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High-Frequency Homologous Recombination in Vaccinia Virus DNA

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A recombinant vaccinia virus genome was constructed in which the viral thymidine kinase (*tk*) gene was placed between direct repeats of a 1.5-kilobase-pair DNA sequence of heterologous origin. When forced to replicate in tk^- cells in the presence of methotrexate (i.e., under tk^+-selective conditions), the recombinant maintained its tk^+ phenotype. Under nonselective conditions, however, the *tk* gene was frequently excised by both inter- and intramolecular recombination events because the repeated sequences provided substantial targets for homologous DNA recombination. Unique DNA products of intramolecular recombination were detected in the cytoplasm of infected cells soon after the onset of viral DNA replication, and their appearance was blocked by inhibitors of DNA synthesis. During repeated passage of the virus under nonselective conditions, the tk^+ fraction decreased with first-order kinetics at a rate that reflected the frequency of recombination per cycle of virus replication. Eventually, a residual population of stable tk^+ viruses remained, and analyses of the genome structures of individual members of this population showed that some of them appeared to be the products of nonhomologous DNA recombination.

Vaccinia virus (VV) is the prototype orthopoxvirus, a class of large DNA-containing animal viruses that replicates in the cytoplasm of infected cells (5). The genome of VV is a molecule of double-stranded DNA that contains about 187 kilobase pairs (kb) and encodes about 250 viral proteins (17). Several known VV genes have been assigned map positions on the viral DNA. These include the genes for the viral thymidine kinase (*tk*) (11, 22), DNA polymerase (13), and the large subunit of the guanylyltransferase enzyme (16), as well as many genes the identity of which is not yet clear (2, 4, 7, 8).

The technique of marker rescue of viral mutants with cloned fragments of wild-type VV DNA was instrumental in mapping many of these VV genes (18, 20). This approach depends on recombination between the viral sequences in the DNA fragment and the homologous sequences in the viral genome. The same method has been used to construct VV recombinants that contain and express foreign DNA sequences and, thus, to harness VV both as a eucaryotic expression vector and as the basis of potential recombinant vaccines against a variety of pathogens (14). Despite the increasingly widespread use of this technique, however, little is known about the mechanism of homologous DNA recombination in VV-infected cells.

It seems reasonable to speculate that, like VV DNA replication, recombination occurs in the cytoplasm and is mediated by enzymes some or all of which are virus-specified. These considerations make VV DNA recombination particularly attractive as an experimental system because it should be accessible to both genetic and biochemical approaches. Moreover, the safety and genetic stability of potential vaccines based on VV recombinants could be enhanced by an understanding of VV DNA recombination and by finding ways to prevent its occurrence. The marked difference in the neurovirulence of tk^+ and tk^- mutants of VV (3) emphasizes the importance of genetic stability in potential recombinant vaccines.

One of the difficulties inherent in studies of DNA recombination is that the relative infrequency of the event often restricts one to the use of genetic methods to analyze the recombination products. I sought to solve this problem by constructing a VV genome that contained a large tandem repeat which would constitute a target for high-frequency recombination. To overcome the intrinsic instability of such a target, I inserted the VV *tk* gene between the two copies of the tandemly repeated sequence. Because the viral *tk* gene can be made essential, nonessential, or lethal, depending on the conditions of incubation of the infected cells, this DNA arrangement creates a situation in which the frequency of recombination can be precisely measured and partially controlled by the investigator. As long as tk^+ selection pressure is maintained, replicating virus will be constrained from eliminating the *tk* gene by recombination. On the other hand, under nonselective conditions replicating virus will be free to recombine, and under tk^- selection it will be forced to do so.

MATERIALS AND METHODS

Cells and viruses. Thymidine kinase-deficient 143 cells (kindly provided by B. Moss, National Institutes of Health, Bethesda, Md.) were used exclusively for the growth and plaque assay of VV (strain WR) and its recombinants as described previously (10, 21). The cells were maintained as monolayer cultures in minimal essential medium containing 5% fetal bovine serum. To impose tk^- selection pressure, 5-bromo-2′-deoxyuridine (65 μM) or trifluorothymidine (16 μM) was added to the medium. To impose tk^+ selection pressure, the following additions were made: methotrexate (0.6 μM), adenosine (50 μM), guanosine (50 μM), thymidine (16 μM), and glycine (100 μM) (6). Selective conditions were imposed at least 1 h before virus growth or plaque assay and were maintained throughout the course of the infection. Multiple passages of virus under nonselective conditions were performed by infecting cultures of 3×10^6 cells at a

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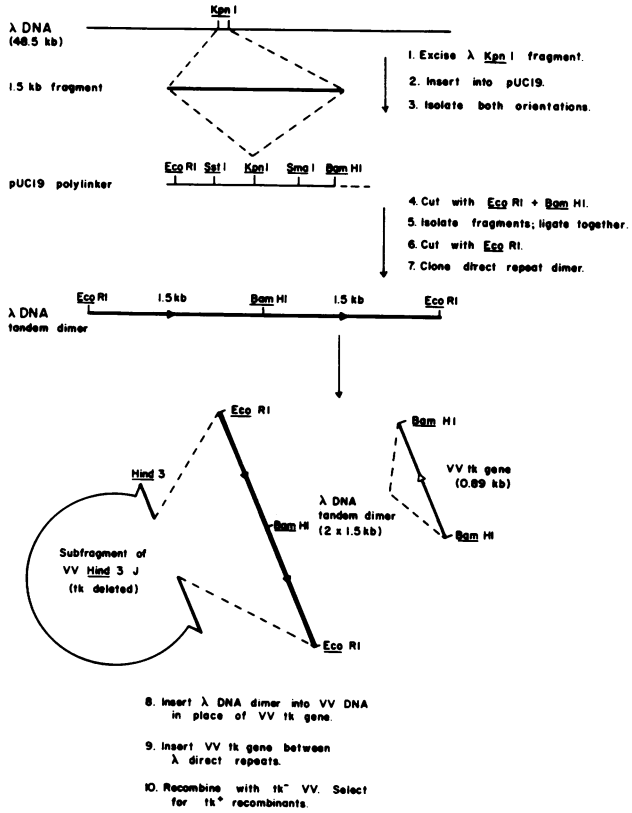

FIG. 1. Construction of VV.λ*tk*λ. The 1,503-bp *Kpn*I fragment of bacteriophage λ DNA was inserted at the *Kpn*I site of pUC19, and two plasmids that carried this fragment in different orientations were isolated (steps 1 to 3). The λ fragments were then excised from these plasmids by digestion at restriction sites that flanked the *Kpn*I site and ligated together to form dimers (steps 4 to 7). By this approach, homodimers formed inverted repeats, but heterodimers formed direct repeats. The direct repeat dimer was inserted into a plasmid carrying a subfragment of VV *Hin*dIII-J DNA from which all of the *tk*-coding sequences except for the first five and the last two codons had been deleted by *Bal* 31 exonuclease digestion (step 8). Finally, the 888-bp *Sau*3A fragment of VV DNA that contains the VV *tk* gene was inserted between the two copies of the λ DNA fragment (step 9), and the recombinant plasmid was recombined with a tk^- deletion mutant of VV to produce tk^+ recombinant virus (step 10).

multiplicity of infection (MOI) of about 5, 0.5, or 0.05 PFU per cell. The infections were allowed to proceed for 24 h between passages. Multiple passage under tk^+ selection was performed at an MOI of 0.05 PFU per cell.

Plasmid construction and manipulation. An *Escherichia coli* plasmid carrying the *Hin*dIII J fragment of VV DNA (which contains the VV *tk* gene) in the *Hin*dIII site of pBR322 was kindly provided by B. Moss (2). A version of this plasmid from which most of the *tk*-coding sequences were deleted was constructed by *Bal* 31 exonuclease digestion from the *Eco*RI site near the middle of the *tk* gene. An *Eco*RI linker was inserted at the position of the deletion, and the limits of the deletion were determined by sequence analysis. The 888-base-pair (bp) *Sau*3A fragment that encompasses the *tk* gene (base pairs 410 to 1298) was subcloned into the *Bam*HI site of pBR322, a site from which it could be excised by digestion with *Bam*HI. All plasmid DNA manipulations were performed as described by Maniatis et al. (15). Plasmid DNA was recombined with the replicating DNA of a tk^- deletion mutant of VV (T. E. Ryan and L. A. Ball, manuscript in preparation); and recombinant viruses were selected, screened by DNA hybridization, and plaque purified as described previously (1, 21).

Preparation of viral DNA and analysis by Southern blot hybridization. Viral cores were released from VV-infected cells by detergent lysis and were recovered by centrifugation (9). DNA was isolated from the core preparations by digestion with proteinase K followed by extractions with phenol-chloroform and precipitation with ethanol (9). Total cytoplasmic DNA was isolated by the same procedure except that the cell lysates were not centrifuged before DNA extraction. Inhibitors of viral gene expression and DNA replication were used as described previously (10). DNAs were resolved by electrophoresis on 0.7% agarose gels, denatured by soaking in 0.4 M NaOH–0.6 M NaCl, and transferred by capillary blotting in the same solution to positively charged nylon membranes (GeneScreen Plus; Du Pont Co., Wilmington, Del.). After neutralization, the membranes were hybridized to DNA probes labeled with ^{32}P by nick translation, and radioactive bands were detected by autoradiography (15).

RESULTS

Construction and isolation of VV.λ*tk*λ. A 1,503-bp *Kpn*I fragment from bacteriophage λ DNA (base pairs 17058 to 18560) was chosen to provide the target for high-frequency homologous recombination. I constructed a bacterial plasmid in which an 888-bp *Sau*3A fragment of VV DNA that encompassed the *tk* gene was flanked by direct repeats of this λ sequence. The λ*tk*λ sandwich was inserted into a deleted version of the VV *tk* gene from which all but the first five and the last two codons were removed (Fig. 1). The resulting plasmid was then recombined into a tk^- deletion mutant of VV, and tk^+ recombinants were selected. After five successive plaque purifications under tk^+-selective conditions, one such recombinant was designated VV.λ*tk*λ.

The projected DNA structure of this recombinant in the region of the λ*tk*λ sandwich is shown in Fig. 2. Because the promoter and termination-polyadenylation sites of the *tk* gene overlap the adjacent VV genes (12, 23), duplication of the termini of the 888-bp *tk* fragment in the regions immediately flanking the λ*tk*λ sandwich was unavoidable. Thus, base pairs 410 to 512 and 1022 to 1298 of the VV *Hin*dIII J fragment were duplicated at the 5′ and 3′ ends of the *tk* gene, respectively (Fig. 2). To diminish the possibility that these repeats would direct homologous recombination events that would excise the λ sequences, the *tk* gene fragment was inserted in the reverse of its usual orientation. The abundant symmetrically placed restriction enzyme cleavage sites were derived from the pUC19 multiple cloning site (Fig. 1).

Characterization of VV.λ*tk*λ. The expression of the VV *tk* gene in this unusual sequence environment was examined by measuring the *tk* activity in lysates of tk^- cells that were prepared at various times after infection with wild-type VV or VV.λ*tk*λ. The results indicate that both the absolute level and the kinetics of *tk* expression are indistinguishable in the two cases (Fig. 3). Other VV recombinants that contained the same 888-bp *tk* fragment relocated to a different site in the viral genome also expressed *tk* in a manner that was very similar to that of the wild-type virus (Ryan and Ball, in preparation). As expected, no increase in enzyme activity was detectable in cells infected with the tk^- mutant from which VV.λ*tk*λ was derived.

The ability of VV wild type, tk^-, and λ*tk*λ viruses to form plaques on monolayers of tk^- cells was compared under tk^+-

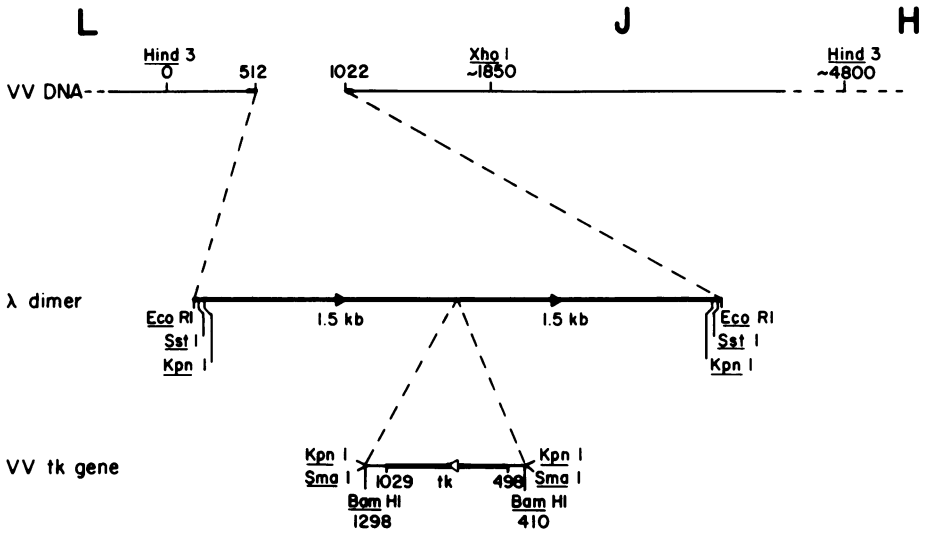

FIG. 2. Diagram of the genome region of VV.λ*tk*λ that contained the λ DNA repeats and the inverted VV *tk* gene. The 4.8-kb *Hin*dIII J fragment is near the middle of the VV genome and normally contains the *tk* gene between base pairs 498 and 1029 from the *Hin*dIII L-J fragment junction (12, 23). In VV.λ*tk*λ, base pairs 513 to 1021 were replaced by two direct repeats of a 1.5-kb fragment of bacteriophage λ DNA separated by an 888-bp fragment that encompassed the VV *tk* gene (base pairs 410 to 1298 of *Hin*dIII-J). The filled arrows indicate the orientation of the λ fragments (base pairs 17058 to 18560), and the open arrowhead indicates the 5′ to 3′ orientation of the *tk* gene. The numbers refer to the base pair positions measured from the *Hin*dIII L-J fragment function.

and tk^--selective and nonselective conditions. The wild-type and tk^- viruses were able to form plaques under tk^+- and tk^- selective conditions, respectively, with the same efficiency as under nonselective conditions; they failed to form plaques under the adverse selection conditions (Table 1). However, VV.λ*tk*λ was unusual in that imposing either tk^+ or tk^- selection pressure diminished only partially its ability to form plaques. Under tk^- but not tk^+ selection, the efficiency of plaque formation by VV.λ*tk*λ increased with longer times of incubation of the plaque assay, from an initial value of about 65% (Table 1) to a maximum of about 100%. No plaques were formed if both tk^+ and tk^- selection pressures were imposed simultaneously.

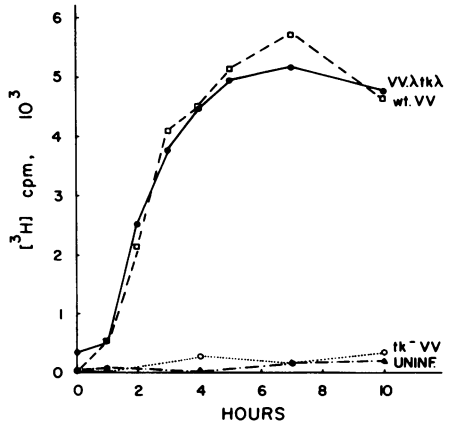

FIG. 3. Kinetics of *tk* expression in infected cells. Thymidine kinase-deficient 143 cells were infected with wild-type (wt) VV or VV.λ*tk*λ at an MOI of 10 tk^+ PFU per cell or with the tk^- parent of VV.λ*tk*λ at an MOI of 10 tk^- PFU per cell. At the indicated times after infection, cytoplasmic extracts were prepared and assayed for *tk* activity by measurement of the phosphorylation of [^{3}H]thymidine (10).

The trivial possibility that VV.λ*tk*λ could form plaques under both selection conditions because of incomplete plaque purification was eliminated by repeated purification and by the observation that individual tk^+ plaques always yielded about the same 1:2 ratio of secondary plaques when used to inoculate monolayers of tk^- cells under tk^+ and tk^- conditions, respectively. On the other hand, individual tk^- plaques yielded secondary plaques only under tk^--selective or nonselective growth conditions. These results suggest that during the few rounds of viral replication that are necessary to produce a visible plaque, the genome of VV.λ*tk*λ undergoes recombination to produce tk^- virus. Under tk^+-selective conditions, tk^- virus generated in this way can replicate by being complemented by the residual tk^+ viral genomes in the infected cells.

Genome structure of VV.λ*tk*λ. Direct evidence to confirm the interpretation presented above was derived from analyses by Southern blot hybridization of the genome structure(s) of VV.λ*tk*λ. DNA preparations from cores of the virus that were grown under either tk^+- or tk^--selective conditions were digested with *Kpn*I, which cuts at each end of both λ sequences, or with *Sst*I, which cuts at the outer

TABLE 1. Relative plaquing efficiencies (%)

Virus	Relative plaquing efficiencies (%) under the following conditions[a]:		
	NS	tk^+	tk^-
Wild type	(100)	100	<0.01
tk^- deletion	(100)	<0.001	100
VV · λ*tk*λ	(100)	35	65

[a] Wild-type VV, a tk^- deletion mutant, and VV · λ*tk*λ were assayed for their relative plaquing efficiencies on monolayers of tk^- cells under nonselective (NS) or tk^+- or tk^--selective conditions. The stocks of VV · λ*tk*λ used in this and other experiments were prepared by growth under tk^+-selective conditions. For each virus, the number of plaques detectable after 40 h of incubation under selective conditions was expressed as a percentage of the number formed under nonselective conditions, shown as (100).

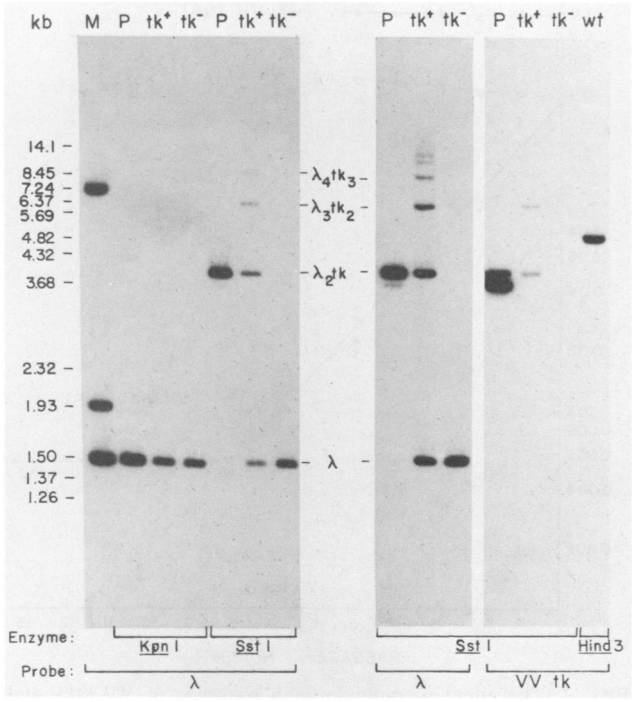

FIG. 4. Southern blot analysis of the genome structure of VV.λ*tk*λ. DNA was extracted from cores of VV.λ*tk*λ grown under either tk^+- or tk^--selective conditions (as indicated), digested with *Kpn*I or *Sst*I (as indicated), resolved by agarose gel electrophoresis, and hybridized with a radioactive probe specific for the λ *Kpn*I fragment or with a nick-translated plasmid that contained an internal region of the VV *tk* gene (base pairs 530 to 771 of *Hin*dIII J) (as indicated). Lanes marked P received digests of the bacterial plasmid used to construct VV.λ*tk*λ, the structure of which is shown in Fig. 1. The lane marked wt received a *Hin*dIII digest of wild-type VV DNA; only the 4.8-kb *Hin*dIII J fragment hybridized to the *tk*-specific probe. The lane marked M received molecular size markers, which are given beside the figure, in kilobase pairs. The markers were a *Bst*EII digest of bacteriophage λ DNA mixed with the 1.5-kb *Kpn*I fragment of λ DNA. This fragment spans the junction between the 7.24- and 1.93-kb *Bst*EII fragments which therefore hybridized to the λ probe.

ends of either the λ*tk*λ sandwich or any sequence arrangement derived therefrom by homologous recombination (Fig. 2). After agarose gel electrophoresis and Southern transfer, the digests were hybridized with probes that were specific for DNA fragments that contained the λ sequences or for those that contained the VV *tk* sequences (Fig. 4). *Kpn*I digestion showed that virus grown under either tk^+ or tk^- selection contains the λ sequences only in the form of the input 1,503-bp λ fragment. In neither case did any detectable sequence rearrangements occur within the λ fragments. Digestion with *Sst*I, on the other hand, showed that viruses grown under tk^+ or tk^- selection differed in their DNA structures. Virus grown under tk^- selection contained only a single copy of the λ fragment and no *tk* gene; its DNA structure can be represented as VV.λ, which is the predicted result of homologous inter- or intramolecular recombination between the λ direct repeats in VV.λ*tk*λ (Fig. 5). Virus grown under tk^+ selection also showed the 1,503-bp *Sst*I fragment that was characteristic of the tk^- product of homologous recombination, but in addition it yielded a series of larger DNA bands that contained both λ and VV *tk* sequences. The smallest of this series corresponded to the input λ*tk*λ fragment (3.9 kb), while the larger bands had the structures λ*tk*λ*tk*λ (6.2 kb), λ*tk*λ*tk*λ*tk*λ (8.6 kb), and so on (Fig. 4; other data not shown). These larger *Sst*I fragments are predicted products of repeated rounds of homologous intermolecular recombination (Fig. 5).

These results confirm that stocks of VV.λ*tk*λ that are grown under tk^+ selection comprised a dynamic mixed population of tk^+ and tk^- viruses, the genome structures of which result from homologous recombination between the λ direct repeats. To determine how flexible the composition of this virus population was and how rapidly the distribution of genome structures was achieved, several individual plaques of VV.λ*tk*λ were isolated under tk^+- or tk^--selective or nonselective conditions and used as sources of viral DNA after the minimum possible further replication. The results of Southern blot analysis of *Sst*I digests of several such DNA preparations are shown in Fig. 6. Three separate virus stocks grown under nonselective conditions or five stocks grown under tk^- selection all contained single copies of the 1,503-bp λ fragment. However, three independent virus stocks grown under tk^+ selection yielded ladders of λ-containing DNA fragments that were not only identical in their relative abundances among the three stocks but that were also indistinguishable from the DNA fragments derived from a stock of VV.λ*tk*λ that was amplified repeatedly under tk^+ selection. These observations demonstrate that a few rounds of replication of VV.λ*tk*λ under tk^+ selection are sufficient for recombination to establish and maintain a steady-state distribution of tk^+ and tk^- viral genome structures.

Evolution of VV.λ*tk*λ during multiple passages. The evolution of VV.λ*tk*λ was examined by measuring the relative abundance of tk^+ virus during several low-multiplicity passages under different growth conditions. Under tk^+ selection, tk^+ virus constituted 35 to 40% of the total, and this value was maintained during repeated passage (Fig. 7). Multiple passage under nonselective conditions, however, resulted in a progressive log-linear decrease in the abundance of tk^+ virus. During each of the first eight passages under nonselective conditions, 40% of the residual tk^+ virus lost its tk^+ phenotype. The same behavior was observed at three different MOIs. Wild-type virus that was subjected to multiple passages under nonselective conditions showed a fully stable 100% tk^+ phenotype.

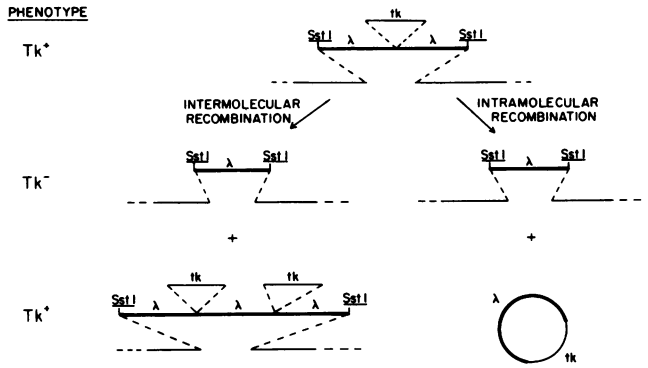

FIG. 5. Diagrammatic representation of the structures of the predicted DNA products of inter- and intramolecular recombination between the λ direct repeat in VV.λ*tk*λ. Only the *Sst*I cleavage sites are indicated; the location of other restriction enzyme cleavage sites in the predicted products can be determined from Fig. 2.

Analysis of the genome structures of VV.λ*tk*λ during the first six nonselective passages showed a progressive decrease in the abundances of all the *tk*-containing *Sst*I fragments (Fig. 8). The 1,503-bp λ fragment generated from tk^- viral DNA did not diminish during these nonselective passages.

After eight nonselective passages of VV.λ*tk*λ, the abundance of tk^+ virus stabilized at about 5×10^{-3}. One hundred individual isolates of tk^+ virus from nonselective passage 13 were examined for the stability of their tk^+ phenotype and for the presence and arrangement of the λ DNA fragments (data not shown). Forty-six of these isolates were indistinguishable from the VV.λ*tk*λ starting virus. They had unstable tk^+ phenotypes and the original λ*tk*λ arrangement of genes. Forty-two were indistinguishable from wild-type VV. They had stable tk^+ phenotypes, no λ sequences, and an intact *tk* gene in the correct orientation in the *Hin*dIII J fragment of their genomes. DNA of this structure would result from a double intermolecular crossover between the duplicated copies of the terminal sequences of the 888-bp *tk* fragment (Fig. 2). Because of the inverted orientation of the 888-bp *tk* DNA fragments in VV.λ*tk*λ, single intermolecular crossover events would produce only nonviable DNA structures that contained two copies of the left or right halves of the VV genome. On the other hand, a double intermolecular crossover event would result in the elimination of both λ fragments and the regeneration of wild-type VV DNA. Ten mutants achieved a stable tk^+ phenotype by somehow eliminating one copy of the λ sequences, and two did this by relocating the *tk* gene without any flanking λ sequences to a site about 45 kb away from its original position, in the *Hin*dIII F fragment of the genome. Remarkably, the *Bam*HI sites at each end of the *tk* gene fragment were retained in the new location, but the adjacent *Kpn*I sites were not. The site of insertion was at or near one of the two *Bam*HI sites in the *Hin*dIII F fragment. In the DNA of this latter class of mutant, one copy of the λ fragment remained at the site of the original insertion in *Hin*dIII J. Because the members of these mutant classes may have been siblings and were subjected to different selection pressures during the multiple passages, their relative abundance may not accurately reflect the intrinsic frequency with which they arose in the viral population.

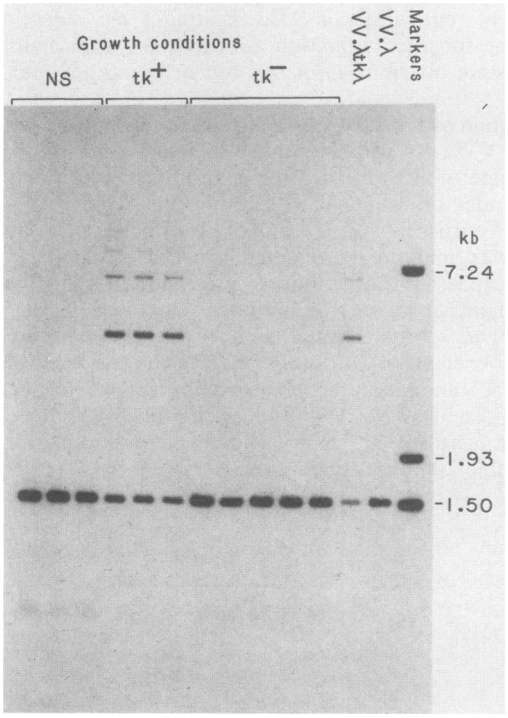

FIG. 6. Southern blot analysis of the genome structures of several isolates of VV.λ*tk*λ. Individual plaques were picked from VV.λ*tk*λ grown under nonselective (NS) or tk^+- or tk^--selective conditions (as indicated) and amplified under the same growth conditions for 36 h, which is the minimum extent necessary to provide sufficient viral DNA for analysis by Southern blot hybridization. Viral DNA was digested with *Sst*I, resolved by agarose gel electrophoresis, and hybridized with a radioactive probe specific for the λ *Kpn*I fragment. The lanes marked VV.λ*tk*λ and VV.λ received digests of DNA from virus stocks that had been amplified repeatedly under tk^+- or tk^--selective conditions, respectively. The molecular size markers to the right of the gel are as described in the legend to Fig. 4.

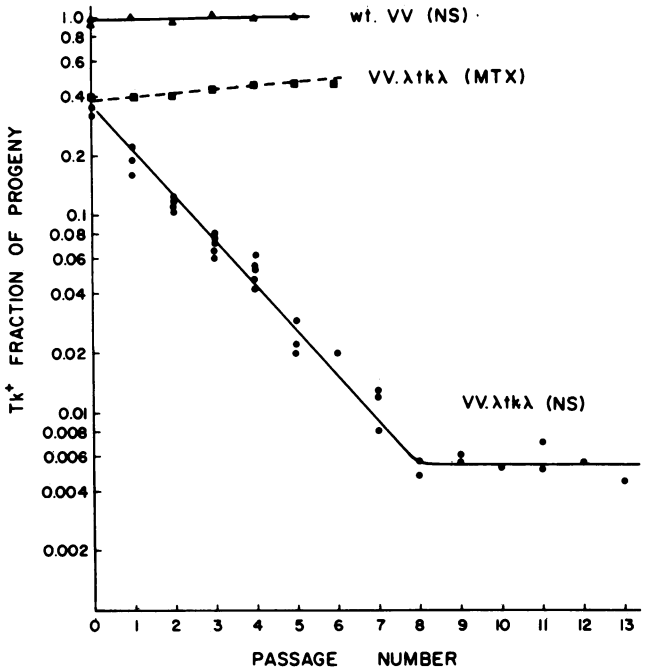

FIG. 7. The effect of multiple 24 h passages of VV.λ*tk*λ and wild-type (wt) VV on the fraction of viral progeny that retained a tk^+ phenotype. VV.λ*tk*λ was subjected to repeated passage at an MOI of about 5, 0.5, or 0.05 PFU per cell under nonselective (NS) conditions or at an MOI of 0.05 PFU per cell under tk^+ selection (MTX). Wild-type VV was subjected to repeated passage at an MOI of 0.5 PFU per cell under nonselective conditions. After each passage, the fraction of viral progeny that retained a tk^+ phenotype was determined by plaque assays under tk^+-selective and nonselective conditions. The results of several plaque assays are shown.

Intramolecular recombination in VV.λ*tk*λ. Of the λ-containing DNA fragments generated by *Sst*I digestion of VV.λ*tk*λ DNA, the larger members (> 6.2 kb) provided clear evidence of intermolecular recombination, as described above. On the other hand, viral genomes that contained the smallest (1.5-kb) fragment could have been generated by either inter- or intramolecular recombination (Fig. 5). The possibility that recombination occurs in the latter manner (i.e., between the two copies of the λ sequence within a single viral DNA molecule) was examined by searching for

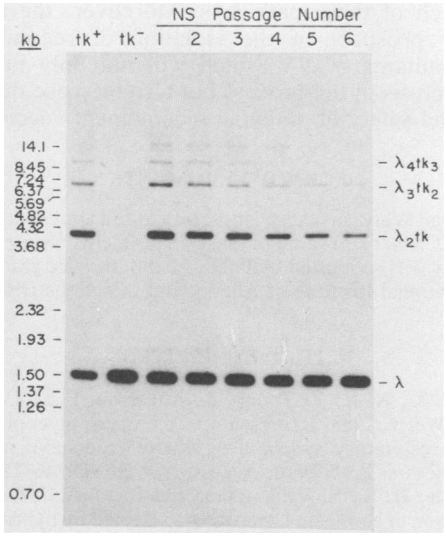

FIG. 8. Southern blot analysis of the genome structures present in VV.λ*tk*λ as it evolved during multiple passage under nonselective (NS) conditions. DNA was extracted from cores of VV.λ*tk*λ that were maintained under tk^+ or tk^- selection (as indicated) or that were subjected to the indicated number of passages under nonselective growth conditions. The DNA samples were digested with *Sst*I and resolved by agarose gel electrophoresis, and λ-containing DNA fragments were detected by hybridization to a radioactive probe specific for the λ *Kpn*I fragment. The molecular size markers were as described in the legend to Fig. 4, and their migration positions are indicated on the left.

the unique predicted DNA product of this mode of recombination, a 2.4-kb DNA fragment containing single copies of the λ and *tk* sequences (Fig. 5). Such a molecule is unlikely to be packaged in viral cores, so total cytoplasmic DNA was extracted from cells at various times after infection with VV.λ*tk*λ and analyzed by gel electrophoresis and Southern blot hybridization. At about the onset of viral DNA replication (3.5 h after infection), the predicted 2.4-kb DNA fragment was detected (Fig. 9). It contained both λ and *tk* sequences as well as cleavage sites for *Eco*RI and *Pst*I (data not shown) but not for *Sst*I (Fig. 9). Its kinetics of accumulation preceded those of replicated viral DNA, suggesting that intramolecular recombination predominantly occurs early during DNA replication. In some experiments a DNA molecule that had the properties of a relaxed but covalently closed circular 2.4-kb fragment was the predominant form of the recombination product (data not shown). Inhibition of viral gene expression by cycloheximide or actinomycin D as late as 2 h after infection or of viral DNA replication by hydroxyurea or cytosine arabinoside completely blocked the appearance of the 2.4-kb DNA fragment (data not shown). These results indicate that intramolecular recombination can occur in VV DNA even between sequences that are only 2.4 kb apart.

DISCUSSION

I used the VV *tk* gene to stabilize, by tk^+ selection pressure, a 1.5-kb direct repeat near the middle of the VV genome. Without stabilization, such sequence arrangements were so readily eliminated from the viral DNA by homologous recombination that isolation of the corresponding virus was impossible (19; unpublished data). Even in the case of VV.λ*tk*λ, recombination occurred with such high frequency that the majority of infectious virus particles that could be recovered from single plaques grown under tk^+ selection contained DNA that had already undergone recombination with consequent loss of the *tk* gene. The replication of such tk^- viral DNA was presumably achieved via complementation by *tk* expression from VV genomes that had not yet undergone recombination or from the tk^+ progeny of intermolecular recombination events. Viral stocks of VV.λ*tk*λ therefore contained a mixed population of viruses, the genome structures of which were in a dynamic recombinational steady state with each other, with the composition of the steady state being rapidly achieved and maintained by the selection pressure that was imposed. All the genome structures that were major components of this mixture were predicted products of intermolecular homologous recombination, including some presumably highly unstable structures with up to five tandem copies of the λ*tk* sequence motif.

The only potentially stable major component of the population of viruses that constituted VV.λ*tk*λ was the tk^- end product of recombination (VV.λ), the DNA of which re-

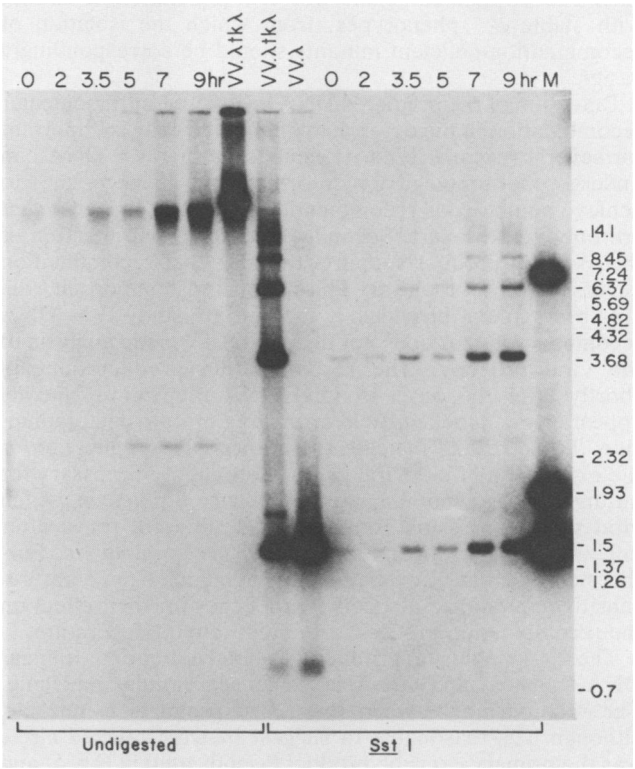

FIG. 9. Southern blot analysis of total cytoplasmic DNA extracted from cells at the indicated times after infection with VV.λ*tk*λ at an MOI of 3 tk^+ PFU per cell under nonselective conditions. The DNA was resolved by agarose gel electrophoresis before and after digestion with *Sst*I (as indicated), and λ-containing DNA fragments were detected by hybridization to a radioactive probe specific for the λ *Kpn*I fragment. The lanes marked VV.λ*tk*λ and VV.λ received DNA from cores of virus that were grown under tk^+ and tk^- selection, respectively. The lane marked M received molecular size markers, as described in the legend to Fig. 4. The autoradiograph was deliberately overexposed to reveal the 2.4-kb product of intramolecular recombination.

tained only a single copy of the λ sequence. During repeated low-multiplicity passage of VV.λ*tk*λ under nonselective conditions, this component increased in abundance until it constituted 99.73% of the total virus population. The remaining 0.27% of the population was of interest because it contained viruses that had achieved a stable tk^+ phenotype, and among this population viral mutants that are defective in homologous recombination might be expected to be found. However, results of analyses to date have instead revealed three other ways in which the virus can achieve a stable tk^+ phenotype: (i) by homologous recombination within the VV sequences that are repeated at each end of the *tk* fragment, resulting in the reconstruction of the wild-type genome; (ii) by elimination of one of the flanking λ copies by an unknown mechanism; and (iii) by spontaneous relocation of the 888-bp *tk* gene fragment to a site in the *Hin*dIII-F region of the genome, a process that appears to involve nonhomologous recombination. Detailed analyses of the DNA structures of these recombinants may contribute to an understanding of nonhomologous recombination in VV-infected cells. Meanwhile, to facilitate the search for recombination-deficient mutants among the residual population of tk^+ viruses, I am currently constructing a version of VV.λ*tk*λ which lacks the potential to regenerate wild-type VV by homologous recombination. Multiple nonselective passage of the new construct should leave a much smaller residual population of viruses with stable tk^+ phenotypes, from which the isolation of recombination-deficient mutants should be correspondingly easier.

Detection of the predicted DNA product of intramolecular recombination in the cytoplasm of infected cells is important for several reasons. First, it establishes that VV DNA can undergo the topological contortion that is necessary to achieve homologous recombination between sequences that are only 2.4 kb apart. Second, the cytoplasmic location of the excision product supports the idea that recombination occurs in the cytoplasm. Third, and most important, the 2.4-kb fragment provides a method to study VV DNA recombination that does not depend on a genetic analysis of the viral progeny. The recombination product appears shortly after the onset of viral DNA replication, and its appearance is blocked by hydroxyurea or cytosine arabinoside. These results indicate that either DNA replication or the expression of late viral genes, or both, are necessary for intramolecular recombination to occur. Studies in which viral gene expression is inhibited at different times after infection may distinguish between these possibilities. Furthermore, it should now be possible to examine temperature-sensitive mutations in specific viral genes for their effect on the recombination process at the restrictive temperature.

The 2.4-kb excision product was detected both as a linear DNA fragment and as a relaxed closed circular molecule. The relationship between these two products is unclear, although it is reasonable to suggest that the relaxed circle was the primary excision product (as indicated in Fig. 5) and was then converted to a variable extent into a linear molecule by random cleavage by the DNase activities that were present in the infected cells. Further structural studies of these two forms of the recombination product will be necessary to resolve this question.

In conclusion, this approach has made homologous DNA recombination a major pathway of DNA metabolism in VV-infected cells and thus rendered it accessible to study by biochemical methods. The DNA products of both inter- and intramolecular recombination can be directly identified and quantitated, and it may prove possible to detect intermediates in each of these pathways. Moreover, the approach offers the prospect of the isolation of recombination-deficient mutants of VV which will not only facilitate a genetic analysis of the process but also increase the genetic stability and safety of potential recombinant vaccines.

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