Heteroduplex DNA Formation Is Associated With Replication and Recombination in Poxvirus-Infected Cells

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ABSTRACT

Poxviruses are large DNA viruses that replicate in the cytoplasm of infected cells and recombine at high frequencies. Calcium phosphate precipitates were used to cotransfect Shope fibroma virus-infected cells with different DNA substrates and the recombinant products assayed by genetic and biochemical methods. We have shown previously that bacteriophage lambda DNAs can be used as substrates in these experiments and recombinants assayed on *Escherichia coli* following DNA recovery and *in vitro* packaging. Using this assay it was observed that 2–3% of the phage recovered from crosses between point mutants retained heteroduplex at at least one of the mutant sites. The reliability of this genetic analysis was confirmed using DNA substrates that permitted the direct detection of heteroduplex molecules by denaturant gel electrophoresis and Southern blotting. It was further noted that heteroduplex formation coincided with the onset of both replication and recombination suggesting that poxviruses, like certain bacteriophage, make no clear biochemical distinction between these three processes. The fraction of heteroduplex molecules peaked about 12-hr postinfection then declined later in the infection. This decline was probably due to DNA replication rather than mismatch repair because, while high levels of induced DNA polymerase persisted beyond the time of maximal heteroduplex recovery, we were unable to detect any type of mismatch repair activity in cytoplasmic extracts. These results suggest that, although heteroduplex molecules are formed during the progress of poxviral infection, gene conversion through mismatch repair probably does not produce most of the recombinants. The significance of these observations are discussed considering some of the unique properties of poxviral biology.

HETERODUPLEX DNAs are widely believed to be key intermediates in the production of recombinant molecules. The idea that heteroduplex molecules might be formed during replication and recombination was first deduced from genetic experiments involving T-even phage (LEVINTHAL 1954) and then incorporated into the early recombination models of WHITEHOUSE (1963) and HOLLIDAY (1964) in an attempt to rationalize certain properties of the recombinants recovered from fungal meioses (reviewed in ORR-WEAVER and SZOSTAK 1985; HASTINGS 1988). Since then, heteroduplex intermediates have been invoked to explain a diversity of phenomena ranging from high negative interference in bacteriophage crosses (WHITE and FOX 1974) to the response of site-specific recombination systems to mutant target sites, and most recombination models proposed over the last few decades require that heteroduplex DNAs, of varying length, be formed during the recombination process (MESELSON and RADDING 1975; NASH *et al.* 1981; SZOSTAK *et al.* 1983). Many of these genetic experiments have been complemented by biochemical studies of the properties of artificial heteroduplexes. These DNAs have provided insights into the structure of mismatched helices (PATEL *et al.* 1984) and have been used to demonstrate that heteroduplex DNAs can be repaired by mismatch-repair systems present in organisms as diverse as bacteria (CLAVERYS and LACKS 1986; MODRICH 1987), *Saccharomyces cerevisiae* (BISHOP *et al.* 1987), and mammalian cells (WEIBAUER and JIRICNY 1990). A recent study provided an interesting fusion of these genetic and biochemical methods wherein denaturant gel methods for the direct detection of mismatched DNA were used to confirm the genetic deduction that heteroduplexes are formed, and sometimes repaired, during meiotic recombination in *S. cerevisiae* (LICHTEN *et al.* 1990).

We have been investigating the mechanism by which poxviruses recombine DNA. Poxviruses are large (>160 kb) DNA viruses that replicate nearly autonomously in the cytoplasm of infected cells (HRUBY, GUARINO and KATES 1979) and catalyze very high frequencies of replication and recombination, *in trans*, in a DNA sequence-independent manner (DELANGE and MCFADDEN 1986; EVANS, STUART and MCFADDEN 1988). Like most other eucaryotic viruses, the mechanism of poxviral recombination is not well understood nor has the role of host and viral gene products in recombination been clearly established. Bearing in mind the association between heteroduplex

formation and recombination summarized above we previously suggested that mismatched DNA was also formed during recombination in poxvirus-infected cells because such an intermediate might account for the high negative interference noted in certain crosses (PARKS and EVANS 1991). Here we show, directly, that poxviral recombination systems do catalyze formation of heteroduplex DNA and thus extend the range of organisms in which heteroduplex formation appears linked with genetic recombination.

MATERIALS AND METHODS

Bacteriophage lambda methodology: Bacteriophage lambda strains containing point mutations in the *c*I gene flanked by *Nam*53 or *Oam*29 mutations were obtained from M. LIEB. Rearrangement of *c*I, *Nam*53, and *Oam*29 mutations was accomplished by performing crosses in *Escherichia coli* essentially as described (DAVIS, BOTSTEIN and ROTH 1980). Bacteriophage lambda DNA was prepared from cleared lysates by polyethylene glycol precipitation followed by CsCl density gradient purification, phenol:$CHCl_3$ extraction and ethanol precipitation (YAMAMOTO *et al.* 1970). Double-strand DNA sequencing and custom synthetic oligonucleotides were used to determine the sequence of *c*I mutations employed in Table 1. *In vitro* packaging used two-strain packaging extracts prepared from *E. coli* strains BHB2688 and BHB2690 (HOHN 1979). Packaged phage were plated on *E. coli* strains K802 (*hsdR*$^-$ *hsdM*$^+$ *galT*22 *galK*2 *supE*44 *lacY*1 *metB*1 *mcrA*$^-$ *mcrB*$^-$ *rfbD*), DHE128 (K802 *mutS*201::Tn*5*) or RK1036 (*thyA*$^+$ *hsdR*$^-$ *hsdM*$^+$ *recA*1). To minimize the problem of coincident plaques in heteroduplex assays we tried to limit the number of plaques to about 100 per plate at which it has been estimated that the probability of superposition would be about 10^{-3} (KELLENBERGER, ZICHICHI and EPSTEIN 1962).

Plasmids and plasmid purification: Plasmids pMJ76 and pMJ77 were obtained from M. LICHTEN and were originally constructed by N. SCHULTES. These were remonomerized, transformed separately into strain RK1400 (DOHERTY, MORRISON and KOLODNER 1983), and subsequently purified using a large-scale alkaline lysis procedure followed by centrifugation to equilibrium on ethidium bromide/CsCl density gradients. Plasmid pRDK41 (DOHERTY, MORRISON, and KOLODNER 1983) was prepared in a similar manner.

Heteroduplex DNA preparation: Most of the M13 mp11 phage derivatives used for the construction of heteroduplex substrates were obtained from R. KOLODNER and propagated using standard methods (MESSING 1983). M13 mp19-PRMP was constructed by inserting a 401-bp fragment of DNA encoding a partial cDNA copy of the influenza A/Puerto Rico matrix protein gene into the *Sma*I site of M13 mp19. M13 phage were propagated on *E. coli* strain RS5033 [(PO of *HfrH*) *metB*1 *rel-1 str-400 azi-7 lacM*5286 *f80dII thi-1 lacBK*1 *dam-4*] to avoid N^6-deoxyadenosine methylation. Form I heteroduplex DNAs were prepared as described by MUSTER-NASSAL and KOLODNER (1986).

Tissue culture and virological methods: The Leporipoxvirus Shope fibroma virus (SFV strain Kasza) and the Orthopoxvirus vaccinia (WR strain) were obtained from G. MCFADDEN. SFV was propagated on rabbit cornea SIRC cells (American Type Culture Collection) in DMEM (GIBCO) supplemented with nonessential amino acids (GIBCO) and 10% fetal calf serum (Boknek) at 37° in a 5% CO_2 atmosphere. Vaccinia was propagated in spinner culture on HeLa cells (American Type Culture Collection) in SMEM (GIBCO) supplemented with nonessential amino acids and 5% horse serum (Flow). Antibiotics were not used for maintenance passages and periodic tests for mycoplasma were negative.

Heteroduplex and recombinant detection: Conditions required to resolve heteroduplex DNAs were identified using denaturing-gradient polyacrylamide gels as described by MYERS, MANIATIS and LERMAN (1987). The 300-bp *Sac*I-*Eco*RV fragments from pMJ76 and pMJ77 were recovered from low melting agarose (SeaKem), treated with calf-intestinal phosphatase, and end-labelled using polynucleotide kinase and [γ-^{32}P]ATP. Artificial heteroduplexes were prepared by denaturing labeled fragments in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA at 98° for 2 min followed by cooling to 20° at 0.4° min^{-1}. Polyacrylamide gels (11%) were run at 60° at 60 V for 17 hr in a Bio-Rad Protean II assembly with buffer recirculation. Temperature was maintained by circulating hot water through the assembly core.

In order to detect *in vivo* heteroduplex formation 60 mm dishes of SIRC cells were infected with SFV at a multiplicity of infection of five and then transfected with pMJ76 and/or pMJ77 using calcium phosphate as described previously (PARKS and EVANS 1991). At appropriate intervals dishes were washed and the monolayers lysed with a sodium dodecyl sulfate/pronase mixture. Released DNA was then sheared to reduce the viscosity, purified, and digested with *Sac*I and *Eco*RV. Twenty μg of restricted DNA was subjected to electrophoresis at 38% fixed denaturant concentration. DNA fragments were electroblotted to modified nylon (Zetaprobe, Bio-Rad) and hybridized at 60° with the 300-bp ^{32}P-labeled *Sac*I-*Eco*RV fragment according to Bio-Rad's suggestions. Probe DNA was prepared by labeling the *Sac*I-*Eco*RV fragment with random primers and [α-^{32}P]dATP (FEINBERG and VOGELSTEIN 1983).

Recombinants recovered from cells transfected with pRDK41 were detected by digesting recovered DNA with *Xho*I, separating the digestion products on a 0.8% agarose gel and Southern blot analysis performed using ^{32}P-labeled pRDK41 probe exactly as described (EVANS, STUART and MCFADDEN 1988). Newly replicated DNA was detected by digesting the recovered DNA with both *Xho*I and *Dpn*I prior to electrophoresis and blotting.

Heteroduplex incision, DNA polymerase and DNA repair assays: SFV extracts were prepared by infecting 150-mm dishes of SIRC cells with SFV at a multiplicity of infection of ten and whole-cell extracts prepared 14 hr postinfection using the method of DARBY and BLATTNER (1984). (This time corresponds to 12 hr post-transfection in transfection experiments.) Nuclei were removed by centrifugation at 600 × *g* for 10 min. Cytoplasmic protein and viral replication complexes ("factories") were precipitated by addition of 50% saturated $(NH_4)_2SO_4$ followed by centrifugation at 15,000 × *g* for 10 min. After careful removal of all the supernatant, extracts were either used immediately or frozen in liquid N_2 and stored at −70°. DNA polymerase assays were performed essentially as described by EVANS and LINN (1984). Mismatch-repair reactions (150 μl) contained 15 μg protein, 3.2 μg heteroduplex DNA and salts (MUSTER-NASSAL and KOLODNER 1986). Reactions were incubated for 60 min prior to recovery of DNA and digestion with *Cla*I plus the appropriate restriction enzyme. Digested DNA was then subjected to electrophoresis on 0.8% agarose gels, stained with ethidium bromide, and photographed. To assay for mismatch-specific incision the DNA was recovered from repair reactions, restricted with *Cla*I, denatured, and annealed to a −40 M13 sequencing primer. Radioactive

TABLE 1

Enhanced recovery of mixed infective centres on *mutS E. coli*

Cross[a]	K802				K802 *mutS*::Tn*5*			
	Clear (cI^-)	Turbid (cI^+)	Mixed ($cI^{+/-}$)	Het[b] %	Clear (cI^-)	Turbid (cI^+)	Mixed ($cI^{+/-}$)	Het %
*ts*307 × *ts*U51	1170	89	11	0.9	870	72	28	2.9
*ts*307 × *ts*U16	2040	90	10	0.5	1300	74	26	1.9

[a] DNA sequencing showed *ts*307 to be a T → G transversion at map position 37252, *ts*U51 a G → A transition at position 37480, and *ts*U16 an A → G transition at position 37402 (data not shown).
[b] Het = (mixed)/(mixed + turbid + clear) × 100%.

runoff copies were then prepared and analyzed as described by EVANS and KOLODNER (1988).

RESULTS

Heteroduplex phage lambda recombinants: Our previous study was based upon the ability to transfect genetically marked bacteriophage lambda DNAs into cells infected with SFV (PARKS and EVANS 1991). Following replication and recombination the DNA was recovered as high molecular weight head-to-tail concatemers and packaged, *in vitro*, into infectious phage particles as originally proposed by KOBAYASHI and IKEDA (1978). These phage can be plated on appropriate *E. coli* hosts and recombinants analyzed on the basis of plaque morphology phenotype using mutations located in the phage *c*I gene (LIEB, ALLEN and READ 1986). Although it was obvious from a variety of controls that recombinant formation required infecting virus and was not a product of the packaging extracts, it remained possible that DNAs recovered from infected cells contained heteroduplex that was subsequently exposed to repair by *E. coli* mismatch-repair systems. This would be expected to occur with varying efficiencies (depending upon the specific mismatch) and without strand bias in the absence of *dam*-methylated GATC sites (DOHET, WAGNER and RADMAN 1985) except for crosses involving very small patch (VSP) repair markers (LIEB, ALLEN and READ 1986).

To further investigate this hypothesis, packaged phage were plated on wild-type or *mutS* cells since the *mutS* mutation inactivates both the *dam*-directed and VSP repair systems of *E. coli* (RADMAN 1988). A number of plaques were observed on both cell types which, on close inspection, displayed the mottled phenotype characteristic of heteroduplex phage infections. Because of the difficulty in distinguishing turbid (cI^+) from mottled ($cI^{+/-}$) infections all the turbid-appearing plaques were picked and replated in order to accurately determine the fraction containing both cI^+ and cI^- phage. This fraction provided an estimate of the heteroduplex frequency. The data presented in Table 1 show clearly that a significant proportion of the phage recovered from SFV-infected cells were heteroduplex at at least one of the two mutant *c*I sites entering the cross. When phage were plated on mismatch-repair-proficient *E. coli* between 0.5% and 0.9% of all phage formed heterogeneous plaques and this fraction increased to 2–3% when plated on *mutS* cells. This was a sufficiently large effect that when these heteroduplex phage were plated on *E. coli* and misscored as "cI^+ recombinants" we observed that the apparent recombinant frequency changed depending upon host repair proficiency (Table 2). It can be seen in Table 2 that when no attempt was made to differentiate mottled from turbid plaques the recombinant frequency was ~1.5-fold higher on *mutS* cells. This was presumably due to the conversion in repair-pro-

TABLE 2

Enhanced recovery of heteroduplex phage on *mutS E. coli*

Cross	Distance (bp)	K802			K802 *mutS*::Tn*5*		
		Clear (cI^-)	Mottled + turbid ($cI^{+/-}$ and cI^+)	R[a]	Clear (cI^-)	Mottled + turbid ($cI^{+/-}$ and cI^+)	R
cIcp7 × *cIcp7*	0	650	0	0	849	2	0.2
cIcp7 × *cIc60*	102	581	6	1.0	813	12	1.5
cIts307 × *cItsU51*	238	682	10	1.5	576	30	5.0
cItsU16 × *cIcp60*	305	413	14	3.3	431	20	4.4
cItsU16 × *cIcp7*	408	236	10	4.1	339	16	4.5
cItsU16 × *cIam12*	490	353	13	3.6	298	18	5.7
cIts307 × *cIc60*	668	644	16	2.4	649	39	5.8

[a] R = (mottled + turbid)/(mottled + turbid + clear) × 100%.

ficient cells of some $cI^{+/-}$ phage back to a parental cI^{-} phenotype with concomitant loss of a "cI^{+}" class of "recombinants." Interestingly this effect was not dependent upon any particular choice of marker (Table 2) and suggested that, not only can heteroduplex be formed in infected cells, but can be formed at a variety of sites.

Denaturing gel analysis of heteroduplex DNA: The approach described above was complicated by the need to infer the existence of heteroduplex from an indirect genetic analysis. It is, however, possible to directly detect the presence of heteroduplex sites using methods developed by LERMAN and co-workers (FISHER and LERMAN 1983). Under the appropriate conditions of denaturant and temperature, less stable heteroduplex containing DNA fragments migrate more slowly than homoduplex DNAs on polyacrylamide gels and thus can be differentiated (MYERS, MANIATIS and LERMAN 1987). This approach has been used to investigate the formation of heteroduplex during *S. cerevisiae* recombination at the ARG4 locus (LICHTEN *et al.* 1990). Separation of heteroduplex from homoduplex fragments is readily achieved under a fairly wide range of denaturant concentrations at 60° (Figure 1). Using artificial heteroduplexes prepared by annealing ARG4 and arg4(*Nsp*I^{-}) clones we found that 60 ± 2° and 38% denaturant provided a good separation of DNA fragments containing G/G and C/C mismatches (Figure 1) and these conditions were used in subsequent experiments designed to detect heteroduplex products formed during SFV-catalyzed recombination.

Plasmids containing the ARG4 and arg4(*Nsp*I^{-}) alleles cloned as *Pst*I fragments in pMLC12 were transfected separately or together into SFV-infected cells. After recovering the cellular, viral, and transfected DNA mixture, the region containing the mutant and wild-type alleles was excised as a 300-bp *Sac*I-*Eco*RV fragment, subjected to electrophoresis on a partially denaturing gel, electroblotted, and probed with a ^{32}P-labeled *Sac*I-*Eco*RV fragment. Examination of the autoradiograph showed clearly the formation of heteroduplex products of both the C/C and G/G types (Figure 2). Heteroduplex formation appeared dependent upon viral infection and required the presence of both parental plasmids. Inspection of the autoradiograph also showed that heteroduplex molecules formed a significant proportion of the *Sac*I-*Eco*RV fragments recovered from infected cells and no bias in favour of one of the heteroduplex types was seen since densitometer traces showed that G/G and C/C heteroduplexes were recovered with equal efficiencies (data not shown).

Timing of heteroduplex formation: A close relationship between viral replication and recombination has been seen in cells infected with vaccinia virus (BALL 1987; MERCHLINSKY 1989), SFV (EVANS, STUART and MCFADDEN 1988) or a number of other viruses and phage (LEVINTHAL 1954; LUDER and MOSIG 1982; YOUNG *et al.* 1984; WEBER *et al.* 1988).

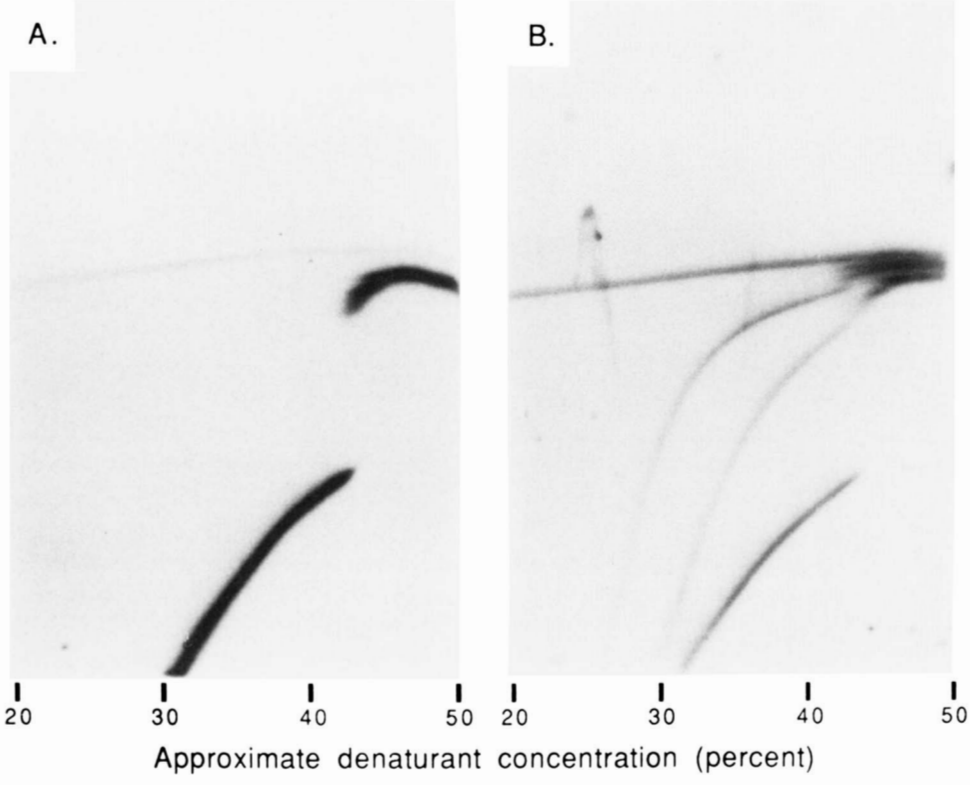

FIGURE 1.—Electrophoretic behavior of 300-bp heteroduplex and homoduplex *Sac*I-*Eco*RV fragments derived from plasmids pMJ76 and pMJ77. Duplicate polyacrylamide gels were prepared containing linear gradients of formamide and urea. End-labeled homoduplex (panel A) or heteroduplex (panel B) DNAs were applied to the top of each gel and subjected to electrophoresis as indicated in MATERIALS AND METHODS. Two novel species migrating slowly in the heteroduplex sample can be seen. These are heteroduplex DNAs containing C/C and G/G mismatches of which the more slowly migrating is the C/C heteroduplex (LICHTEN *et al.* 1990). In both panels A and B the species migrating slowest (and insensitive to denaturant) is single-stranded DNA and the species migrating fastest is homoduplex.

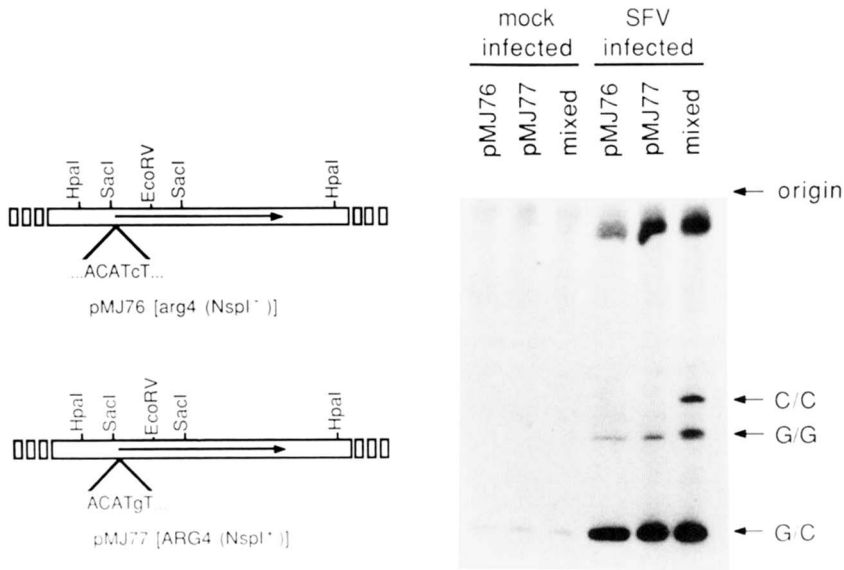

FIGURE 2.—Formation of heteroduplex molecules during poxviral recombination *in vivo*. Virus- or mock-infected cells were transfected with plasmids containing the clones shown at left. These differ by a single G to C transversion at position 552 (GenBank entry YSCARG4) in the cloned region of the *S. cerevisiae* ARG4 locus. After DNA recovery the 300-bp fragment spanning this site was released by digestion with *Sac*I and *Eco*RV and the DNA mixture subjected to electrophoresis on a fixed 38% denaturant gel. Resolved fragments were electroblotted to modified nylon and detected by Southern blotting. In the presence of SFV two new heteroduplex bands can be seen migrating at a position predicted from the results obtained with artificial heteroduplexes (Figure 1). Formation of heteroduplex fragments required the presence of both plasmids (lane 6, "mixed") and was not seen in mock-infected cells (lane 3). (The faint band migrating in all three lanes slightly ahead of the G/G heteroduplex species is probably due to incomplete *Eco*RV digestion.)

How does the timing of heteroduplex formation correspond to the time of onset of replication and recombination in poxvirus-infected cells? Parallel dishes of infected cells were transfected with appropriate plasmid substrates and DNA recovered for analysis at various times post-transfection. Three different assays were then used to monitor heteroduplex formation, DNA replication, and recombination. Heteroduplex formation in cells cotransfected with pMJ76 and pMJ77 was determined using the denaturing gel method described above. Recombination was assayed using cells transfected with pRDK41 which contains *Xho*I linker insertions at different sites in duplicated copies of the pBR322 tetracycline gene (DOHERTY, MORRISON and KOLODNER 1983). One can follow virus-mediated recombination between these sites by looking for the presence of novel *Xho*I restriction fragments on Southern blots (EVANS, STUART and MCFADDEN 1988). Finally, newly replicated DNA was detected by digesting DNA recovered from pRDK41 transfected cells with *Xho*I and *Dpn*I prior to electrophoresis and Southern blotting. Newly replicated DNA is resistant to *Dpn*I since DNA synthesized in mammalian cells lacks the *dam*-methylation required for cleavage by this restriction enzyme.

Figure 3 shows that significant amounts of heteroduplex molecules first appeared about 10 hr post-transfection, were most abundant at 12 hr then declined over the next 12 hr. At the point of maximal recovery, 23% of *Sac*I-*Eco*RV fragments contained heteroduplex and this declined to 3% by 24 hr post-transfection. The kinetics of heteroduplex formation shown in Figure 3 were similar, but not identical, to those of recombination and replication illustrated in Figure 4. Maximal recovery of recombinant molecules was observed 14 hr post-transfection, coincident with the time at which the DNA was becoming *Dpn*I resistant. This was about 2 hr after the peak of heteroduplex recovery seen in Figure 3 and 2–4 hr after the maximal rate of heteroduplex synthesis. The stability of recombinant molecules was also different from that of heteroduplex DNAs. Unlike heteroduplex molecules the amount of recombinant DNA declined only slightly between 14 and 24 hr (Figure 4). Thus there appeared to be a close temporal linkage between all three processes although maximal heteroduplex DNA recovery appeared to precede the recovery of replicated and recombinant molecules.

What is the cause of heteroduplex instability? Once heteroduplex molecules have been formed they face several possible fates. In the presence of mismatch-repair systems heteroduplexes would disappear and, if the repair were in the appropriate direction, one could observe the appearance of recombinant molecules through gene conversion. In the absence of DNA repair, extensive DNA replication would destroy heteroduplex molecules and one would only observe the production of recombinants if heteroduplex tracts were short (*i.e.*, did not overlap both marked sites) or if heteroduplex formation were as-

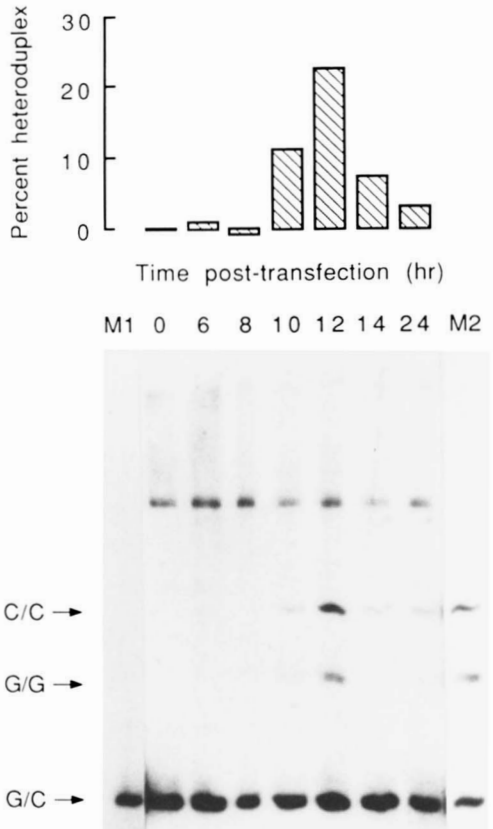

FIGURE 3.—Kinetics of heteroduplex formation and repair. Dishes of SFV-infected SIRC cells were cotransfected with pMJ76 and pMJ77 and DNA recovered at the indicated times. Heteroduplex formation was then monitored as described in Figure 2. The amount of heteroduplex was determined by cutting the radioactive blot into pieces and counting labeled regions of heteroduplex and homoduplex in aqueous scintillation fluid (inset). M1 = homoduplex marker, M2 = artificial heteroduplex marker.

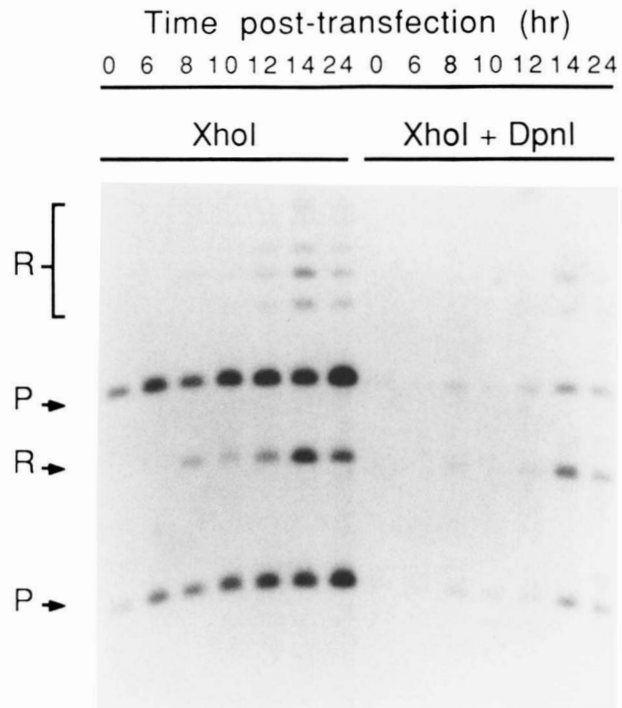

FIGURE 4.—Kinetics of recombinant formation and DNA replication. Dishes of SFV-infected SIRC cells were transfected with plasmid pRDK41 in parallel with the experiment described in Figure 3. After recovery the DNA mixture was digested with *Xho*I, subjected to electrophoresis, transferred to nitrocellulose, and hybridized with a pRDK41 probe. Recombinational rearrangement of the heteroallelic *tet*-10 and *tet*-14 linker insertions in pRDK41 would be expected to generate 1.24-, 4.36-, 7.48-kb and other (larger) recombinant restriction fragments (R) in addition to the 5.60-kb and 3.12-kb parental DNA fragments (P). *Xho*I cut DNA was also treated with *Dpn*I in order to detect newly synthesized, *Dpn*I resistant, molecules. Comparison with Figure 3 shows that maximal heteroduplex recovery preceded the recovery of replicated and recombinant DNA by about 2 hr.

sociated with crossing over. In the absence of both repair and replication heteroduplex DNAs would be expected to persist at levels that reflect the frequency with which they were formed. The phage experiments described in Tables 1 and 2 indicated that significant amounts of heteroduplex do persist to be recovered at the end of the infectious cycle but the data presented in Figure 3 showed that this represented only a fraction of the heteroduplex originally formed. Clearly heteroduplex DNAs were subjected to repair and/or replication after synthesis. Which of these processes (or both) are responsible for this instability?

Several additional pieces of data suggest that the disappearance of heteroduplex was mostly a consequence of continued DNA replication rather than DNA repair. These results are summarized in Figures 5, 6 and 7. In one set of experiments, cells were cotransfected with *Nam*53 *cIts*307 and *cIc*60 *Oam*29 DNAs in order to more accurately determine when DNA replication (and recombination) terminated in infected cells. This could be monitored by following the recovery of packaged phage during the progress of viral infection. Both phage DNA synthesis (assayed as total phage/μg recovered DNA) and recombinant formation (assayed as N^+O^+ or cI^+ phage) persisted through 14 hr post-transfection (Figure 5). In a second set of experiments we assayed directly for induced DNA polymerase in cytoplasmic extracts. This provided a control for the mismatch-repair assays described below and also avoided certain difficulties associated with measuring replication end products rather than replicative potential. Figure 6 shows that the appearance of DNA polymerase was coincident with the recovery of heteroduplex DNA and remained detectible in these extracts through 14 hr post-transfection. Clearly SFV-infected cells retained a replicative capacity throughout the period during which heteroduplex formation and loss were observed.

In contrast to these experiments designed to detect continued DNA replication we were unable to detect any form of induced (or constitutive) DNA-repair activity in poxvirus-infected cells. Cytoplasmic extracts were prepared from SFV-infected cells 14 hr postinfection. This time corresponded to 12 hr post-transfection and represents a compromise time at

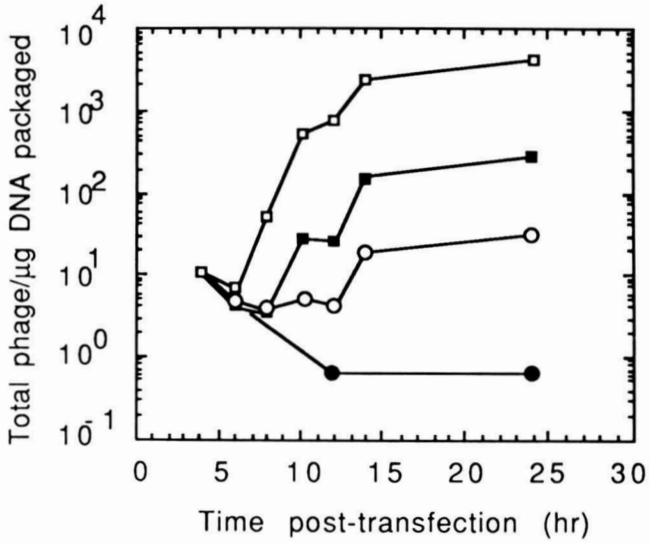

FIGURE 5.—Persistence of DNA replication and recombination in SFV-infected cells. SFV-infected SIRC cells were cotransfected with *Nam*53 *cIts*307 and *cIc*60 *Oam*29 lambda DNAs then DNA recovered at the times indicated. *In vitro* packaged phage were then plated on the *supE* strain K802 to determine the relative number of phage genomes present (□——□) or on the *sup*° strain RK1036 to determine the proportion recombinant for *N* and *O* markers (■——■). The number of cI^+ recombinants was determined by inspecting the plaques recovered on RK1036 (○——○). Also shown are the number of N^+O^+ recombinants observed among phage recovered from mock infected cells (●——●).

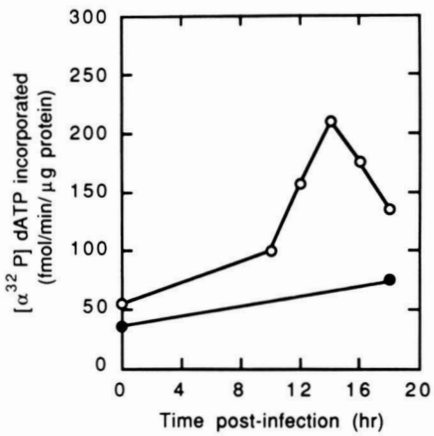

FIGURE 6.—Persistence of SFV DNA polymerase in SFV-infected cells. Extracts from mock (●——●) or SFV-infected (○——○) SIRC cells were prepared as described in MATERIALS AND METHODS and assayed for induced DNA polymerase by incorporating [α-^{32}P]-labeled dATP into an activated calf-thymus DNA substrate. The presence of replicating virus induced a peak of polymerase activity 14 hr postinfection (equivalent to 12 hr post-transfection). Endogenous DNA polymerase levels rose reproducibly in mock-infected cells probably in response to the addition of fresh media at the end of mock adsorption.

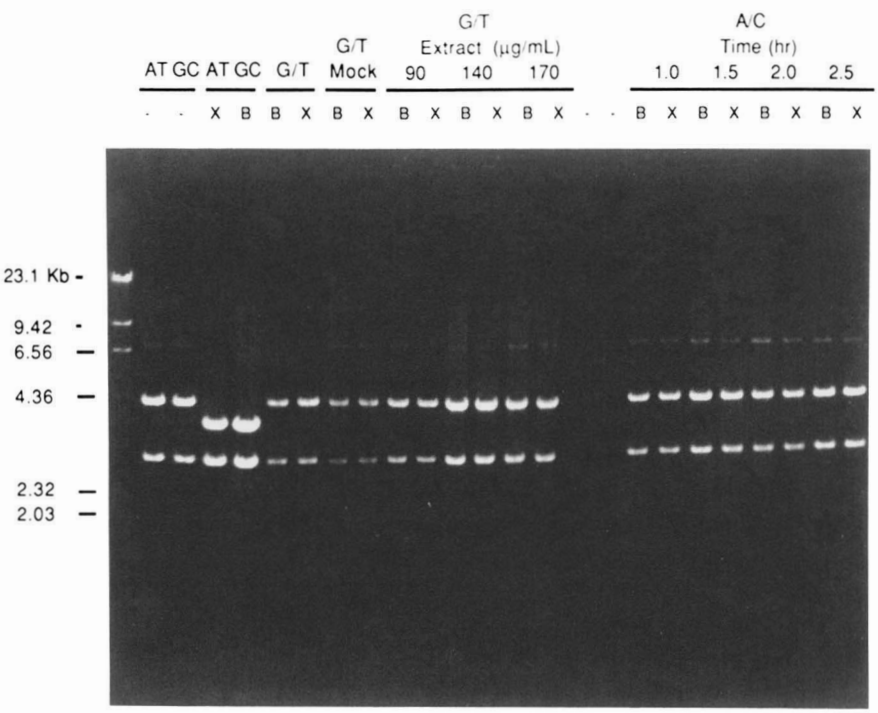

FIGURE 7.—*In vitro* assay for the presence of mismatch repair activity in cytoplasmic extracts of SFV-infected cells. Form I heteroduplex DNA was incubated for 1 hr with varying amounts of extract (G/T substrate) or with 70 µg/ml extract for varying amounts of time (A/C substrate). Nucleic acids were then recovered and digested with *Cla*I plus either of two restriction enzymes expected to cut repaired, but not heteroduplex, DNA as indicated (X = *Xba*I, B = *Bam*HI). No evidence for site restoration can be seen in any of the digests nor could double-strand breaks have formed at the mismatched site. Control lanes are as follows: lane 1, lambda *Hin*dIII size marker; lanes 2 and 3, homoduplex DNA digested with *Cla*I alone; lanes 4 and 5, homoduplex DNA digested with *Cla*I plus *Xba*I or *Bam*HI (3.72-kb fragment indicates expected repair product); lanes 6 and 7, G/T heteroduplex treated with *Cla*I plus *Xba*I or *Bam*HI; lanes 8 and 9, G/T heteroduplex incubated with mock-infected extract prior to restriction.

which both heteroduplex and recombinant formation were detected by Southern blot analysis (Figures 3 and 4). Mismatch-repair assays were performed in which the extracts were incubated with heteroduplex DNA substrates containing different point mismatches or 1, 4, 9 or 401 base insertional heteroduplexes. These substrates included DNA containing G/T mismatches [which are known to be well repaired in *S. cerevisiae* (MUSTER-NASSAL and KOLODNER 1986) and mammalian cells (WEIBAUER and JIRICNY 1990)] as well as the G/G mismatches seen disappearing in Figure 3. No evidence for repair was noted in any of these experiments (Figure 7) nor were double-strand breaks detected. These experiments were repeated

using extracts prepared by a different method or using different reaction conditions, all without success. Cytoplasmic extracts prepared from vaccinia-infected HeLa cells were also unable to catalyze mismatch repair although, as with SFV (Figure 6), we detected expected amounts of the induced DNA polymerase (CHALLBERG and ENGLUND 1979). Because it is possible that some labile component of the mismatch correction reaction was inactivated during extract preparation we also investigated whether biochemically simpler mismatch-specific nicking activities were present in infected cells. A 401-bp insertion-heteroduplex substrate was first incubated with SFV-infected cell extracts. Then, the DNA was recovered, restricted, and runoff copies prepared using DNA polymerase and a synthetic primer. Again, despite extensive incubation, no evidence for mismatch-specific incision could be seen (data not shown). Therefore we conclude that any DNA repair systems in the cytoplasm of poxvirus-infected cells must have been either biochemically unstable or present at very low levels.

DISCUSSION

The recovery of partial heterozygotes from T-even phage crosses (HERSHEY and CHASE 1951) led LEVINTHAL to propose that heteroduplex DNAs might be formed during bacteriophage replication and recombination (LEVINTHAL 1954). Since then, heteroduplex progeny have been recovered from other phage crosses (reviewed by LURIA 1962; FOX 1978) while phenomena like postmeiotic segregation (PMS), and direct methods for the detection of heteroduplex, also provides evidence that heteroduplex molecules are formed during meiosis in many fungi (KITANI, OLIVE and EL-ANI 1962; PAQUETTE and ROSSIGNOL 1978; LICHTEN *et al.* 1990). This diversity of organisms that catalyze heteroduplex formation raises the question of whether heteroduplex molecules might also be formed during the replication and recombination of mammalian viruses. It has been known for more than 30 years that DNA viruses can recombine (WILDY 1955; FENNER and COMBEN 1958) but little is known about the mechanism(s) of viral recombination and thus far the only experiment to suggest that heteroduplex intermediates might be produced during viral recombination comes from experiments with herpes simplex virus. BROWN and RITCHIE (1975) noted that about 5% of progeny from crosses between syn^+ and syn^- herpes strains formed plaques of mixed syncytial/nonsyncytial phenotype and thus may have derived from infection by a $syn^{+/-}$ heteroduplex particle. Unfortunately, whether these plaques derived from recombinant viruses containing mismatched DNAs has never been subjected to additional experimentation.

Because so little is known about the mechanism of viral recombination we have been studying this process in poxvirus-infected cells. Transfection experiments, designed to investigate the effect of marker distance on recombinant formation in virus-infected cells, showed that high negative interference characterized crosses between markers spaced less than ~100 bp apart (PARKS and EVANS 1991). Considering the complex methodology, there are two hypotheses that might account for this observation. Firstly, mottled plaques arising from infection by $cI^{+/-}$ heteroduplex lambda phage can be misscored as cI^+ recombinants since it is sometimes difficult to distinguish the two phenotypic classes of plaques by superficial inspection. When marker separation is reduced to the point that the frequency of this event exceeds the crossover (or conversion) frequency one might observe "enhanced" recombinant frequencies. Secondly, biased VSP repair of heteroduplex spanning the *cIam6* marker could also have generated an excess of cI^+ progeny in certain short-distance crosses (LIEB, ALLEN and READ 1986) although, for this to be true, it would still be necessary to hypothesize that heteroduplex tracts formed in poxvirus-infected cells are considerably shorter than those formed in lambda-infected *E. coli*. Which of these hypotheses is correct is not clear but both postulate the existence of a heteroduplex intermediate and led us to examine whether heteroduplexes were to be found in the DNA recovered from transfected SFV-infected cells. Using phage lambda DNA as a substrate we observed that a significant fraction of the packaged phage recovered from infected cells produced mottled plaques on *E. coli* which is a phenotype characteristic of lambda $cI^{+/-}$ heteroduplexes plated on mismatch-repair defective indicator strains (HUISMAN and FOX 1986). Replating experiments showed that 2–3% of phage genomes originally plated on a *mutS* host were heteroduplex at one of the mutant *cI* sites (Table 1). Not surprisingly, fewer mottled plaques were recovered on repair-proficient cells than on *mutS* cells and the efficiency of recovery varied from one *cI* allele to another (Tables 1 and 2) reproducing, in a complicated way, the observations of DOHET, WAGNER and RADMAN (1985).

Direct assays for the presence of heteroduplex molecules confirmed that by 24 hr post-transfection about 3% of molecules contained G/G or C/C heteroduplexes (Figures 2 and 3). As with our earlier observations on poxvirus-mediated recombination (EVANS, STUART, and MCFADDEN 1988) this reaction required both parental DNAs and was not observed in mock-infected cells (Figure 2). Equal amounts of the two types of heteroduplexes were recovered from transfected cells at all times in the infection but lacking additional information on the relationship between recombinant products one cannot draw any conclusions concerning the symmetry or asymmetry of strand exchange. The frequency of heteroduplex re-

covery was consistent with the recombination frequencies that characterize phage DNA crosses in poxvirus-infected cells (Figure 5) and was surprisingly close to the heteroduplex frequency determined by phage plating (Table 1). This agreement between the two methods suggests that relatively few heteroduplex phage were lost on plating through random-strand loss or through residual DNA repair in a *mutS* strain, and confirms the observations of HUISMAN and FOX (1986) using artificial phage heteroduplexes plated on *mutL E. coli.*

At 24 hr, the frequency with which heteroduplex molecules were recovered from SFV-infected cells was more than 100-fold higher than what is seen among the unselected progeny of lambda crosses (KELLENBERGER, ZICHICHI and EPSTEIN 1962) and about 10-fold higher than the frequency of internal heterozygotes recovered from normal T4 infections (SÉCHAUD *et al.* 1965). SFV frequencies are comparable to the frequency of $syn^{+/-}$ plaques recovered from herpes simplex coinfections (BROWN and RITCHIE 1975) and fall within the range of PMS frequencies observed at high PMS alleles in fungi like *S. cerevisiae* (LICHTEN *et al.* 1990) and *Ascobolus immersus* (PAQUETTE and ROSSIGNOL 1978). Comparisons between the amount of heteroduplex formed transiently in SFV-infected cells and in other organisms are more difficult since most studies have tended to examine the end products of recombination and probably underestimate the frequency. The only available estimates of how much heteroduplex might form during recombination are based on genetic arguments that must make assumptions concerning the origin of gene conversion and the properties of DNA repair systems. Within these constraints PAQUETTE and ROSSIGNOL (1978) estimated that 35% of all meioses saw hybrid DNA at the *A. immersus b2* 17 allele which, while it probably represents an upper limit on the frequency of heteroduplex formation in fungi, is clearly comparable to the maximal frequency seen in SFV-infected cells (Figure 3).

Experiments designed to investigate the kinetics of heteroduplex formation showed that mismatch formation and DNA replication were closely associated events (Figures 3, 4 and 6). It is tempting to explain this linkage between heteroduplex formation and DNA replication with a modified version of the recombination-primed replication model of T4 (LUDER and MOSIG 1982; FORMOSA and ALBERTS 1986). This model is consistent with the reaction kinetics, the observation that inhibiting poxviral polymerases inhibits recombination, and the observation that the number of exchanges per transfected lambda genome [about 1 productive exchange per 500 bp (PARKS and EVANS 1991) or ~200 exchanges per genome] is roughly the same as the amount of lambda DNA

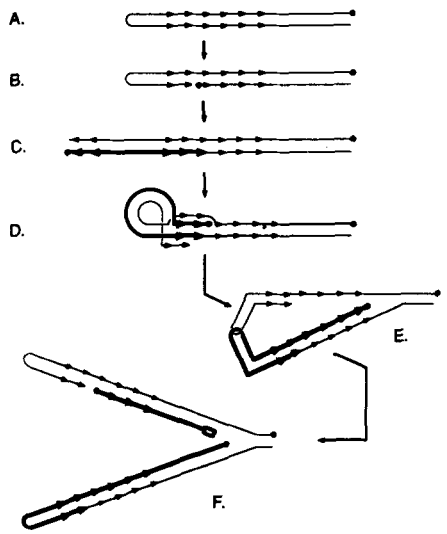

FIGURE 8.—Poxviral replication initiation by recombinational priming. A typical poxviral telomere is shown along with the short, direct repeats (arrows) found adjacent to the terminal hairpins in some of these viruses (A). An endonucleolytic nick within one of the repeats generates a 3′-end (B, —●) that can be extended through the hairpin by DNA polymerase (C). Precise invasion of the new end back into the repeats generates a displacement loop which can serve as a replication origin (D). Imprecise invasion would generate variation in repeat number (not shown). If one permits pairing of the 5′-end with the displaced strand, straighten out the ends, and form intrastrand base pairs, one obtains the molecule seen in (E) after DNA polymerase extension from the 3′-end. Decatenation and initiation of discontinuous replication following primer synthesis (open box), would generate structure (F). For simplicity only one topological linkage is shown in (E) although, in reality, the molecule might be expected to contain additional links or interstrand base pairs.

synthesis (~600 fold, Figure 5). It is also consistent with the fact that experiments that might have recovered poxviral recombination mutants did not do so (BALL 1987), suggesting that such genes are essential to cytoplasmic viruses. However several pieces of evidence indicate that the situation is more complex. While it is clear that inhibiting poxviral DNA polymerases blocks recombination (BALL 1987; EVANS, STUART and MCFADDEN 1988; MERCHLINSKY 1989) these experiments are complicated by the effects of polymerase on DNA copy number and by the need to retain some polymerase activity to stabilize DNA in infected cells. More importantly electron microscopy (ESTEBAN, FLORES and HOLOWCZAK 1977) and pulse-labeling experiments (POGO, O'SHEA and FREIMUTH 1981) suggest that poxviral replication initiates in the terminal hairpins of the virus and no evidence has yet been reported for the existence of multiply branched structures like those seen in T4 infections.

These somewhat contradictory observations can be unified by modifying the mechanisms of poxviral replication initiation proposed by MOYER and GRAVES (1981) and MOSS (1990) to include an intramolecular, end invasion within the terminal repetitions following terminal incision (see Figure 8). DNA polymerase

extension from a nick located near the telomere, through the hairpin end, would generate molecule (C) containing an inverted repetition of a portion of the viral telomere. A standard recombinational invasion of the newly synthesized 3′-end back into the telomere with a polarity like that of *E. coli* recA or *S. cerevisiae* SEP1 proteins (COX and LEHMAN 1981; KAHN *et al.* 1981; KOLODNER, EVANS and MORRISON 1987) generates intermediate (D) which could now serve as a primed replication origin. DNA polymerase extension from the 3′-end and base pairing between the 5′-terminal end and the D-loop generates molecule (E) which is a starting point for replication of the viral genome *via* a process requiring the semiconservative, discontinuous DNA synthesis observed by ESTEBAN and HOLOWCZAK (1977). The rearrangement of base pairing and resolution of linked telomeres required to go from (D) to (F) creates topological problems but the type I topoisomerases encoded by these viruses provide an obvious solution to this difficulty.

Many disparate properties of poxviral biology are explained by this hypothesis and we will only discuss some of the most obvious. First this mechanism rationalizes why poxviruses seem to initiate replication near the telomeres (since that is where invasive ends are formed) but can also nonspecifically replicate and recombine transfected DNAs through intermolecular invasions between multiple DNA copies transfected into infected cells. Second, random end invasion readily explains the permanent instability of the telomeric repeats found in poxviruses like vaccinia since invasion may not precisely align repeated elements leading to variations in copy number from isolate to isolate. Third, end invasion also explains why linear-duplex molecules containing small (0.1 μm) and large (1.7 μm) double-stranded terminal loops, as well as large Y-shaped forms, are found in the early stages of vaccinia infection (ESTEBAN, FLORES and HOLOWCZAK 1977). The structures containing terminal loops resemble intermediate (E) in various states of replication while the Y-shaped molecules are predicted to be kinetically late forms arising from topological resolution of the linked hairpins [intermediate (F)]. Fourth, this mechanism, plus a shift from intramolecular to intermolecular invasion as viral copy number increases, can also explain why only head-to-head dimers are recovered early in the course of vaccinia infection (MOYER and GRAVES 1981) but a mixture of concatameric forms later in infection (MERCHLINSKY and MOSS 1989). Finally, this model predicts that conditional recombination mutations would appear to be replication defective while unconditional mutants should not be viable.

Although this hypothesis readily explains many aspects of poxviral biology it should be remembered that it derives from an analysis of the behaviour of calcium-phosphate transfected DNAs, not the virus providing gene products in *trans*. Is one justified in extending the model to a replicating poxvirus? Several groups have investigated the structure of vaccinia and SFV telomeres (BAROUDY, VENKATESAN and MOSS 1982; DELANGE *et al.* 1986) and data from these experiments shows that many mismatched and extrahelical nucleotides are found adjacent to the "flip-and-flop" hairpin ends of packaged viruses. Such structures could be created by recombination between imperfectly homologous sequences and a mechanism like that shown in Figure 8 provides an obvious way in which they can be maintained. An interesting corollary suggests that, because they retain heteroduplex termini through many plaque purifications, poxviruses are not subjected to high efficiency mismatch repair otherwise repeated rounds of conversion would tend to "homogenize" telomeric sequences. We conclude that, although experiments with nontelomeric viral markers need to be performed, the available data supports the idea that poxviruses retain heteroduplex following replication and are not subjected to efficient repair.

How are recombinants formed in poxvirus infected cells? As outlined above they are probably an accidental product of the way in which poxviruses replicate DNA and contain large amounts of heteroduplex because replication occurs in an environment lacking a mismatch-repair capacity. The latter point is based on our inability to detect any form of mismatch-repair activity in infected-cell extracts despite the fact that these extracts contain active DNA polymerase (Figure 6) and catalyze recombination *in vitro* at frequencies exceeding 1% (M. LAUZON and D. EVANS, manuscript submitted for publication). The simplest explanation is that mismatch-repair activities cannot be detected because they are not present in poxvirus-infected cytoplasmic extracts. It is difficult to prove this hypothesis but the absence of specific repair activities is consistent with the parity of G/G *vs.* C/C recovery seen in Figure 3 [which is in clear contrast to the disparity observed in repair-proficient *S. cerevisiae* (LICHTEN *et al.* 1990) and *E. coli* (DOHET, WAGNER and RADMAN 1985)] and with the stability of telomeric heteroduplex cited above. Within the constraints of a number of recombination models (HOLLIDAY 1964; MESELSON and RADDING 1975; LUDER and MOSIG 1982) the biochemical data remain broadly compatible with two basic mechanisms of recombinant formation. One possibility is that heteroduplex formation is associated with crossing over and it is the crossing over which generated these recombinants. This association has been well documented in fungi and phage and is consistent with the efficiency of plasmid-x-poxvirus recombination documented by a number of

authors (BALL 1987, SPYROPOLOUS *et al.* 1988). An alternate possibility is that, assuming heteroduplex tracts are short (PARKS and EVANS, 1991) but formed with high frequency in SFV-infected cells, multiple rounds of random strand-transfer associated with replication are responsible for recombinant formation. Unfortunately the nature of viral infections and viral plaques makes it difficult to differentiate these two models because one cannot easily recover recombinationally related molecules from coinfections. We are presently trying to isolate the recombinational intermediates that differentiate the various models.

Although the mechanism of poxvirus recombination remains uncertain it is clearly different from that of mammalian viruses like SV40 and adenovirus. Several experiments illustrate these differences but those most relevant to this study were a series of adenovirus experiments performed by VOLKERT, MUNZ and YOUNG (1989). In one experiment the authors rescued multiply marked DNA segments into virus and looked for close exchanges among the recombinants. Since a number of studies have shown that mammalian cells can repair mismatched DNA the failure to recover multiply recombinant adenovirus particles was interpreted to mean that heteroduplex intermediates were probably not formed during adenoviral recombination. This result confirmed and extended earlier studies which were also unable to detect any excess of close exchanges in "multifactor" crosses performed in SV40 (WAKE and WILSON 1980) and adenovirus-transfected cells (VOLKERT and YOUNG 1983). This difference between poxviruses and nuclear viruses is perhaps not too surprising. SV40 and adenovirus replicate by mechanisms that are very different from poxviruses (CHALBERG and KELLY 1989) and, as VOLKERT and YOUNG (1983) have pointed out, recombination of these viruses may be dependent upon host gene products. In contrast, poxviruses replicate in isolated structures in the cytoplasm of infected cells which might have provided immunity from nuclear repair enzymes and an opportunity to evolve different ways of generating recombinants.

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