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High Levels of Genetic Recombination among Cotransfected Plasmid DNAs in Poxvirus-Infected Mammalian Cells

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The frequency of recombination between transfected plasmid DNAs was measured by using cultured cells infected with a variety of poxviruses. Plasmid derivatives of pBR322 containing *Xho*I linker insertion mutations in the tetracycline gene were used to assess recombination frequencies in rabbit cells infected with the leporipoxviruses Shope fibroma virus and myxoma virus and the orthopoxvirus vaccinia virus. Recombination frequencies were calculated by Southern blotting, which detects novel plasmid restriction fragments generated by genetic recombination, and by a plasmid rescue procedure in which the reconstruction of an intact tetracycline gene in the transfected rabbit cell was monitored by transformation back into *Escherichia coli*. The highest recombination frequencies were measured in cells infected with Shope fibroma virus and myxoma virus, and a minimum recombination frequency of at least one recombination event per 7 kilobases was calculated within 24 h posttransfection under these conditions. The deduced recombination frequency in vaccinia virus-infected cells was at least fivefold lower and was not detectable in mock-infected cells, suggesting that the induced recombination activity detected by these methods was under viral control. The results of kinetic studies, analysis with methylation-sensitive restriction enzymes, and the use of phosphonoacetic acid, a specific inhibitor of poxvirus DNA polymerase, indicated that recombination between transfecting DNAs occurred concomitantly with DNA replication but that the two processes could be partially uncoupled. We conclude that the dramatic expansion of recombination activities in the cytoplasm of poxvirus-infected cells is virus specific and offers a good model system with which to analyze the mechanism of recombination in a eucaryotic environment.

In genetic recombination, segments of DNA are exchanged between interacting genomes in either a reciprocal or a nonreciprocal fashion (20, 29, 37, 40). Many of the large bacteriophages are subject to high levels of genetic recombination, and in some cases this process is mediated by phage-encoded gene products. These include site-specific systems such as those found in the lambdoid phages (26), as well as general recombination systems such as those that catalyze T4 (14), λ Red (35), and T7 (38) recombination pathways. There is abundant evidence that replicating mammalian DNA viruses are also subject to genetic recombination (reviewed in reference 30). Genetic maps of many of these viruses have been constructed and have been shown to be colinear with physical maps. Other lines of evidence also indicate that mammalian DNA viruses can be subjected to high levels of recombination: for example, the good efficiency of marker rescue techniques and the high frequencies of gene conversion and duplication-deletion events that have been reported for many viruses (30). What is not clear, however, is whether these processes are catalyzed by host or viral gene products or both.

It is possible that viruses which replicate in cell nuclei need not encode functions needed for general homologous recombination, although site-specific functions could very well be virus specific. Abundant experimental evidence suggests that eucaryotic cell nuclei can be highly recombination proficient for exogenous genomes, since DNAs artificially introduced into a cell nucleus can be subjected to extraordinarily high levels of rearrangements (1, 2, 6, 13, 16, 21, 32–34). Not all mammalian DNA viruses replicate in the nucleus, however, and whether these viruses encode the enzymes which catalyze viral recombination remains an open question.

Viruses of the *Poxviridae* family replicate in the cytoplasm of infected cells and are subjected to high levels of recombination (3). These viruses have been shown by genetic and biochemical means to encode many of the functions needed for DNA replication, and it is quite probable, given the large size of poxvirus genomes, that they encode recombination functions as well (7, 18, 23, 25, 43). It has previously been demonstrated that when circular plasmids are transfected into poxvirus-infected cells, the input DNA is efficiently replicated in a sequence-independent fashion into high-molecular-weight, head-to-tail concatemers (8). There are several ways to explain this result, but one hypothesis is that extensive generalized recombination in concert with replication generated the amplified, plasmid-derived tandem arrays in the cytoplasm of the transfected cells. In this study, by marking homologous plasmid DNAs with restriction site polymorphisms (10), we demonstrated that cotransfected plasmids are subjected to very high levels of intramolecular and intermolecular recombination within poxvirus-infected cells and that recombination is dependent upon the presence of the infecting virus. These observations suggest either that poxvirus genomes encode viral recombination functions or

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that the presence of replicating poxviruses can somehow induce extensive levels of host recombination processes. The dramatic expansion of recombination activity in the cytoplasm of poxvirus-infected cells also suggests that these cells may be an excellent source of enzymatic activities involved in general genetic recombination.

MATERIALS AND METHODS

Strains and viral stocks. Plasmids were prepared from *Escherichia coli* strains provided by R. Kolodner. These were RDK1233(pRDK41), RDK1388(pRDK35), and RDK 1389(pRDK39), all of which are ampicillin-resistant transformants of JC10287 [Δ(*srlR-recA*)*304 thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44*] (10). In transformation assays (39) we used *E. coli* RDK1400 (*recA13 thr leuB6 thi thyA trpC1117 hsdR12 hsdM12* Str^r). Vaccinia virus IHD-W (provided by S. Dales) was propagated on BGMK monkey cells, and Shope fibroma virus (SFV) (Kasza) and myxoma virus (Lausanne) were propagated on SIRC rabbit cells. Both cell lines were from the American Type Culture Collection, Rockville, Md. Viral stocks were prepared and purified as described previously (42).

Enzymes, chemicals, and media. Restriction enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., Boehringer Mannheim Biochemicals, Indianapolis, Ind., or New England BioLabs, Beverly, Mass., and used as directed by the manufacturers. *E. coli* DNA polymerase I was the gift of A. R. Morgan and was purified by the procedure of Jovin et al. (15). T4 DNA ligase was the gift of R. Kolodner and was purified by a modification of the procedure of Panet et al. (27). Proteinase K was purchased from Beckman Instruments, Inc., Palo Alto, Calif. Bacterial media were from Difco Laboratories, Detroit, Mich. SIRC and BGMK cell lines were cultured as monolayers in Dulbecco modified Eagle medium (Gibco, Burlington, Ontario, Canada) supplemented with 5% fetal calf serum (Flow Laboratories, Mississauga, Ontario, Canada). Phosphonoacetic acid (PAA) was from Sigma Chemical Co., St. Louis, Mo.

Nucleic acids. Plasmids were prepared from cells grown to stationary phase overnight in Fraser's medium supplemented with 50 μg of thymine per ml and 180 μg of ampicillin per ml. Plasmids were isolated by a large-scale alkaline lysis procedure (4), and supercoiled DNA was purified by using two successive ethidium bromide-CsCl gradients.

Transfection of virus-infected cells. Monolayers containing 2×10^6 to 3×10^6 SIRC cells were infected as described previously (8) and then transfected 1 h (vaccinia virus) or 3 h (myxoma virus and SFV) later by the addition of 50 ng of $CaPO_4$-precipitated plasmid DNA. Unadsorbed DNAs were removed by washing the monolayer first with phosphate-buffered saline and then with phosphate-buffered saline plus 5 mM EDTA 3 h after addition of DNA, and fresh medium was then added. When plasmid mixtures were applied, they were first coprecipitated and 50 ng of total DNA was added per 2×10^6 to 3×10^6 cells. As described previously, no carrier DNA is required for the efficient uptake of $CaPO_4$ plasmid DNA into the cytoplasm of poxvirus-infected host cells (8). After the incubation times indicated, total cellular DNA was recovered from infected or transfected cells by enzymatic digestion, phenol extraction, and ethanol precipitation. All times given in the figure legends are times (in hours) posttransfection.

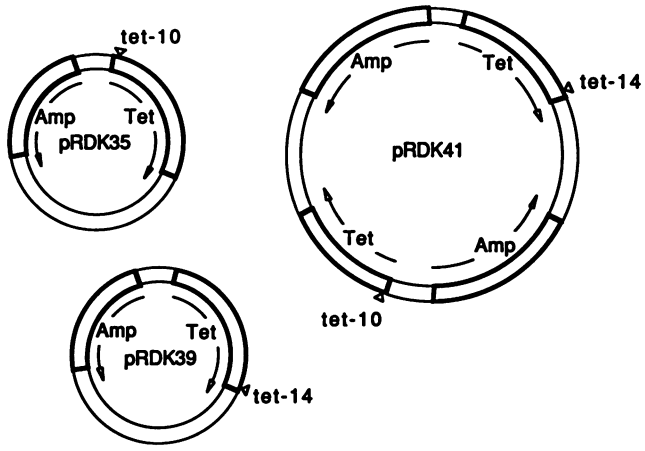

FIG. 1. Structure of plasmids used to quantitate recombination in transfected cells. The three plasmids are derivatives of pBR322, and their construction is described elsewhere (10). The *tet-10* and *tet-14* alleles are *Xho*I linker insertions at *Taq*I sites that inactivate the tetracycline gene. The two loci are separated by 1.24 kb.

Analysis of recombinant products by Southern blotting. Total cellular DNA was digested with restriction enzymes as indicated in the Figure legends, and 50 to 500 ng was applied per well and electrophoresed on 0.7% agarose gels. The DNA was transferred to nitrocellulose (36), blotted with nick-translated ^{32}P-labeled pBR322 and λ DNA probes (specific activity, ca. 10^8 cpm/μg), and exposed to Kodak AR film (Eastman-Kodak Co., Rochester, N.Y.) with Cronex intensifier screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Bacteriophage λ *Eco*RI-*Hin*dIII digests served as molecular weight markers. Complete restriction digests were assured by examining ethidium bromide-stained agarose gels prior to Southern blotting. Viral DNA restriction fragments were visible superimposed upon a heterogeneous background of cellular DNA digestion products (see Fig. 3 and 4).

When indicated, autoradiographs were scanned with an LKB Ultroscan XL Laser densitometer (LKB Instruments, Inc., Rockville, Md.). Integration was performed either by the cut-and-weigh method or by using integration values calculated by the densitometer. The percent recombination was calculated by using the following formula:

$$\% \text{ recombination} = \frac{\Sigma \text{ (integrated area of recombinant plasmid restriction fragments)} \times 100}{\Sigma \text{ (integrated areas of parental and recombination plasmid restriction fragments)}}$$

Analysis of recombinants by transformation in *E. coli*. DNA recovered from transfected cells was digested to completion with *Eco*RI, repurified by phenol extraction in the presence of 100 μg of tRNA per ml, ethanol precipitated, and circularized at a DNA concentration where $j = 10i$ with T4 DNA ligase (17). After purification by phenol extraction and ethanol precipitation, the DNA was transformed into competent *E. coli* (22) and plated in soft agar on LB plates

containing 100 μg of ampicillin per ml. Transformants were patched onto LB plates containing 100 μg of ampicillin per ml and 20 μg of tetracycline per ml to determine the recombination frequency. The recombination frequency was calculated from the following formula:

$$\% \text{ recombination} = \frac{(\text{ampicillin- + tetracycline-resistant colonies}) \times 100}{\text{ampicillin-resistant colonies}}$$

RESULTS

Experimental design. The plasmids used for this study are illustrated in Fig. 1. Plasmids pRDK35 and pRDK39 contain *Xho*I linkers inserted into *Taq*I sites within the tetracycline gene of pBR322 (10). Plasmid pRDK41 is a circular dimer containing pRDK35 joined in tandem with pRDK39 and is used to monitor intramolecular recombination events (10). Genetic recombination occurring within the tetracycline genes of these plasmids can be detected by selection for a functional tetracycline gene or by restriction mapping (10, 39). The use of restriction mapping with *Xho*I to detect a simple, single crossover event between the monomeric constructs pRDK35 and pRDK39 is illustrated in Fig. 2. Such an event occurring within the tetracycline gene would generate (after digestion with *Xho*I) restriction fragments of 1.24 and 7.48 kilobases (kb). Gene conversions or multiple rounds of recombination would generate these, and other, predictable recombinant fragments which can be readily detected by Southern blotting.

Recombination between transfecting plasmids is dependent upon poxvirus infection. In preliminary experiments monolayers of SIRC cells were infected with 1 to 3 PFU of SFV, myxoma virus, or vaccinia virus per cell, transfected with plasmid DNA, washed, and incubated for 24 h, and total DNA recovered. It has been shown previously that under these conditions a broad variety of circular input plasmid DNAs can be replicated in a sequence-independent fashion into high-molecular-weight concatemeric arrays by *trans*-acting factors supplied by the infecting poxvirus (8). After complete digestion with *Xho*I, DNA fragments were separated by agarose gel electrophoresis and Southern blots were probed with ^{32}P-labeled pBR322 DNA. The results of these transfection experiments with *Xho*I linker variants of pBR322 permitted a number of conclusions to be made. Recombinant products were readily recovered from poxvirus-infected cells 24 h after transfection (after replication of the endogenous viral genomes was essentially completed) but not at 3 h after transfection (before viral DNA replication) or at either time in uninfected cells (Fig. 3 and 4). This is most readily apparent in the laddered array of recombinant fragments observed, for example, in myxoma virus- and SFV-infected cells transfected with a mixture of pRDK35 and pRDK39 or with pRDK41 alone (Fig. 3B, lanes 16 and 17; Fig. 4B, lanes 7 and 8). This distribution of new fragment sizes can be rationalized owing to the formation of tandem, head-to-tail repeats of plasmid vector DNA flanked by multiple orientations of *tet-10* and/or *tet-14* *Xho*I linker insertion sites. Both intermolecular and intramolecular events occurred with approximately equal frequency, since ladders of similar distribution and intensity were observed in cells transfected with a mixture of pRDK35 and pRDK39 or with the dimeric pRDK41. The generation of intermolecular recombinants is clearly dependent upon the presence of both pRDK35 and pRDK39, because when cells were transfected with either plasmid alone, only 4.36-kb fragments of parental configuration could be recovered at 24 h (e.g., Fig. 3B, lanes 14 and 15).

The observed recombination was dependent upon poxvirus infection in two ways. First, as shown below, recombinant fragments were derived from newly replicated DNA, and this replication was necessary to maintain and amplify the plasmid copy number. In the absence of infection only a small fraction of the input plasmid sequences survived for 24 h in culture (Fig. 3B, lanes 5 through 8), although at 3 h posttransfection both infected and uninfected cells had taken up equal amounts of plasmid DNA (Fig. 3B, compare lanes 1 through 4 with lanes 10 through 13). It was also apparent that detectable levels of recombination occurred only in the poxvirus-infected cells (Fig. 3B, compare lanes 7 and 8 with lanes 16 and 17). Although some input plasmid DNA survived for 24 h in uninfected cells, overexposed Southern blots showed that this DNA still retained only a parental configuration of *Xho*I restriction sites (Fig. 3C). Note that this observation does not imply that uninfected SIRC cells are incapable of homologous recombination, but rather that within the 24-h period examined, recombination frequencies in uninfected cells were below levels detectable by the methods described here. The absence of carrier DNA in these transfections and the small amounts of input plasmid DNA may both have contributed to the apparently low levels of recombination in the uninfected SIRC cells. Furthermore, the observed amplification of transfecting plasmid DNA is

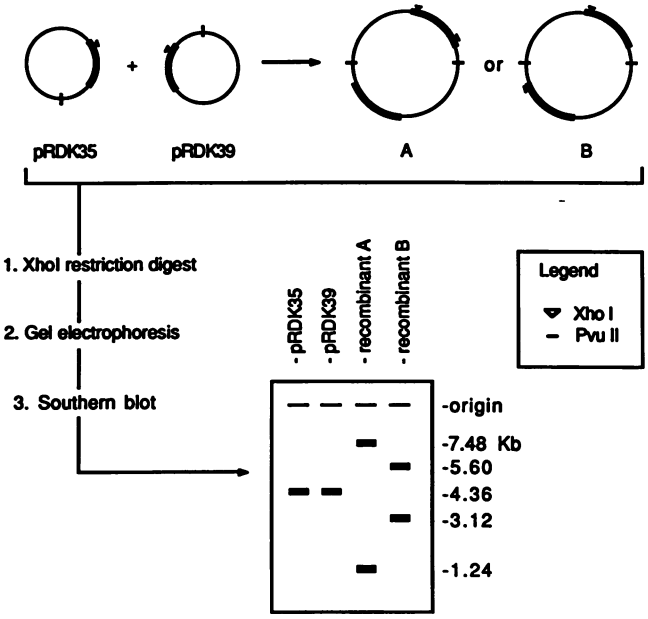

FIG. 2. Protocol used to quantitate recombination by Southern blotting. Only the initial consequences of intermolecular, single recombination events are illustrated. A single reciprocal intermolecular recombination event between pRDK35 and pRDK39 would generate a dimeric product. The formation of these molecules can be detected by digestion with *Xho*I, agarose gel electrophoresis, and Southern blotting with a ^{32}P-labeled vector probe. New restriction fragments 7.48 and 1.24 kb in length are the expected products of a reciprocal event occurring within the tetracycline gene, whereas 3.12- and 5.60-kb fragments are characteristic of events occurring outside the tetracycline gene. Further rounds of reciprocal and/or nonreciprocal recombination would also generate these species, plus other characteristic higher-order restriction fragments. For example, recombination between the dimers A and B could generate additional *Xho*I fragments of 8.72 and 9.96 kb (not shown).

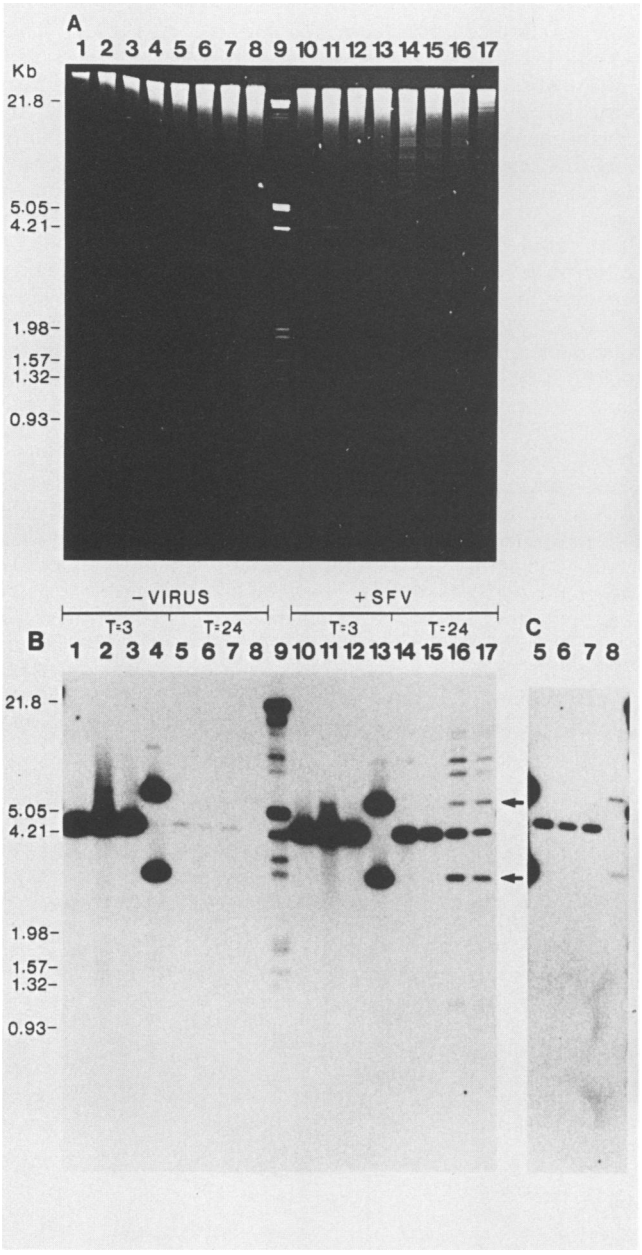

FIG. 3. Recombination between transfecting plasmid DNAs in uninfected and SFV-infected SIRC cells. Monolayers of SIRC cells were infected with SFV or mock infected and transfected with the DNAs indicated below, and the DNA was recovered at 3 or 24 h posttransfection as described in Materials and Methods. DNA was digested with *Xho*I, electrophoresed on 0.7% agarose gels, stained with ethidium bromide (A), and then blotted and hybridized with ^{32}P-labeled pBR322 and exposed for 15 h to detect recombinant vector forms (B). Size markers (lane 9) are derived from λ *Eco*RI-*Hin*dIII digests, and some of the sizes are indicated on the left. Harvest times (in hours) and type of infection are shown between panels A and B. Transfecting DNAs were pRDK35 (lanes 1, 5, 10, and 14), pRDK39 (lanes 2, 6, 11, and 15), pRDK35 plus pRDK39 (lanes 3, 7, 12, and 16) and pRDK41 (lanes 4, 8, 13, and 17). (C) Seven-day exposure of lanes 5 through 8 to illustrate that the level of recombination in uninfected cells is low under these conditions of transfection. Note that pRDK41 is one of the recombinants that can be formed between pRDK35 and pRDK39. The 3.12- and 5.60-kb restriction fragments characteristic of this event (lane 16) and of unrecombined pRDK41 (lanes 4 and 13) are illustrated by arrows.

believed to occur in the cytoplasm of the poxvirus-infected host cells (8), a site which may very well be devoid of detectable recombination enzyme activities in the uninfected cell controls.

The three poxviruses were not equally proficient at this reaction. SFV (Fig. 3B, lanes 16 and 17) and myxoma virus (Fig. 4B, lanes 7 and 8) clearly catalyzed higher levels of recombination than did vaccinia virus (Fig. 4B, lanes 16 and 17). Densitometric analysis showed that the fraction of DNA present as recombinant fragments 24 h after transfection of SFV- and myxoma virus-infected cells was 57 to 63% and 61 to 66%, respectively. A similar analysis involving an over-

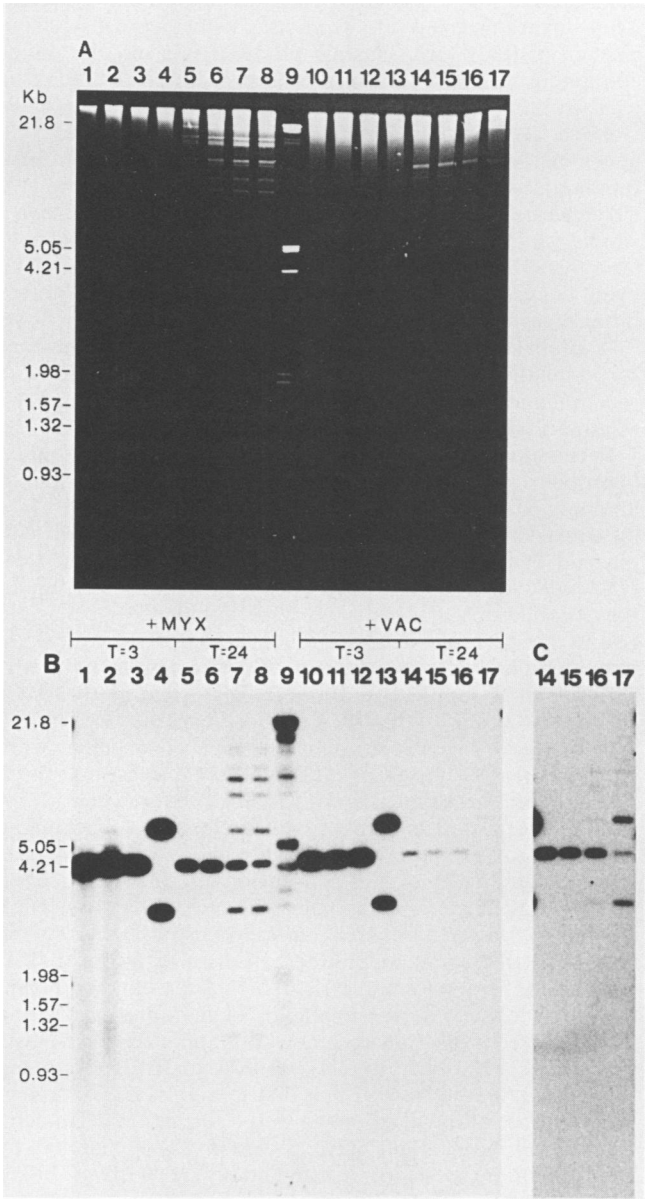

FIG. 4. Recombination between transfecting plasmid DNAs in myxoma virus- and vaccinia virus-infected SIRC cells. The experiments were performed as indicated in Fig. 3, and the results are presented in an identical manner. DNAs are pRDK35 (lanes 1, 5, 10, and 14), pRDK39 (lanes 2, 6, 11, and 15), pRDK35 plus pRDK39 (lanes 3, 7, 12, and 16), and pRDK41 (lanes 4, 8, 13, and 17). (C) Seven-day exposure of lanes 14 through 17 to better illustrate the detectable levels of recombination in vaccinia virus-infected cells.

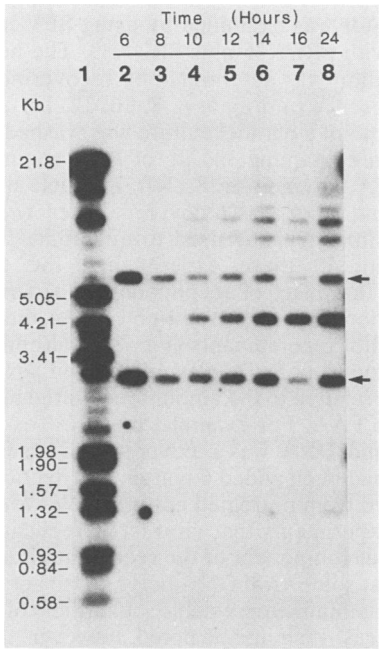

FIG. 5. Kinetics of recombination following transfection of pRDK41 DNA into SFV-infected SIRC cells. Monolayers of SIRC cells were infected with SFV and transfected with pRDK41, and the DNA was recovered at the times indicated (lanes 2 through 8). DNAs were digested with *Xho*I, electrophoresed, and blotted as described in Materials and Methods. A bacteriophage λ *Eco*RI-*Hin*dIII digest (lane 1) was used as a size marker. Arrows indicate the two parental pRDK41-derived *Xho*I fragments of 3.12 and 5.60 kb (Fig. 1).

exposure of the same Southern blots showed only at most 25 to 36% of the DNA to be recombinant in vaccinia virus-infected cells (Fig. 4C, lanes 16 and 17, respectively). Cells were shown to be productively infected to a comparable degree in each case, since similar amounts of virus-derived *Xho*I fragments were readily detected by ethidium bromide staining prior to Southern transfer (Fig. 3A and 4A). This lower degree of recombinational activity in vaccinia virus-infected cells cannot therefore simply be explained by the failure of vaccinia virus to replicate in the host SIRC cells.

The actual recombination frequency was difficult to estimate, given that multiple events might have masked the origin of some recombinants. An approximate minimum frequency that ignores the possible effects of phenomena such as positive interference (and disregards the fact that recombination between identical plasmids is not monitored) was estimated from the distribution of plasmid fragment sizes by assuming that it was generated by a Poisson distribution of events. If $P(r) = (\mu^r \times e^{-\mu})/r!$, where μ is the average number of events per population, r is the number of events per individual = 1, 2, 3, 4 ..., and $P(r)$ is probability of r events per individual in the population, then, given that the two products of 3.12 and 5.61 kb (Fig. 3A, lane 16) composed 28% of the total DNA population and required a minimum of one crossover or conversion event per 3.12 kb (i.e., outside the tetracycline gene), $P(1) = [\mu^1 \times e^{-\mu}]/1! = 28\%/100$. Solving for μ gives $\mu = 0.44$ (per 3.12 kb), implying a minimum recombination frequency of about one event per 7.1 kb in SFV-infected cells. A similar calculation generates $\mu = 0.39$ and 0.07 for myxoma and vaccinia viruses, respectively.

Timing of plasmid recombination. The kinetics of recombination in SFV-infected cells transfected with a mixture of pRDK35 and pRDK39 or with pRDK41 alone were further examined. DNA was recovered at various times after transfection, and recombination was assayed by *Xho*I digestion as described above. Essentially identical results were obtained by using a mixture of monomeric plasmids or pRDK41 alone, so that only the results with pRDK41 are shown (Fig. 5).

Recombinant plasmid DNA was first detected about 8 h posttransfection, and a limit pattern was reached approximately 4 h later (Fig. 5, lanes 3 through 5). The first recombinants detected were the products expected for single recombination events (for example, the 4.36-kb monomer product), and subsequent events generated higher-molecular-weight tandem repeats of pBR322 flanked by the *tet-10* and/or *tet-14* *Xho*I linker insertion sites. Whether recombination terminated 10 to 12 h after transfection could not be determined by this protocol because further rounds of recombination would not be expected to appreciably alter these profiles (Fig. 5, lanes 5 through 8). Tandem repeats in excess of 13 to 15 kb were observed, although this may have reflected an equilibrium limit rather than the actual termination of recombination events per se.

Properties of newly recombined DNA. The recombined plasmid DNA sequences were found as newly replicated, high-molecular-weight DNA within the poxvirus-infected cells. Figure 6A illustrates the structural organization of plasmid DNA recovered from SFV-infected cells. At 3 h posttransfection, transfected plasmids were still found as

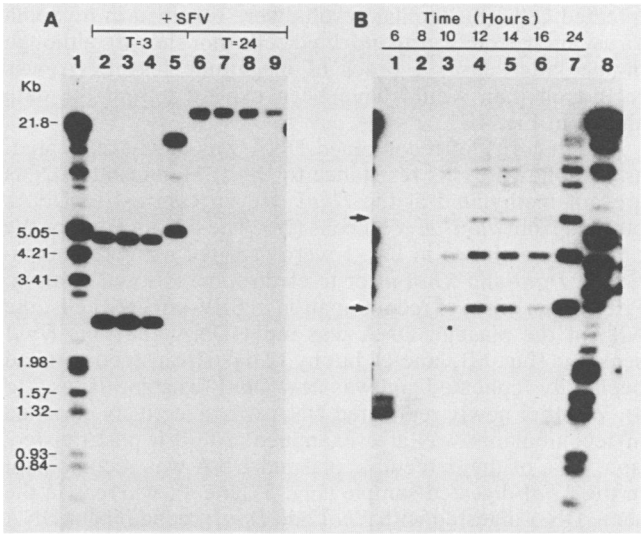

FIG. 6. Recombinant products found within high-molecular-weight, newly replicated concatemeric arrays in SFV-infected cells. SIRC cells were infected with SFV and transfected as described in Materials and Methods. (A) DNA was recovered at the indicated times and fractionated, without a restriction digestion step, on 0.7% agarose gels. DNAs were pRDK35 (lanes 2 and 6), pRDK39 (lanes 3 and 7), pRDK35 plus pRDK39 (lanes 4 and 8), and pRDK41 (lanes 5 and 9). Lane 1 is a bacteriophage λ *Eco*RI-*Hin*dIII digest as a size marker. (B) Cells were transfected with pRDK41, and DNA was recovered at the indicated times. DNAs were digested with *Xho*I-*Dpn*I, fractionated, and Southern blotted as in Fig. 5. Note that by 10 to 12 h all the plasmid-derived DNA, including parental-sized fragments, was newly replicated, as shown by the resistance to *Dpn*I. Arrows indicate the two *Xho*I fragment sizes (3.12 and 5.60 kb) of pRDK41.

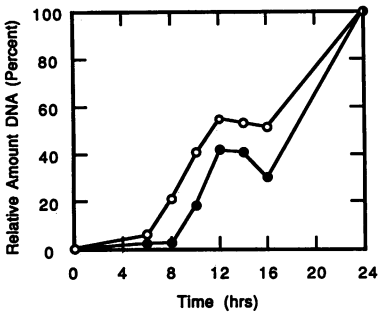

FIG. 7. Approximately synchronous plasmid and viral replication in SFV-infected cells. The autoradiograph shown in Fig. 6B was scanned with a densitometer, and the amount of newly replicated plasmid DNA was quantitated by integration of the densitometric trace. The amount of viral DNA was quantitated by densitometric analysis of a photograph of the original ethidium-stained gel on which viral *Xho*I fragments were visible superimposed upon a uniform background of cellular DNA (not shown). In both cases, the amount of DNA present was normalized to the amount of plasmid or viral DNA found at 24 h. The scale is arbitrary and does not reflect the fact that several orders of magnitude as much viral DNA is present by 24 h posttransfection as there is replicated-recombined plasmid DNA. Symbols: ○, viral DNA; ●, plasmid DNA.

unlinked, intact circles, although some conversion of form I to form II DNA was observed (Fig. 6A, lanes 2 through 5). By 24 h, this DNA could be recovered only as a high-molecular-weight polymer in excess of 40 kb in length (Fig. 6A, lanes 6 through 9), which is in agreement with previous observations of other transfected plasmids in poxvirus-infected cells (8). Similar results were obtained in myxoma virus- and vaccinia virus-infected cells (not shown), although in the latter case, 1 order of magnitude less DNA was recovered than would have been expected from the data shown in Fig. 4B.

The majority of recombined DNA was newly replicated, as was shown by its resistance to *Dpn*I. Mammalian DNAs are not methylated at the *Dpn*I site, in contrast to DNAs purified from *dam*$^+$ *E. coli* cells (28). The DNAs used for the time course shown in Fig. 5 were digested with a combination of *Dpn*I and *Xho*I prior to electrophoresis and blotting. Prior to the onset of recombination in SFV-infected cells, the bulk of the plasmid DNA was input DNA and thus *Dpn*I sensitive (Fig. 6B, lane 1), but by 12 h posttransfection it had been fully replicated and was now *Dpn*I resistant (lanes 2 to 4). Whether newly replicated DNA was selectively enriched in recombinants was also examined. At 10 h posttransfection, 46% of the detectable plasmid DNA was recombinant in the *Xho*I-digested sample (Fig. 5, lane 4), whereas in the same DNA digested with *Xho*I and *Dpn*I, recombinant DNA forms composed ca. 61% of the *Dpn*I-resistant *Xho*I fragments (Fig. 6B, lane 3). This suggests that a slight excess of recombinant products may have been present in newly replicated DNA.

Role of viral replication. The observations shown in Fig. 3, 4, and 6 suggest that recombination and viral replication are closely associated events. The initiations of viral and plasmid replication were approximately coincident (Fig. 7) (see also reference 8) and occurred 6 to 8 h posttransfection in SFV-infected cells in these experiments. This period was also precisely coincident with the onset of recombination (Fig. 5).

To determine whether replication and recombination were inextricably linked, the effect of adding a viral DNA polymerase inhibitor was examined by using SFV-infected cells transfected with pRDK35 plus pRDK39. The normal extent of recombination was determined by recovering DNA samples in the absence of drug at 6, 8, 10, 12, 14, 16, and 24 h. At each time point a parallel culture was washed, exposed to fresh medium containing 300 μg of PAA per ml (to inhibit poxvirus DNA polymerase [8, 24]), and held until the 24-h time point, and DNA was recovered. After *Xho*I digestion, blotting densitometry was used to quantitate the extent of recombination. In these experiments, the time course showed that the onset of recombination occurred 6 to 8 h posttransfection (Fig. 8B). Addition of PAA did not prevent the formation of recombinants (Fig. 8A), although it greatly reduced the total amount of plasmid derived DNA that could be recovered relative to the amount recovered at 24 h in cells unexposed to PAA. For example, only 12% as much hybridizable plasmid DNA was recovered at 24 h from cells to which PAA had been added 6 h after transfection relative to that recovered from untreated cultures. We would conclude from these experiments that viral DNA replication may not be an essential component of the recombination process per se, but at least some viral DNA polymerase activity must be present to maintain appreciable quantities of transfected DNA sequences. It should be noted, however, that since the kinetics of PAA inhibition have not been defined in this system, a requirement for low levels of DNA replication during the observed recombination cannot be rigorously excluded.

The observed products are true recombinants. The observation that transfected DNAs can be subjected to extraordinarily high levels of mutation (5, 31, 41) requires some caution in assuming that the recombinant products detected here by blotting are true recombinants. For example, muta-

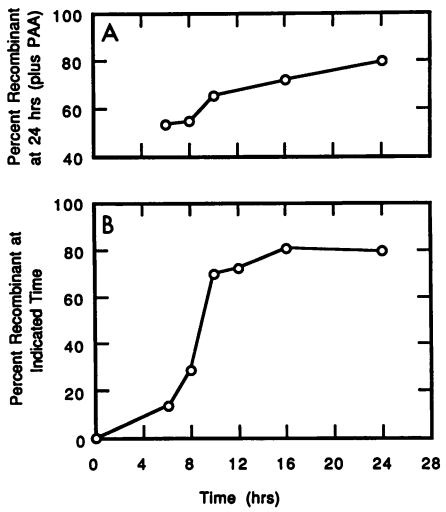

FIG. 8. Effect of PAA on recombination of transfected plasmid DNA in SFV-infected cells. SIRC cells were infected with SFV and transfected with a mixture of pRDK35 and pRDK39. At the indicated times, samples of DNA were recovered and the frequency of recombination was determined by densitometry as described in Materials and Methods. (A) Fraction of plasmid DNA that can be shown to be recombinant at the time indicated. Note that in cells continuously exposed to PAA, recombination still continues. For example, the percentage of plasmid DNA found in recombinant forms goes from 14% at 6 h (panel B) to 55% 18 h later (panel A) in the presence of the inhibitor, although further amplification of both viral and plasmid sequences has been blocked.

TABLE 1. Comparison of recombination frequencies measured by Southern blotting and *E. coli* transformation assays[a]

Infection	Time (h) posttransfection	*E. coli* transformation method				Blotting method[b]	
		No. Amp^r[c]	No. patched	No. Amp^r Tet^r	% Tet^r	% Recombinant	% Tet^r
None	3	12,104	150	0	<1%	ND[d]	0
	24	86	41	0	≤2	ND	0
SFV	3	5,140	150	0	<1	ND	0
	24	686	148	26	18	57	14
Myxoma virus	3	14,300	150	0	<1	ND	0
	24	1,120	150	21	14	61	15
Vaccinia virus	3	9,688	150	0	<1	ND	0
	24	171	39	0	≤3	25	6

[a] DNA was recovered from cells transfected with a mixture of pRDK35 and pRDK39 as described in the legends to Fig. 3 and 4. Purified DNAs were digested with *Eco*RI, circularized, and transformed into *E. coli*, and the percent recombination was calculated as described in Materials and Methods.
[b] Quantitated by densitometric analysis of autoradiograms.
[c] Number of transformants per microgram of DNA.
[d] ND, Not detectable.

tion of the *Xho*I sites could potentially generate some, although not all, of the observed products. This seems unlikely, given the controls shown in Fig. 3 and 4, in which infected cells transfected with pRDK35 or pRDK39 alone retained only the input plasmid restriction pattern. However, to confirm the validity of the quantitation method, we have also calculated the recombination frequency by transforming recombined DNAs extracted from infected-transfected SIRC cells back into *recA E. coli* and measured the fraction of transformants that are tetracycline resistant relative to the total number of ampicillin-resistant transformants (Table 1).

Recombination frequencies measured by the transformation method underestimate the true recombination frequency, since only a subset of the recombinant products are tetracycline resistant. In general, only one-quarter of the recombinants measured by densitometry are Tet^r. This is best illustrated by considering the hypothetical case of quantitatively recombined DNA in which the distribution of *Xho*I sites has been completely randomized. Only 25% of pBR322 genomes would be Tet^r, although 100% are recombinant. Taking this correction into account, it is clear from Table 1 that transformation assays were also able to detect the products of high-frequency in vivo recombination and that, quantitatively, transformation and Southern blot assays gave almost identical results. This transformation assay was not sensitive enough to detect the lower level of recombinants formed in vaccinia virus-infected cells, although statistically the result is not in conflict with frequencies expected on the basis of the Southern blot data. Both SFV and myxoma virus infections, however, induced statistically significant ($P \geq 99.5\%$) increases in the recovery of amp^r tet^4 recombinants, in excellent agreement with the data from Southern blot analyses. Thus, we can conclude that the novel plasmid *Xho*I fragments detected by blotting are an accurate reflection of extremely high levels of bona fide genetic recombination in these poxvirus-infected cells.

DISCUSSION

The use of DNA substrates containing polymorphic restriction sites has proved very useful in experiments designed to investigate the process of recombination in a number of organisms both in vivo and in vitro (10, 34). Poxviruses provide a unique model system with which to investigate the mechanisms of eucaryotic recombination because, first, they replicate in the cytoplasm of infected mammalian cells at a site distinct from the site where the events of host nuclear recombination occur and, second, they are believed to encode most of the enzymatic machinery for viral RNA and DNA metabolism (7, 18, 23, 25, 43). It has been shown elsewhere that when exogenous circular plasmid DNAs are transfected into poxvirus-infected cells, the input DNA is replicated by a mechanism under the control of *trans*-acting viral factors that does not apparently require a virus-specific origin sequence (8). The major product of this replication process in the cytoplasm of the transfected cells is concatemeric head-to-tail arrays of high-molecular-weight vector DNA unlinked to viral sequences (8), unless the input DNA contains viral sequences required for telomeric resolution, which generates linear hairpin-terminated minichromosomes (9, 19). In this study we show that plasmids containing polymorphic *Xho*I restriction sites can be used to demonstrate that this amplification of transfecting plasmid sequences is also associated with the induction of extremely high levels of genetic recombination activities in the cytoplasm of the infected cells.

It is clear that the recombination we observe is strictly dependent upon poxvirus infection. This result may seem surprising, given previous reports that DNAs transfected by the $CaPO_4$ method are often subjected to high levels of recombination even in uninfected cells. By using either a blotting method to detect recombinant plasmid *Xho*I fragment sizes or a transformation assay to detect the generation of the wild-type tetracycline resistance gene, recombination is not detectable in the uninfected SIRC cells within the 24-h span of these experiments. Although it is possible that the rabbit SIRC cells used in this study are less proficient at recombination than many of the rodent or primate cell lines that have been used in other studies, it is more likely that the transient assay used here is less sensitive to nuclear recombination events than are other recombination assays, because the parameters have been optimized to assess specifically cytoplasmic events. It should be noted that efficient transfection of even nanogram quantities of input plasmid into poxvirus-infected cells does not require carrier DNA (8), and this may also contribute to the low recombination frequencies in the uninfected controls reported here.

The amount of induced recombination is virus specific and follows the order mxyoma virus ≈ SFV > vaccinia virus. This order also matches the observed capacity of these three viruses to catalyze nonspecific replication of transfected DNAs (8). All three viruses grow well in SIRC cells, as can

be shown by calculating viral burst sizes and by examining the relative levels of virus-specific restriction fragments on ethidium bromide-stained agarose gels (Fig. 3A and 4A). This eliminates poor levels of vaccinia virus replication in SIRC cells as a trivial explanation of this observation. The simplest alternative explanation is that viral gene products are catalyzing the events of genetic recombination described here, which would account for the virus specificity. Although this is an attractive idea, other experiments must be performed before such a conclusion can be verified. It could always be argued, for example, that the presence of replicating virus induces host functions that then catalyze plasmid recombination and that the two leporipoxviruses are somehow more proficient at this induction than is vaccinia virus.

The observed frequency of recombination in poxvirus-infected cells can be very high. Of the pBR322-derived genomes recovered from SFV- or myxoma virus-infected cells, 18% were Tet^r, which approaches the theoretical limit (assuming no bias in the directionality of gene conversion) of 25%. This indicates that the two *Xho*I markers have, by 24 h, been segregated virtually on a random basis, to the extent that further rounds of recombination cannot be measured. Quantitatively this agrees quite well with the frequency calculated from Southern blots, assuming that all *Xho*I-resistant genomes of $\geq$7.48 kb contain a functional tetracycline resistance gene and suggests that high frequencies of mutagenesis are not somehow involved in the events quantitated by blotting (Table 1). A minimal recombination frequency can be estimated from a Poisson analysis of the *Xho*I fragment distribution and suggests that at least one recombination event takes place per ca. 7.1, 8.0, and 45 kb of transfected DNA in SFV-, myxoma virus-, and vaccinia virus-infected cells, respectively. How this compares with viral recombination frequencies is more difficult to assess, since detailed comparisons of the genetic and physical maps of all three viruses have not been made. Ensinger and Rovinsky (12) and Drillien and Spehner (11) have mapped temperature-sensitive mutations in vaccinia virus with respect to both genetic and physical maps. They obtained a minimal recombination frequency of between 0.9% and 1.8% per 1 kb (or one event per 55 to 110 kb), which is somewhat lower than the plasmid frequency we observed between transfected plasmids in vaccinia virus-infected cells. This suggests that whatever the mechanism of recombination, transfected plasmid and endogenous viral DNAs may be subjected to the same processes, although the endogenous replicating viral genomes may be less accessible to the induced recombination activities than naked transfected DNAs are. The difficulties in precisely correlating physical maps of closely linked temperature-sensitive mutations of vaccinia virus with deduced recombination frequencies (E. Niles and R. Condit, personal communication) may be related to the accessibility of DNA template to these activities, rather than their induction per se. The data presented in this communication also suggest that although the physical sizes of these three viruses are very similar (vaccinia virus is ca. 190 kb, and SFV and myxoma virus are ca. 160 kb), genetically the two leporipoxviruses might appear to be manyfold larger than vaccinia virus by standard genetic mapping techniques involving pairwise crosses of viral mutants. This latter prediction assumes that the differences between plasmid recombination rates in leporipoxvirus and orthopoxvirus-infected cells are also reflected in the viral genomes and is an issue which remains to be resolved.

It is worth noting the close association of replication and recombination. The original observation that transfected plasmid DNAs can be recovered from poxvirus-infected cells as high-molecular-weight concatemers specifically in the head-to-tail orientation suggested that either a rolling-circle mode of replication or a high level of generalized genetic recombination was taking place (8). The two events may be associated processes, given the significant excess of recombinants in newly replicated DNA and the coincident timing of recombination and replication (Fig. 7), but whether the two processes are inextricably linked is difficult to deduce from our data. Inhibition of the SFV viral DNA polymerase with PAA did not inhibit subsequent recombination events, although it markedly reduced the total amount of plasmid DNA that could be recovered from infected cells. This result would suggest that recombination can, at least under conditions of PAA inhibition, be separated from replication, but it remains to be determined whether the two events can be rigorously segregated. Recombination may very well turn out to be an important component of poxvirus replication, as has been observed in bacteriophages such as T4.

Finally, we would note that because plasmid DNA replication is as sensitive to PAA as is viral DNA replication (8), the transfected DNA is located in a cellular location accessible to SFV polymerase, presumably in a cytoplasmic site where the viral factories themselves are located. Given the observation that plasmid recombination frequencies are at least comparable to viral recombination frequencies and that the plasmid DNAs seem to function as templates for *trans*-acting viral activities required for replication, recombination, and telomeric resolution, it is reasonable to speculate that transfected DNAs are exposed to the same recombination processes as are the viral DNAs. Transfected DNAs should turn out to be useful probes for studying the process of poxvirus recombination, and poxvirus-infected cells may turn out to be an ideal source of the gene products which catalyze these events.

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