properties of DNA polymerase interactions in the mixed infection, is an indirect one. The link involves some reasonable assumptions and some rules formulated on the basis of results of other investigations. The detailed description of these studies is beyond the scope of this paper and will be given elsewhere. However, experiments which illustrate certain of these rules as they apply to the viruses used in the remainder of this paper are summarized below.

In the first experiment, aliquots of 4 × 10⁶ BHK-21 cells in suspension were infected with from 2 to 100 p.f.u./cell of HSV-1 [HFEM] P18cl. After incubation of virus–cell mixtures at 37 °C for 1.5 h, samples were withdrawn and processed for the measurement of infectious centres. Unadsorbed virus was then removed and the infected cultures were re-incubated at 37 °C until 30 h after infection, at which time they were harvested, homogenized, and titrated for their content of infectious virus. The results are shown in Fig.1 (a). More than 70% of infected cells gave rise to infectious centres with multiplicities in the range 2 to 100 p.f.u./cell (not shown) and the maximum yield of infectious progeny was independent of applied multiplicity under these conditions (Fig. 1 a). Infections of cell monolayers gave an analogous result, except that the yield was about 300 p.f.u./cell.

Fig. 1 (b) shows the proportion of syn. P⁰ and syn+. P⁸ progeny in the yields from dual infections as a function of the proportion of these viruses in the inoculum. Monolayers of 4 × 10⁶ BHK-21 cells were inoculated with artificial mixtures of a syn (HSV-1 [HFEM] P18cl) and a syn+ (HSV-1 [B2006]) virus to give a total applied multiplicity in the range 40 to 50 p.f.u./cell and ratios of syn to syn+ in the inoculum from 0.1 to 5.0 (abscissa). The total infectious virus yield and the proportion of syn and syn+ viruses amongst the progeny was measured after 24 h incubation at 37 °C. The total yield was independent of the composition of the infection and was about 200 to 240 p.f.u./cell; the ratio of syn to syn+ virus in the yield was the same as the ratio of syn to syn+ virus in the inoculum (Fig. 1 b). We have noted in previous work the errors involved in predicting effective multiplicities of viruses participating...
Fig. 1. (a) Maximum yields of infectious virus (ordinate) as a function of the applied multiplicity of infection (abscissa) from BHK-21 cells infected in suspension with HSV-1 [HFEM] P18cl. (b) Composition of infectious virus yield (ordinate, ratio of syn/syn+ viruses in the progeny yield) from dual infections between HSV-1 [HFEM] P18cl (syn) and HSV-1 [B2006] (syn+) as a function of the composition of the inoculum (abscissa, ratio of syn/syn+ viruses in inoculum).

in mixed infections at high multiplicities from the results of low multiplicity titrations (Honess et al., 1980). The correspondence between the composition of input and progeny shown in Fig. 1 (b) is only observed with viruses having equivalent particle/infectivity ratios. In the experiments to follow, the effective contributions of viruses to mixed infections were estimated from their contributions to the total virus yield in the absence of inhibitors.

**Complementation between P^r and P^s viruses: the response to the composition of the mixed infection**

A series of experiments was performed in which monolayers of BHK-21 cells were infected with artificial mixtures of HSV-1 [HFEM] P18cl (i.e. syn.P^r) and HSV-1 [B2006] (syn+.P^s), with HSV-1 [HFEM] P18cl and HSV-2 [HG52] (syn+.P^s) or with HSV-1 [HFEM] (syn.P^s) and HSV-2 [HG52] P7cl (syn+.P^r). These artificial mixtures were composed to give relative multiplicities of P^r to P^s partners ranging from < 1 to 9 to > 9 to 1 and absolute multiplicities from 5 to > 250 p.f.u./cell. The yields from infections incubated in the absence of PAA and in the presence of 300 μg/ml (HSV-1 .P^r) or 250 μg/ml (HSV-2 . P^r) of PAA were titrated and the yields of P^r and P^s partners measured by virtue of their linked plaque morphology markers. Recombination between syn and P^r loci in a homotypic cross is significant (21 ± 2.8%, i.e. about 10% of progeny from an equiparental cross between syn.P^r and syn+.P^s are syn.P^r; Honess et al., 1980); however, measurements of the virus yields in plaque assays in the presence of PAA (125 μg/ml) showed that the recovery of the P^r marker amongst the progeny was accurately estimated by the recovery of the syn marker. For each infection, these experiments therefore gave four measurements: the yield of P^r progeny and of P^s progeny from infections in the absence of PAA and the yield of P^r and P^s progeny from identical infections performed in the presence of PAA; let these measurements be R, S and R_p, S_p p.f.u./cell. The composition of the mixed infection (abscissae, Fig. 2) was expressed as R/R + S, the total virus yield in the presence of PAA as a fraction of the control yield as R_p + S_p/R + S, and the corresponding figures for P^r and P^s viruses separately as R_p/R and S_p/S (ordinates, Fig. 2). Results from 18 experiments with homotypic mixed infection and six experiments with heterotypic mixed infections presented in this way are collected in Fig. 2 (a and b respectively).
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Yields from single infections of $P^s$ viruses were unaffected by PAA and from single infections of $P^r$ viruses were less than 0.01 of the control values. In both homotypic (a) and heterotypic (b) complementation it is clear that the $P^r$ partners are rendered relatively more sensitive and the $P^s$ partners relatively more resistant than the corresponding single infections. Moreover, the addition of the $P^r$ partner has a disproportionately large effect on the resultant sensitivity of the mixed infection and the growth of the resistant partner. Reversal of the parental origin of the $P^r$ determinant in mixed infections between syn.$P^s$ (HSV-1 [HFEM]) and syn.$P^r$ (HSV-1 [syn.$P^r$]) viruses gave comparable results with respect to the PAA resistance of the mixed infection as a function of the proportions of $P^s$ and $P^r$ partners (not shown). From mixed infections in the absence of PAA which yield a fraction, $r = R/R + S$, of
Table 1. Measures of mean deviation $(x + y)$ of observed resistance of virus yield from values expected on the assumption that resistance is determined by the proportion of various resistant oligomers $(A)$ and that the frequency of occurrence of these oligomers is governed by the terms of the appropriate binomial probability distribution $(B)$

<table>
<thead>
<tr>
<th>Polymerization state of active enzyme $(n)$</th>
<th>A: composition of oligomers with complete or fractional activity in the presence of PAA</th>
<th>B: expected fraction of resistant activity assuming binomial distribution with fraction $r$ of resistant partner and $s$ of sensitive partner</th>
<th>HSV-1 (18 observations)</th>
<th>HSV-2 (6 observations)</th>
<th>HSV-1 and HSV-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$(P^r)$ monomer fully active</td>
<td>$r$ (curve 1, Fig. 2)</td>
<td>$0.244$</td>
<td>$0.197$</td>
<td>$0.232$</td>
</tr>
<tr>
<td>2</td>
<td>$(P^r)^2$ dimer fully active, others inactive</td>
<td>$r^2$</td>
<td>$0.172$</td>
<td>$0.175$</td>
<td>$0.172$</td>
</tr>
<tr>
<td>3</td>
<td>$(P^r)^3$ trimer fully active, others inactive</td>
<td>$r^3$ (curve 2, Fig. 2)</td>
<td>$0.241$</td>
<td>$0.250$</td>
<td>$0.243$</td>
</tr>
<tr>
<td>4</td>
<td>$(P^r)^4$ tetramer fully active, $(P^r)^2$, $(P^r)^2$ tetramer 1/2 active, others inactive</td>
<td>$r^4 + 3 r^2 s^2$</td>
<td>$0.276$</td>
<td>$0.202$</td>
<td>$0.258$</td>
</tr>
<tr>
<td>6</td>
<td>$(P^r)^6$ hexamer fully active, others inactive</td>
<td>$r^6$</td>
<td>$0.333$</td>
<td>$0.325$</td>
<td>$0.331$</td>
</tr>
<tr>
<td>6</td>
<td>$(P^r)^6$ and $(P^r)^3$ hexamers fully active, others inactive</td>
<td>$r^6 + 6 r^2 s^3$</td>
<td>$0.225$</td>
<td>$0.273$</td>
<td>$0.237$</td>
</tr>
<tr>
<td>6</td>
<td>$(P^r)^6$ hexamer fully active, $(P^r)^3$, $(P^r)^3$ hexamer 1/2 active, others inactive</td>
<td>$r^6 + 10 r^2 s^3$</td>
<td>$0.214$</td>
<td>$0.265$</td>
<td>$0.226$</td>
</tr>
<tr>
<td>8</td>
<td>$(P^r)^8$ octamer fully active, others inactive</td>
<td>$r^8$</td>
<td>$0.399$</td>
<td>$0.340$</td>
<td>$0.385$</td>
</tr>
<tr>
<td>8</td>
<td>$(P^r)^8$ and $(P^r)^3$, $(P^r)^3$ octamers fully active, others inactive</td>
<td>$r^8 + 8 r^2 s^3$</td>
<td>$0.269$</td>
<td>$0.310$</td>
<td>$0.279$</td>
</tr>
<tr>
<td>8</td>
<td>$(P^r)^8$ octamer fully active, $(P^r)^4$, $(P^r)^4$ octamer 1/2 active, others inactive</td>
<td>$r^8 + 35 r^4 s^4$ (curve 3, Fig. 2)</td>
<td>$0.159$</td>
<td>$0.202$</td>
<td>$0.169$</td>
</tr>
<tr>
<td>8</td>
<td>$(P^r)^8$ octamer fully active, $(P^r)^6$, $(P^r)^6$ octamer 3/4 active, others inactive</td>
<td>$r^8 + 21 r^6 s^3 + 35 r^4 s^4$</td>
<td>$0.224$</td>
<td>$0.138$</td>
<td>$0.204$</td>
</tr>
</tbody>
</table>
the resistant partner, \( s = S/R + S \), of the sensitive partner, a fraction \( P' \) of polymerase polypeptide will be specified by the resistant partner and \( P_s \) by the sensitive partner, where \( r/r + s = P'/P' + P_s \) (see Discussion). For non-defective viruses, the total yield of DNA polymerase protein per cell is a constant independent of the applied multiplicity or the composition of the infection and the synthesis of the polymerase protein appears to be unaffected by PAA in single infections of both \( P' \) and \( P_s \) viruses (Honess & Watson, 1977). We therefore assume that the fraction of the control virus yield from a mixed infection which is obtained in the presence of PAA is a measure of the fractional resistance of the functional polymerase population. If this population were monomeric and non-interactive the expected resistance of the mixed infection would be given by \( P'/P' + P_s \) or \( r/r + s \), i.e. curves 1 of Fig. 2. This solution is clearly a very poor fit to the experimental results and it is therefore necessary to propose that the activity of \( n \)-monomer units, acting together as an oligomer or independently as monomers, is required for the synthesis of a single unit of virus DNA. In a mixed infection the frequencies of formation of oligomers of \( n \)-subunits having different compositions with respect to resistant and sensitive subunits should be given by the appropriate terms in the binomial expansion of \((P' + P_s)^n\), which is numerically equivalent to \((r + s)^n\). The probabilities of a given template genome, which requires \( n \)-monomer units to replicate it (acting simultaneously or sequentially), encountering various proportions of resistant and sensitive monomer units is predicted by the same frequency distribution. We have fitted the data of Fig. 2(c, d) to a number of curves expressing various forms of the binomial expansion for different values of \( n \) (Table 1, column A gives the assumptions stated in terms of the oligomeric theory; column B gives the relevant terms of the appropriate binomial expansion). The fit of observed values to these theoretical curves was measured by summing the magnitudes of deviations of observed values from the theoretical curves in both \( x \) and \( y \) dimensions (irrespective of the sign of the deviation) and then calculating mean \( x + y \) deviations per observation from these totals. The values for a number of the possible solutions are collected in Table 1. The curves for a relatively simple solution (i.e. a trimer with only the \((P')^3\) homotrimer having activity in the presence of the drug) and a more complex one (i.e. an octamer with the \((P')^8\) complex fully active, the \((P')^4 \cdot (P_s)^4\) complex with half activity and other complexes being inactive in the presence of PAA) are added to panels (c) and (d) of Fig. 2 as curves 2 and 3 respectively. These two solutions give almost equally good fits to the available data (Table 1, best fits are underlined) and, given the nature of the observations, the simplest solution (i.e. the trimer) is to be preferred.

**Differences in the sensitivity of \( P' \) and \( P_s \) viruses in mixed infections: effects of the multiplicity of infection**

In the results shown in Fig. 2 the relative resistance of the mixed infection and of the \( P' \) and \( P_s \) partners are displayed separately in panels (a) and (b). In all cases, the growth of the \( P' \) partner is relatively more resistant than the \( P_s \) partner. A number of explanations for this phenomenon can be envisaged but they may be divided generally into deterministic and probabilistic classes. In the group of deterministic explanations we could include the existence of an exclusion effect in a minority of infected cells producing a larger than expected fraction of singly infected cells, or a greater affinity of resistant or sensitive polymerase subunits for their homologous genomes. In the group of probabilistic explanations we may consider the underestimation of cell-to-cell or intracellular heterogeneities in the distribution of complementing gene products. Observations on infectious centres from cells infected with approximately equal multiplicities of infection of \( > 5 \) of \( \text{syn} \) and \( \text{syn}^+ \) viruses do not show a significant fraction of singly infected cells, an exclusion effect therefore seeming unlikely. The plausibility of the other alternative explanations was assessed by an examination of the multiplicity dependence of the enrichment effect. The results from these experiments are
Fig. 3. Measurements of the relative enrichment of the \( P^r \) partner in yields from infections incubated in the presence of PAA relative to the contribution of the \( P^r \) partner to yields from identical infections incubated in the absence of PAA (i.e. \( R_p/S_p + R/S \), ordinate) as a function of the composition of the mixed infection (i.e. \( R/R + S \), abscissa) and the total multiplicity of infection (5 to 250). Multiplicities of infection were: ○, 5 to 25; ●, 25 to 50; ■, 50 to 100; □, 100 to 250.

summarized in Fig. 3, where the heterogeneity in the response of \( P^r \) and \( P^s \) partners is expressed as the change in the ratio of resistant and sensitive progeny in the yield from cells incubated in the presence of PAA (i.e. \( R_p/S_p \)) relative to that from cells in the absence of PAA (i.e. \( R/S \); thus, relative enrichment of \( P^r \) progeny, ordinate of Fig. 3, is \( R_p/S_p \div R/S \)).

Imperfect complementation (or the heterogeneity in the response of \( P^r \) and \( P^s \) partners indicated by the enrichment of the \( P^r \) partner in yields in the presence of PAA) is reduced at high multiplicities. However, even at rather high multiplicities of infection (> 50) and with infections having equal contributions from \( P^r \) and \( P^s \) partners, a significant enrichment of the \( P^r \) partner was observed.

**DISCUSSION**

The evidence locating the determinant of HSV PAA resistance and sensitivity in a component of the virus DNA polymerase is now compelling (see Introduction). In this paper, we have used the \( P^r \) phenotype to obtain some information on the functional form of the DNA polymerase in vivo by an analysis of complementation in mixed infections between resistant and sensitive viruses. The paucity of data on the functions of herpesvirus gene products is likely to limit the interpretation of information from analyses of the nucleotide sequence and transcription of the genome. Qualitative and quantitative analysis of complementation in vivo and attempts to reconstitute elements of the transcription or replication systems of the virus in vitro therefore remain of some importance. For the former approach, a semi-quantitative understanding of the regulation of virus macromolecular synthesis during a normal infection is essential; for the latter, some criteria for judging the validity of partial reconstitutions of in vivo characteristics by in vitro systems would be invaluable. Results from this paper are relevant to both of these problems.

The demonstration of homo- and heterotypic complementation between \( P^r \) and \( P^s \) phenotypes confirms and extends our previous results (Honess & Watson, 1977). This complementation was disproportionately affected by the contribution of the \( P^s \) partner to the mixed infection and showed an unexpected asymmetry in the growth of \( P^r \) and \( P^s \) partners. The former result was incompatible with a non-interactive monomeric polymerase molecule and suggested that an oligomeric enzyme may be the functional unit in vivo. The recent demonstration of intragenic complementation between ts mutants with defects in the DNA polymerase locus (Purifoy & Powell, 1981) is obviously consistent with this notion. An
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Analysis of the stoichiometry of the intragenic complementation was undertaken by the application of a set of simple rules which describe the quantitative aspects of gene expression in single and mixed infections between non-defective isolates of herpes simplex viruses. We will justify these rules more fully elsewhere (R. W. Honess et al., unpublished results), but the most important of these rules also seem reasonable a priori. Thus, beyond the multiplicity necessary to infect all susceptible cells in a culture, the kinetics of protein synthesis are multiplicity-dependent but the final yield of a given gene product is a constant, independent of the initial multiplicity of infection. In a mixed infection, the proportional contribution of allelic forms of a given virus protein (e.g. polypeptides of resistant and sensitive polymerase) to the yield of this protein is equal to the proportional contribution of viruses specifying these alleles to the yield of progeny virus. These general rules, the observation that the synthesis of the polymerase protein by both resistant and sensitive strains is not affected by PAA (Honess & Watson, 1977), and the assumption that the fraction of control virus yields obtained in the presence of PAA is equal to the fraction of functionally resistant polymerase molecules, permits the transformation of data on virus yield to an expression of relative resistance of the polymerase population. This transformation could then be interpreted in terms of an interaction between sensitive and resistant polymerase monomers to a functional oligomer. The simplest solution which gave a good fit to the observations was the behaviour predicted assuming the functional polymerase was a trimer, with the (P^r)^3 trimer being fully and exclusively active in the presence of PAA. The intuitively more attractive dimer or tetramer could not be ruled out, but theoretical curves for a more complex solution [e.g. an octamer, with the (P^r)^8 octamer fully active and the (P^r)^4. (P^s)^4 octamer having half the activity in the presence of the drug that it has in the absence of the drug] provided an excellent simulation of the experimental observations. On the alternative theory, i.e. that the DNA polymerase activity remains monomeric but that replication requires multiple polymerase monomers to interact with a single template molecule, the present results indicate that at least three such interactions are required.

A criterion for a valid partial reconstruction of the virus DNA polymerase activity in vitro may be suggested from these results. Mixtures of enzyme activities from resistant and sensitive viruses should have a resultant sensitivity which mimics the results of Fig. 2. This behaviour is not observed under the normal assay conditions for the HSV DNA polymerase in vitro; artificial mixtures formed from crude extracts of cells infected with P^r and P^s viruses gave resultant sensitivities which were simply proportional to the relative contributions of the activity from the P^r extracts (results not shown).

The yields from all mixed infections incubated in the presence of PAA were enriched for the P^r partner. The imperfect complementation manifested by this heterogeneity in the behaviour of P^r and P^s partners in mixed infections was reduced at increased multiplicities of infection, but was still significant at multiplicities of > 50. Results from measurements of infectious centres in cells infected with fractional multiplicities are entirely consistent with the expectation that viruses in a mixed infection will be distributed amongst susceptible cells as independent Poisson variables. Accordingly, the residual fraction of single infected cells could only explain the present results at relatively low multiplicities of infection (i.e. 5 to 20 with a low proportion of the P^r partner, Fig. 3) and observations of infectious centres from cells mixedly infected with approx. 2 to 4 p.f.u. of a syn and a syn + virus do not show a significant fraction of singly infected cells. Similarly, we have no clear evidence for the saturation of critical cellular sites by relatively low multiplicities; uptake of virus to the nucleus and rates of virus protein synthesis increase with increasing multiplicities up to applied multiplicities of tens of thousands of particles (i.e. hundreds of p.f.u.) per cell (R. W. Honess et al., unpublished results). These arguments all lead us to reject an underestimation of cell-to-cell heterogeneities in the distribution of P^r and P^s viruses as a complete explanation for the observations. We also eliminate the possibility that the resistant polymerase has an
intrinsically higher affinity for the homologous resistant genome since transfer of the \( P^r \) locus into a heterologous strain does not affect the enrichment of the \( P^r \) partner. Thus, mixed infections between HSV-1 [syn\(^+\).\( P^4 \)] and HSV-1 [HFEM] gave similar yields and an enrichment of the \( P^r \) partner comparable to that observed in the mixed infection between HSV-1 [HFEM] P18cl and HSV-1 [B2006]. The existence of an intracellular heterogeneity in the distribution of resistant and sensitive gene products relative to resistant and sensitive genomes therefore remains as the most likely explanation for the results. This explanation envisages a random distribution of parental genomes in the nucleus, but transport of transcripts to the cytoplasm and the return of nuclear-acting virus gene products by the shortest radial routes. Gene products returning to the nucleus would be expected to differ in their effective diffusion rates across the circumference of the nucleus due to differences in size, polymerization and differences in binding to relatively less mobile polymers (e.g. DNA). The multiplicity dependence of asymmetric complementation should therefore provide a measure of the intracellular mobility of the gene product under examination. We observed significant asymmetry in the replication of \( P^r \) and \( P^s \) genomes in the presence of PAA at combined multiplicities of infection of more than 50. This suggests that the mobility of DNA polymerase is restricted to a relatively small fraction of the nuclear volume surrounding the genome which gave rise to it.

REFERENCES

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