In Vitro Resolution of Poxvirus Replicative Intermediates into Linear Minichromosomes with Hairpin Termini by a Virally Induced Holliday Junction Endonuclease

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Available evidence suggests that one or more late viral gene products are involved in processing poxvirus replicative intermediates into mature progeny hairpin-terminated genomes. Cloned versions of the Shope fibroma virus (SFV) replicated telomere in the inverted repeat configuration were used as substrates to assay lysates from poxvirus-infected cells for protein fractions that participate in the resolution of the circular substrate plasmid into a linear minichromosome with viral hairpin termini. An activity in a crude protein fraction obtained from vaccinia virus-infected cells at late times during the replicative cycle was capable of accurately resolving all poxviral inverted repeat replicative intermediates tested. The resolved linear products are identical to the products of *in vivo* resolution and possessed symmetrical nicks which mapped at the borders of the inverted repeat sequence. Strand-specific nicks were also identified, which mapped within the telomere resolution target sequence known to be required for telomere resolution *in vivo*. The resolving activity that we have identified is specific to virus-infected cells at late times during replication and cleaves cloned poxviral telomeric substrates in a fashion expected of a classic Holliday junction-resolving enzyme in addition to possessing a telomere resolution target-specific nicking activity. Although a Holliday junction-resolving activity would also be expected to play a role in the recombination induced by poxvirus infection, the appearance of the activity described here only after the commencement of viral late protein synthesis suggests that it functions strictly at late times. Other non-viral Holliday junction analogs can also be cleaved by this extract, suggesting that this component of the resolution activity may also play a role in other viral processes that require cleavage of a branched DNA structure. Thus, we have identified a poxviral activity that may be a part of a protein complex which resolves concatemeric replicative intermediates of viral DNA as well as participate in general recombination late during infection.

Poxviruses such as vaccinia virus and Shope fibroma virus (SFV) replicate exclusively in the cytoplasm of infected host cells and are believed to encode most, if not all, of the gene products required for the viral replicative cycle (45). The poxviral DNA genomes that have been studied are linear double-stranded molecules that possess covalently closed hairpin termini (11, 21) and have coding capacities for over 200 gene products (22). The cytoplasmic site of infection and apparent ability to replicate in enucleated cells (52) suggests that all of the enzymatic activities required for poxviral DNA replication are virally encoded (for a recent review, see reference 68). In addition to enzymes required for DNA precursor biosynthesis, such as thymidine kinase (25), thymidylate kinase (60), and ribonucleotide reductase (57, 58), poxviruses have been found to encode and express genes for DNA polymerase (29, 34), DNA ligase (59, 61), topoisomerase (55, 56), and a number of nucleases (32, 50, 54). It has been shown through genetic and molecular biological means that poxvirus DNA is subject to extensive genetic recombination during its replicative cycle (2, 18, 20, 62). Efficient recombination has also been observed between coinfecting viral genomes (18) and between viral genomic DNA and homologous sequences in transfected plasmid DNA (48). This observation has proven useful for the mapping of viral genes (72) and for the construction of recombinant poxviruses for use as vectors (33). The induced recombination activity is not specific for viral DNA se-

quences because unrelated cotransfected bacterial plasmids are subject to both intermolecular and intramolecular recombination in poxvirus-infected cells (17). Analysis of the viral products of recombination have demonstrated that both single and double crossover events occur with high frequency (2, 62) and the unstable tandem repeat products of single crossover events are rapidly processed via second crossover events into more-stable structures (62).

The induction of recombination activity in poxvirus-infected cells shows a stringent requirement for viral DNA replication. Cells infected with poxvirus mutants that are conditionally lethal for DNA replication are unable to catalyze intramolecular recombination at the nonpermissive temperature (38). Similarly, infected cells that have been treated with inhibitors of viral DNA replication are unable to recombine transfected plasmids (17) or tandem repeats within the genomic DNA (2). To date, it has thus been impossible to unlink the processes of DNA replication and recombination in poxvirus-infected cells. However, mutant poxviruses that are conditionally lethal for late protein synthesis but competent for DNA replication are capable of recombining transfected DNA (38), suggesting that all of the enzymatic activities required for recombination are synthesized at early times during infection. The autonomous nature of the poxvirus replicative cycle makes it likely that the observed recombination activities are catalyzed by virus-encoded enzymes, although none of the gene products responsible have been identified yet.

The replication of poxvirus DNA leads to the formation of transient high-molecular-weight concatemers of viral DNA

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(8, 41, 47). The precise structures of these replicative intermediates have not been completely characterized, but it is clear that most, if not all, of the DNA exist as a linear array (8, 41). Although the presence of branched or circular intermediates has not been excluded, little evidence exists to verify the occurrence of such structures. The arrays of viral DNA are joined end to end such that the junction between genomes exists in the form of an inverted repeat (10). It has been shown that a cloned version of this inverted repeat can be extruded in a cruciform configuration in vitro if the purified plasmid is subjected to negative superhelical torsion (11, 13, 40). At its base, an extruded cruciform is structurally identical to a Holliday junction, which is a key intermediate in most models of general genetic recombination (24). The final stage in homologous recombination requires the cleavage of a Holliday junction and the subsequent ligation of the newly generated recombinant duplexes. Endonucleases that display specificity for Holliday junctions and branched DNA structures have been isolated from phage T4 (30), phage T7 (12), *Saccharomyces cerevisiae* (16, 28, 66, 73), human placenta (26), HeLa cells (71), and calf thymus (15). The function of these endonucleases is relatively well established for bacteriophages. At late times in the replicative cycle, phage T4 primes replication by strand invasion with the concomitant formation of branched DNA structures (43). Maturation and packaging of replicated DNA requires the resolution of these branched intermediates into unit-sized progeny genomes (19, 27). The replication of phage T7 DNA results in the formation of long concatemeric structures that must be cleaved in order to be packaged (63, 65). Both phage T4 and T7 undergo homologous recombination at high frequency (14, 65), and phage mutants defective in these resolvase activities are deficient in homologous recombination (31, 51). At the nonpermissive temperature, these mutants accumulate branched recombination intermediates (69) and are deficient in maturation and packaging of replicated DNA (49, 63). No clear role has been assigned to the enzymes in *S. cerevisiae*, although it is proposed that they serve some function in recombination (28).

Vaccinia virus encodes several nucleases, one of which is capable of cleaving single-stranded DNA and ligating some of the cleavage products (32, 40, 53). This enzyme, designated nicking-joining enzyme, cleaves branched DNA structures at single-stranded regions but displays no specificity for the base of the branched substrate, and its role in viral replication or recombination remains to be clarified.

Poxviral telomere resolution bears important similarities to site-specific recombination (for a recent review, see reference 10) and hence elucidation of the mechanism of the resolution of concatemeric arrays of viral DNA into hairpin-terminated daughter molecules may shed light on the biochemistry of site-specific recombination as well. In an attempt to develop an in vitro model for the resolution of poxvirus replicative intermediates, we have identified an endonuclease activity induced at late times in poxvirus-infected cells that displays specificity for the branched DNA structures of extruded cruciforms. This endonuclease activity resolves cloned versions of the SFV replicative intermediate in vitro to yield hairpin-terminated minichromosomes that are indistinguishable from those generated in vivo. In addition, asymmetric nicks are observed within the viral telomere resolution target sequence (TRT), suggesting that some of the specificity of resolution in vivo can be reproduced by these extracts in vitro.

MATERIALS AND METHODS

Cells and viruses. BGMK cells (African green monkey kidney), obtained from the American Type Culture Collection (Rockville, Md.) were propagated as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (GIBCO Laboratories). HeLa S3 cells, provided by V. Paetkau, were maintained as suspension cultures in Joklik modified Eagle's medium (Flow Laboratories), supplemented with 10% equine serum (GIBCO Laboratories). Vaccinia virus (WR) and SFV (Kasza) were obtained from the American Type Culture Collection. Viruses were propagated by infection of BGMK monolayers (SFV) or HeLa suspension cultures (vaccinia virus) at a multiplicity of one infectious unit per cell. Virus stocks from these infected cells were generated as described previously (5, 74). The vaccinia virus temperature-sensitive mutant *ts53* was generously provided by R. Condit. The conditions for growth and infection of this mutant have been previously described (5).

Plasmids and strains. Bacterial plasmids that contain inverted repeats were propagated in the recombination-deficient *Escherichia coli* DB1256 (11), and all other plasmids were maintained in *E. coli* HB101. The pAT34 plasmid, provided by David Pulleyblank, contains an insert of the self-complementary alternating purine-and-pyrimidine sequence d(AT)_n:d(AT)_n (23). The construction and purification of plasmids that carry inverted repeats of SFV telomere sequence, pSCB1a and pSAD-2, have been described previously (11). Variants of the SFV telomere sequence generated by site-specific mutagenesis have been described previously (64).

Preparation of cell lysates. Cytoplasmic or whole-cell lysates were prepared by a modification of the procedure for preparing nuclear extracts competent for in vitro transcription (35). HeLa S3 cells, 3 to 6 liters grown to a density of 3×10^5 to 5×10^5 cells per ml, were concentrated by centrifugation to 5×10^6 cells per ml and infected with vaccinia virus at a multiplicity of 5 to 10 infectious units per cell. After a 1-h adsorption, the cells were diluted 10-fold and the infection was allowed to proceed for 14 to 16 h. The infected cells were harvested by centrifugation, washed with ice-cold 0.01 M phosphate-buffered saline pH 7.4 and resuspended in 4 packed cell volumes (PCVs) of an ice-cold solution consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5 mM dithiothreitol (DTT). After the cells were allowed to swell for 15 min on ice, they were broken by 20 strokes with a Dounce homogenizer. A cytoplasmic fraction was obtained by pelleting the nuclei and cell debris for 10 min at $600 \times g$. These cytoplasmic fractions or whole-cell lysates were then mixed with 4 PCVs of a solution consisting of 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 2 mM DTT, 25% sucrose, and 50% glycerol. After 10 min of gentle stirring at 4°C, the salt concentration was raised by the addition of 1 PCV of a saturated (NH₄)₂SO₄ solution which also served to lyse the nucleic in whole-cell extracts. This homogenate was stirred for 20 min at 4°C, and then chromatin and cell debris were removed by centrifugation at 47,500 rpm for 4 h at 4°C in a Beckman Ti70 fixed-angle rotor. Supernatants from the high-speed spin were precipitated by the addition of 0.35 g of solid (NH₄)₂SO₄ per ml of homogenate and neutralized with 10 μ l of 1 N NaOH per g of (NH₄)₂SO₄. The precipitate was pelleted by centrifugation at $15,000 \times g$ for 30 min in a Sorvall SS34 rotor at 4°C and resuspended in 1/10 the volume of the original high-speed supernatant in buffer A (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 0.01%

Nonidet P-40, 2 mM DTT, 10% glycerol). The suspension was dialyzed against 100 volumes of buffer A for 10 h with two changes of buffer. This preparation was assayed for activity, the KCl concentration was increased to 250 mM, and the homogenate was chromatographed on DEAE-Seph-rose. The flowthrough fraction was again dialyzed against buffer A for 6 h and then was concentrated by ultrafiltration in Centricon 10 concentrators (Amicon). This preparation was then aliquoted, quick-frozen in a dry ice-ethanol bath, and stored at -70°C . In some cases, the infection was performed in the presence of 40 μg of cytosine- β -D-arabino-furanoside (araC) per ml to inhibit viral DNA replication and late gene expression. When the cell lysates were made after infection with vaccinia virus *ts53*, the virus adsorption was for 2 h and the infection was allowed to proceed at either the permissive temperature of 32°C or the nonpermissive temperature of 39.5°C .

Enzymes and drugs. DNA restriction and modification enzymes used in the construction of plasmids were obtained from Bethesda Research Laboratories and used as directed by the manufacturer. Topoisomerase I purified from calf thymus was a generous gift from A. R. Morgan. Topoisomerase I was used for the relaxation of plasmid DNA specifically to resorb cruciforms that were extruded by negatively supercoiled plasmid DNA. Relaxation of plasmids was accomplished by incubating plasmid DNA with 100 μg of ethidium bromide per ml on ice in darkness. After a 15-h incubation, the mixture was made to final concentrations of 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM MgCl_2 , 1 mM DTT, and 0.1 mM EDTA and extracted six times with ice-cold isobutanol saturated with 50 mM Tris-HCl (pH 7.6) and once with ice-cold chloroform. DNA was then relaxed by the addition of 5 U of calf thymus topoisomerase I, and the reaction was allowed to warm slowly to room temperature at which time 1 U of topoisomerase was added and the reaction was allowed to proceed for 30 min at 37°C . Topoisomerase and salts were removed by extraction with phenol and chloroform and dialysis against 10 mM Tris-HCl (pH 8.0).

Phage T7 gene 3 endonuclease was purified from *E. coli* W3110 harboring pAR2510 (provided by W. Studier) which carries the T7 gene 3 under the control of the LacUV promoter inserted into plasmid pBR322 (12). Expression of this gene is induced by the addition of isopropylthio- β -D-galactoside to 0.4 mM to a culture of exponentially growing cells at a density of 5×10^8 cells per ml. Cleared cell lysates were prepared as described previously (12). The lysate was applied to an 8-ml phosphocellulose column equilibrated with buffer 1 (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM β -mercaptoethanol, 4% glycerol). Gene 3 endonuclease was eluted in steps with 0.6 M NH_4Cl in buffer 1 (fraction 2). The phosphocellulose fraction was diluted with 3 volumes of buffer 1 and loaded on to a 4-ml column of CM52 cellulose equilibrated with 0.15 M NH_4Cl . The column was then developed with a 0.15 to 0.6 M NH_4Cl gradient. Fractions with gene 3 endonuclease activity which eluted at 0.3 M NH_4Cl (fraction 3), were diluted with 2 volumes of buffer 1 and applied to a 0.2-ml column of Biogel HTP DNA-grade hydroxyapatite equilibrated with 0.1 M NH_4Cl in buffer 1. The peak fractions of gene 3 endonuclease collected from a 0.1 to 1.0 M NH_4Cl gradient were pooled, concentrated against polyethylene glycol 8000, and dialyzed against a solution consisting of 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 50% glycerol (HTP pool). The activity of the T7 endonuclease was assayed in 20- μl reaction mixtures containing 1 μg of target DNA, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 1 mM

DTT, 4 mM spermidine, and 50 μg of denatured gelatin per ml, and cleavage reactions were conducted for 5 min at 37°C .

Specific endonuclease activity in vaccinia virus-infected cell lysates was assayed in 50- μl reaction mixtures containing 1 μg of target DNA, 50 mM Tris-HCl (pH 8.0), 100 mM KCl or potassium glutamate, 0.1 mM EDTA, 0.01% Nonidet P-40, 2 mM DTT, 10% glycerol, 2 mM MgCl_2 , and 1 mM ATP for 15 min at 30°C . DNA cleavage assays were stopped by the addition of 0.5% sodium dodecyl sulfate (SDS), 20 mM EDTA, and 2 mg of proteinase K (Sigma) per ml. The products of this assay were extracted with phenol and chloroform and then ethanol precipitated. The resuspended DNA was then cleaved with restriction enzymes to identify the sites of cleavage, electrophoresed through a 0.7% agarose gel, and stained with ethidium bromide. Specific nicks created in substrate DNAs by the infected-cell lysate were mapped by cleaving the nicked DNA with *HindIII* and then 3' end labelling the fragments with [α - ^{32}P]dATP and Klenow fragment or 5' end labelling with [γ - ^{32}P]ATP and polynucleotide kinase. These labelled fragments were boiled and electrophoresed through 0.4-mm-thick 8% polyacrylamide gels in the presence of 7 M urea. In some cases, the labelled hairpin fragment was purified by electrophoresis through an 8% nondenaturing polyacrylamide gel. The labelled fragment was detected by autoradiography, excised as a gel slice, eluted by diffusion into 10 mM Tris-HCl (pH 8.0), and ethanol precipitated. The purified fragment was then analyzed by electrophoresis through a 0.4-mm-thick 8% polyacrylamide gel in the presence of 7 M urea. Chemical sequencing was performed as described previously (36), using chemicals and [α - ^{32}P]dATP obtained from New England Nuclear Research Products.

Determination of hairpin structures. Hairpin-terminated structures among the cleavage products generated by the infected-cell lysate were detected by stopping the cleavage reactions with a solution of 0.2% SDS, 20 mM EDTA, and 2 mg of proteinase K per ml. After a 2-h digestion at 37°C , the DNA was extracted with phenol-chloroform (1:1) and chloroform, precipitated with ethanol, and washed with 70% ethanol. DNA was resuspended and digested with 5 U of *HindIII* for 1 h at 37°C . The products of this cleavage were boiled for 5 min quick-chilled by immersing the tubes in ice water, and then electrophoresed through nondenaturing agarose gels. Apyrase, araC, diminazene aceturate, actinomycin D, and cordycepin triphosphate were all obtained from Sigma Corp. In vivo assays for the resolution of SFV replicative intermediates in infected or transfected cells were performed as described previously (11).

RESULTS

Assays for the resolution of viral replicative intermediates. To probe poxvirus-infected cell lysates for activities involved in the resolution of viral replicative intermediates, we utilized SFV-derived plasmid substrates that have previously been reported for in vivo resolution assays (11, 64). After transfection into infected cells, cloned poxvirus replicative intermediates are replicated initially into high-molecular-weight *Dpnl*-resistant concatemer structures (11, 41). Resolution of these concatemer structures in vivo by *trans*-acting viral factors yields a ladder of replicated hairpin-terminated linear minichromosomes. Cleavage of the fully and partially resolved linear molecules at a unique *HindIII* site produces molecules that during gel electrophoresis migrate as unit-length linear plasmid DNA, a faster-migrating fragment that has had one of its hairpin termini cleaved off

FIG. 1. Schematic representation of the plasmid substrate and products of the resolution assay. (A) Substrate pSCB1a is a pUC19-derived plasmid carrying a 1.5-kb inverted repeat version of a replicative-form SFV telomere (11). The axis of dyad symmetry in the inverted repeat is denoted by the recognition site for the restriction enzyme *AflII*. The TRTs required for resolution of the inverted repeat into progeny hairpin termini are indicated by the stippled and shaded boxes within the inverted repeat. The lineform and extruded cruciform configurations of pSCB1a are shown. The pUC vector sequence is shown as a single line, and the SFV insert is shown as a double line. (B) The linear hairpin-terminated minichromosome that results from resolution of plasmid pSCB1a. Cleavage of the linear plasmid at the unique *HindIII* site gives diagnostic fragments of 3,436 and 750 bp. (C) Ethidium bromide-stained gel showing the resolving activity present in the infected-cell lysate. Lane 1, pUC19 linearized with *HindIII*; lane 2, pSCB1a linearized with *HindIII*; lane 3, pSCB1a cut with *HindIII* and *AflII* to generate marker fragments (a portion of the 4,186-bp linear fragment is resistant to digestion with *AflII* because of the persistence of the cruciform structure in the substrate plasmid); lanes 4, 5, and 6, pUC19 reacted with the infected-cell cytoplasmic lysate at 30°C for 5, 15, and 30 min, respectively and then cleaved with *HindIII*; lanes 7, 8, and 9, pSCB1a reacted with an infected-cell cytoplasmic lysate at 30°C for 5, 15, and 30 min, respectively, and then cleaved with *HindIII*.

and a very rapidly migrating hairpin molecule. We have mimicked this assay in vitro by utilizing plasmid pSCB1a, which contains 1.5 kb of SFV telomere sequences as a substrate (Fig. 1A). When plasmids that contain large inverted repeats are subjected to superhelical torsion, the inverted repeat can be extruded into a cruciform structure

(Fig. 1A). Correct resolution of this plasmid by activities in an infected-cell lysate would produce a linear hairpin-terminated minichromosome species (Fig. 1B). Cleavage of this linear plasmid with *Hind*III would yield a 3,436-bp fragment with one hairpin end plus a 750-bp self-complementary hairpin molecule (Fig. 1B). Relaxation of the circular template DNA by topoisomerase or random nicking would result only in linearization of the plasmid by *Hind*III. Similarly, random double-strand breaks induced by sequence-nonspecific endonucleases in vitro would lead to a variety of cleavage products that would appear as a smeared pattern after electrophoresis. Only a site- or sequence-specific cleavage of the plasmid could yield the correct single hairpin-terminated 3.4- and 0.75-kb fragments after cleavage by *Hind*III.

Infected-cell lysates resolve cloned replicative intermediates. Preliminary experiments using cleared cytoplasmic lysates from poxvirus-infected cells to resolve viral telomeric plasmids in vitro met with limited success, in part because of the high level of nonspecific virus-induced nuclease activities. However, the recent observation that the TRT is also a strong late promoter suggested that resolution and the late transcriptional machinery may be linked (64), and hence protein extracts enriched for viral late transcription factors were prepared and tested for resolution in vitro. A cytoplasmic extract generated from 6 liters of HeLa cells that had been infected with vaccinia virus for 15 h was used initially to test for resolving activities.

Since the resolution targets of vaccinia virus and SFV are conserved and these viruses can efficiently resolve the other telomeres (11), the plasmid substrates used for the assays described below were from our library of SFV-derived constructs. The result of treating pSCB1a with this lysate for various periods of time and then cleaving it with *Hind*III is illustrated in Fig. 1C. After a 5-min incubation, two discrete fragments of 3.4 and 0.75 kb are visible and these increase in intensity after incubation for 15 and 30 min (Fig. 1C, lanes 7, 8, and 9). Little random degradation of the plasmid DNA appears to occur over the course of the assay, although the persistence of a portion of the full-length linearized plasmid DNA indicates that not all of the substrate was cleaved. A plasmid DNA (pUC19) that carries no viral sequence or large inverted repeats was rapidly relaxed by virally induced topoisomerase activity in the lysate (not shown), but no discrete cleavage products or random degradation was detected after treatment with *Hind*III (Fig. 1C, lanes 4, 5, and 6).

The specific resolving activity that we observed in our initial experiment was not detectable in lysates made in parallel with identically prepared cultures of uninfected cells (Fig. 2, lane 3) or in cultures that had been infected with vaccinia virus for only 3 h (Fig. 2, lane 4). Plasmid DNAs were resolved into linear monomers by lysates from cells infected for 16 h (Fig. 2, lane 6) but not by lysates from cells that had been infected with vaccinia virus for 16 h in the presence of the viral DNA replication inhibitor araC (Fig. 2, lane 5). Lysates made from cells that had been infected with the thermolabile vaccinia virus mutant *ts53* were active for the resolution of cloned replicative intermediates if the infection was allowed to proceed at the permissive temperature of 32°C but no activity was detectable in lysates that were made from cells infected at the nonpermissive temperature of 40°C (data not shown). Since *ts53* is defective in late gene expression because of a thermolabile RNA polymerase subunit (67), we conclude that late gene products must at least contribute to the resolution activity observed. Lysates

FIG. 2. Ethidium bromide-stained 0.7% agarose gel showing the linearization of the substrate plasmid pSCB1a by a vaccinia virus-infected cell lysate. Lane 1, uncut plasmid DNA; lane 2, plasmid DNA linearized with *Hind*III; lane 3, plasmid DNA reacted with an uninfected-cell lysate at 30°C for 30 min; lane 4, plasmid DNA reacted at 30°C for 30 min with a lysate made from cells that had been infected for 3 h; lane 5, plasmid DNA reacted at 30°C for 30 min with a lysate made from cells that had been infected for 16 h in the presence of araC; lane 6, plasmid DNA reacted for 30 min with a cell lysate made from cells that had been infected for 16 h. The DNA in lane 6 was pooled from three reactions to make the resolved fragments more visible. The position of the resolved hairpin-terminated fragments is indicated by the arrows.

made from two other cell lines (BGMK and SIRC) infected with vaccinia virus or SFV were found to contain too much endogenous nuclease activity to accurately monitor the virus-specific resolving enzyme by this extraction procedure. These observations indicate that the resolving activity is poxvirus specific, is induced after the initiation of DNA replication, and is dependent on late protein synthesis.

Characteristics of the resolving activity. The resolving activity identified in poxvirus-infected cell lysates cleaves plasmid DNA in a fashion indistinguishable from the resolution of cloned inverted repeats in the cruciform configuration by the phage T7 gene 3 endonuclease. Cleavage of the cruciform extruded by pSCB1a with the T7 endonuclease also generates a linear minichromosome molecule (Fig. 3, lane 4). When this linearized molecule is cleaved with *Hind*III, discrete 3,436- and 750-bp molecules are produced (Fig. 3, lane 5) that are identical to those produced by digestion with infected-cell lysate and then with *Hind*III (Fig. 3, lane 6). The requirements for the lysate to accurately resolve the inverted repeat plasmid were also investigated (Fig. 3). The initial complete reaction mixture used to screen for resolution activity contained dATP, dTTP, dCTP, dGTP, ATP, TTP, CTP, and GTP as well as 2 mM MgCl₂. Upon identification of the activity, however, we observed that the plasmids are efficiently resolved without the addition of deoxynucleoside triphosphates (Fig. 3, lane 7) or nucleoside triphosphates (Fig. 3, lane 8) or ATP (Fig. 3, lane 9). Clearing the lysate of endogenous ATP with apyrase also had a minimal effect only (data not shown). Although ATP does not appear to be strictly required, its presence does stimulate the reaction. The chain-terminating ATP analog cordycepin triphosphate does not stimulate the activity but has no inhibitory effect (data not shown), suggesting that RNA polymerization per se is not necessary for this resolution activity. Similarly, the addition of RNase A and T₁ has no effect upon resolution of the plasmid substrate into linear minichromosomes (data not shown). A divalent metal ion is required because the chelation of Mg²⁺ with EDTA abrogates the reaction (Fig. 3, lane 10). The need for Mg²⁺ is not

FIG. 3. Characterization of the resolvase activity in infected-cell lysates with plasmid pSCB1a. All reactions were carried out for 30 min at 30°C, and the products were fractionated on a 0.7% agarose gel and visualized by staining with ethidium bromide. Lane 1, uncut plasmid; lane 2, *Hind*III-linearized standard; lane 3, plasmid cleaved with *Hind*III and *Afl*II; lane 4, plasmid cleaved with the phage T7 gene 3 endonuclease; lane 5, plasmid digested with phage T7 gene 3 endonuclease and then cleaved with *Hind*III; lane 6, plasmid reacted with lysate and then *Hind*III; lane 7, deoxyribonucleotides omitted from the standard reaction; lane 8, both ribonucleotides and deoxyribonucleotides omitted from the reaction; lane 9, ATP, ribonucleotides, and deoxyribonucleotides all omitted from the reaction; lane 10, ATP, ribonucleotides, and deoxyribonucleotides all omitted, and the reaction was made to 5 mM in EDTA; lane 11, complete reaction mix; lane 12, complete reaction mix with a 100 μ M concentration of the poxviral topoisomerase inhibitor diminazene aceturate added.

stringent, however, and MnCl_2 can substitute for MgCl_2 (data not shown). Higher concentrations of MgCl_2 or the addition of spermidine stimulates cleavage of the single-stranded regions of the hairpin loops of the substrates, making it more difficult to monitor resolution activity. The addition of diminazene aceturate, a viral topoisomerase inhibitor (55), had no effect on endonuclease activity (Fig. 3, lane 12), although it significantly inhibited the relaxation of the supercoiled pUC plasmids in these extracts (data not shown). This was an unexpected observation considering that relaxed plasmids function as less efficient substrates for the *in vitro* resolution reaction (see below).

The resolution activity is sensitive to heat and to proteinase K but not to RNases. Two drugs tested that proved to be potent inhibitors of the resolving activity were actinomycin D (at 10 $\mu\text{g}/\text{ml}$) and ethidium bromide (at 10 $\mu\text{g}/\text{ml}$). We have not elucidated the mechanisms of action of these inhibitors, but they may act by altering the helical pitch of the target DNA and preventing the binding of critical proteins or by quantitatively intercalating into the substrate plasmids and forcing the resorption of extruded cruciforms.

Cleavage products that are consistent with a Holliday junction endonuclease. The circular substrate plasmid used in this assay contains a large inverted repeat of poxviral telomeric DNA sequences. Sufficient negative superhelical tension will cause the inverted repeat to be extruded from the plasmid to create a four-armed DNA junction that mimics the structure of the Holliday junction intermediate obligatory to most models of homologous recombination (13, 40). Resolution of this Holliday junction analog in a sequence-independent fashion can be accomplished by the nicking of opposing strands at the base of the junction with phage enzymes specific for branched DNA structures to yield a linear hairpin structure that is indistinguishable from the product of *in vivo* resolution (37). Nicking of the single-stranded hairpins at the apex of the extruded cruciform can also result in

FIG. 4. Snapback analysis of the products generated from reaction of pSCB1a with the infected-cell lysate. After cleavage of the resolved plasmid with *Hind*III, the reaction tubes were immersed in a boiling water bath for 5 min and then quick-chilled by plunging the tubes into ice water. The DNA fragments were then fractionated on nondenaturing 0.7% agarose gels and visualized by ethidium bromide staining. Lane 1, undenatured DNA; lane 2, DNA denatured and quick-chilled. The 750- and 3,436-bp snapback products are indicated.

the formation of discrete digestion products after cleavage with *Hind*III. The distinguishing feature between these two events is that the products formed by the nicking of hairpins will not be covalently linked, while the product of cleavage across the base of the cruciform will be a single self-complementary molecule. Thermal denaturation followed by quick-chilling allows the two types of products to be distinguished because the self-complementary molecule with hairpin termini will rapidly reanneal and so will migrate as an undenatured full-length fragment during gel electrophoresis. The product of hairpin nicking will fail to reanneal and its migration will be drastically altered during nondenaturing gel electrophoresis.

The fragments produced by treating pSCB1a with infected-cell lysate and then digesting with *Hind*III rapidly reanneal after boiling (Fig. 4, lane 2), suggesting that at least some of the fragments produced are hairpin molecules. The hairpin fragments produced by the infected-cell lysate are identical to the fragments created when the pSCB1a that is extruding a cruciform is cleaved by the phage T7 gene 3 endonuclease (data not shown). Relaxation of plasmid DNA prior to treatment with an infected-cell lysate makes most, but not all, of the DNA resistant to cleavage (Fig. 5A, lane 1). This implies that the bulk of the resolution activity detected for this replicative intermediate structure may be targeted for the structure of the substrate DNA rather than on any specific sequence. By the same token, the residual level of resolution of completely relaxed plasmid substrates may well represent an independent activity that has more-stringent sequence specificity (this is currently under study). We tested the infected-cell lysates for sequence-independent cleavage of branched DNA structures by utilizing plasmid pAT34 which contains a self-complementary stretch of the sequence d(AT):d(AT). This plasmid is known to readily extrude a cruciform that is susceptible to cleavage by T7 gene 3 endonuclease (23). When tested with extracts from virus-infected cells, the induced activity cleaved pAT34 in a manner similar to that of pSCB1a (Fig. 5B, lanes 2, 3, and 4), indicating that the cleavage was not dependent upon the presence of specific poxvirus sequence. A similar result was found when a nonmobile Holliday junction analog made of annealed oligonucleotides was used as the substrate (data

FIG. 5. (A) Plasmid pSAD-2 (9) carrying an inverted repeat insert of the core SFV TRT sequence was relaxed with topoisomerase, treated with cell lysate, and cleaved with *Hind*III (lane 1) or reacted with cell lysate and then with *Hind*III without prior relaxation (lane 2). In each case, the cleavage products were 3' end labelled with [α - 32 P]dATP and Klenow fragment. The end-labelled fragments were fractionated on an 8% nondenaturing polyacrylamide gel and visualized by autoradiography. The smaller pSAD-2 hairpin fragment generated by *Hind*III cleavage of the linearized plasmid is indicated with an arrow. (B) Plasmid pAT34 (23) which contains a self-complementary d(AT)-d(AT) insert is resolved by the infected-cell lysate. Lane 1, pAT34 linearized with *Bgl*II; lanes 2, 3, and 4, pAT34 reacted with the infected-cell lysate at 30°C for 5, 10, and 20 min, respectively, and then cleaved with *Bgl*II. The hairpin fragment released by *Bgl*II cleavage is indicated by the arrow.

not shown). Thus, unlike the sequence-specific resolution of replicative intermediates observed *in vivo*, the major resolving activity detected in these lysates *in vitro* displays no sequence preference, consistent with a potential role in general recombination.

The major cleavage sites introduced into a cloned viral replicative intermediate were mapped on plasmid pSAD-2, which contains an inverted repeat arrangement of the core TRT from SFV. The inverted repeats contained by plasmids such as pSAD-2 can exist in a lineform configuration or can be extruded into a cruciform (Fig. 6A). Resolution of pSAD-2 can therefore occur by nicking opposing strands across the a-c or b-d axis at the base of the cruciform. The major product identified by denaturing gel electrophoresis of *in vitro*-resolved pSAD-2 that had been reacted with the lysate and then cleaved with *Hind*III and 3' end labelled was a set of fragments about 160 bases in length that were indicative of substrate molecules that have been nicked at site a across the a-c axis within the inverted repeat insert (Fig. 6B, lane 3). In addition, specific nicking occurred in the region of the single-stranded hairpin loop (site e) 85 bases from the labelled *Hind*III site and internally at sequences corresponding to the TRT (site f). In addition to the 85-base size standard, the marker lane (Fig. 6B, lane 2), shows a minor band at the position corresponding to a 95-nucleotide fragment. This appears to be a nonspecific band or it may be a site within the plasmid DNA that is for some reason particularly labile. pSAD-2 cleaved with the cytoplasmic

lysate shows a doublet of bands at this position (Fig. 6B, lane 3). This cleavage occurs within the TRTs, but the significance of these nicks is not clear at this time. Nicks are introduced into pSAD-2 in a symmetric fashion on both the 3' and 5' strand of the inverted repeat insert at sites a, b, c, and d (Fig. 6C), suggesting that the Holliday junction is resolved by nicking of the opposing strands across either the a-c or b-d axis at the base of the junction. Specifically, symmetrical cleavage of either axis is indicated by the formation of comparably sized fragments when either the 3' or 5' strand of pSAD-2 is labelled after digestion with infected-cell lysate and either *Hind*III (Fig. 6C, lanes 3 and 4) or *Eco*RI (Fig. 6C, lanes 5 and 6). These cleavage sites in pSAD-2 introduced by the poxvirus-induced endonuclease coincide precisely with the nicks made by the phage T7 gene 3 endonuclease (Fig. 6C, lane 7). The position of the faster-migrating bands (Fig. 6C, lanes 4 and 6) is not consistent with the initial nicks being introduced at the apex of the hairpin loop; instead, the nicking appears to be located approximately 10 nucleotides 5' to the hairpin loop within the TRT region of the inverted repeat. The origin of this series of nicks is unclear; however, they may have been introduced by the labelling procedure (for instance, the labelling of internal nicks), since they are not reproduced when the cleaved DNA is labelled at the 3' end.

Mapping of *in vitro* cleavages within the viral TRT. Fine mapping of the nicks induced in the plasmid pSAD-2 at the sites most distal to the axis of symmetry indicates that cleavage occurs directly at the point at which the two sides of the inverted repeat lose complementarity (Fig. 7A). This cleavage also coincides with the nicks caused by the phage T7 endonuclease and illustrates the fact that branch migration to the end of these rather short inverted repeats only partially relaxes the negative supercoiling of the target plasmid and hence the crossover structures are relatively nonmobile at the most distal complementary nucleotides that permit free branch migration. Maxam-Gilbert sequencing (36) illustrates that these cleavages are very precise and are not followed by random degradation (Fig. 7B).

Some faster-migrating bands that are indicative of cleavages within the TRT appear in Fig. 7B, lane 6. Importantly, these TRT cleavages are not symmetrical, as are those produced by the Holliday crossover resolving activity. Instead, as can be seen in lanes 6 and 7 in Fig. 7B, when the lower strand (illustrated in Fig. 7A) is probed by 3' end labelling from the *Hind*III site, only the TRT copy distal to the dyad axis is cleaved, while the proximal TRT copy remains intact. When the upper strand is assessed, for example, by 5' end labelling from the *Hind*III site, the converse is observed (Fig. 6C, lane 4), indicating that each copy of the TRT is cleaved only once on the strand 5' from the central axis of symmetry. Thus, these cleavages are highly specific for the viral TRT and cleavage occurs in a fashion distinct from a standard Holliday crossover endonuclease. Another distinction can be made in the salt sensitivities of these two kinds of cleavages. As shown in lane 7 of Fig. 7B, changing the salt conditions of the reaction buffer from 50 mM potassium glutamate to 100 mM KCl reduced the TRT cleavages, while the Holliday crossover cleavages were unaffected. Thus, by these criteria the TRT cleavages behave as would be predicted for sequence-specific telomere resolution, while the Holliday crossover cleavages could also potentially reflect nonspecific recombination activities. The sites of the major cleavages introduced into plasmids pSAD-2 and pSCB1a by this latter activity thus are structure specific rather than sequence specific, suggesting that it will

FIG. 6. Positions of site-specific nicks produced by the infected-cell lysate on the cloned SFV telomere. (A) Substrate plasmid pSAD-2 with the inverted repeat in the line form and in the cruciform configuration. Paired cleavages that would lead to resolution of the extruded cruciform are indicated by the arrows labelled a, b, c, and d, nicking at the hairpin loops is indicated by the arrows labelled e, and nicks occurring within the TRT sequences are indicated by the arrows labelled f. (B) Lanes 1 and 4, pUC19 digested with *HinfI* size standards. The two fragments used for size determination are the 75-base fragment at the bottom of the gel and the 214-base fragment visible at the top of the gel. Lane 2, Substrate plasmid pSAD-2 cut with *HindIII* and *AflIII* to indicate the position of the 85-nucleotide band produced by creating nicks at the single-stranded hairpin loop. Lane 3, pSAD-2 reacted with infected-cell lysate, cut with *HindIII*, and 3' end labelled. The major cleavage site corresponding to the Holliday crossover cleavages at site a appears at 160 nucleotides from the labelled *HindIII* site, and a possible TRT cleavage occurs at 95 nucleotides from the *HindIII* site. Cleavages at sites b, c, and d are not detected by this protocol but are detected in panel C. A minor amount of the labelled product mapping to the hairpin region may be the result of a low level of hairpin nicking, but the specificity of this cleavage was not investigated. (C) Lane 1, pUC19 cleaved with *HinfI*; lane 2, Φ X174 DNA cleaved with *HinfI* for size standards; lanes 3 and 4, pSAD-2 digested with infected-cell lysate, cleaved with *HindIII*, and finally 3' end labelled (lane 3) to detect site a or 5' end labelled (lane 4) to detect site d; lanes 5 and 6, pSAD-2 digested with infected-cell lysate, cleaved with *EcoRI*, and finally 3' end labelled (lane 5) to detect site c or 5' end labelled (lane 6) to detect site b; lane 7, pSAD-2 cut with phage T7 gene 3 endonuclease, cleaved with *HindIII*, and finally 3' end labelled to detect site a. All samples were electrophoresed through a 0.4-mm-thick 8% polyacrylamide gel containing 7 M urea and then visualized by autoradiography.

resolve branched DNA structures and is probably not restricted to only viral telomere replicative intermediates. Plasmids identical to pSAD-2 but with single-base mutations that abrogate *in vivo* resolution yet maintain the palindromic nature of the insert were tested in the assay and found to be converted to minichromosomes with comparable efficiency to that of pSAD-2 (data not shown), further indicating a lack of sequence specificity for the Holliday crossover endonuclease activity. We have also searched the newly available genomic sequence of vaccinia virus (22) for open reading frames that display similarity to the phage T4 and T7 resolving enzymes, but no significant homologies were identified.

DISCUSSION

During the process of DNA replication, the hairpin termini of poxviral genomes are transiently converted into palindromic fusion sequences that are the targets for virus-specific resolution activities that convert the inverted repeat conformation into daughter hairpin termini (for a recent review, see reference 10). This resolution process can be monitored by the use of cloned poxvirus replicative intermediates that are replicated and resolved into linear hairpin-terminated minichromosomes when transfected into poxvirus-infected cells (11, 39, 41). This observation has allowed the delineation of the minimal target DNA sequences required for the resolution of poxvirus replicative intermediates *in vivo*. The complex process of telomere resolution is dependent upon the presence of two copies of a highly conserved 20-bp sequence, TRT (9, 39). Resolution of the telomere replicative intermediate also requires that the two copies of the TRT be oriented such that they are inverted with respect to one another (39). The significance of inverted repeat nature of the replicative intermediate is clearly demonstrated by the observation that altering the orientation of the TRTs or inserting an asymmetric interruption between the TRTs abrogates or severely decreases the efficiency of the resolution process (37, 39). Two models for the resolution of replicative intermediates have been put forth that are consistent with these data (10). One model involves site-specific nicking and strand exchange within the TRTs and directed branch migration toward the axis of symmetry between the two TRTs. The second model invokes branch migration and the formation of a cruciform structure in the replicative intermediate. This secondary structure must be resolved by nicking the opposing strands of the Holliday junction at the base of the cruciform. The final products of either reaction

are the same, and both models mandate strand exchange coupled with branch migration to effect strand isomerization into daughter hairpin structures. However, the enzymes required to catalyze each reaction are different, and the second model proposes that a transient cruciform structure would be an obligatory intermediate in the process.

In vitro, the cloned replicative intermediate of the poxviral telomere is capable of extruding a cruciform structure that at its base is functionally analogous to a Holliday junction recombination intermediate and whose extruded arms are identical to the viral hairpin termini (11, 13, 40). We have identified an activity present in poxvirus-infected cell extracts that shows a specificity for cleaving Holliday junctions in such extruded cruciforms. The presence of a virus-induced Holliday junction endonuclease in infected-cell lysates is not unexpected, considering the tremendous amount of virus-induced recombination that occurs *in vivo* (2, 17, 62). Most models of homologous recombination invoke the presence of such a crossover-specific nuclease to resolve the Holliday junctions that result from strand exchange and branch migration. The activity that we have identified in this communication is able to resolve Holliday crossover junctions associated with cloned replicative intermediates into linear hairpin-terminated minichromosomes that are indistinguishable from progeny molecules generated *in vivo* by replication and resolution. The resolution activity is virally induced and requires both viral DNA replication and the synthesis of late gene products, but further analysis will be required to ascertain whether it is required for both recombination and resolution or is restricted to just one process. Resolution *in vivo* requires viral late gene products but is not strictly dependent upon active DNA replication (41), while recombination can be detected at both early and late times in conjunction with DNA synthesis (17). Results from lysates made from cells infected with the thermolabile vaccinia virus mutant *ts53* at the nonpermissive temperature and from cells infected with wild-type virus in the presence of a DNA synthesis inhibitor such as araC indicate that the resolving activity we have identified is not induced until the synthesis of late gene products begins. Proof that the activity is due to a viral gene product with late kinetics will require identification of the cognate gene, and at this point we cannot exclude the possibility that the activity is expressed early but is only unmasked at late times in these extracts. The presence of a Holliday junction endonuclease in uninfected HeLa cells has been reported (71); however, we have been unable to detect the cellular activity in the extracts prepared by using this protocol either from uninfected cells or at early

FIG. 7. Fine mapping of nicks induced by the infected-cell lysate on the substrate plasmid pSAD-2. (A) Sequence of the pSAD-2 insert with the major Holliday crossover cleavage sites a, b, c, and d and the TRT cleavages at site f denoted by vertical arrows. The axis of dyad symmetry in the insert is indicated by the horizontal arrows, and the TRTs are boxed. The vertical arrows at the outer

times after virus infection. The potential presence of both cellular and viral activities in the infected-cell extracts would complicate the interpretation of some of the biochemical data; however, it has been observed that most of the induced biochemical activities are directly attributable to viral gene products in cells infected with vaccinia virus at a high multiplicity (6). Whether our inability to detect the cellular endonuclease is due to its lability or physical removal from these extracts is not known.

Although the activity specific for Holliday crossovers described here is consistent with a model of replicative intermediate resolution that calls for the extrusion of a cruciform from the inverted repeat there is no direct evidence for extruded poxviral cruciforms *in vivo*. Relaxed plasmid DNA substrates that do not possess extruded cruciforms are less efficiently resolved to minichromosomes than are supercoiled templates by these extracts, but resolution can still be detected. This may indicate the presence of viral proteins that lead to unwinding of the inverted repeat TRTs and extrusion of a cruciform in these extracts or it may merely be an indication of how difficult it is to completely remove all cruciform structures from the substrate plasmids. Interestingly, the very active poxvirus-encoded topoisomerase that is present within these extracts quickly relaxes all input plasmid DNAs and so we might expect to see some competition between the nicking and resolution of the plasmid cruciform structures and the relaxation of the substrate plasmids with concomitant resorption of the cruciform structures. However, inhibition of the viral topoisomerase with diminazene aceturate resulted in no detectable increase in the amount of resolved linear molecules (Fig. 3). *In vivo*, relaxed plasmids that are transfected into infected cells are replicated and resolved as efficiently as are transfected supercoiled molecules that extrude cruciforms (11). This suggests that cruciform extrusion mediated by superhelical tension alone is probably not an essential aspect of resolution of the inverted repeat structure *in vivo*. Indeed, it is predicted that the large linear replicated concatemeric DNA molecules that form the natural substrate for viral resolving activities would not be subjected to sufficient superhelical tension to cause quantitative extrusion of cruciforms. The possibility that viral proteins could serve to force the forma-

extremities of the inverted repeat denote the symmetrical Holliday crossover cleavages at sites a, b, c, and d, and the asterisks indicate the cleavage sites mapped in panel B. The vertical arrows within the TRT denote the strand-specific cleavages within the TRT, and the asterisks indicate the cleavages that are mapped in panel B. (B) Mapping of the cleavage sites induced by the infected-cell lysate in pSAD-2 by Maxam-Gilbert sequencing. Lanes 1, 2, 3, and 4, pSAD-2 was cleaved with *Hind*III, 3' end labelled, and cleaved with *Eco*RI. The labelled SFV fragment was isolated by nondenaturing polyacrylamide gel electrophoresis and subjected to Maxam-Gilbert sequencing reactions (lanes 1 to 4). Lane 5, pSAD-2 cleaved with *Hind*III and *Afl*III and then 3' end labelled to mark the position corresponding to bands created by the introduction of nicks at the single-stranded hairpin region. Lane 6, pSAD-2 digested with infected-cell lysate in a reaction mixture containing 50 mM potassium glutamate, cleaved with *Hind*III, and finally 3' end labelled. The major cleavage product migrates as a 160-nucleotide fragment corresponding to cleavage at the sequences indicated by asterisks in panel A. Some minor cleavage products can be observed migrating more rapidly through the gel. Lane 7, pSAD-2 treated identically to the sample in lane 6 except the resolution reaction was carried out in the presence of 100 mM KCl. The higher salt conditions decrease the spectrum of cleavages that are detected within the TRTs.

tion of an unusual secondary structure in the replicative intermediate remains a possibility. This is particularly germane in light of the finding that a single copy of the TRT is capable of acting as a strong viral promoter at late times during infection (64). Melting and unwinding of promoter sequences by the viral transcriptional machinery (70) could, in fact, serve to induce the formation of a secondary structure such as a cruciform that would be cleavable by the branched structure-specific nuclease described here.

The resolution process *in vivo* is strictly dependent upon the sequence and orientation of the TRTs in the replicative intermediate (10). Strand exchange between the arms of the inverted repeat and branch migration between the two TRTs are required for resolution of the replicative intermediates (39) and in fact is the only way to explain the regeneration of the "flip" and "flop" pattern of hairpin termini that are observed in all poxviruses that have been examined (10). In order to explain the segregation of daughter hairpin structures from a precursor inverted repeat substrate, transient cleavage must occur at or near the TRTs at some time during the resolution process *in vivo* (39). Thus, the sequence-specific, asymmetric cleavages that we observed created within the TRTs by these virus-infected extracts are particularly significant and suggest that at least some aspects of the resolution mechanism *in vivo* have been maintained. At the present time, we are unable to deduce whether the Holliday crossover endonuclease is linked with the TRT-cleaving activity, either physically in a complex or functionally in a common resolution pathway. One possibility is that the role of the Holliday junction endonuclease activity in virus replication is not only the sequence-specific resolution of replicative intermediates but also the resolution of any branched junctions that are incurred during replication and recombination of viral DNA. Further analysis, especially the generation of mutants in the cognate viral gene(s), will be required to establish the precise roles of these two activities in the viral replicative cycle.

In several significant ways, the poxvirus replicative cycle is more like that of the bacteriophages T4 and T7 than that of nuclear mammalian viruses. Convolved interactions occur among the pathways for phage T4 transcription, replication, recombination, and packaging such that they utilize many common enzymes and attempts to uncouple these processes with mutational blocks or inhibitors often result in pleiotropic effects on DNA metabolism (44). Strand invasion and recombination in phage T4 has been demonstrated to result in the formation of primers for DNA synthesis and mutants that are deficient in strand invasion demonstrate an arrest of DNA replication after the early rounds of synthesis (7). Recombination can occur in the absence of replication, but it occurs with a much lower frequency than when DNA replication is allowed to proceed (43). Phage T4 gene 49, which encodes a Holliday junction-resolving enzyme (27, 42), is expressed at late times (19) and is required to trim the branched structures that comprise the nascent genomic DNA and cleave the concatemeric structures into single unit-length genomes in the packaging reaction (27). Bacteriophage T7 also encodes a Holliday junction-cleaving enzyme that cleaves concatemeric DNA in a packaging reaction (12). DNA replication and recombination occur concurrently in poxvirus-infected cells, and to date, attempts to separate the two processes with conditional lethal mutants (38) or inhibitor drugs (17) have been unsuccessful. Efficient recombination in poxviruses is dependent upon DNA synthesis but there is no obvious requirement for late gene expression *per se* which usually commences after DNA

replication is initiated (38). This is in contrast to the resolution of the replicative intermediates which occurs only under conditions that are permissive for late gene expression (41). No poxvirus mutants have yet been identified that are solely deficient in recombination or resolution and all temperature-sensitive viruses that have been found to be defective in recombination are also deficient in DNA synthesis (38). Most of the viral mutants identified that are defective in replicative intermediate resolution are also deficient in the expression of late genes or the stability of late mRNA (41). The single exception is the vaccinia virus mutant *ts9383* which is defective in telomere resolution as a result of a mutation in the small subunit of the mRNA capping enzyme (4).

The pleiotropic nature of the defects in viral recombination and replicative intermediate resolution are similar to the wide-ranging defects in DNA metabolism displayed by phage mutants that are defective in recombination or replication proteins (43). During the phage T4 replicative cycle, it has been shown that both replication and recombination are carried out by multisubunit enzymes or protein machines (1). Many of the proteins that reside within these complexes are utilized for both replication and recombination activities. The multicomponent nature of these complexes means that the constituent enzymes must associate with low affinity in order to allow a mixing and matching of activities between different complexes in different places. This intrinsic linkage of activities and efficient use of enzymes results in functional complexes that are inherently unstable and refractory to purification. This situation may also be the case for sequence-specific resolution of poxviral replicative intermediates. For example, the poxviral transcription complex utilizes different subunits at early and late times during infection (67). The transcriptional assembly includes a variety of subunits including the viral capping enzyme (3) and possibly a host RNA polymerase II subunit (46), and thus recruitment of the multisubunit transcription complex to the TRT may target this sequence to a resolution or strand exchange factor that is associated with the transcription complex. The activity that we have identified may be one part of that complex but it is likely that more factors remain to be identified before *bona fide* resolution can be accurately reconstituted *in vitro*.

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