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Intramolecular Homologous Recombination in Cells Infected with Temperature-Sensitive Mutants of Vaccinia Virus

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I have used a plasmid containing two copies of the *Saccharomyces cerevisiae his3* gene to study intramolecular homologous recombination in vaccinia virus-infected cells. Recombination of the plasmid was monitored by restriction enzyme digestion and Southern blot hybridization in cells infected with representatives from each of 32 complementation groups of temperature-sensitive mutants of vaccinia virus at the nonpermissive temperature. The DNA replication-negative mutants *ts*42 and *ts*17 did not replicate nor detectably recombine the input plasmid. All except one of the mutants that synthesized normal amounts of viral DNA and protein replicated and recombined the plasmid in a manner indistinguishable from wild-type virus. The remaining mutant, *ts*13, only poorly replicated and recombined the input plasmid. Thus, the processes of replication and recombination could not be separated by using this battery of mutants. Viral mutants defective in late protein synthesis were unable to resolve the vaccinia virus concatemer junction in plasmids but carried out intramolecular homologous recombination with plasmids as efficiently as did wild-type virus at the conditionally lethal temperature. This result distinguishes homologous recombination, which requires early gene products, from resolution of concatemer junctions, which requires additional late gene products.

Poxviruses are large, double-stranded DNA viruses which replicate in the cytoplasm of infected cells. Vaccinia virus contains a genome of approximately 185,000 base pairs with 10,000-base-pair inverted terminal repeats (38) and covalently continuous hairpin termini (3). Due to the cytoplasmic locale and nuclear independence (35) of vaccinia virus replication, the gene products needed for vaccinia virus DNA metabolism are probably virus encoded. Genes for thymidine kinase (14, 37), DNA polymerase (15, 31, 34), and ribonucleotide reductase (29, 32) have been identified thus far.

Recombination was demonstrated initially for poxviruses by analyzing the genetic products after coinfecting cells with different vaccinia viruses (10, 11). Subsequently, recombination was shown after transfection of DNA into poxvirus-infected cells (25, 28). More recently, marker rescue with recombinant DNA has been used to locate viral gene products (7, 33, 37). Also, homologous recombination has been used to introduce foreign DNA into the vaccinia virus genome for expression of proteins (17, 26). Such recombinant viruses are being evaluated as live vaccines.

The study of vaccinia virus recombination at the molecular level has only recently been pursued. Viral genomes were shown to rapidly process direct repeats embedded within the genome forming intramolecular and intermolecular recombination products soon after the removal of selective pressure used to maintain the repeat structures (2). Single and double crossover products were detected after recombination between transfected plasmids and resident viral genomes (30). Also, intermolecular and intramolecular recombinants were detected in vaccinia virus-infected cells cotransfected with plasmids containing polymorphic restriction sites (9). In each case, high levels of recombination were observed in the cytoplasm of infected cells.

In order to delineate viral genes used for recombination, I assayed for intramolecular crossover within a plasmid containing two copies of the *Saccharomyces cerevisiae his3* gene in cells infected with conditionally lethal mutants of vaccinia virus at the nonpermissive temperature. Replication and recombination of transfected plasmid was detected in all but four cases: uninfected cells, cells infected in the presence of the vaccinia virus DNA polymerase inhibitor cytosine arabinoside, cells infected with stringent DNA-negative mutants, and cells infected with *ts*13, a mutant which still synthesized normal amounts of viral but not plasmid DNA. Recombination was observed in cells infected with every mutant which replicated the input plasmid DNA, including the late defective mutants blocked in concatemer junction processing, demonstrating that resolution of concatemer junctions does not solely rely on homologous recombination. However, the results obtained from this battery of mutants do not allow one to separate the processes of homologous recombination and DNA replication. Thus, recombination and replication may be inexorably linked. Conversely, the mutant library only contains 32 complementation groups and may not include those with lesions in gene products specific for either process.

MATERIALS AND METHODS

Cells and viruses. A human embryonic line transformed with adenovirus 5, 293 (13), and a continuous line of African green monkey cells, BSC-1, were maintained in Eagle minimal essential medium (MEM) (Quality Biologicals) supplemented with 10% fetal calf serum (GIBCO Diagnostics, Madison, Wis.). The set of vaccinia virus WR temperature-sensitive mutants provided by R. Condit (4, 5) were described elsewhere (22). Infections were performed with crude stocks derived from BSC-1 cells infected at a low multiplicity (less than 0.01 PFU per cell) as described elsewhere (22). Infections with cytosine arabinoside were performed as described elsewhere (22).

Analysis of transfected DNA in infected cells. The plasmid pBYA209 (1) was purified by large-scale alkaline lysis (18), treatment with RNase, and incubation with 0.6 vol of 20% polyethylene glycol–2.5 M NaCl for 1 h on ice before centrifugation. The sample was suspended in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, extracted with phenol–chloroform (1:1), and precipitated with ethanol before

suspension in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA.

Confluent six-well dishes of 293 cells were infected with vaccinia virus or the appropriate temperature-sensitive mutant at 2 PFU per cell or were mock infected with medium. After 30 min at room temperature, the inoculum was replaced with 5 ml of medium containing 10% fetal calf serum and the cells in each well were transfected in 0.5 ml containing 0.8 μg of calcium phosphate-precipitated pBYA209. The precipitate was formed by mixing an equal volume of 2XHBS (280 mM NaCl, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.1], 2 mM Na_2HPO_4) and 250 mM $CaCl_2$ containing pBYA209. The cells were incubated at 39.5 or 40°C, and the medium was replaced after 4 h. The cells were harvested by trituration with medium, pelleted by low-speed centrifugation, and rinsed with phosphate-buffered saline, and the DNA was isolated after cytoplasmic fractionation or whole cell lysis.

Cytoplasm was isolated by suspending the cells in 300 μl of membrane lysis buffer (100 mM NaCl, 50 mM KCl, 20 mM Tris hydrochloride [pH 8.0], 0.1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 0.5% Nonidet P-40), incubating them on ice for 10 min, and removing the nuclei by low-speed centrifugation. The supernatant was brought to 0.6 mg of proteinase K per ml and incubated at 37°C for 6 h before extraction with phenol, phenol-chloroform (1:1), and chloroform. The samples were precipitated with ethanol and suspended in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA.

Whole cell lysates were prepared by suspending cells in 50 μl of 0.15 M NaCl–0.02 M Tris hydrochloride (pH 8.0)–0.01 M EDTA and adding the suspension to 300 μl of 0.02 M Tris hydrochloride (pH 8.0)–0.01 M EDTA–0.75% sodium dodecyl sulfate-0.6 mg of proteinase K per ml. After 6 h at 37°C, the samples were passed through a 25-gauge needle to reduce viscosity and extracted with phenol, phenol-chloroform (1:1), and chloroform before precipitation with ethanol and suspension of the pellets in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA.

The samples were digested with *Xba*I (New England BioLabs) or *Xba*I and *Dpn*I (New England BioLabs, Inc. or Boehringer Mannheim Biochemicals) before electrophoresis through SeaKem ME agarose (FMC Corp.) in E buffer (18). The DNA was transferred to GeneScreen Plus (Du Pont Co.) and hybridized with pBYA209 labeled with ^{32}P by nick translation (Bethesda Research Laboratories, Inc.) as suggested by the manufacturer. Filters were exposed to Kodak XAR film.

RESULTS

Intramolecular homologous recombination in infected cells. In order to delineate the viral gene products which participate in vaccinia virus recombination, I monitored intramolecular homologous recombination within a plasmid in cells infected with a set of vaccinia virus conditionally lethal mutants at the nonpermissive temperature. The plasmid pBYA209 (1), a yeast pBR322 chimera that contains two copies of the *S. cerevisiae his3* gene (Fig. 1), was transfected into cells infected with the viral mutants, and the DNA was analyzed by restriction enzyme digestion and Southern blot hybridization. If intramolecular recombination occurs, the *his3* gene will be rearranged so that the relative position of the *Xba*I sites will change. Digestion of the input pBYA209 with *Xba*I will generate two fragments of 6.3 and 5.65 kilobases (kb), whereas after recombination two additional DNA fragments of 9.4 and 2.55 kb would be detected.

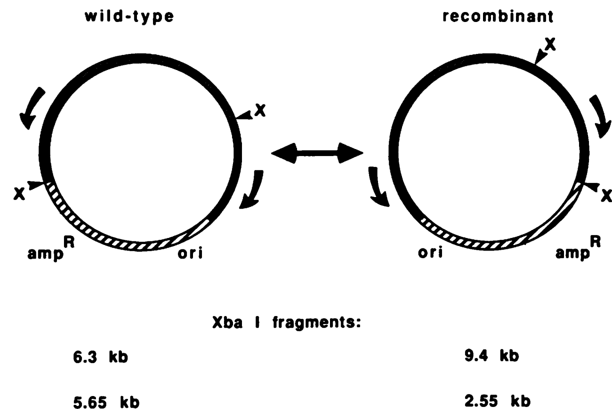

FIG. 1. Schematic representation of the products of intramolecular recombination. The construction of the input plasmid was described earlier (1). Rearrangement of the *his3* genes will generate the conformer illustrated on the righthand side (recombinant). The x refers to the *Xba*I sites. Symbols: ▨, yeast DNA; ▧, pBR322; ■, *his3* gene.

A calcium phosphate precipitate of pBYA209 was added to the 293 cells infected with 1 of 32 vaccinia virus temperature-sensitive mutants used in this study. The cells were incubated at 39.5°C for 24 h, and the cytoplasmic DNA was isolated. Each sample was incubated with *Xba*I and with *Dpn*I, which digested the input-methylated plasmid made in *Escherichia coli* but not DNA replicated in mammalian cells. The DNA fragments were electrophoresed through 1.5% agarose, transferred to a nylon membrane, and hybridized with pBYA209 labeled with ^{32}P by nick translation. DNA samples isolated after infection with the temperature-sensitive mutants from the first 12 complementation groups that include representatives of the three major phenotypes (DNA negative, late defective, and normal) are shown in Fig. 2. Since the DNA-negative mutant *ts*42 did not replicate the input DNA, no *Dpn*I-resistant molecules were detected. The mutants with normal phenotype, *ts*19, *ts*37, *ts*40, *ts*45, *ts*46, *ts*49, *ts*52, and *ts*57, make normal amounts of viral DNA and proteins. Although these mutants synthesized variable levels of plasmid, each mutant generated nearly equivalent amounts of the four DNA fragments diagnostic of recombination (Fig. 2). Similar results were obtained for all but one of the other normal phenotype temperature-sensitive mutants (data not shown). The single exception, *ts*13, will be discussed in a subsequent section. The late defective mutants, *ts*21, *ts*53, and *ts*56, synthesize reduced amounts of late viral proteins. However, the levels of DNA replication and the formation of the recombination-specific fragments was similar to the normal phenotype mutants (Fig. 2). The sole member of the abortive late phenotype group, *ts*22, as well as the other representatives of the late defective complementation group, *ts*7 and *ts*63, also replicated and recombined the input plasmid (data not shown).

Recombination is not observed in the absence of DNA replication. The previous experimental protocol was designed to monitor recombination only when the plasmid is replicated, since the samples were treated with *Dpn*I. To measure recombination for the DNA-negative mutants, *Dpn*I treatment was omitted and DNAs from cells infected with the mutants and from uninfected or infected cells were digested with *Xba*I alone. Hybridization with pBYA209 labeled with ^{32}P by nick translation revealed a high degree of recombination in the wild-type or *ts*21-infected cells but little

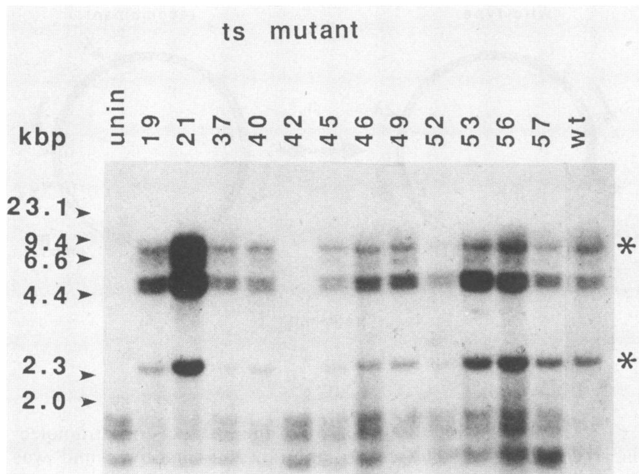

FIG. 2. Detection of intramolecular recombination in cells transfected with pBYA209 and infected with temperature-sensitive mutants of vaccinia virus. 293 cells were transfected with pBYA209 and infected with either wild type (wt) or one of the temperature-sensitive mutants, or mock infected with media (unin). After 24 h at 39.5°C, the DNA was isolated and analyzed by digestion with *Xba*I and *Dpn*I, electrophoresis through 1.5% agarose, and Southern blot hybridization with pBYA209 labeled with ^{32}P by nick translation as the probe. The DNA fragments due to recombination are indicated by asterisks.

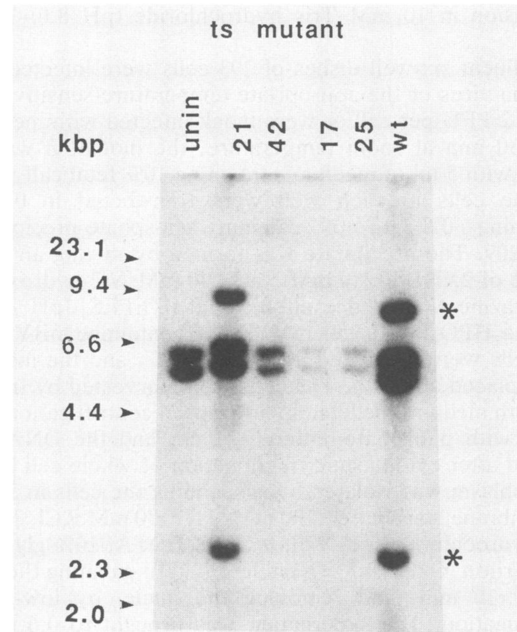

FIG. 3. Intramolecular recombination in cells transfected with pBYA209 and infected with DNA-negative, temperature-sensitive mutants of vaccinia virus. 293 cells were transfected with pBYA209 and infected with wild type (wt), the DNA-negative mutants *ts*42, *ts*17, or *ts*25, the late defective mutant *ts*21, or mock infected with media (unin). After 24 h at 39.5°C, the DNA was isolated and analyzed by digestion with *Xba*I, electrophoresis through 1.5% agarose, and Southern blot hybridization with pBYA209 labeled with ^{32}P by nick translation as the probe. The DNA fragments arising after recombination are indicated by asterisks.

or no recombination in uninfected cells or those infected with *ts*42, *ts*17, and *ts*25 (Fig. 3). Longer exposures clearly revealed small amounts of the 9.4- and 2.55-kb fragments in cells infected with *ts*25, indicative of recombination. However, plasmid replication was detected in cells infected with *ts*25 as determined by *Dpn*I resistance (see Fig. 4).

The phenotype of the mutants defective in DNA replication, *ts*42, *ts*17, and *ts*25, was also investigated at the permissive temperature in order to determine if the defect in recombination was due to the conditional-lethal lesion. Confluent monolayers of 293 cells were infected with each of the mutants or with wild-type virus, transfected with pBYA209, and incubated for 24 h at 40°C (the nonpermissive temperature) or for 30 h at 31°C (permissive temperature). The samples were digested with *Dpn*I and *Xba*I, transferred to nylon membrane, and hybridized with pBYA209 labeled with ^{32}P by nick translation (Fig. 4). At 31°C, infection with any of the mutants or wild type generated the four DNA fragments indicative of recombination. At 40°C, however, no *Dpn*I-resistant DNA is detected in cells infected with *ts*42 or *ts*17. For mutant *ts*25, which has been classified as a DNA-negative mutant (5), or wild-type virus, detectable levels of plasmid replication and recombination occurred as indicated by all four *Dpn*I-resistant *Xba*I digestion products. Also, the relative amounts of the four *Dpn*I-*Xba*I DNA fragments in *ts*25-infected cells is similar to that observed for the normal and late defective mutants (Fig. 2). Therefore, the degree of recombination for the replicated plasmid in cells infected with *ts*25 is analogous to that for wild type, although the absolute levels of replicated plasmid is lower than those for wild type. By using this battery of conditionally lethal mutants, I have been unable to detect intramolecular homologous recombination in the absence of plasmid DNA replication.

The replication and recombination of pBYA209 was also measured in cells infected with wild-type virus in the presence of 40 μg of cytosine arabinoside per ml, an inhibitor of vaccinia virus DNA polymerase. The cells were infected with wild-type virus and transfected with pBYA209 as described earlier. The DNA was isolated after 24 h at 40°C and analyzed by Southern blot hybridization after digestion with *Xba*I or *Dpn*I and *Xba*I with pBYA209 labeled with ^{32}P by nick translation as the probe (Fig. 4). As indicated by the absence of *Dpn*I-resistant fragments, no DNA replication of the plasmid was detected in these cells. Digestion with *Xba*I generated only the two fragments present in input DNA. Thus, even for infection with wild-type virus, no recombination was observed in the absence of DNA replication.

Replication and recombination of pBYA209 in *ts*13-infected cells. The amount of *Dpn*I-resistant plasmid in cells infected with the normal and late defective mutants varied considerably. The *ts*63 and *ts*11 exhibited low levels of plasmid replication, and *ts*13 appeared not to replicate plasmid to detectable amounts. For the experiment depicted in Fig. 5, *ts*63, *ts*11, and *ts*13, as well as *ts*21 and wild-type virus were used to infect monolayers of 293 cells at a multiplicity of 5 PFU per cell. Replication and recombination of pBYA209 at the nonpermissive temperature was measured as described previously. *ts*21 and wild-type virus replicated and efficiently recombined the input DNA (Fig. 5A). The *ts*63 mutant, with a late defective phenotype, and *ts*11, with a normal phenotype, replicated albeit at reduced levels and recombined the pBYA209. However, uninfected cells and *ts*13-infected cells did not synthesize detectable amounts of *Dpn*I-resistant DNA, although the total quantity of transfected DNA (as determined by the *Dpn*I-sensitive material near the bottom of the gel) is comparable to that seen for the other samples. Digestion with *Xba*I of the DNA isolated from cells infected

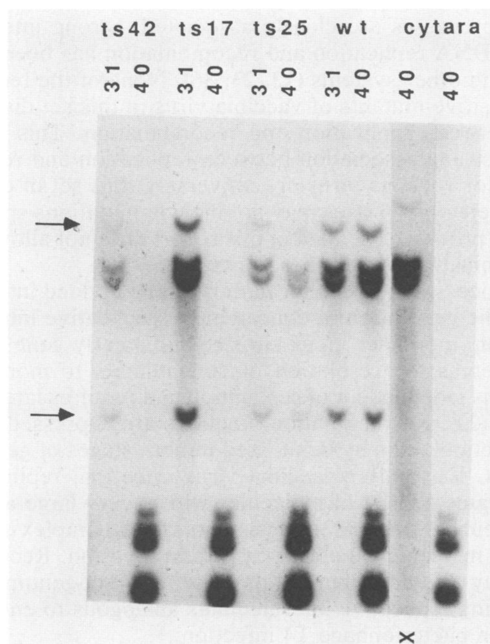

FIG. 4. Intramolecular recombination in cells transfected with pBYA209 and infected with temperature-sensitive mutants or wild-type vaccinia virus at the permissive and conditionally lethal temperatures. The 293 cells were transfected with pBYA209 and infected with the temperature-sensitive mutants *ts*42, *ts*17, or *ts*25 defective in viral DNA replication, with wild-type (wt) virus, or with wild-type virus in the presence of 40 μg of cytosine arabinoside (cytara) per ml. After 24 h at 40°C or 30 h at 31°C, the DNA was isolated and analyzed by digestion with *Dpn*I and *Xba*I, electrophoresis through 1.0% agarose, and Southern blot hybridization with pBYA209 labeled with ^{32}P by nick translation as the probe. The DNA isolated from the cells treated with cytosine arabinoside was digested with *Xba*I (lefthand lane marked with an X) or *Dpn*I and *Xba*I. The slower-migrating DNA band in cytara digested with *Xba*I does not migrate with the 9.4-kb fragment arising from recombination. The DNA fragments arising after recombination are indicated by the arrows.

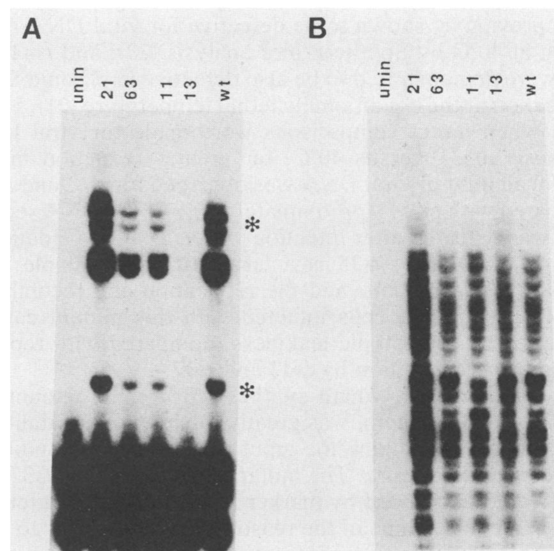

FIG. 5. Analysis of replication and recombination of transfected pBYA209 and replication of viral DNA in cells infected with temperature-sensitive mutants of vaccinia virus. 293 cells were transfected with pBYA209 and infected with either wild type (wt), one of the normal phenotype temperature-sensitive mutants *ts*11 and *ts*13, the late defective temperature-sensitive mutants *ts*21 or *ts*63, or mock infected with medium (unin). After 24 h at 39.5°C, the DNA was isolated and analyzed by digestion with *Xba*I and *Dpn*I, electrophoresis through 1% agarose and (A) Southern blot hybridization with pBYA209 labeled with ^{32}P by nick translation as the probe. The DNA fragments arising after recombination are indicated by asterisks. (B) The probe was removed by treatment with 0.5 M NaOH, and the filter was rehybridized with vaccinia virus DNA labeled with ^{32}P by nick translation.

with *ts*13 generated only the 6.3- and 5.65-kb fragments present in input plasmid as observed for *ts*42 and *ts*17 (data not shown).

In order to assay the replication of the viral DNA, the filter was treated with 0.5 M NaOH to remove the pBYA209 probe and then rehybridized with vaccinia virus DNA labeled with ^{32}P by nick translation. There are similar amounts of viral DNA in all except the uninfected sample (Fig. 5B). The greatly reduced levels of plasmid replication in the *ts*13-infected cells was not paralleled with a reduction in viral DNA replication.

DISCUSSION

High levels of recombination have been observed in vaccinia virus-infected cells (2, 9, 30). The aim of this study was to delineate the gene products necessary for homologous recombination in vaccinia virus-infected cells by testing the ability of conditionally lethal mutants to carry out intramolecular homologous recombination at the nonpermissive temperature. Advantage was taken of the previous observation that replication of plasmids in vaccinia virus-infected cells is not sequence-specific but nevertheless requires vaccinia virus gene products (21). The infected cells were transfected with a plasmid containing a pair of *S. cerevisiae his3* genes which, if rearranged via recombination, would alter the *Xba*I restriction digest pattern. Although the absolute level of the plasmid replication varied among the mutants, in every example where plasmid replication was detected, all four *Xba*I fragments indicative of intramolecular homologous recombination were observed and the ratio of the 2.55- and 9.4-kb fragments to the 6.3- and 5.65-kb fragments was nearly unity. Comparable amounts of the four *Xba*I fragments implies that recombination has generated an equimolar mix of the conformers. Since input plasmids are converted to high-molecular-weight, head-to-tail multimers in vaccinia virus-infected cells (6, 20), the *Dpn*I-*Xba*I digestion products may arise from intramolecular homologous recombination of a circular plasmid or by intramolecular or intermolecular homologous recombination of large tandem arrays of replicated plasmid. Either form of recombination will generate the same end products.

Replication and recombination of pBYA209 was observed for all but four scenarios. Replication or recombination was not observed in uninfected cells, in cells infected in the presence of the inhibitor of vaccinia virus DNA polymerase cytosine arabinoside, or the DNA-negative mutants *ts*42 and *ts*17. Also, *ts*13, a mutant which synthesized normal levels of viral DNA and protein, had dramatically diminished levels of replicated and recombined pBYA209.

Although the DNA-negative mutant *ts*25 was observed to recombine the input plasmid, this was associated with some plasmid replication. The phenotypes for the DNA-negative mutants *ts*42, *ts*17, and *ts*25 were investigated more closely by blot hybridization of viral DNA isolated from cells infected at the nonpermissive temperature. All three mutants

were previously shown to be defective for viral DNA replication at 40°C by Southern blot analysis (22), and *ts*42 and *ts*17 were demonstrated to be also defective in plasmid DNA replication at the conditionally lethal temperature (21). However, when direct comparisons were made for viral DNA synthesis at 31 versus 40°C, far greater reduction in the overall amount of viral DNA was observed for *ts*42 and *ts*17, compared with *ts*25. Approximately 10% of the DNA seen at 31°C was detected after infection with *ts*25 at 40°C (data not shown). Therefore, *ts*25 is a less stringent example of a DNA-negative mutant, and the replication and recombination of plasmids in cells infected with this mutant can be explained by phenotypic leakiness, compared with replication and recombination by *ts*42 and *ts*17.

The mutant *ts*13, which synthesized normal amounts of viral DNA and protein, was greatly inhibited in its ability to replicate and recombine the input plasmid at the conditionally lethal temperature. The mutant *ts*13, as well as *ts*37 and *ts*57, were not mapped by marker rescue, although attempts were made (33). Some of the reasons for the inability to map mutants by marker rescue include phenotypic leakiness, multiple site mutations, or lesions in recombination functions (33). *ts*37 and *ts*57 replicated and recombined exogenous DNA, implying that the blocks to marker rescue are not due to problems in recombination (Fig. 2). However, *ts*13, with greatly reduced levels of replication and recombination of the input DNA, may represent a lesion in a recombination function. Longer exposures revealed that *ts*13 infected-cells replicated the input plasmid DNA approximately 50-fold less than cells infected with the wild-type virus did and contained all four fragments indicative of recombination. The ability of *ts*13 to selectively replicate virus but not plasmid DNA is curious and will be the subject of further investigations.

The results in this manuscript demonstrate that homologous recombination occurs at high levels in cells infected with wild-type vaccinia virus and with many conditionally defective viral mutants at the nonpermissive temperature. In cells infected with the late defective group of mutants, efficient recombination of plasmids was detected at the nonpermissive temperature, although plasmids containing the concatemer junction were poorly resolved (21). It was previously demonstrated that resolution of the concatemer junction is independent of replication (22). The data in this manuscript demonstrates that resolution of the concatemer junction is not simply due to homologous recombination.

Cells infected with the late defective mutant *ts*21 at the conditionally lethal temperature accumulate linear multimers containing both head-to-head and head-to-tail concatemer junctions (22). The mix of concatemer junction conformers was observed as early as 6 h postinfection (M. Merchlinsky, unpublished data) and can readily be explained by recombination between homologous terminal sequences in replicative intermediates. Furthermore, apparent translocation of terminal sequences (8, 16, 24, 27) or generation of mirror image deletions (19) can be explained by invoking recombination between homologous terminal sequences in replicative intermediates.

Recombination was always detected for infections in which plasmid DNA was replicated. Previous work has alluded to the strong connection between recombination and replication. The products of intramolecular recombination appeared shortly after the onset of vaccinia virus DNA replication and were not detected in the presence of inhibitors of viral DNA replication (2). Also, recombination products generated from transfected plasmids occurred concomitantly with poxvirus DNA replication (9), suggesting that the two processes are closely associated. Strong interplay between DNA replication and recombination has been documented in other systems (12, 23, 36). None of the temperature-sensitive mutants of vaccinia virus in this set discriminated between replication and recombination. This could reflect a strong association between replication and recombination for vaccinia virus or, conversely, this set of conditionally defective viruses may not include mutations specific for either process. The data in this report does not allow one to discriminate between these possibilities.

The process of genome formation can be divided into two stages. The generation of concatemeric replicative intermediates from monomer input virus requires early gene products, whereas the resolution of concatemers to monomer genomes is independent of replication and requires late gene products (22). Recombination functions are expressed soon after infection and may be utilized in both stages of genome formation. Early after vaccinia virus infection, replicative intermediates consist of molecules with a very large apparent molecular weight (22) consistent with a complex multibranched molecule generated by strand invasion. Recombination may also contribute to the maturation of genomes by debranching replicative intermediates analogous to endonuclease 7 in bacteriophage T4 infection.

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