JOURNAL OF VIROLOGY, Mar. 1988, p. 1046–1054
0022-538X/88/031046-09$02.00/0

Vol. 62, No. 3

Delineation of the Viral Products of Recombination in Vaccinia Virus-Infected Cells

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Received 14 August 1987/Accepted 21 November 1987

Plasmids containing the vaccinia virus thymidine kinase gene, its flanking DNA sequences, and the *Escherichia coli* β-galactosidase gene were used in conjunction with a thymidine kinase-deficient virus to examine the viral products of recombination. Progeny derived from single-crossover events could be distinguished from those generated by gene conversion or double-crossover events when the β-galactosidase gene was separated from the thymidine kinase gene by the flanking sequences. Using methotrexate to select for recombinant virus and a chromogenic indicator to detect β-galactosidase, the generation of viral recombinants was measured over a 48-h period. Recombinant progeny were first observed at 12 h and increased to a maximum of 2.5% at 48 h. Single-crossover products, as determined by β-galactosidase expression, reached a maximum of 57% of the recombinant population at 24 h and thereafter declined. DNA hybridization analysis was used to examine genomic structures of the progeny of the initial viral plaques, plaques purified three times, and those subject to a 10^4-fold amplification. These analyses confirmed that single-crossover events within either the 5′- or 3′-homologous flanking sequences generated unstable recombinant structures. These structures were shown to contain a single copy of the intact thymidine kinase gene within the corresponding copy of the duplicated thymidine kinase flanking sequences, separated by the β-galactosidase gene and plasmid DNA. Significantly, these duplicated structures could undergo further recombination to produce repeats of either the intact or the deleted thymidine kinase sequences. These intermediate structures ultimately degenerated to produce either the parental thymidine kinase-deleted or the wild-type genome. The wild-type genome was also shown to be generated directly by gene conversion or double-crossover events.

Recombination involves the breakage and crosswise reunion of nucleic acid strands within homologous sequences. Early genetic studies of *Escherichia coli* with the transducing bacteriophage lambda demonstrated that the products of recombination were often phenotypically unstable (25). Subsequent experiments indicated that recombination could produce direct nontandem duplications of the homologous sequences through single-crossover events between circular phage and *E. coli* DNAs and that these duplications were unstable (6). In contrast, the products of double-crossover or gene conversion events defined by the reciprocal or nonreciprocal exchange of genetic information were stable (6).

Recombination in poxviruses was initially shown to occur by coinfecting cells with two distinct viruses and demonstrating that plaques formed by the progeny expressed a combination of the distinct genetic markers (16, 17). More recently, recombination between exogenous and viral DNAs in vaccinia virus-infected cells has been used extensively in the mapping of mutations and in the insertion of foreign genes (22, 26, 38). Nevertheless, the process of recombination in vaccinia virus remains obscure (3).

In the work described in this report, the structures and the relative stabilities of the viral products of recombination in vaccinia virus-infected cells were examined. The formation of unstable gene duplications and their condensation by further recombination are described. These results are discussed as they relate to the mechanism and experimental applications of recombination in vaccinia virus-infected cells.

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MATERIALS AND METHODS

Cells and viruses. The monkey kidney cell line BSC-40 was obtained from R. Condit, and the thymidine kinase (TK)-deficient human cell line (Hu143TK$^-$) was obtained from T. Sieck (2, 5, 32). Both cell lines were cultured as monolayers at 37°C under 5% CO_2 in Dulbecco modified Eagle medium containing 10% fetal calf serum. Vaccinia virus WR was obtained from J. Kates.

The TK-negative mutant TK-79 was derived by mutagenesis with nitrosoguanidine and was selected by its ability to replicate in the presence of 5-bromodeoxyuridine (BUdR [29]). Both viral isolates were plaque purified and amplified, and the titers of the resulting stocks were determined on monolayers of BSC-40 cells.

The HB101 strain of *E. coli* was used as the host for the growth of all plasmids (4, 23).

Recombination in vaccinia virus-infected cells. Confluent monolayers of BSC-40 cells on 60-mm dishes were infected with TK-negative vaccinia virus at a multiplicity of 0.1 PFU per cell in 1 ml of growth medium. Virus was allowed to absorb onto cells for 30 min, at which time the medium was removed, the monolayers were washed with phosphate-buffered saline (pH 7.4), and 4 ml of growth medium was added. In the experiments described below, this time is arbitrarily defined as the starting point of infection. A 20-μg portion of plasmid DNA in 500 μl of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (20 mM HEPES, 150 mM NaCl, 0.7 mM Na_2HPO_4, 5 mM KCl, 6 mM glucose [pH 7.0]) was precipitated by the addition of 31 μl of 2 M $CaCl_2$ at 23°C for 45 min. This calcium phosphate precipitate was added directly to growth medium 30 min after the start of infection (19, 35, 40). Cells were harvested 24 h later by scraping plates, and virus was

released by three freeze-thaw cycles. Viral titers were determined on Hu143TK$^-$ cell monolayers either in liquid medium or under 1% Noble agar containing growth medium, and plaques were visualized 72 h later by staining with either 0.1% crystal violet or 0.009% neutral red solution respectively. Titers of TK-positive recombinant virus were determined on Hu143TK$^-$ cell monolayers under Noble agar containing growth medium, 5 μM methotrexate (MTX), 15 μM thymidine, 50 μM adenosine, 50 μM guanosine, and 100 μM glycine, and visualized 72 h later by neutral red staining (7, 10). Titers of TK-negative virus were determined on Hu143TK$^-$ cell monolayers under 1% Noble agar containing growth medium and 60 μg of BUdR per ml (7). At 24 h after cells were infected, β-galactosidase-producing virus was visualized by the addition of a second agar overlay containing 300 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml.

Viral DNAs. After 48 to 72 h, viral progeny were picked from 60-mm plates containing no more than 100 plaques and placed in 1 ml of Dulbecco modified Eagle medium. Viral plaques were purified by three repetitions of this procedure. When specified, purified plaques were amplified by growth for 24 to 48 h on monolayers of the Hu143TK$^-$ cells and harvested by scraping. Viral cores were purified from the progeny of either individual plaques or infected-cell monolayers, and the DNA was purified from the cores by procedures previously described (8, 14). The structures of recombinant genomes were determined by DNA hybridization analysis (34). All autoradiographs were exposed for 1 h, unless otherwise stated, at −70°C with preflashed Kodak X-Omat film and Du Pont Cronex intensifying screens.

Plasmid substrates for recombination. Plasmid pTK-1 consists of a 1.8-kilobase-pair (kb) *Hin*dIII-to-*Pvu*II fragment containing the *tk* gene and its flanking sequences derived from the *Hin*dIII J fragment of the vaccinia virus genome. This fragment was inserted into the *Nde*I site of pUC19 deleted for *lacZ* and polylinker sequences by removal of a 445-base-pair (bp) *Hae*II fragment. All subsequent plasmids used in these experiments are derivatives of pTK-1 (Fig. 1). pTKΔ417 was generated by the removal of 417 bp of the 531-bp coding sequence of the vaccinia virus *tk* gene by *Ssp*I digestion. Plasmid pTK+Z was generated by inserting a fragment containing the *E. coli lacZ* gene under the transcriptional control of the vaccinia virus 7.5K gene promoter (7.5KZ) into the *Hin*dIII site of pTK-1 (37). Plasmid pTKZ-1 was generated by inserting the 7.5KZ fragment into the *Fok*I site 81 bp distal to the translation termination codon of the vaccinia virus *tk* gene (39).

Materials. Restriction endonucleases and enzymes were purchased from Bethesda Research Laboratories, Inc., Boehringer Mannheim Biochemicals, or New England BioLabs, Inc., and used as specified by the manufacturer. MTX, thymidine, adenosine, guanosine, X-Gal, HEPES, and BUdR were purchased from Sigma Chemical Co. Nitrocellulose filters were purchased from Schleicher & Schuell, Inc. Radioisotopes and scintillation fluid were purchased from New England Nuclear Corp.

RESULTS

Viral recombinants can be generated at a frequency of 0.5 to 2.0% between plasmids containing vaccinia virus sequences and replicating viral DNA in the cytoplasm of vaccinia virus-infected cells (22, 26, 29, 38). In the work described in this paper, plasmids containing vaccinia virus sequences and two genetic markers were constructed to study viral recombination. The vaccinia virus *tk* gene was used to select recombinant progeny, and the *E. coli lacZ*

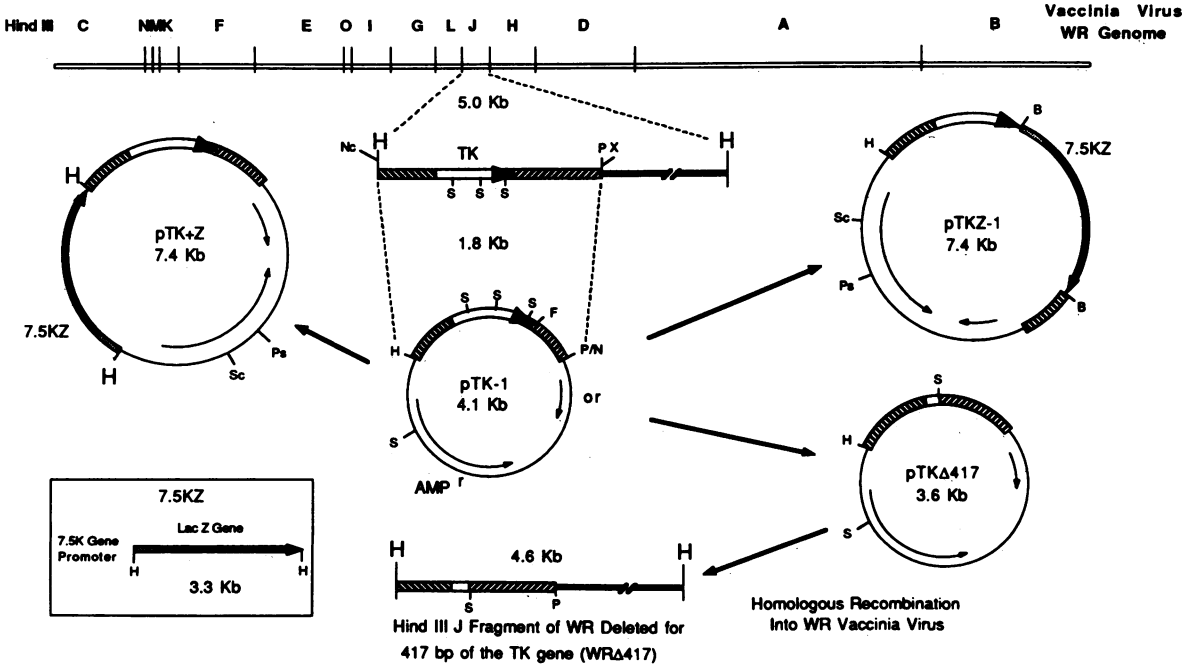

FIG. 1. Derivation of plasmids and *tk*-deleted virus, WRΔ417. Plasmids were derived from the *Hin*dIII J fragment of the vaccinia virus genome as described in Materials and Methods. Symbols: ▭, *tk* gene coding sequences; ▨, *tk* gene flanking sequences; ▬, vaccinia virus sequences; ——, pUC19 sequences; ➡, *lacZ* gene coding sequences. Cleavage sites: B, *Bgl*II; F, *Fok*I; H, *Hin*dIII; N, *Nde*I; Nc, *Nco*I; P, *Pvu*II; Ps, *Pst*I; S, *Ssp*I; Sc, *Sca*I; X, *Xho*I. Abbreviations: AMP, β-lactamase; or, bacterial origin of replication.

gene was used to distinguish different types of recombinants within this population.

Conditions for the selection of recombinant viral progeny. Plaque formation by either TK-79, a virus which carries a point mutation abolishing TK activity, or wild-type WR virus on Hu143TK$^-$ cell monolayers was assessed at a range of MTX concentrations (Table 1). Either 1 or 10 μM MTX completely blocked the formation of TK-79 plaques when up to 10^6 PFU were plated, whereas no effect was seen on plaque formation by wild-type virus. As expected, the inclusion of 60 μg of BUdR per ml in the growth medium blocked plaque formation by wild-type virus, while not interfering with the formation of TK-79 plaques (Table 1) (26, 38). Mixing experiments selecting either for or against *tk* expression demonstrated that a 10^4-fold excess of drug-sensitive PFU did not alter the number of plaques formed by the drug-resistant virus (data not shown). These data demonstrate the efficacy of the selections for identifying viral populations which are distinguished by TK activity.

Derivation of virus and plasmids for studying recombination. To avoid the complication of TK-79 reverting to the wild type and to provide a diagnostic genomic structure, a *tk*-deleted virus, WRΔ417, was generated by the excision of 417 bp of coding sequence (Fig. 1). This virus was characterized by its ability to form plaques in the presence of BUdR, the analysis of its genomic structure, and the ability of the intact *tk* gene to restore wild-type activity in marker rescue experiments.

Plasmid pTK+Z contains the *lacZ* gene controlled by the 7.5K vaccinia virus promoter, the viral *tk* gene, and its flanking sequences (Fig. 1). Recombination by a single-crossover event in either of the vaccinia virus sequences adjacent to the *tk* gene would result in insertion of the entire plasmid, giving rise to a virus containing both markers. However, recombinants generated by gene conversion or double-crossover events involving both regions flanking the *tk* gene result in a virus containing only the intact *tk* gene. In contrast, plasmid pTKZ-1 contains the *lacZ* gene distal to the *tk* gene but within the flanking vaccinia virus sequences (Fig. 1). Thus, both genetic markers should cosegregate in all progeny from recombination between plasmid pTKZ-1 and WRΔ417 virus, regardless of whether they were generated by single-crossover or gene conversion events.

Time course of appearance of viral recombinants. Confluent cell monolayers were infected for 30 min at a multiplicity of 0.1 PFU per cell with WRΔ417, after which the viral inoculum was removed and replaced with growth medium. Plasmid pTK+Z DNA was added as a calcium phosphate precipitate to the medium after an additional 30 min, and individual cell monolayers were scraped and pelleted by centrifugation at 6-h time intervals. Titers of the resulting virus were determined in the presence and absence of MTX, as well as in the presence of MTX plus X-Gal, to determine the percentage of viruses which were recombinants and those which had incorporated both the *tk* and *lacZ* genes (Fig. 2). Recombinant virus was first observed at 12 h, continued to increase in number for the 48-h duration of the experiment, and reached a maximum value of 2.5% of the total viral progeny (Fig. 2). The fraction of recombinants which contained both genetic markers attained a maximum value of 57% at 24 h and thereafter declined, suggesting that they were formed by single-crossover events to generate unstable recombinant structures (Fig. 2).

TABLE 1. Conditions for selection of recombinant viral progeny

Virus (PFU/plate)	No. of plaques[a] generated in presence of:				
	BUdR (60 μg/ml)	MTX at:			
		0 μM	0.1 μM	1.0 μM	10 μM
TK-79 (10^6)	>1,000	>1,000	>1,000	0	0
TK-79 (10^5)	>1,000	>1,000	>1,000	0	0
TK-79 (10^4)	>1,000	>1,000	>1,000[b]	0	0
TK-79 (10^3)	280	250	30[b]	0	0
WR (10^5)	3	>1,000	>1,000	>1,000	>1,000
WR (10^4)	2	>1,000	>1,000	>1,000	>1,000
WR (10^3)	0	820	740	790	820
WR (10^2)	0	100	90	110	110

[a] Average values from five plates are given; no more than 1,000 plaques were counted per plate.
[b] Small and irregular plaques.

Segregation of genetic markers in viral recombinants. Both circular and linear forms of plasmids pTK-1, pTKZ-1, and pTK+Z were used as substrates for recombination with virus WRΔ417, and the progeny generated after 24 h were assayed for the presence of the *tk* and *lacZ* genetic markers (Table 2). Approximately half of the recombinants generated with circular plasmid pTK+Z contained both genetic markers; however, only 0.5% of the TK-positive recombinant

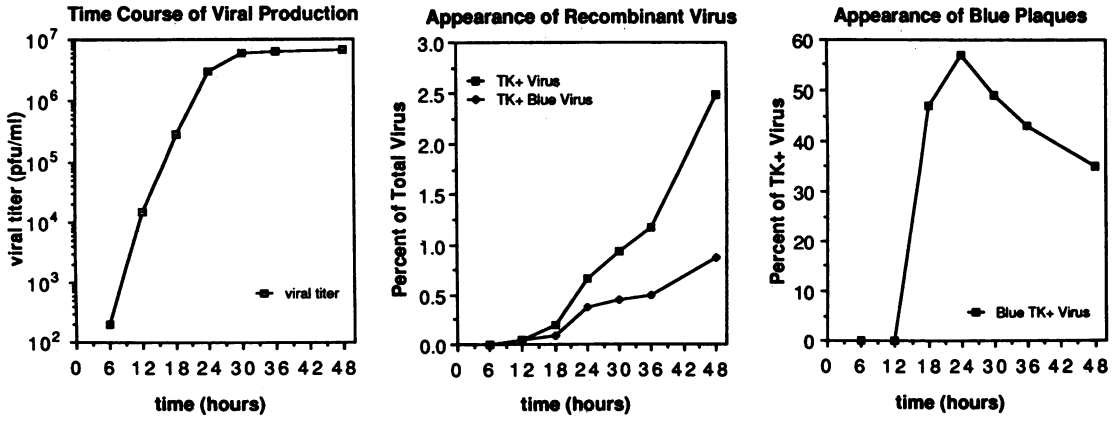

FIG. 2. Time course of appearance of viral recombinants. Monolayers of BSC-40 cells were infected with WRΔ417 virus and subsequently transfected with pTK+Z plasmid as described in Materials and Methods. Virus was isolated from replica plates at 6-h time intervals and plated on monolayers of Hu143TK$^-$ cells in the absence of drug to determine the viral titer and in the presence of MTX and X-Gal to identify recombinant virus. Duplicate plates made at three 10-fold serial dilutions were counted for each point shown.

TABLE 2. Segregation of genetic markers in viral recombinants

Plasmid	Viral titer (PFU/ml)[a]	Titer of TK-positive virus (PFU/ml)	% Recombinants	% of recombinants that formed blue plaques[b]
No DNA	4.1×10^6	0	0	0
pTK-1	3.1×10^6	1.0×10^5	3.2	0
pTK+Z[c]	4.2×10^6	7.2×10^4	1.7	42
pTK+Z (linear)[d]	7.7×10^6	7.7×10^4	1.0	0.5
pTKZ-1	3.3×10^6	1.7×10^4	0.5	93
pTKZ-1 (linear)	3.5×10^6	3.5×10^4	1.0	91

[a] Average values from duplicate plates made at three 10-fold serial dilutions are given.

[b] The percentage of recombinants that formed blue plaques was determined by dividing the number of blue plaques by the total number of plaques that formed in the presence of MTX and X-Gal.

[c] Average values for both orientations of 7.5KZ in all plasmids containing the *lacZ* gene are given, since they are statistically indistinguishable.

[d] Average values for both *Sca*I- and *Pst*I-linearized plasmids are given, since they are statistically indistinguishable.

virus contained the *lacZ* marker when pTK+Z DNA was linearized within plasmid sequences. In contrast, *tk* and *lacZ* markers cosegregated in more than 91% of all TK-positive recombinant progeny generated by using both linear and circular forms of pTKZ-1. There was no significant variation in overall frequency of recombination between circular and linear forms of the plasmids (Table 2). The percentage of recombinants that formed blue plaques indicates that the progeny generated by single-crossover or gene conversion events occur at equal frequencies when circular plasmids are used, but only those arising from gene conversion or double-crossover events are viable with linear plasmid substrates.

Molecular analysis of recombinant virus. The insertion of plasmid pTK+Z into the genome of WRΔ417 by a single crossover within either the 5'- or 3'-flanking sequences would result in a recombinant genome which contains a single copy of the intact *tk* gene within the corresponding copy of the duplicated *tk* flanking sequences, separated by plasmid DNA and the *lacZ* gene (Fig. 3, structures c and d). Gene conversion within either of these structures could result in further duplication of either the TK-positive or TK-deleted sequences (Fig. 3, structures e and f). In each of these possible intermediate structures, the proximity of direct, nontandem duplications would promote a second single-crossover event to generate either the parental TK-deleted or wild-type TK-positive genomic structures, lacking vector and *lacZ* DNAs (Fig. 3, structures a and b). These events would be characterized by distinctive alterations in the phenotype, as well as by the genotype of the ensuing recombinants as a function of the segregation of the two genetic markers.

Recombinants expressing both *tk* and *lacZ* genes. To characterize the duplicated genomic structures shown in Fig. 3, 21 plaques expressing both genetic markers generated in a 24-h period of recombination between plasmid pTK+Z and virus WRΔ417 were determined. In each case, titers of the viral progeny were determined in the presence of MTX and X-Gal, and genomic DNA was purified from the remainder of the sample without further amplification. On average, 91% of the resulting TK-positive virus expressed *lacZ* upon being replated. Purified genomic DNAs were digested with restriction endonuclease *Hin*dIII, resolved by agarose gel electrophoresis, and subjected to hybridization analysis with the vaccinia virus *tk* gene and its flanking sequences. The DNAs from six representative plaques are shown in the top panel of Fig. 4. DNAs from the parental TK-negative and wild-type viruses were included as controls in lanes 1 and 2 and showed single hybridizing bands of 4.6 and 5.0 kb, respectively. These controls were included in lanes 1 and 2 of each panel of Fig. 4 and 5. More complicated patterns of hybridization were produced from DNAs isolated from recombinant virus originating from single-crossover events (Fig. 4, top panel, lanes 3 through 8). In lanes 3 and 4 the hybridization patterns were consistent with genomic structures derived from 5' and 3' crossover events that had not undergone further rounds of recombination. A 5' crossover event generated fragments of 4.6 and 4.1 kb (Fig. 3, structure c; Fig. 4, top panel, lane 3), and a 3' crossover event generated fragments of 5.0 and 3.6 kb (Fig. 3, structure d; Fig. 4, top panel, lane 4). Although a total of 21 plaques were examined by hybridization analysis, only three showed hybridization patterns consistent with these structures. The majority of the plaques examined produced hybridization patterns indicative of further rounds of recombination (Fig. 3) (see above). Figure 4, top panel, lane 5, shows three bands of approximately equal intensities at 5.0, 4.6, and 4.1 kb, which represent a mixed population of predominantly 5' single-crossover and TK-positive duplicated structures (Fig. 3, structures c and e). Conversely, Fig. 4, top panel, lane 6, shows three bands of approximately equal intensity of 5.0, 4.6, and 3.6 kb, which represent a mixed population of predominantly 3' single-crossover and TK-negative duplicated structures (Fig. 3, structures d and f). A more complex pattern, represented by the appearance of all four bands, was observed most frequently (Fig. 4, top panel, lane 7). This banding pattern probably represents a mixed population of each of the four gene-duplicated structures in Fig. 3, structures c to f. An example of a TK-positive gene-duplicated structure having 5.0- and 4.1-kb hybridizing fragments was observed in approximately one-third of the plaques (Fig. 3, structure e; Fig. 4, top panel, lane 8). In each of these analyses, the contribution from either the parental TK-negative or the wild-type genome within the population is minimal, since 91% of the viral progeny originating from these plaques express the *lacZ* gene.

Hybridization analysis of genomic DNA isolated directly from viral plaques resulting from recombinant virus formed within 24 h of the addition of plasmid DNA to vaccinia virus-infected cells indicates that most plaques already contained a complex mixture of recombinant progeny. These data indicate that during vaccinia virus replication, direct, nontandem gene duplications are very unstable. To examine this instability, the alterations in the structure of viral recombinants as a function of multiple rounds of replication were studied. Individual viral progeny that formed blue plaques in the presence of MTX and X-Gal were picked from cell monolayers infected with the 21 original recombinants and replated under the same conditions. This process of picking and replating was repeated three times. As above, the titer of each viral recombinant was determined in the presence of MTX and X-Gal, and genomic DNA was purified from the remainder of the sample (Fig. 4, middle panel, lanes 3 to 7). On average, 82% of the resulting TK-positive virus expressed the *lacZ* gene on being replated. All of these isolates produced either mixed banding patterns (Fig. 4, middle panel, lanes 3, 4, and 5) similar to those described in Fig. 4, top panel, lanes 6 and 7, or TK-positive gene-duplicated structures which now predominate the population (Fig. 4, middle panel, lanes 6 and 7; top panel, lane 8). The accumulation of stable wild-type genomes, which would reveal a 5.0-kb fragment, from unstable TK-positive gene-

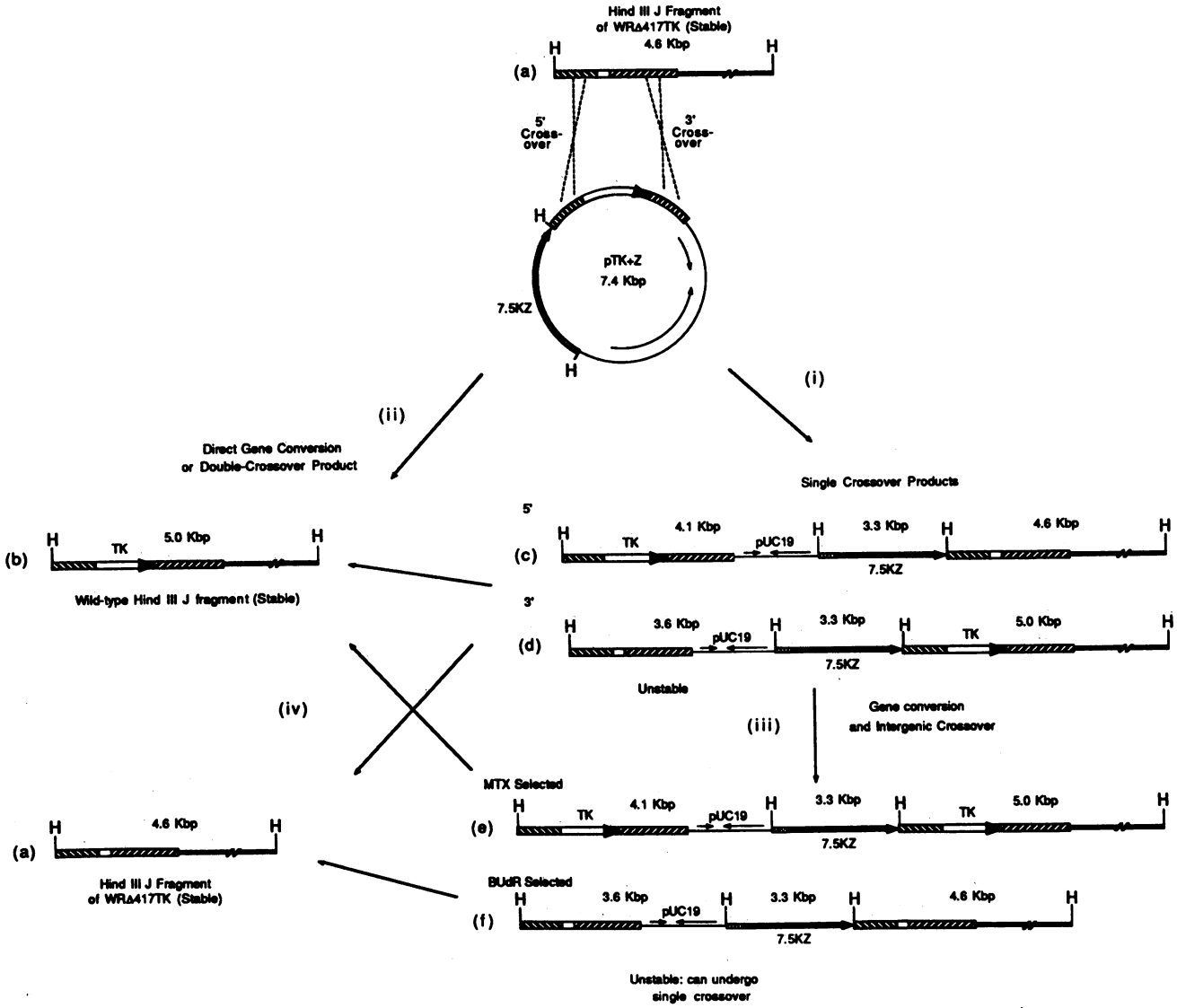

FIG. 3. Proposed recombinant viral structures. Plasmid pTK+Z recombines with homologous sequences of WRΔ417 virus by (i) single crossover to generate a direct, nontandem duplication of *tk* flanking sequences, separated by the plasmid and *lacZ* marker DNAs, and (ii) gene conversion or double crossover to generate a wild type (WR) genome. Gene conversion between homologous *tk* gene-flanking sequences in products c and d will result in (iii) the duplication of the intact TK gene and flanking sequences or the duplication of the *tk*-flanking sequences alone, both of which are separated by the plasmid and *lacZ* marker DNAs. Furthermore, crossover between duplicated vaccinia virus sequences in products c to f will result in (iv) either the wild-type (WR) or parental *tk*-deleted (WRΔ417) genome.

duplicated structures is demonstrated by the reduced band intensity of the 4.1-kb fragment (Fig. 4, middle panel, lane 7). No banding patterns representing 5′ or 3′ crossover products without evidence of further rounds of recombination persisted in this population.

Recombinants expressing only the *tk* gene. To demonstrate that the gene-duplicated structures continue to undergo genomic rearrangement by recombination, the viral stocks plaque purified three times in the presence of MTX were amplified 10^4-fold in the presence of the drug. The titer of each of these amplified stocks of virus was determined in the presence of MTX and X-Gal, and genomic DNA was purified from the remainder of the sample. On average only 4.2% of the viral progeny expressed the *lacZ* gene upon being replated, supporting the contention that the gene-duplicated structures are less stable than the wild type. Hybridization analysis of five representative plaques is shown in Fig. 4, bottom panel. The most common banding pattern corresponds to a population of TK-positive viral genomes that have almost completely lost all duplicated structures (Fig. 4, bottom panel, lanes 6 and 7). Only 0.2% of the virus whose DNA is shown in Fig. 4, bottom panel, lane 7, expressed the *lacZ* gene upon being replated in the presence of MTX and X-Gal. A less common banding pattern additionally contains the intact TK-positive gene-duplicated structure (Fig. 4, bottom panel, lane 5). The remainder of the viral stocks produced mixed banding patterns (Fig. 4, bottom panel, lanes 3 and 4). Although recombinants with this mixed banding pattern were frequent in both earlier sets of isolates, they were relatively rare in the amplified set of recombinants.

The molecular analysis of recombinant virus demonstrates

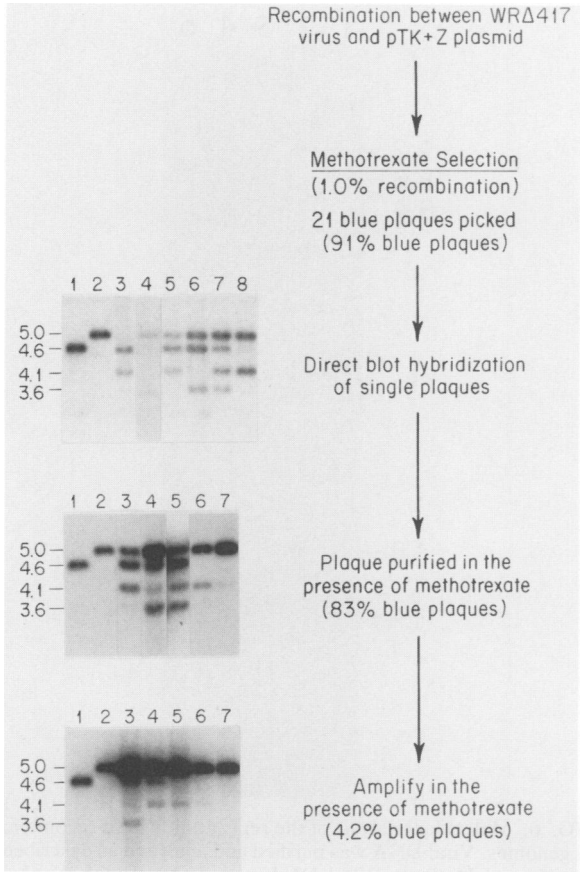

FIG. 4. Molecular analysis of viral recombinants expressing the *tk* gene. Viral DNA was purified and analyzed as described in Materials and Methods. Viral DNA digested with *Hin*dIII was subjected to gel electrophoresis (0.7% agarose), transferred to nitrocellulose filters, and hybridized with the *Hin*dIII-*Pvu*II fragment of pTK-1. In all panels, lane 1 contains DNA from the parental TK-negative virus and lane 2 contains DNA from TK-positive wild-type virus. Lanes 3 to 8 of the top panel contain DNA from plaques that originated from virus isolated directly from infected-cell monolayers that were transfected with plasmid pTK+Z. The top panel is a composite of a single gel in which lane 4 was exposed three times longer than the remaining lanes, which were exposed for 24 h. Lanes 3 to 7 of the middle panel contain DNA from virus that was plaque purified three times in the presence of MTX and originated from virus used in the top panel. The middle panel is a composite of a single gel. Lanes 3 to 7 of the bottom panel contain DNA from virus that was amplified 10,000-fold in the presence of MTX and originated from virus used in the middle panel. The average percentage of virus that formed blue plaques in the presence of X-Gal on replating of individual plaque isolates is given.

that the gene-duplicated structures generated by single-crossover events can undergo further rounds of recombination to generate the wild-type TK-positive structure (Fig. 3). It is noteworthy that one-half of the progeny in the initial recombinant population, as well as 10 to 20% of the progeny of *lacZ*-expressing plaques, formed TK-positive, *lacZ*-negative plaques. These results indicate that gene conversion or double crossover predominantly produce this latter type of virus and that plasmid insertion-excision events produce it to a lesser extent. Implicit in this analysis is the assumption that all TK-positive white plaques are of the same genomic structure. Viral progeny that formed white plaques in the presence of MTX and X-Gal were picked from cell monolayers infected with either the initial recombinant population or the original 21 TK-positive, *lacZ*-expressing recombinants selected from this population. In each case, when the isolates were replated in either MTX plus X-Gal or BUdR plus Xgal, only TK-positive, *lacZ*-negative progeny were observed. Genomic DNA purified from these isolates all demonstrated the same banding pattern, which corresponds to the predicted wild-type TK-positive structure (Fig. 5, lane 3).

Recombinants not expressing the *tk* gene. Gene-duplicated structures formed by single-crossover events can undergo further rounds of recombination which ultimately produce stable wild-type structures. The predominance of the TK-positive gene-duplicated structure in a population of viruses that form blue plaques when grown in the presence of MTX is indicative of both the relative stability of the recombinants and the growth selection used (Fig. 3, structure e). To demonstrate the formation of the alternate TK gene-duplicated structure, recombinants were grown in the presence of BUdR (Fig. 3, structure f). Viral progeny that formed both blue and white plaques in the presence of BUdR plus X-Gal were picked from cell monolayers infected with either the initial recombinant virus or the 21 original TK-positive, *lacZ*-expressing recombinants and replated for three rounds under the same conditions. The titer of each viral recombinant was determined in the presence of MTX plus X-Gal and BUdR plus X-Gal, and genomic DNA was purified from the remainder of the sample. All 20 of the isolates examined were unable to form plaques in the presence of MTX. On average, 87% of the plaques recovered from the 15 TK-negative, *lacZ*-positive isolates expressed the *lacZ* gene upon being replated, whereas none of the 5 TK-negative *lacZ*-negative isolates expressed the *lacZ* gene. DNA hybridization analysis of the five TK-negative, *lacZ*-negative isolates produced a single hybridizing band of 4.6 kb (Fig. 5, lane 4). These hybridization results, together with the ab-

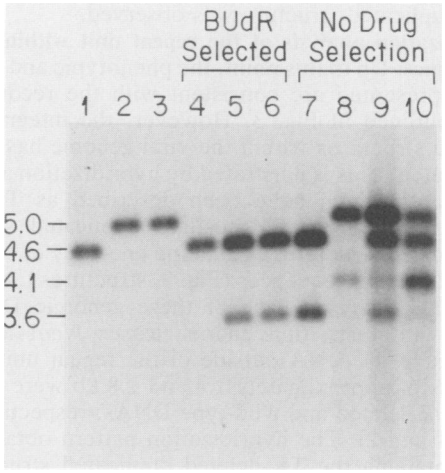

FIG. 5. Molecular analysis of viral recombinants generated under different selection conditions. Viral DNA was purified and analyzed as described in Materials and Methods. Viral DNA digested with *Hin*dIII was subjected to gel electrophoresis (0.7% agarose), transferred to nitrocellulose filters, and hybridized with the *Hin*dIII-*Pvu*II fragment of pTK-1. Each lane contains DNA from the following recombinants: the parental TK-negative virus (lane 1), the TK-positive wild-type virus (lane 2), a TK-positive, *lacZ* negative virus (lane 3), a TK-negative, *lacZ*-negative virus (lane 4), a TK-negative, *lacZ*-positive virus (lanes 5 and 6), and a *lacZ*-positive virus grown under nonselective growth conditions (lanes 7 to 10).

sence of *lacZ* expression, indicate that these viruses have the parental *tk*-deleted genomic structure (Fig. 3, structure a; Fig. 5, lane 4). The 15 TK-negative, *lacZ*-positive isolates were determined to have a *tk*-deleted gene-duplicated structure, demonstrating 4.6- and 3.6-kb hybridizing fragments (Fig. 3, structure f; Fig. 5, lanes 5 and 6). Within the resolution of this analysis, no variance in the size of the 4.6-kb band was observed for all 20 of the TK-negative viral isolates, suggesting that the gene conversion and intermolecular double-crossover events that gave rise to these structures must not only begin but also end within homologous sequences. Otherwise, bands ranging in size from 5.0 to 4.6 kb would have been observed.

Recombinants produced under nonselective growth conditions. Drug selection conditions used thus far have allowed us to determine the structure of all possible *tk*-positive and *tk*-deleted recombinant viral genomes (Fig. 3). To identify recombinant structures that could have been missed through drug selection and to determine whether strong polarity in favor of either the TK-positive or the TK-negative duplication exists, *lacZ*-expressing recombinants were picked in the absence of MTX or BUdR and were replated for five rounds in the absence of selective media to allow gene-duplicated structures to be generated. The titers of each viral recombinant were determined in the presence of MTX plus X-Gal or BUdR plus X-Gal, and genomic DNA was purified from the remainder of the sample. On average 81% of the plaques recovered expressed the *lacZ* gene upon being replated. The DNAs from four representative plaques are shown in Fig. 5, lanes 7 to 10. The progeny of half of the isolates formed plaques in only BUdR or MTX and had the predicted *tk*-deleted or *tk*-positive duplicated structure (Fig. 5, lanes 7 and 8, respectively). The remaining half of the isolates contained progeny that could form plaques in the presence of either BUdR or MTX and had a mixture of banding patterns or genomic structures that ranged between those shown in lanes 9 and 10. No new genomic structures were observed, and no strong polarity toward the TK-positive or the TK-deleted duplicated structure was observed.

Hybridization analysis of the repeat unit within recombinant genomes. Up to this point, the phenotypic and structural analyses presented are consistent with the recombination process outlined in Fig. 3. However, the integrity of the duplicated structures within the viral genome has yet to be demonstrated. This is illustrated by hybridization analysis of recombinants which have been described as TK-deleted duplicated structures, TK-positive duplicated structures, and duplicated structures containing one copy of each of the intact and deleted sequences (Fig. 3, structures f, e, and c or d). For this analysis, each of these genomic DNAs was digested with restriction endonucleases *Nco*I and *Xho*I, which cleave the DNA outside of the repeat unit (Fig. 1). Fragments of approximately 1.4 and 1.8 kb were generated from the *tk*-deleted and wild-type DNAs, respectively (Fig. 6, lanes 1 and 2). The hybridization pattern obtained from DNA containing the TK-deleted duplicated structure produced two bands at approximately 8.4 and 1.4 kb (Fig. 6, lane 3). DNA containing one copy of each of the intact and deleted *tk* sequences produced three bands at approximately 8.8, 1.8, and 1.4 kb (Fig. 6, lane 4), whereas that containing the TK-positive duplicated structure showed two bands at approximately 9.2 and 1.8 kb (Fig. 6, lane 5). These results clearly demonstrate the integrity of the duplicated structures, as indicated by the high-molecular-weight bands. Moreover, they confirm the proposed degeneration of these structures to yield either a mixture of TK-deleted and wild-type progeny or exclusively one of the two, as indicated by the lower-molecular-weight bands.

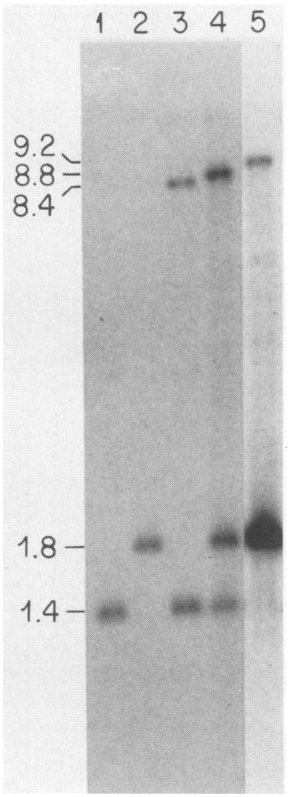

FIG. 6. Molecular analysis of the repeat unit within recombinant viral genomes. Viral DNA was purified and analyzed as described in Materials and Methods. Viral DNA was digested with *Nco*I and *Xho*I, which cut outside of the repeat unit. This DNA was then subjected to gel electrophoresis (0.7% agarose), transferred to nitrocellulose filters, and hybridized with the *Hin*dIII-*Pvu*II fragment of pTK-1. Each lane contains DNA from the following recombinants: the parental TK-negative virus (lane 1), the TK-positive wild-type virus (lane 2); a TK-negative, *lacZ*-positive virus unable to form plaques in the presence of MTX (lane 3); a TK-positive, *lacZ*-positive virus (lane 4), and a TK-positive, *lacZ*-positive virus unable to form plaques in the presence of BUdR (lane 5). The figure is a composite made from a single gel in which lane 5 was exposed for 4 h.

DISCUSSION

Recombination in vaccinia virus was first reported almost 30 years ago by Fenner and Comben (16, 17). This initial study identified the products of recombination between two genetically distinct viruses. Recently, homologous recombination between plasmid and viral DNAs has been widely used to introduce DNA sequences into the vaccinia virus genome, and yet an understanding of this process has not been pursued.

In the work described in this paper, plasmids containing vaccinia virus sequences and two genetic markers were used to define the viral products of recombination. Analysis of recombinants between transfected plasmid and viral DNAs in vaccinia virus-infected cells has shown that single-crossover events readily occur between homologous sequences, leading to the insertion of the entire plasmid and the generation of duplications of these homologous sequences. The

duplicated sequences undergo further rounds of recombination to ultimately generate viral genomes that contain one copy of the homologous sequence. These resulting stable recombinant genomes can also be generated directly by either gene conversion or double-crossover events. The recombinant structures and their relative stabilities are thus consistent with models derived from molecular and genetic studies of bacteriophages and fungi (20, 24, 31, 36).

At 24 h after the addition of plasmid to vaccinia virus-infected cells, an average of 1% of the viral progeny have recombinant genomes. Interestingly, the fraction of recombinants in the population increased from 0.25% at 18 h to 2.5% at 48 h. In general, the frequency of recombination varies with the conditions and multiplicity of infection, as well as with the amount and form of plasmid DNA introduced, and ultimately depends on the interaction between homologous sequences. In fact, the frequency of recombination is significantly increased when plasmid and intact viral genomic DNAs are coprecipitated onto infected cells (15, 21). The frequency of recombination within the viral genome can reach 100% when the homologous sequences are covalently linked (3, 29; D. Evans, personal communication). In this case, both the sizes of and distance between homologous sequences will affect the frequency of recombination, assuming the absence of preferred recombination sites.

Approximately 50% of the recombinant viruses formed within 24 h contain only one copy of the homologous sequences and were generated either directly by gene conversion or double crossover or indirectly by two rounds of single crossover. The remaining recombinants contain duplications of the homologous sequences that were generated by single crossovers. Only a small proportion of the recombinants that contain a single copy of the homologous sequences are probably generated by two rounds of single crossover. Additionally, recombinants that contain either one or two copies of the homologous sequences appear coincidentally. These data indicate that the probability of a single crossover is roughly equal to that of either a gene conversion or a double crossover, which is consistent with results obtained with yeasts (28, 33). Still, the overall frequency of recombination in vaccinia viruses does not change appreciably when the formation of recombinants resulting from single-crossover events is prevented by linearization of plasmids in nonhomologous sequences (27).

Tetrad analysis of fungi has demonstrated the nonreciprocal transfer of information from one duplex to another, distinguishing gene conversion from double-crossover-mediated recombination (20). Analysis of only the viral products of recombination between homologous plasmid and vaccinia virus sequences precludes the distinction of these two pathways. However, the frequency of these events suggests that gene conversion is the predominant mechanism unless the primary crossover event in the generation of a double crossover significantly enhances the frequency of the second crossover (13, 20, 24, 36). The 417-bp insertion that reconstitutes the vaccinia virus *tk* gene falls within the average size of a gene-converted segment in yeast cells and *Drosophila melanogaster* and probably does not limit the frequency of the conversion event (1; A. J. Hilliker, S. H. Clark, and A. Chovnick, *in* K. B. Low, ed., *Recombination of Genetic Material*, in press). In addition, the conversion of structures generated by single crossovers (Fig. 3, structures c and d) to those containing two copies of identical sequence (Fig. 3, structures e and f) occur without bias in the absence of selective growth conditions.

The stabilities of each of the genomic structures generated from recombination between plasmid and viral DNA were defined by subsequent rounds of recombination and were reflected in their relative abundance among the viral progeny. The most unstable of the structures were those which formed directly from either 5′ or 3′ crossovers and demonstrated the ability to undergo either gene conversion to create identical duplications or an additional single crossover to produce single copies of the homologous sequences. Structures containing identical duplications can undergo only single crossovers to produce a single copy of the homologous sequences. Virus containing a single copy of the homologous sequences did not demonstrate further genomic rearrangements, whereas all the duplicated structures demonstrated instability. It has been shown that vaccinia virus replication involves the synthesis of branched concatemeric intermediates having both specific origins of replication near the genomic termini and nonspecific origins throughout (9, 11, 30). These results would suggest an essential function of recombination in viral replication and may preclude the identification of recombination-deficient virus.

Knowledge of the structures that are generated through recombination between homologous plasmid and viral sequences can be used to facilitate the study of other aspects of vaccinia virus. Genes that encode functions essential for the viral cycle in tissue culture an be identified by using plasmids that contain two markers, one that interrupts and another that flanks the coding sequences of the gene of interest. Progeny derived from recombination with this plasmid must express both markers if an essential gene is involved. Additionally, the study of sequence dosage effects for viral properties such as virulence or drug resistance can be facilitated by generating multiple copies of the sequences of interest.

As a virus which replicates in the cytoplasm of the host cell, vaccinia virus presents a unique opportunity for molecular and genetic analysis of the functions associated with viral replication and morphogenesis in a eucaryote. The development of techniques which permit the introduction of DNA sequences into the viral genome by homologous recombination has allowed the mapping of randomly generated mutations and initiated the genetic characterization of many of these functions (8, 13, 21, 26, 38). Clearly, a method for site-directed alteration of any specific viral DNA sequences is now required. The definition of single-crossover events leading to the creation of nontandem duplications and the condensation of these sequences by additional recombination presented in this work are directly applicable. An understanding of these events has been combined with growth selection schemes to create a two-step procedure for the site-specific alteration of DNA sequences in yeast cells (33). As demonstrated in this work, the *tk* gene can be used to confer such a growth advantage, and alternate markers exist (18). The application of these techniques may allow the manipulation of viral sequences in a manner currently unavailable.

ACKNOWLEDGMENTS

B.E.R. and D.D.S. are supported by the National Institutes of Health (Public Health Service grants 20730 and 8943-2, respectively). L.K.C. is grateful for support awarded by the Medical Foundation.

We appreciate insightful comments on the manuscript by Don Coen, Neil DeLuca, and Hiroshi Nakai; valuable discussions with David Evans; the construction of plasmid pTK-1 by Gail P. Mazzara; and assistance with the preparation of the manuscript from Marcy Ketcham.

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