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Effect of Marker Distance and Orientation on Recombinant Formation in Poxvirus-Infected Cells

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Little is known about the mechanism of poxvirus recombination even though construction of recombinant viruses by recombination-dependent methods is a widely adopted technique. We have shown previously that transfected DNAs are efficiently recombined while replicating in cells infected with Shope fibroma virus. Because recombinant DNA can be recovered from infected cells as a high-molecular-weight head-to-tail concatemer, it was possible to transfect genetically marked lambda DNAs into infected cells and assay recombinants as bacteriophage particles following in vitro packaging. This approach was used in this study to examine how marker distance and marker orientation influence recombination in Shope fibroma virus-infected cells. Simple two-factor crosses were readily modelled by using a mapping function derived from classical phage studies and showed low negative interference ($I = -2.8 \pm 0.5$) in crosses involving markers >100 bp apart. More complex four- and five-factor crosses showed that the recombination frequency per unit distance was not constant (rising as the marker separation was reduced from 100 to 1 bp) and that crosses performed in poxvirus-infected cells are subject to high negative interference. One consequence is that marker orientation does not dramatically influence the outcome of most Shope fibroma virus-catalyzed crosses in clear contrast to what is observed in adenovirus or simian virus 40-infected cells. These results can be interpreted to indicate that similar statistical and physical constraints influence both viral and phage recombination and suggest that heteroduplexes may be important intermediates in the poxvirus recombination process.

Poxviruses are large mammalian DNA viruses that replicate in the cytoplasm of infected cells (6, 26). Viral genomes encode a number of proteins that modify host responses to infection and catalyze viral growth, replication, and transcription. What is curious is that, although the first linkage maps of vaccinia virus were deduced over 30 years ago (14) and the construction of recombinant poxviruses by marker rescue (27, 40) requires recombination, the mechanism of viral recombination remains obscure. It is known that inhibiting poxviral DNA polymerases blocks recombination (3, 12, 24), which supports the idea that the enzymes involved are probably early gene products (24) that play an essential role in replication (3, 12, 24). It is also known that, like other DNA viruses and bacteriophage, replicating poxviruses undergo high-frequency intermolecular and intramolecular homologous recombination (3, 12, 31). Whether host enzymes are cofactors in these reactions remains unknown. Unfortunately, attempts to isolate recombination-defective viruses have been unsuccessful (3), which has precluded a genetic analysis of the recombination mechanism.

An alternate approach to studying poxviral recombination mechanisms involves analyzing the effects of marker distance, type, and orientation on recombinant formation. In this respect a fundamental feature of poxviral recombination that remains to be determined is the influence of marker distance on recombinant formation. It is known that both very close and far-distant poxviral mutations are difficult to map by homologous recombination (11, 32, 36). A high-frequency recombination mechanism can explain the difficulty with distant markers, but it is not at all clear why close markers are so difficult to map. In this communication we present experiments designed to quantitate further the relationship between physical and genetic distances.

It had been shown previously that circular DNAs, transfected into Shope fibroma virus (SFV)-infected cells, are replicated to produce high-molecular-weight concatemers (10). It was subsequently shown with Southern blotting and *Escherichia coli* transformation assays that during replication the DNA was also recombined (12). A convenient property of SFV-infected cells is that transfected DNAs are recovered as head-to-tail concatemers which, if they were to contain a *cos* site, could be encapsidated into lambda phage particles. This property of SFV-infected cells has permitted us to cotransfect well-characterized, genetically marked lambda DNAs into infected cells and analyze the recombinant products by plaque analysis. Results from these crosses provide insights into how poxvirus-infected cells recombine point mutations and also allow us to compare viral and bacteriophage recombination processes.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. Bacterial strains are listed in Table 1. NZY medium (0.5% NaCl, 0.2% $MgCl_2 \cdot 6H_2O$, 0.5% yeast extract [Difco], 1% casein hydrolysate [GIBCO]) supplemented as needed with Bacto-Agar (Difco) was used for all phage and bacterial experiments. The mismatch repair-deficient *E. coli* strain DHE129 was constructed by P1 transduction (using P1 *cml clr-100*) of the *mutS201*::Tn*5* allele from ES1574 to K802 (25). The presence of the *mutS201*::Tn*5* mutation was confirmed by measuring the rate of mutation to streptomycin resistance.

Lambda strains containing point mutations in the *c*I gene flanked by *Nam*53 or *Oam*29 mutations were kindly provided by M. Leib (University of Southern California). A map of these mutations is shown in Fig. 1. Temperature-sensitive

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source
BHB2688	*sup*^{0} *recA* (λ *Eam*4 Δ*b*2 *red*3 *imm*^{434} c*Its Sam*7)/λ	W. Wong
BHB2690	*sup*^{0} *recA* (λ *Dam*15 Δ*b*2 *red*3 *imm*^{434} c*Its Sam*7)/λ	W. Wong
CSH56	*ara* Δ(*lac pro*) *supD nalA thi*	J. Wood
DHE129	K802 *mutS201*::Tn*5*	This work
ES1574	*his-4 argE3 leuB6 proA2 thr-1 thi-1 rpsL31 galK2 lacY1 ara-14 xyl-5 mtl-1 kdgK51 supE44 tsx-33 mutS201*::Tn*5*	R. Kolodner
K802	*hsdR hsdM*$^+$ *galT22 galK2 supE44 lacY1 metB1 mcrA mcrB rfbD1*	W. Wong
RK1036	*hsdR hsdM*$^+$ *recA1*	R. Kolodner
RK1007	*dam-3 dcm-6 thr-1 leu-6 lacY1 galK2 galT22 ara-14 tonA31 tsx-78 supE44*	R. Kolodner
SMR10	*E. coli* C-1a (λ*cos*2 Δ*B xis*1 *red*3 *gam am*210 c*Its*857 *nin*5 *Sam*7)/λ	S. Rosenberg

*c*I mutants were distinguished from wild-type *c*I$^+$ strains by plating at permissive and nonpermissive temperatures of 32 and 42.5°C, respectively. Amber mutants were distinquished from *c*I$^+$ strains by plating on *supE* or *supD* hosts. *supE* suppressed all amber mutations used here except c*Iam*504. This mutation was suppressed (albeit poorly) by *supD* (20). Phage were plaque purified two or three times, and phage stocks were prepared from confluent lysates by standard methods (9).

Bacteriophage constructions. Rearrangement of *c*I, *Nam*53, and *Oam*29 mutations was accomplished by performing phage crosses in *E. coli* essentially as described previously (9). Late-log-phage *E. coli* were grown in maltose-supplemented (0.2%) NZY broth and coinfected with a 1:1 ratio of phage at a multiplicity of infection of 20 PFU/cell. At 90 min postinfection, cells were lysed with $CHCl_3$ and plated on an appropriate selective host. Most crosses utilized K802 but because the c*Iam*6 marker is a site of action of very-short-patch repair in *E. coli* (21) the frequency of recovering c*Iam*6 recombinants in K802 was low. To avoid this c*Iam*6 crosses were performed in DHE129 since the *mutS* mutation disables very-short-patch repair (29). *Nam*53 c*Iam*6 phage were distinquished from *Nam*53 *c*I$^+$ phage by plating on CSH56 since *supD* suppresses *Nam*53 but not c*Iam*6. Double-strand DNA sequencing with the primer d(CTTGAGGACGCACGTCG) was used to check the identity of *c*I mutations used in very close crosses (see Table 4). This primer anneals to bases 37888 to 37904 in the lambda sequence (15).

Preparation of lambda DNA. Lambda DNA was prepared by the method of Yamamoto et al. (44) with minor modifications. Cleared lysates of strain K802 were prepared and debris was removed by low-speed centrifugation. Phage were precipitated with polyethylene glycol 8000 (Fisher) and centrifuged to equilibrium in CsCl (Pharmacia). The resulting phage band was recovered and dialyzed twice against 0.5 M NaCl–10 mM Tris (pH 7.5)–10 mM $MgSO_4$. Phage were phenol extracted in the presence of 20 mM EDTA–0.5% sodium dodecyl sulfate (SDS), re-extracted with $CHCl_3$-isoamyl alcohol (24:1), and ethanol precipitated. Purified DNA was redissolved in 10 mM Tris HCl [pH 8.4]–1 mM EDTA. Unmethylated DNA was prepared on RK1007, and the lack of methyl groups was demonstrated by digestion with *Eco*RV (Bethesda Research Laboratories) and resistance to *Dpn*I.

In vitro λ packaging extracts. Two-strain in vitro packaging extracts were prepared as described before (17) from BHB2688 and BHB2690 grown in NZY broth. Typical packaging efficiencies were 1×10^7 to 10×10^7 PFU/μg of ligated lambda DNA. Single-strain extracts were prepared from SMR10 (30) and packaged pure lambda concatemers with a similar efficiency. However, DNA in mixtures was packaged with much lower efficiencies in SMR10 extracts so the two-strain extracts were used for all except control experiments.

Tissue and virus culture. Rabbit SIRC cells were obtained from the American Type Culture Collection and maintained at 37°C in Dulbecco modified Eagle medium supplemented with nonessential amino acids and 10% fetal calf serum in a 5% CO_2 atmosphere. Periodic assays for mycoplasma were negative. The Kasza strain of SFV was obtained from G. McFadden. Viral stocks were grown and titers were determined on SIRC cells, on which they formed characteristic foci (43).

Transfection of virus-infected cells. Calcium phosphate precipitates were used to transfect DNA into infected cells. One day prior to transfection, 60-mm dishes were seeded with 10^6 SIRC cells. On the next day the near-confluent monolayers were washed with Dulbecco phosphate-buffered saline (PBS) and overlaid with 0.5 ml of 10^7 PFU of SFV per ml. Dishes were rocked at 22°C every 15 min for 2 h, virus was removed, monolayers were covered with 4 ml of fresh medium, and the dishes were returned to 37°C. A mixture containing 125 ng of each DNA to be transfected was prepared by the slow addition of 500 μl of 2× HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (pH 7.10) to 500 μl of DNA dissolved in 0.25 M $CaCl_2$ (42). Two hours after washing off the virus, 400 μl of this precipitate was added to each dish and left in contact with the cells for 3 h at 37°C. The calcium phosphate precipitate was removed by washing twice with EDTA (1) and once with PBS and replaced by 4 ml of fresh medium per dish. Dishes were then returned to the incubator for an additional 21 h. Time courses (data not shown) indicated that no further recombinants could be detected with additional incubation. DNA was recovered from transfected cells by adding 0.5 ml of SDS-pronase solution (10 mM Tris [pH 7.5], 10 mM EDTA, 0.5% SDS, 500 μg of pronase per ml) to the monolayer followed by overnight digestion at 37°C. DNA was then gently phenol-$CHCl_3$ extracted, ethanol precipitated, and resuspended in 100 μl of 10 mM Tris HCl–1 mM EDTA (pH 8.4) per dish.

Assaying recombinant formation. A 50-μl portion of DNA from each transfection was added to 35 μl of packaging extract and packaged in vitro in the presence of 3.5 mM ATP. Normally these phage were plated on *E. coli* K802 to determine total phage numbers and on RK1036 to select for N^+O^+ recombinants. Using the two-strain packaging method and plating on K802, we typically obtained $\sim 3 \times 10^3$ PFU/μg of recovered cellular DNA. Recombinant frequencies were calculated by averaging the results from two or three separate transfection experiments, analyzing a total

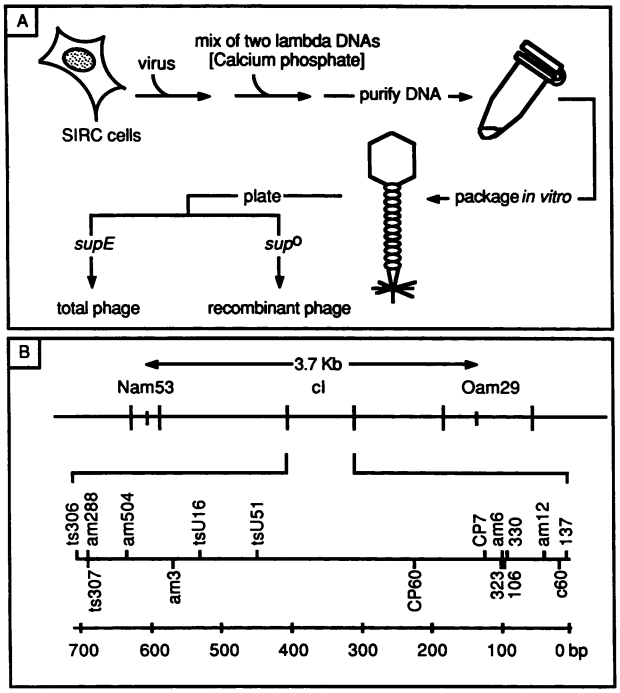

FIG. 1. Outline of the assay and mutations used to analyze the effect of distance on recombinant formation. (A) The assay in schematic form. Infected SIRC cells were cotransfected with phage lambda DNAs bearing plaque morphology or selectable mutations or both. After incubation, total cellular DNA was isolated and the transfected DNA was recovered by in vitro packaging. Resulting phage were plated on appropriate *E. coli* hosts to assay for recombinant formation. (B) Relative positions of the mutations used in this analysis. The map is oriented correctly with respect to the standard lambda map. Exact marker positions are known from sequence analysis or have been mapped by four-factor crosses.

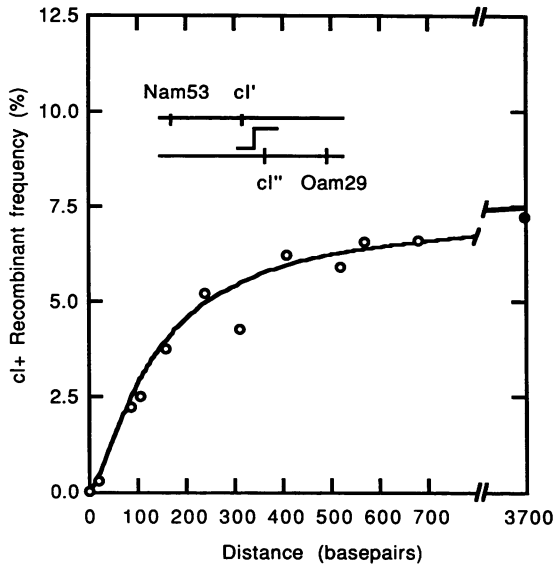

FIG. 2. Pairwise combinations of *Nam53 cI'* and *Oam29 cI"* DNAs were crossed in SFV-infected cells, using the marker configuration shown in the inset. DNA was recovered and packaged in vitro, and the frequency of cI^+ recombinants was determined by scoring turbid plaques on *E. coli* K802 (○). To extend the plot beyond the *cI* gene boundary, we also show the frequency with which recombination takes place over the 3,700-bp *N-O* interval (●). This was based on the number of N^+O^+ phage generated in such crosses relative to the total number of phage recovered. A very high frequency of recombination was visible over short physical distances, while the distance dependence was lost when marker spacing exceeded ~350 bp.

$\geq 10^3$ plaques per experiment. Close crosses associated with lower recombinant frequencies required analysis of considerably more plaques (see Table 4). It should be noted that recombinant frequencies are not necessarily recombination frequencies (33).

RESULTS

Experimental design. An outline of the method used here to detect recombinant molecules is shown in Fig. 1. The method has been used previously to detect recombinants formed in vivo and in vitro (8, 18). Transfected DNAs were recovered from infected cells and packaged in vitro, and recombinants were analyzed by plating phage on appropriate *E. coli* hosts. To ensure that the assay detected viral recombination processes, we performed a number of preliminary experiments to eliminate reversion, complementation, and recombination in the packaging extracts as sources of "recombinants."

Reversion frequencies were very low, making it possible to accurately detect low-frequency recombination events. *Nam53* reversion frequencies were calculated by transfecting infected cells with 250 ng of *Nam53* DNA, recovering the DNA at 24 h, packaging, and plating on RK1036 (N^+ phage) or K802 (total phage). The *Nam53* to N^+ reversion frequency was 0.5×10^{-4}. With an identical assay, *Oam29* reverted at a frequency of 1.1×10^{-4}. To determine the frequency of *cI* marker reversion, *Nam53 cICP7* and *cICP7 Oam29* DNAs were cotransfected and the frequency of cI^+ phage was scored among N^+O^+ recombinants on RK1036. cI^+ phage were noted at a frequency of 4.7×10^{-4}. These frequencies are several orders of magnitude lower than most of the recombinant frequencies described below.

Complementation between nonrecombinant phage was not possible considering the low multiplicity of infection used in the phage platings. We usually obtained $\sim 2 \times 10^6$ PFU of packaged phage per ml and plated 10 μl on 3×10^7 *E. coli*. This is a multiplicity of infection of $\sim 6 \times 10^{-4}$. The two-strain packaging extract used here can also contribute endogenous phage (30). We were not able to detect these phage in our extracts, and even the published concentrations are too low to provide complementing phage functions.

Since the in vitro packaging extracts were produced from whole cells, it was also possible that enzymes responsible for recombination in lambda-infected *E. coli* might recombine DNA during packaging. To test for recombination during packaging, *Nam53 cIts307* and *cIc60 Oam29* DNAs were separately transfected into SFV-infected SIRC cells, recovered, and combined in equal proportions before packaging. N^+O^+ recombinant phage were recovered at a frequency of 3.2×10^{-4} and $N^+cI^+O^+$ phage were not detectable. These values are similar to the reversion frequencies, and we conclude that in vitro recombination does not take place in these packaging extracts.

Effect of marker distance on recombinant formation in unselected crosses. To determine the effect of marker distance on the overall recombination frequency, we performed the cross illustrated in Fig. 2. These crosses were performed

in a type I configuration (Fig. 2, inset), using *c*I alleles *ts*307, *ts*U16, *ts*U51, *CP*60, *CP*7, and *c*60, and scored cI^+ phage plating on *E. coli* K802. The crosses were designed to detect any recombinants produced by an odd number of crossovers between the *c*I markers or recombinants generated by a gene conversion event involving one of the *c*I markers. Figure 2 shows clearly the high level of recombination that takes place in SFV-infected cells. The proportionality between recombination frequency and distance disappeared above ~350 bp, indicating a loss of genetic linkage over an unusually short physical distance. A limiting recombinant frequency of 6.4 ± 0.4% cI^+ phage was noted in the crosses involving *c*I markers ≥408 bp apart. This value is in good agreement with the 7.4 ± 0.7% frequency of recombination between *Nam*53 and *Oam*29 measured as N^+O^+ phage on RK1036 versus total phage on K802.

Formally, the recombinant frequencies described above should be multiplied by 2 to compensate for an inability to detect the expected reciprocal cI^- double mutants. This would be justified based on Southern blot analysis of recombinant products isolated from poxvirus-infected cells (12). Furthermore, under some circumstances one can identify all four possible *c*I genotypes by plating on *supE* or sup^0 hosts at permissive and nonpermissive temperatures. In one such cross *Nam*53 c*Its*U16 and c*Iam*6 *Oam*29 DNAs were cotransfected and N^+O^+ recombinants were selected by plating on RK1036. Plaques were picked and replated to identify +/+, c*Its*U16/+, +/c*Iam*6, and c*Its*U16 c*Iam*6 phage. We obtained 11, 23, 15, and 4 of each phage, respectively. Considering the tendency to misidentify c*Its*U16 c*Iam*6 phage, this result is consistent with the blotting data. Nevertheless, we have not routinely applied this correction since it may not apply to all crosses. Furthermore, no experimental data exist showing that viral recombination is truly reciprocal at the molecular level and we do not wish to create such an impression by mathematical manipulations.

Effect of marker distance on recombinant formation in four-factor crosses. A number of additional crosses were performed to examine more closely the effect of marker distance on recombinant formation. In particular we wanted to see whether marker effects and negative interference are features associated with SFV recombination. In these and subsequent experiments, packaged phage were plated on *E. coli* RK1036 (sup^0). The crosses were designed so that *Nam*53 and *Oam*29 parental phage would be eliminated from analysis. This selection reduced the difficulty of detecting low-frequency recombinants expected in some of these crosses since only phage that have recombined (N^+O^+ recombinants) were able to survive the screen.

Figure 3 shows the results of such an analysis. At distances in excess of 100 bp, a simple dependence of recombinant frequency on distance was noted. Averaging the results for all crosses involving markers ≥305 bp apart, a value of 0.022 ± 0.003% cI^+ recombinants per base pair of separation was obtained. One concern was that this high frequency of recombination might have been an artifact introduced by the presence of N^6-methyladenine and 5-methylcytosine in the lambda substrates. However, DNAs prepared on *dcm dam E. coli* behaved in a manner identical to methylated substrates (Fig. 3), showing that this parameter did not influence these experiments.

An interesting feature of the data shown in Fig. 3 was the observation that over short distances (<100 bp) an excess of cI^+ recombinants was recovered. This is most obvious in the upper panel, where ordinate values rose sevenfold from 0.022% recombinants bp^{-1} over long distances to >0.2% in

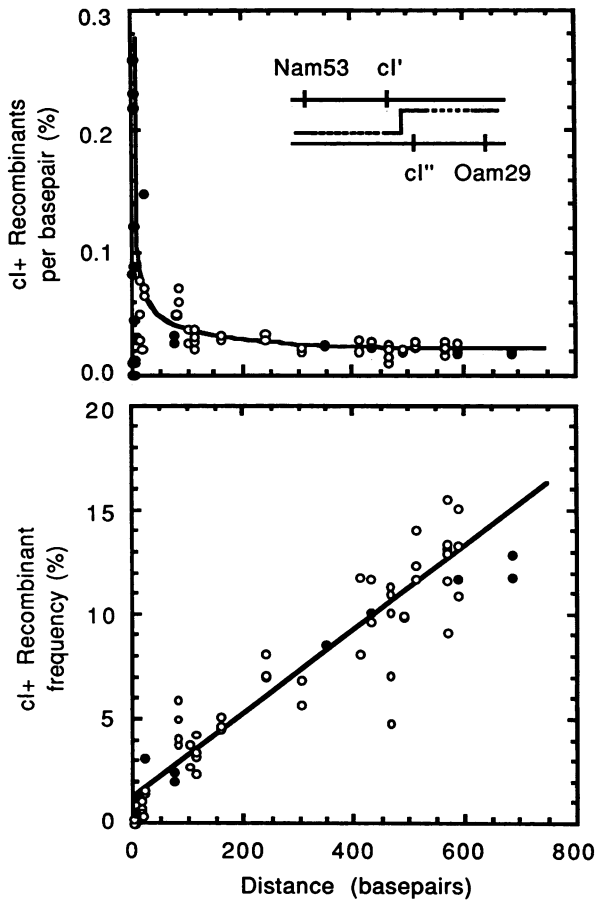

FIG. 3. Effect of marker distance on recombinant formation in selected crosses. Pairwise combinations of *Nam*53 *c*I′ and *c*I″ *Oam*29 DNAs were crossed as shown in the inset. Packaged phage were plated on *E. coli* RK1036 to select for N^+O^+ recombinants and the *c*I phenotype was determined by inspection. The lower panel shows the effect of marker distance on the recombination frequency. The upper panel shows the same data normalized per unit distance. Each point represents a different transfection analyzing ≥1,000 N^+O^+ plaques. In most crosses methylated substrates were used (○), but DNA prepared on *E. coli* RK1007 (*dam-3 dcm-6*) was also used to ensure that the presence of modified bases did not alter the recombination frequency (●).

certain crosses involving markers <30 bp apart. This was a phenomenon dependent only on base separation since *c*I alleles generating excess recombinants in short-distance crosses behaved quite normally in long crosses. For example, two separate selected crosses between c*ICP*7 and c*Iam*12 mutations located 82 bp apart generated 5.5% recombinants (range, 5.0 to 5.9%) or 0.067% recombinants bp^{-1}. Yet in all crosses at distances ≥305 bp involving c*ICP*7, we obtained 0.023 ± 0.004% recombinants bp^{-1} and in crosses involving c*Iam*12 we obtained 0.023 ± 0.003% recombinants bp^{-1}. Excess recombinants were also recovered from short crosses with a variety of *c*I alleles. These included the crosses described above as well as crosses involving c*Iam*6 × c*ICP*7 (22 bp) and c*Its*U51 × c*Its*U16 (80 bp). We concluded that this phenomenon was not due to allele-specific marker effects.

The excess of recombinants noted in short crosses held true down to separations of only a few bases. Figure 4

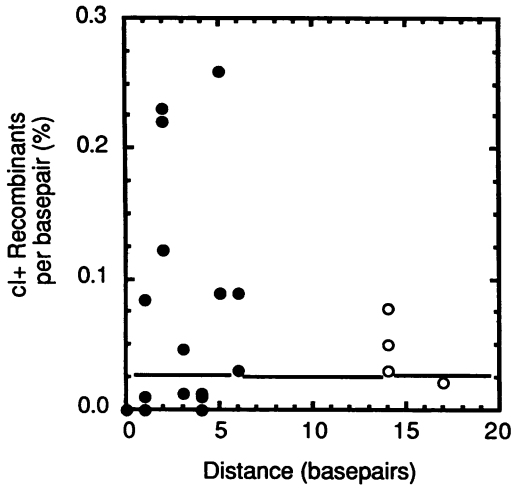

FIG. 4. Effect of very close markers on recombinant formation in selected crosses. Crosses were performed as described in the legend to Fig. 3 and the very close crosses (≤6 bp) used *c*I alleles *c*I323, *cIam6*, *c*I106, and *c*I330. Each point represents a different transfection analyzing 1,000 to 10,000 N^+O^+ plaques. The horizontal line represents the average frequency of recombinant formation when *c*I markers were separated by ≥305 bp. Symbols: ○, methylated DNA; ●, unmethylated DNA.

illustrates the behavior of markers separated by <20 bp. Recombinants were recovered from crosses involving mutations only 1 to 6 bp apart and at higher frequencies than would be expected considering the physical distance between these markers. Again, the high frequencies appear to be a property of the distance between markers rather than marker specific. For example, *cIam6* behaved quite normally in long-distance crosses with *cIts*U16, *cIam288*, and *cIts307*. The recovery of recombinants from crosses involving such close markers is perhaps the most curious feature of poxviral recombination and is in clear contrast to what is seen in a number of eukaryotic recombination systems. Recombinational collapse and map expansion are commonly observed when extremely close markers are crossed in organisms such as *Drosophila* (16) and various fungi (13), an observation usually explained by a co-correction mechanism. The absence of such effects in poxvirus-infected cells suggests that other recombinational processes may be operative.

No obvious allele-specific marker effects were noted in any of the crosses shown in Fig. 3 and 4. The only possible exception was the observation that long crosses (≥305 bp) with *cIts307* produced somewhat fewer cI^+ recombinants than might have been expected (0.019 ± 0.004% recombinants bp^{-1}; $n = 14$). However, a comparison of crosses involving *cIts307* with *cIam288* crosses failed to support this statistically. *cIts307* and *cIam288* are different mutations but both map to nucleotide 688 in the *c*I gene (22). Twelve *cIam288* crosses produced 0.024 ± 0.002% recombinants bp^{-1}, a result not statistically different from *cIts307* crosses.

Two types of negative interference characterize poxviral crosses. Negative interference is a classical phenomenon readily observed in bacteriophage crosses. It refers to the fact that, in a cross of the form $a^+b^+c^+ \times a^-b^-c^-$, the recovery of double recombinants (i.e., $a^-b^+c^-$ and $a^+b^-c^+$ genotypes) often exceeds the number expected considering the frequency of recovery of a^-b^+ and b^-c^+ genotypes. Simple inspection of the data shown in Fig. 3 and 4 suggested that poxviral recombination should display negative interference considering the observation that over distances of less than ~100 bp the normalized recombinant frequency (cI^+ recombinants per base pair) increased with decreasing marker separation. This turned out to be true, but more careful examination of crosses performed in poxvirus-infected cells showed that two different forms of interference were present.

Interference equals $1 - S$, where S is the coefficient of coincidence. Two methods can be used to calculate S depending on the availability of data from two-, four-, or five-factor crosses. We have used both methods and present the results in Tables 2 and 3. Inspection of the crosses in Table 2 showed that two different coefficients of coincidence

TABLE 2. Coefficients of coincidence observed in two- and four-factor poxviral crosses

*c*I markers			Recombinant frequency (%)							
			Unselected crosses[a]				Selected crosses[b]			
A	B	C	R_{AB}	R_{BC}	R_{AC}	S[c]	R_{AB}	R_{BC}	R_{AC}	S
*ts*U16	*CP7*	*c*60	13	5.9	12	4.5	20	6.6	25	0.6
*ts*U16	*CP7*	*am*6					20	3.0	21	1.7
*ts*U16	*CP7*	*am*12					20	11	20	2.5
*ts*307	*ts*U16	*ts*U51	7.5	4.8	10	3.2	9.7	7.9	14	2.4
*ts*307	*ts*U16	*CP7*	7.5	13	13	3.9	9.7	20	23	1.7
*ts*307	*ts*U16	*c*60	7.5	12	13	3.6	9.7	25	23	2.4
*ts*307	*ts*U16	*CP*60					9.7	13	15	3.1
*ts*307	*ts*U16	*am*6					9.7	21	22	2.1
*ts*307	*ts*U16	*am*12					9.7	20	30	0.0
*ts*307	*CP7*	*c*60	13	5.9	13	3.9	23	6.6	23	2.2
*ts*307	*CP7*	*am*6					23	3.0	22	2.9
*ts*307	*CP7*	*am*12					23	11	30	0.8
323	*am*6	106					0.04	0.18	0.06	1,100
323	*am*6	330					0.04	1.7	0.60	840
323	106	330					0.06	0.76	0.60	240
*am*6	106	330					0.18	0.76	1.7	−270

[a] Unselected cI^+ recombinant frequencies were obtained from separate two-factor crosses used to construct Fig. 2.
[b] Selected recombinant frequencies were obtained from separate four-factor crosses used to construct Fig. 3 and 4.
[c] S was calculated from the formula $S = 100 \times [R_{AB} + R_{BC} - R_{AC}]/2 \cdot R_{AB} \cdot R_{BC}$, where $R = 2 \cdot cI^+$ (%).

TABLE 3. Coefficients of coincidence observed in five-factor poxviral crosses

cI markers			Recombinant frequency (%)[a]			
A	B	C	R_{AB}	R_{BC}	R_{dbl}[b]	S[c]
*ts*U16	*ts*U51	*am*6	7.9	18	1.8	1.3
*ts*U16	*CP*60	*am*6	13	(6)	1.7	2.8
*ts*U16	*CP*7	*am*6	20	3.0	1.6	2.7
*ts*U16	323	*am*6	21	0.04	0.65	77

[a] The component recombinant frequencies (R_{AB}, R_{BC}) were obtained from four-factor crosses or extrapolated from Fig. 3 knowing the distance between cI markers (parentheses).

[b] The frequency of obtaining double recombinants (R_{dbl}) was calculated from a five-factor cross of the form *Nam*53 $cI_A cI_C \times cI_B$ *Oam*29 (where cI_B lies between cI_A and cI_C), selecting for N^+O^+ recombinants and scoring cI^+ plaques.

[c] S was calculated from the formula $S = 100 \times R_{dbl}/R_{AB} \cdot R_{BC}$, where $R = 2 \cdot cI^+$ (%).

were present. In unselected crosses, where in all cases R was greater than ~3%, S equalled 3.8 ± 0.5. Application of an N^+O^+ selection to similar crosses (where again R was greater than ~3%) reduced the interference to a value not significantly different from zero ($S = 1.9 \pm 0.9$; $I = -0.9 \pm 0.9$). In contrast, in crosses where at least one pair of markers were separated by less than ~1%, much higher S values were obtained. This result was confirmed by using a more accurate method in which S was calculated directly from experimentally determined values for R_{dbl} (Table 3). The results derived from analysis of close crosses are of particular interest since they show that poxvirus-infected cells, like bacteriophage-infected *E. coli*, contain recombination systems that display high negative interference (HNI).

Repulsion crosses. The observation that recombination frequencies are not proportional to distance over short intervals is a characteristic feature of certain types of bacteriophage crosses. One explanation proposes that recombination can proceed through intermediate splice junctions containing regions of heteroduplex at the splice site. In these circumstances the frequency of recombinant formation is dependent on the efficiency and directionality of mismatch correction as well as the likelihood of the heteroduplex extending over two mutant sites. Most importantly (in the absence of a correction bias), recombination proceeding via heteroduplex intermediates should not be sensitive to marker orientation. This is not true of crossing over. Experiments designed to test this hypothesis are illustrated in the crosses shown in Fig. 5 and Table 4.

Crosses were designed such that at least three "crossovers" were required to generate $N^+cI^+O^+$ recombinants (Fig. 5, inset), a configuration commonly referred to as a type II cross. The same markers were used as in the earlier type I crosses to rule out marker-specific effects and ensure that any observed differences between the two types of crosses were due to marker orientation alone. These experiments again showed a simple linear dependence of recombinant frequency on distance between markers (Fig. 5, lower panel). For markers ≥200 bp apart, an average of 0.012 ± 0.002% cI^+ recombinants bp^{-1} were noted and, as with crosses requiring only a single crossover, excess recombinants were recovered when the cI markers were more closely spaced. This can be seen in Fig. 5, in which a 2.8-fold increase in recombinants per base pair occurred as marker spacing decreased from 200 to 20 bp. The only difference

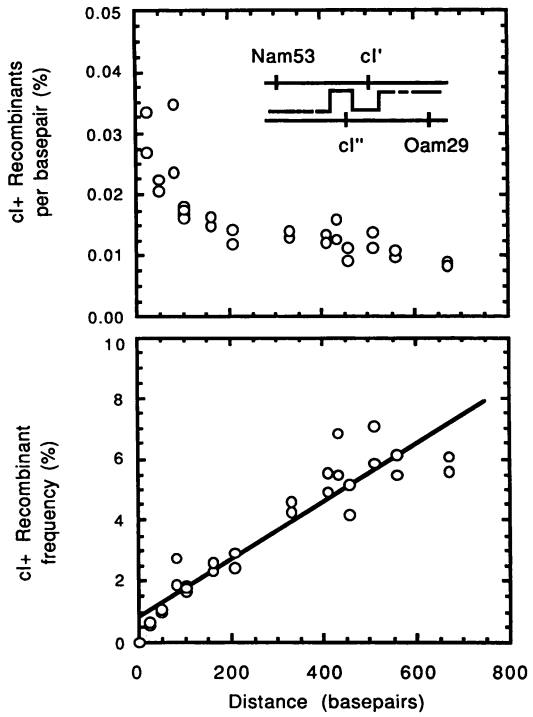

FIG. 5. Effect of marker configuration on recombinant formation. Pairwise combinations of *Nam*53 cI' and cI" *Oam*29 DNAs were crossed as shown in the inset. Packaged phage were plated on *E. coli* RK1036 to select for N^+O^+ recombinants and the cI phenotype was determined by inspection. The lower panel shows the effect of marker distance on recombinant frequency. The upper panel shows the same data normalized per unit distance. Each point represents a different transfection with ≥1,000 plaques analyzed. In all cases methylated DNA substrates were used.

between type I and II crosses was noted when type II crosses were extended to include cI markers spaced 1 to 6 bp apart. In most of these crosses cI^+ phage were not recovered in numbers much in excess of the reversion background (Table 4), which may point to a collapse of recombination under these circumstances. In general, however, the overall similarity between type I and II crosses seen in Fig. 3 and 5 is hard to reconcile with a pure crossover model of recombination.

TABLE 4. Average cI^+ recombinant frequencies for very short distance type I and II crosses

cI markers		Distance (bp)	Recombinant frequency (%)[a]			
			Type I		Type II	
A	B		R	n[b]	R	n
*am*6	323	1	0.015	13,562	<0.02	5,342
330	106	2	0.35	8,331	0.014	7,181
106	*am*6	3	0.12	5,873	0.32	939
106	323	4	0.027	10,928	0.037	8,178
330	*am*6	5	1.05	5,928	0.079	1,258
330	323	6	0.24	6,992	0.074	8,114

[a] The recombinant frequency was calculated from the formula $R = \Sigma\ cI^+$ phage/Σ (cI^+ plus cI^- phage) from all transfections.

[b] n, Number of plaques scored from all transfections.

DISCUSSION

The assay. Transfecting lambda DNA into poxvirus-infected cells provides a simple way to examine the effects of marker distance and orientation on recombinant formation. Lambda DNA is easily prepared and efficiently packaged, and many well-defined mutations are available. Plating phage rather than viruses has the advantage that greater numbers of recombinants can be examined, plaque phenotypes are usually more distinct, and more crosses can be performed in considerably less time. A particular advantage of this approach is that it obviates the need to construct complex viral strains and the experimental results can be compared directly with earlier phage experiments since both used the same markers. Of course, it should always be borne in mind that transfected DNAs may not recombine in exactly the same manner as do viruses since lambda DNA is not transcribed or packaged into nascent virions. Furthermore, heterologous DNAs may lack as yet unidentified, *cis*-acting viral sequences that serve to modulate replication and recombination.

All of the available evidence indicates that this assay measured the production of recombinants within SFV-infected cells. Reversion frequencies were severalfold lower than most recombination frequencies and indicated that the probability of generating an N^+ $c\mathrm{I}^+$ O^+ molecule from a *Nam*53 $c\mathrm{I}^-$ O^+ parent by reversion alone was $<10^{-7}$. This eliminated a high-frequency mutational process as a source of recombinants. Phage were also plated at a very low multiplicity of infection (<0.001 including endogenous phage produced by the packaging extract [30]) to avoid the production of pseudorecombinants through complementation. Although recombination has been detected in lambda packaging extracts (35), recombination occurred at the prophage attachment site and could not have generated N^+ O^+ or $c\mathrm{I}^+$ recombinant molecules. The absence of other recombination activities in the packaging extracts was demonstrated by combining multimeric *Nam*53 *cIts*307 O^+ and N^+ *cIc*60 *Oam*29 DNAs in a single packaging extract and assaying for recombinant phage production. Since N^+ O^+ phage were detected at levels no different from reversion frequencies and N^+ $c\mathrm{I}^+$ O^+ recombinants were not recovered at all, we concluded that general homologous recombination is undetectable in these extracts.

Effect of marker distance on unselected crosses. Examination of the data shown in Fig. 2 suggested that a loss of genetic linkage occurred once marker separations exceeded ~350 bp with a limiting recombinant frequency of 6.4 ± 0.4% $c\mathrm{I}^+$ phage. Within the region of the curve where recombinant frequency was sensitive to distance, ~0.02% $c\mathrm{I}^+$ recombinants bp^{-1} were recovered. If we assume that this is a reciprocal process, the value of 0.04% recombinants bp^{-1} is about 4- to 40-fold higher than that observed in vaccinia virus crosses (0.006% [27a]; 0.001 to 0.003% [32]) and is in good agreement with previous estimates that recombinant frequencies in SFV-infected cells were ~5-fold higher than in vaccinia virus-infected cells (12). Although variations in experimental conditions make comparisons different, recombinant frequencies in SFV-infected cells are comparable to those observed in phage-infected bacteria. By way of comparison, two-factor crosses in T4- and lambda-infected *E. coli* yielded values of 0.014 to 0.043% and 0.013 to 0.056% recombinants bp^{-1}, respectively (6).

An intriguing feature of the unselected crosses is the excellent agreement between the data and classical observations derived from studies of *E. coli* bacteriophage. The curve drawn in Fig. 2 was based on a modified mapping function originally used to rectify lambda crosses (33). For a 1:1 ratio of parental DNAs and with rounds of mating equal to 1, one can derive the mapping function: $r = 0.25\ g\ [1 - e^{-0.5(1\ -\ e^{-2x})}] \cdot 100\%$ (equation 1), where r is the $c\mathrm{I}^+$ recombinant frequency, g is the finite input factor [also called F(p) (19)], and x is the mean number of exchanges or genetic map distance. Values for x and g were iteratively adjusted to generate the curve shown in Fig. 2. Best fits were obtained assuming $x = d/500$ (where d is the marker separation in base pairs) and $g = 0.76$, generating a limiting $c\mathrm{I}^+$ recombinant frequency of 7.5%. The values of x and d imply that one exchange occurred per 500 bp, and from the value of g it can be calculated that the "multiplicity of infection" was between 4 and 5 (19).

The latter value is a curious number. The parameter g reflects the fact that in phage crosses there are physical limits to the number of particles that can coinfect a cell. Under these circumstances statistical considerations imply that, while all cells may be infected and produce progeny, not all cells are infected by both parents. A practical consequence is that recombination frequencies are reduced from 50% since some infected cells contribute progeny while not contributing recombinants. What is difficult to reconcile is the fact that calcium phosphate transfection methods probably introduce far more than four or five lambda genomes into a cell, yet this analysis implied that only four or five genomes participated in the recombination process. Why? One intriguing possibility relates to the fact that poxviruses replicate (and probably recombine) in factories in the cytoplasm of infected cells (26). There may be restrictions on the amount of exogenous DNA that can enter a factory, and it is these restrictions which limit the multiplicity of infection of transfected genomes. If this is correct, the limit would be approximately 190 to 240 kb (4 × 48.5 to 5 × 48.5 kb). The result is speculative but it does partly explain why smaller plasmids recombined at higher frequencies in a previous study (12).

Effect of distance on recombination in four-factor crosses. Selection for flanking marker exchange generated plots showing an approximately linear dependence of recombinant frequency on *c*I marker distance (Fig. 3, lower panel). The result was expected since this selection eliminated some of the statistical and probabilistic considerations that complicate two-factor viral crosses and reduce the frequency of recombinant recovery. The slope of this curve (0.022 ± 0.003% $c\mathrm{I}^+$ recombinants bp^{-1}) was similar to that observed over short distances in two-factor crosses.

Further inspection of Fig. 3 showed that normalized recombinant frequencies were not constant in all crosses. In crosses involving markers 14 to 100 bp apart, more $c\mathrm{I}^+$ phage were recovered than would be expected considering the marker distances. This is most clearly seen in Fig. 3 (upper panel), where the number of recombinants increased from 0.022 to >0.2% bp^{-1} in very close crosses. This was a distance- rather than marker-dependent phenomenon and superficially looked much like classical negative interference. Further crosses (Tables 2 and 3) confirmed that both low negative interference (LNI) and HNI are seen in poxvirus-associated crosses.

Interference in poxvirus crosses. Analysis of the data from unselected crosses involving *c*I markers >100 bp apart showed $I = -2.8 \pm 0.5$ (Table 2). This has the same magnitude ($I = -2$ to -5) and probably arose from the same factors (nonrandom mating and exclusion of some infecting DNA molecules from the replication-recombination pool [2,

5]) that create LNI in phage crosses. Support for this comes from the observation that equation 1, which derives from models incorporating these factors, predicts $I = -2.5$ if one substitutes various values for $d > 100$ bp (33). Furthermore, it would be expected that selection for flanking marker exchange should eliminate these contributions to LNI and this was indeed the case. In N^+O^+ selected crosses, LNI disappeared from both four- and five-factor *c*I crosses.

A second type of interference appears as a clustering of many genetic exchanges over short intervals and has been called HNI. This type of interference is characterized by values of $I \leq -5$ and can be distinguished from LNI by its magnitude and its distance dependence: HNI is only observed in crosses between very close markers. Localized negative interference (or HNI) was first studied extensively by Pritchard (28) in the fungus *Aspergillis*. Since that time it has been observed in a variety of bacteriophage including T2 (34), T4 (5), and lambda (2), but rarely in plants and animals. HNI can be explained in different ways. White and Fox (41) proposed that HNI in lambda crosses is the result of a two-step process. In the first step, a long heteroduplex is formed overlapping close markers but possibly recombinant for flanking markers. The second step involves a reduction to homozygosity by (probably biased) mismatch correction. An alternate explanation has been proposed to explain HNI in T4 crosses whereby end invasion during replication creates multiple events over a short genetic interval (23).

Inspection of Fig. 3 (upper panel) and 4 shows that, for close markers (<100 bp), recombinants were recovered in excess over what would be expected considering the distance. That this is HNI was formally demonstrated by the data presented in Tables 2 and 3, which show that in crosses involving at least one pair of close markers very high negative interference is commonly observed. The best illustration derives from the five-factor cross *Nam*53 *cIts*U16 *cIam*6 × *c*I323 *Oam*29, where $I = -77$ (Table 3). Similar results can be calculated from four-factor crosses (Table 2), although the need to base I on a small difference between two large numbers makes this method quite a bit less accurate and sometimes generated unrealistically high values for I.

This appears to be one of the few demonstrations that HNI is associated with viral crosses. A simple proportionality between recombination frequency and distance was noted in both SV40 and adenovirus crosses (37, 39), although the closest markers used in these experiments (~270 bp) may not have been close enough to detect HNI. Only in the case of vaccinia virus do certain crosses indirectly suggest the existence of HNI. Sridhar and Condit (32) used three-factor crosses to order close mutations in the vaccinia DNA polymerase gene. The result was found to be at variance with DNA sequencing (36), which led Traktman et al. to suggest that in close crosses multiple exchanges prevent the correct ordering of alleles. This is a characteristic feature of HNI and suggests that HNI is not restricted to the transfection assay described here but may influence poxviral crosses in general.

Mechanism of poxvirus recombination. Any model of poxviral recombination must explain a number of experimental observations. These include the failure to recover 50% recombinants, LNI and HNI, lack of marker effects, and the high efficiency with which cI^+ recombinants can be recovered from very close crosses.

The failure to recover 50% recombinants as well as LNI are both readily explained by models deduced from the analysis of phage recombination. Both phenomena are a consequence of statistical considerations associated with the odds of getting two different parental DNAs into an infected cell and the likelihood that these DNAs interact in a way that eventually generates recombinants. In mammalian cells, as in bacteria, there appear to be limits as to how much transfected DNA is available for recombination within the cell. The presence of cells lacking equal amounts of both parental DNAs as well as mating paths that eventually fail to produce recombinants contribute to a reduction in the overall recombination frequency and LNI.

HNI is more difficult to explain and it is possible that it arises from more than one source. One explanation is that HNI in poxviral crosses arises from clustered strand invasions, one of the events thought to contribute to HNI in T4 crosses. Poxviruses lack an identified origin of replication, and it is tempting to propose that priming of replication through random strand invasion circumvents this difficulty while also creating interference. However, the genomes of T4 phage and poxviruses are organized in very different ways, and the highly branched T4 replication intermediates look nothing like the linear concatemers found in poxvirus-infected cells. Both of these observations tend to cast doubts on the hypothesis that HNI seen in poxvirus-associated crosses is related to the HNI seen in T4 crosses.

HNI is most readily explained by proposing that SFV recombination proceeds through formation of short heteroduplex intermediates accompanied by exchange between duplexes (crossing over). (That the mechanism involves true crossing over has never been formally demonstrated, but it is difficult to picture how marker rescue [27, 40] or plasmid-×-virus experiments [3, 31] can proceed without such a step.) There are then several ways in which this heteroduplex could generate HNI in our assay. It may be repaired to cI^+ in the infected cells. Alternatively, heteroduplex DNA can be packaged into phage particles which may or may not be repaired in *E. coli*. Its worth nothing that failure of repair in *E. coli* produces mottled plaques which we have scored here as cI^+ phage because of the difficulty in differenting mottled from pure cI^+ plaques without additional replating. We have, in fact, observed that a significant fraction (~2 to 3%) of all phage recovered from such transfections contain cI^+/cI^- heteroduplexes (Parks and Evans, unpublished data), which strongly supports the contention that heteroduplex is formed during poxvirus-catalyzed recombination.

If this hypothesis is correct, the length of the heteroduplex "splice/patch" cannot be very long. Excess recombinant recovery disappeared by 100 bp, suggesting that few tracts exceed that length. A lower bound can be estimated by nothing that, when marker spacing is much less than the minimum heteroduplex tract length, recombination frequencies should become independent of distance. Inspection of Fig. 4 shows this is probably true for markers ≤6 bp apart, although the experimental noise at such low frequencies makes an exact boundary difficult to determine. Thus, if a heteroduplex tract is involved in recombinant formation, the average length of the tract lies between 10 and 100 bp. This is quite short compared with average heteroduplex tract lengths for *E. coli* (3,000 bp [38]), *Saccharomyces* (1,500 bp [4]), and *Drosophila* (1,200 bp [7]).

Poxviruses do not recombine like adenovirus or SV40. The similarities between SV40 and adenovirus recombination, despite the differences in biology, led Volkert and Young (37) to suggest that recombination of these viruses was partly dependent on cellular rather than viral gene products. Interestingly, these similarities are not shared with poxviruses. This is best illustrated by comparing the results in Fig. 3 and

5 with the data from adenoviral (37) and SV40 (39) crosses. Although the substrates are different, all three studies selected for recombinants that had undergone flanking-marker exchange and all three investigated the effect of internal marker orientation on internal marker recombination frequencies. Examination of Fig. 3 and 5 shows that in SFV-infected cells it made little difference whether one or three crossovers was required to generate a cI^+ recombinant. In type II crosses cI^+ recombinant recovery was reduced only twofold compared with type I crosses. In contrast, recombinant recovery was dramatically reduced in type II crosses in adenovirus- and SV40-infected cells. This difference between small, nuclear, host-dependent viruses and the cytoplasmic poxviruses points to the existence of a distinct poxviral recombination pathway. It would not be surprising if some of the gene products required to catalyze poxvirus recombination are virus encoded.

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