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DNA Strand Exchange Catalyzed by Proteins from Vaccinia Virus-Infected Cells

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Vaccinia virus infection induces expression of a protein which can catalyze joint molecule formation between a single-stranded circular DNA and a homologous linear duplex. The kinetics of appearance of the enzyme parallels that of vaccinia virus DNA polymerase and suggests it is an early viral gene product. Extracts were prepared from vaccinia virus-infected HeLa cells, and the strand exchange assay was used to follow purification of this activity through five chromatographic steps. The most highly purified fraction contained three major polypeptides of 110 ± 10, 52 ± 5, and 32 ± 3 kDa. The purified protein requires Mg^{2+} for activity, and this requirement cannot be satisfied by Mn^{2+} or Ca^{2+}. One end of the linear duplex substrate must share homology with the single-stranded circle, although this homology requirement is not very high, as 10% base substitutions had no effect on the overall efficiency of pairing. As with many other eukaryotic strand exchange proteins, there was no requirement for ATP, and ATP analogs were not inhibitors. Electron microscopy was used to show that the joint molecules formed in these reactions were composed of a partially duplex circle of DNA bearing a displaced single-strand and a duplex linear tail. The recovery of these structures shows that the enzyme catalyzes true strand exchange. There is also a unique polarity to the strand exchange reaction. The enzyme pairs the 3′ end of the duplex minus strand with the plus-stranded homolog, thus extending hybrid DNA in a 3′-to-5′ direction with respect to the minus strand. Which viral gene (if any) encodes the enzyme is not yet known, but analysis of temperature-sensitive mutants shows that activity does not require the D5R gene product. Curiously, v-SEP appears to copurify with vaccinia virus DNA polymerase, although the activities can be partially resolved on phosphocellulose columns.

Homologous recombination is a ubiquitous biological process required for chromosomal segregation, DNA replication, repair, and genetic assortment. Although no single hypothesis accounts for the many experimental observations made for different biological systems, most attempts to explain the mechanism of recombination assume that some form of homologous pairing and strand exchange must occur between recombining DNA molecules (26, 37, 39, 42). This leads to the formation of intermediary joint molecules which might then be subjected to additional enzymatic processing. Because pairing and strand exchange are so fundamental to the process of recombination, much work has focused on isolating and characterizing the enzymes responsible for these reactions.

The first strand exchange enzyme to be discovered was the *Escherichia coli recA* gene product. RecA protein catalyzes the pairing of two homologous DNAs in a reaction which can be partitioned into several biochemical steps (32, 45). These steps include formation of a RecA-coated nucleofilament, a homology search leading to paranemic joint formation, and exchange of strands between homologous duplexes. Turnover of RecA protein requires ATP hydrolysis, but pairing and exchange are not intrinsically ATP dependent (35). Subsequently, strand exchange enzymes have also been isolated from many sources (13), including other bacteria, extracts of bacteriophage T4-infected *E. coli* (54), *Ustilago maydis* (29), *Drosophila melanogaster* (14, 47), human cell lines (27, 38), *Schizosaccharomyces pombe* (1), and *Saccharomyces cerevisiae*. Some of these enzymes are ATP dependent, while others are not, and in some cases more than one activity can be recovered from a given organism. For example *S. cerevisiae* encodes Sep1 (or STPβ) (12, 28, 30, 51), STPα (5, 50), and a third enzyme resembling, and possibly identical to, Sep1 (24). Disrupting the gene encoding Sep1 leads to pleotropic defects in meiotic and mitotic recombination, sporulation, and repair (12, 51), while *recA* mutations create defects in recombination and repair (6, 53). Mutations in some of these genes can also affect DNA replication. For example, T4 *uvsX* mutants cannot catalyze secondary initiation of late DNA replication (3).

The fact that several *E. coli* phage encode recombination functions raises the possibility that large mammalian viruses also encode recombination genes. Poxviruses are obvious candidates because of their large genomes and because they replicate in the cytoplasm of infected cells where many host proteins may be inaccessible. This could account for why poxviruses encode such a complement of replication functions, including DNA ligase, DNA polymerase, and a type I topoisomerase (40, 52). Although it is not known whether poxviruses encode recombination functions, it is known that poxviruses are subjected to high-frequency genetic recombination (2, 49) and induce high levels of *trans*-acting recombination activity in infected cells (18, 44). Interestingly, we have observed that recombinant formation coincides with the onset of viral replication and both replication and recombination are slightly preceded by the formation of large amounts of heteroduplex DNA (21). Considering the example of bacteriophage T4, these observations suggested a mechanism whereby recombination primes the initiation of viral DNA replication (21) and may account for why, with one reported exception (7), most recombination mutants cannot be distinguished from DNA replication mutants (36). The recovery of heteroduplex DNA from virus-infected cells also suggested that viral infection was inducing synthesis of a strand exchange activity. Using an assay designed to detect strand transfer and joint molecule formation, we show

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here that vaccinia virus infection leads to the appearance of a new, cytoplasmic, strand exchange activity. This communication describes the characterization and purification of the enzyme(s) responsible.

MATERIALS AND METHODS

Cell and virus culture. Suspension cultures of HeLa S3 cells (American Type Culture Collection) were maintained at 37°C in minimum essential medium modified for suspension cultures (GIBCO) supplemented with 1% nonessential amino acids, 1% antibiotic-antimycotic, 2% fetal calf serum, and 8% horse serum in a 5% CO_2 atmosphere. The orthopoxvirus vaccinia virus (WR strain) and the leporipoxvirus Shope fibroma virus (strain Kasza) were obtained from G. McFadden and grown and titered on SIRC cells (American Type Culture Collection). Temperature-sensitive vaccinia virus strains bearing mutations mapped to the D5R open reading frame (C17, C24, and E69 [8, 15, 48]) were kindly provided by E. Niles, as were BSC40 cells. Mutant stocks were grown on BSC40 cells, and titers were determined on SIRC cells in Dulbecco modified Eagle medium (GIBCO) supplemented with 1% nonessential amino acids and 5% fetal calf serum. Permissive and nonpermissive temperatures of 32 and 40°C, respectively, were used. Mutant strains formed no plaques at 40°C, thus confirming the phenotype.

Virus infection. HeLa cells were cultured in 3-liter spinner flasks (Bellco). Upon reaching a density of 10^6 cells · ml^{-1} the cells were recovered by centrifugation for 5 min at 1,800 × *g* at 15°C, and the cell pellets from 7 liters of culture were suspended in 350 ml of warm Dulbecco modified Eagle medium without serum. Vaccinia virus was added at a high multiplicity of infection (500 to 1,000 PFU · $cell^{-1}$ [41]), and the suspension was stirred for 2 h at 37°C. The infected cells were then diluted back into the original culture media (which marked time zero in timed infections) and stirred for another 3.5 h at 37°C prior to harvest. Alternatively, BSC40 or HeLa cells were grown to near confluency on 150-cm^2 dishes, washed with phosphate-buffered saline (PBS), and infected with vaccinia virus in 3 ml of PBS at ~1,000 PFU · $cell^{-1}$ for 2 h at room temperature. The PBS was then replaced with 30 ml of complete Dulbecco modified Eagle medium, and the cells were returned to the incubator.

Protein purification. Infected cells grown in spinner culture (16 g [wet weight]) were harvested by centrifugation at 1,800 × *g* for 5 min, washed with ice-cold PBS, and resuspended in 30 ml of cold hypotonic buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM KCl, and 1.5 mM $MgCl_2$. All subsequent steps were performed at 0 to 4°C. Cells were broken with a Dounce homogenizer and fractionated into nuclear and cytoplasmic fractions by centrifugation at 1,800 × *g* for 5 min. After reextracting the nuclear fraction with 5 ml of hypotonic buffer and a second centrifugation, the two supernatants were pooled and the nuclei were discarded. This supernatant was adjusted to 110 ml with 0.4 M KCl, 5 mM spermidine-HCl, 1 mM EDTA (pH 8.0), 0.1% (wt/vol) Brij 58, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF); stirred for 20 min; and centrifuged at 11,000 × *g* for 10 min, and the supernatant was retained (fraction I). Solid ammonium sulfate (24.9 g) was added to fraction I, stirred for 1 h, and centrifuged at 10,000 × *g* for 10 min. The pellet was redissolved in 30 ml of buffer A (20 mM Tris-HCl [pH 7.8]), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 10% [wt/vol] glycerol) and dialyzed overnight against buffer A. Precipitated material was removed by centrifugation (1,800 × *g*, 5 min), and the conductivity of the sample was adjusted with buffer A–1 M NaCl to a final salt concentration of 50 mM. This was fraction II.

Fraction II was applied to a 50-ml single-stranded DNA cellulose column (200 μg of DNA · ml^{-1}) previously equilibrated with buffer A–50 mM NaCl and washed with 400 ml of buffer A–50 mM NaCl, and bound proteins were eluted with a 180-ml 0.05 to 1.0 M NaCl gradient in buffer A. Active fractions eluting from 0.2 to 0.6 M NaCl were pooled (fraction III), dialyzed, and then applied to a 25-ml DEAE-650M column (Toyopearl). The column was washed with 90 ml of buffer A and then eluted with a 90-ml 0.05 to 1.0 M gradient of NaCl in buffer A. Active fractions eluting between 0.08 and 0.4 M NaCl were pooled, concentrated by dialysis against powdered sucrose, dialyzed against buffer A (fraction IV), and then applied to a HW-55F gel filtration column (86 cm by 2 cm^2; Toyopearl) equilibrated with buffer A–50 mM NaCl. Active fractions eluting at $(V_e - V_o)/V_t - V_o) \cong 0.2$ were pooled to give fraction V. Fraction V was applied to a 5-ml heparin-affinity column (Bio-Rad) equilibrated in buffer A–50 mM NaCl, washed with 150 ml of the same buffer, and eluted with a 30-ml 0.05 to 1.0 M gradient of NaCl in buffer A. Active fractions eluting at the start of the gradient were pooled (fraction VI). Finally, 5 ml of fraction VI was diluted to 100 ml with buffer A and K_2HPO_4 (previously adjusted to pH 7.8) to a final concentration of 10 mM phosphate. This was applied to a 20-ml Whatman P-11 column equilibrated with buffer A–20 mM K_2HPO_4, washed with 100 ml of the same buffer, and eluted with a 30-ml 0.02 to 0.8 M phosphate gradient. The active fraction eluting with a conductivity equivalent to 180 mM NaCl was dialyzed against buffer A containing 50% (wt/vol) glycerol and stored at −20°C (fraction VII). Unless stated otherwise, fraction VII was used in all experiments described below.

Small-scale extracts were prepared from infected monolayers as described above, except that the cells on each 150-cm^2 dish was first scraped into 3 ml of PBS, recovered by centrifugation, and then resuspended in 1 ml of hypotonic buffer per dish. Fractions I (from vaccinia virus-infected HeLa cells) or III (from infected BSC40 cells) were then prepared as described but on a scale proportional to the volume of hypotonic buffer.

Nucleic acids. Form I bacteriophage M13mp19 DNA was extracted from *E. coli* JM105 cells by alkaline lysis and centrifuged to equilibrium in CsCl-ethidium bromide density gradients. Single-stranded M13mp19 viral DNA was extracted from phage which had been purified by centrifugation to equilibrium in CsCl density gradients. Linear duplex DNA was prepared by digesting form I DNA with restriction endonucleases (usually *Hin*dIII) and then by phenol-$CHCl_3$ extraction and ethanol precipitation. The construction and sequencing of clones bearing 401-bp fragments of influenza A virus cDNA inserted at the *Sma*I site of M13mp19 have been described elsewhere (55). These clones were obtained by polymerase chain reaction amplification of a highly conserved region of matrix protein gene sequence from the influenza virus strains A/New Jersey/8/76, A/NWS/33, and A/PR/8/34 and contain, distributed unequally over 401 bases of sequence, up to 6% base substitution mutations (55).

Strand exchange assay. Assays were performed in 30-μl reaction mixtures containing 33 mM Tris-HCl (pH 8.3), 20 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg of bovine serum albumin · ml^{-1}, 1.5 μg of M13mp19 linear duplex DNA, 0.75 μg of M13mp19 viral DNA and 30 to 1,000 μg of protein extract · ml^{-1} (30). Reactions were incubated at 30°C for 20 min, and then EDTA, proteinase K, and sodium dodecyl sulfate were added to final concentrations of 50 mM, 600

$\mu g \cdot ml^{-1}$, and 0.1% (wt/vol), respectively. After incubation at 37°C for 10 min, digestion was stopped with the addition of 7 μl of loading buffer (0.25% [wt/vol] bromophenol blue, 10 mM EDTA, and 60% [wt/vol] sucrose), and the samples were fractionated by size on a 0.8% agarose gel in Tris-acetate electrophoresis buffer containing 0.5 μg of ethidium bromide $\cdot ml^{-1}$. The DNA was visualized on a Fotodyne transilluminator and photographed with Polaroid type 665 film. Photographic negatives were scanned with a Bio-Rad densitometer to quantitate DNA distributions.

Other assays. S1 protection assays measured the ability of the strand exchange protein to render heat-denatured DNA resistant to S1 nuclease and therefore precipitable by trichloroacetic acid on Whatman GF/C filters (25). Exonuclease assays measured the release of perchloric acid-soluble nucleotides from duplex DNA (17). Both S1 and exonuclease assays used as substrates *Hin*dIII-digested M13mp19 DNA labelled during growth to a specific activity of 940 cpm $\cdot$ nmol^{-1} with [^{3}H]thymidine. DNA polymerase assays measured the incorporation of [α-^{32}P]dATP into an acid-insoluble form by using a low-molecular-weight preparation of calf thymus DNA template (17). Reaction conditions were identical to those of the strand exchange assay, except for the addition of four deoxynucleoside triphosphates to the DNA polymerase assays at concentrations of 10 μM dATP and 100 μM (each) dGTP, dCTP, and TTP. Protein concentrations were determined by using a commercial Bradford protein assay (Bio-Rad) with bovine serum albumin as a standard. Discontinuous denaturing gel electrophoresis (33) was used to characterize the protein content of different fractions.

Electron microscopy. Fraction V was used in a 30-μl strand exchange reaction, after which the DNA-protein complexes were eliminated by digestion with proteinase K, as described above. The reaction mixture was extracted once with phenol and diluted into 100 μl of hyperphase solution (100 mM Tris-HCl [pH 8.5], 10 mM EDTA, 50% [vol/vol] ultrapure formamide [Schwarz-Mann], 50 μg of cytochrome $c \cdot ml^{-1}$) at a DNA concentration of ~400 ng $\cdot ml^{-1}$. The hyperphase was spread onto a solution containing 10 mM Tris-HCl (pH 8.5), 1 mM EDTA, 20% (vol/vol) formamide, and interfacial material transferred to colloidian-coated copper grids (J. B. EM Services Inc.). After staining with uranyl acetate (50 μM in 90% [vol/vol] ethanol), samples were shadowed at an 8° angle with Pt/Pd and examined by transmission electron microscopy (10).

RESULTS

Vaccinia virus infection induces the appearance of a novel strand exchange activity. Enzymes which catalyze the exchange of strands between homologous DNAs can be detected by using an assay first used to characterize RecA protein. Transfer of a minus strand from the linear duplex DNA to the circular single-stranded viral DNA leads to the formation of joint molecules which can be detected by agarose gel electrophoresis (Fig. 1, left). By using this assay trace levels of a constitutive strand exchange activity in crude HeLa cell cytoplasmic extracts could sometimes be detected, but the amount of activity increased substantially following infection by vaccinia virus (Fig. 1). This induced activity could be detected as early as 30 min postinfection and reached maximal levels 3 to 3.5 h postinfection in HeLa cells. The kinetics of appearance suggests the activity is viral encoded and belongs to an early class of viral gene products. In order to compare the expression of vaccinia virus strand exchange protein (v-SEP) with other early viral genes, the cytoplasmic extracts were also assayed for vaccinia virus

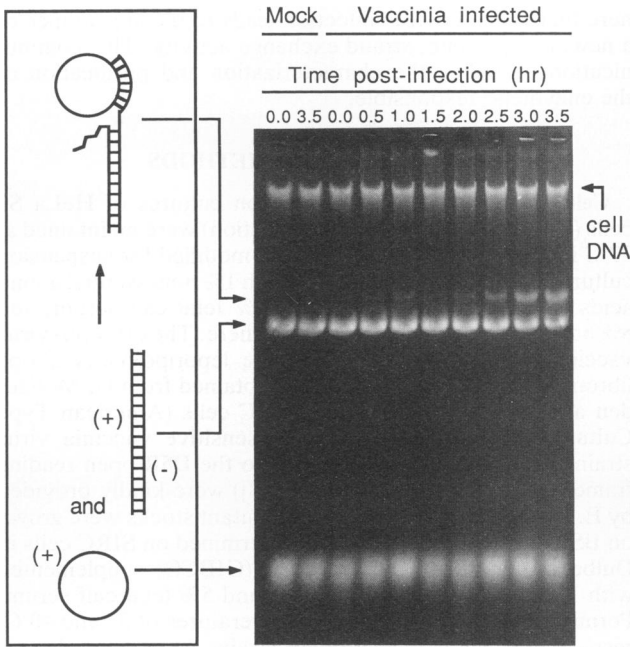

FIG. 1. Induction of strand exchange activity after vaccinia virus infection. HeLa cells were cultivated on 150-cm^2 dishes and infected (or mock infected) with vaccinia virus for 2 h as described in Materials and Methods. Unadsorbed viruses were washed off, and cytoplasmic extracts were prepared at the times indicated by using one 150-cm^2 dish per time point. Standard 30-μl reaction mixtures containing identical amounts of protein (30 μg) per reaction were prepared, and joint molecules were detected by agarose gel electrophoretic analysis. The diagram on the left illustrates the principle of the assay; the results of this experiment are shown at the right. A trace of constitutive activity is visible in the mock-infected cells at both 0 and 3.5 h while maximal levels of induced v-SEP activity (arrow) are seen at 3 to 3.5 h postinfection.

DNA polymerase. Vaccinia virus polymerase expression has been well characterized at the transcriptional and translational levels (34) and can serve as a marker for expression of early poxviral proteins. Polymerase specific activity reached maximal levels about 3 h postinfection (Fig. 2), about the same time that maximal levels of strand exchange activity were seen, which is consistent with the idea that v-SEP is another early viral gene product.

Purification of v-SEP from vaccinia virus-infected cells. Vaccinia virus strand exchange protein can be detected in a variety of infected cell lines, including HeLa, BSC40, and SIRC cells. Active enzyme could not be isolated from purified virions, but a similar activity can be recovered at 14 h postinfection from cells infected with the leporipoxvirus Shope fibroma virus (data not shown). Because HeLa cells can be grown in spinner culture, vaccinia virus-infected HeLa cell extracts were used as starting material for enzyme purification procedures. After preparation of cytoplasmic extracts, the activity was purified by differential precipitation with ammonium sulfate, affinity chromatography on single-strand DNA cellulose and heparin resins, ion exchange on DEAE and phosphocellulose resins, and gel exclusion chromatography. It proved difficult to accurately quantitate the activity in fractions I and II, but on the basis of recovery of activity in fraction III, v-SEP was purified about 170-fold with a yield of 3% (Table 1). Analysis of the active fractions from each step, by denaturing polyacryl-

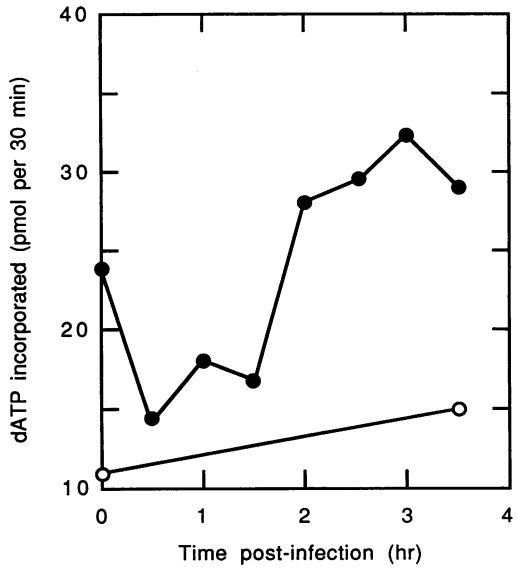

FIG. 2. Kinetics of DNA polymerase induction. The extracts described in the legend to Fig. 1 were assayed for DNA polymerase by using an activated calf thymus DNA template and [α-^{32}P]-labelled dATP. In the vaccinia virus-infected extracts (●) a peak of induced DNA polymerase activity which was not seen in the mock-infected cells (○) appeared about 3 h postinfection. The kinetics of polymerase induction parallel those of v-SEP induction (Fig. 1).

amide gel electrophoresis, showed that three polypeptides with molecular masses of 110 ± 10, 52 ± 5, and 32 ± 3 kDa were enriched by the purification (Fig. 3).

In addition to strand exchange activity, fraction VII was also assayed for DNA renaturing activity and double-strand DNA exonuclease. Under standard v-SEP reaction conditions, by using 1 μg of protein per 30 μl of reaction mixture and 0.9 μg of DNA, 73% of the molecules were rendered S1 resistant in 5 min, while in 20 min, 0.9% (~60 nucleotides per end) of ^{3}H-labelled linear duplex DNA was rendered acid soluble (data not shown). Thus, both activities are present in these preparations.

Characterization of v-SEP reaction requirements. Control experiments showed that no joint molecules were produced if single-stranded or double-stranded substrates were omitted from the complete reaction mixture (Fig. 4) and, like most eukaryotic strand exchange enzymes, v-SEP did not require a high-energy cofactor to catalyze joint molecule formation.

TABLE 1. Purification of v-SEP from vaccinia virus-infected HeLa cells

Fraction	Vol (ml)	Protein (mg)	Total U[a]	Recovered activity (%)	Sp act (U · mg^{-1})	Net purification
I	110	540	35,000[b]	100	67[b]	1
II	50	430				
III	70	9.1	35,000	100	3,900	60
IV	10	7.1	79,000	230	11,000	170
V	40	2.6	17,000	47	6,300	90
VI	10	2.1	10,000	29	4,800	70
VII	1.2	0.10	1,100	3.1	11,000	170

[a] One unit of activity converts 5% of the ethidium-stained DNA from substrates into joint molecules in 20 min at 30°C.
[b] Based on the yield of activity in fraction III.

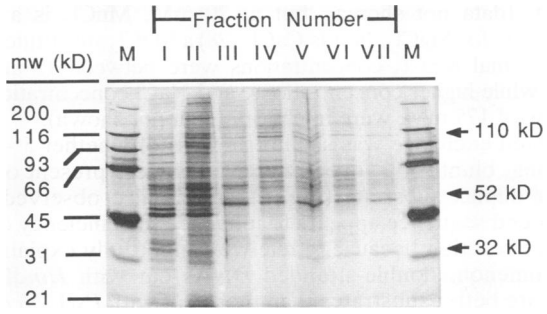

FIG. 3. Purification of v-SEP. Approximately equal amounts of protein from fractions I through VII were precipitated with trichloroacetic acid, applied to a 12% discontinuous denaturing gel, subjected to electrophoresis, and silver stained. The three major proteins in fraction VII bear molecular masses of 110 ± 10, 52 ± 5, and 32 ± 3 kDa.

Depletion of ATP, or addition of the ATP analog ATP-γ-S, had no effect on the efficiency of strand exchange (Fig. 4), nor did the addition of an ATP-regenerating system (30) stimulate the reaction (data not shown). The reaction is Mg^{2+} dependent, and omission of magnesium, or its chelation with EDTA, completely inhibited strand transfer. Trace levels of strand exchange activity were observed with high concentrations of

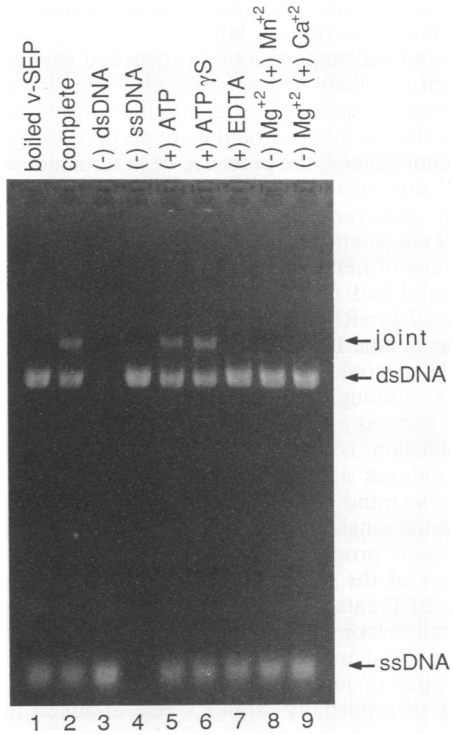

FIG. 4. Strand exchange reaction requirements. Additions or omissions to the standard reaction mixture were used to determine v-SEP reaction requirements. M13mp19 single-stranded (ss) DNA and *Hin*dIII-digested M13mp19 double-stranded (ds) DNA were used as substrates in all these mixtures. The negative control reaction (lane 1) used protein which had been denatured in a boiling water bath for 10 min. ATP or ATPγS was added to a final concentration of 1.5 mM (lanes 5 and 6), and EDTA, $MnCl_2$ or $CaCl_2$ was added to a final concentration of 20 mM (lanes 7, 8, and 9).

$MnCl_2$ (data not shown), but at 20 mM, $MnCl_2$ is a poor substitute for $MgCl_2$. Nor is $CaCl_2$ (28) a $MgCl_2$ substitute (Fig. 4). Optimal $MgCl_2$ concentrations were between 15 and 35 mM, while higher concentrations, and NaCl concentrations in excess of 175 mM, were inhibitory (data not shown).

Strand exchange was seen regardless of whether 5′-overhanging, blunt, or 3′-overhanging ends were present on the linear duplex substrate (Fig. 5). It was also observed that DNA end sequence appears to influence the efficiency of the pairing reaction because, while we cannot fully explain this phenomenon, double-stranded DNAs cut with *Hin*dIII or *Afl*II are better substrates than those cut with *Pst*I, *Nco*I, or *Eco*RI (Fig. 5 and unpublished data). As would be expected, participating molecules must share sequence homology in regions adjacent to the double-stranded ends in order for strand exchange to take place. This was shown by digesting an influenza virus A/PR clone with *Nco*I to produce an M13mp19 linear duplex carrying 252 and 149 bp of 5′-overhanging heterologous sequence on each end. Although this molecule paired readily with A/PR single-stranded DNA (Fig. 5, lane 4), no joint molecules were seen when the pairing partner was single-stranded M13mp19 DNA (Fig. 5, lane 5). The enzyme can, however, accommodate extensive nonhomology between two substrates. Considering the polarity of the strand exchange reaction (see below), clones derived from influenza virus strains A/NJ and A/PR differ by 13 nucleotide substitutions in the first 145 bases of pairable cloned sequence 3′ to the *Nco*I site on the duplex minus strand (55). Nevertheless, a heterologous combination of these clones was paired as efficiently as a homologous pair in standard reactions (Fig. 5, lane 6).

The strand exchange reaction proceeded with a characteristic polarity. Linear duplex molecules containing blocks of non-M13mp19 sequence located at either the 5′ end or the 3′ end of the duplex minus strand were prepared (Fig. 5). Under these circumstances, the presence of heterologous sequences on the 3′ end of the minus strand blocked joint molecule formation between *Hin*dIII-digested A/NWS DNA and M13mp19 single-stranded DNA (Fig. 5, lane 8). In contrast, the presence of heterologous sequences on the 5′ end of the minus strand had no effect on v-SEP-catalyzed pairing between *Nco*I-*Eco*RI-digested A/NWS DNA and M13mp19 DNA (Fig. 5, lane 10). Control experiments showed that both linear DNAs could pair with homologous single-stranded molecules, although the efficiencies varied because of the sequence context effects noted above (Fig. 5, lanes 7 and 9). The conclusion is that, under these reaction conditions, v-SEP catalyzes a polarized pairing reaction in which the duplex plus strand is displaced in a 5′-to-3′ direction by the plus-stranded single strand (see Fig. 8). The significance of this enzymatic property is discussed below.

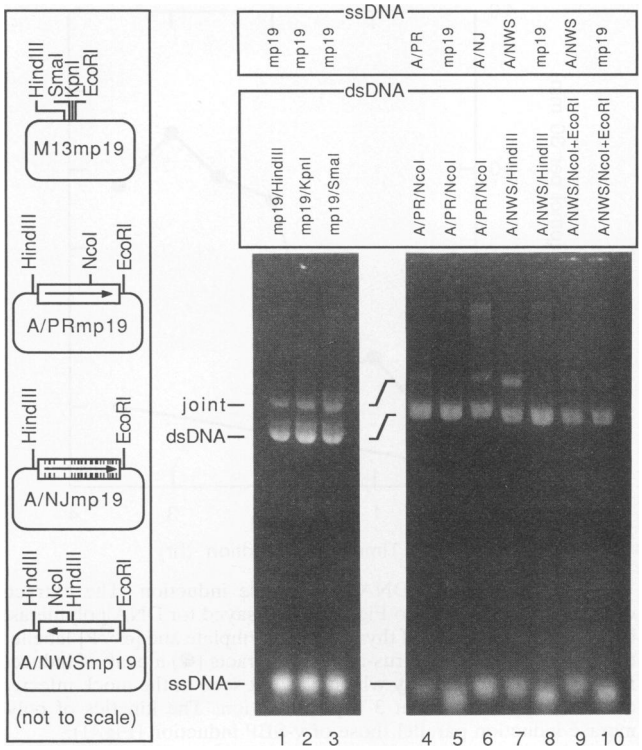

FIG. 5. Effects of ends, homology, and polarity on v-SEP-mediated strand exchange. Standard reaction mixtures were supplemented with different combinations of single-stranded (ss) and double-stranded (ds) substrates, all of which derive from M13mp19. Restriction maps of these DNAs are shown at the left (in the standard vector orientation) with only the relevant restriction sites indicated. The positions of the base substitution mutations which distinguish A/PR and A/NJ clones are marked with vertical bars. Note that digesting A/PRmp19 with *Nco*I leaves cloned DNA on both ends of the duplex minus strand, which is not homologous to M13mp19 DNA. Alternatively, digesting A/NWSmp19 DNA with *Hin*dIII leaves a block of non-M13mp19 sequence on the 3′ end of the duplex minus strand, while digesting A/NWSmp19 with *Nco*I and *Eco*RI leaves non-M13mp19 sequences on the 5′ end of the minus strand. Lanes 1 to 3 show the effects of different ends on the capacity of the double-stranded DNA to serve as a reaction substrate. Lane 4 shows that *Nco*I-digested A/PRmp19 duplex DNA can be paired with A/PRmp19 single-stranded DNA but not with M13mp19 DNA (lane 5). Although numerous base substitution mutations are present, *Nco*I-digested A/PRmp19 duplex DNA can still be paired with A/NJmp19 single-stranded DNA (lane 6). *Hin*dIII-digested A/NWS DNA pairs efficiently with A/NWS single-stranded DNA (lane 7), but it cannot be paired with M13mp19 DNA (lane 8). In contrast, A/NWS DNA cut with *Nco*I and *Eco*RI will pair with both A/NWS (lane 9) and M13mp19 single-stranded DNAs (lane 10).

Structure of the joint molecules. Joint molecules synthesized in v-SEP-catalyzed reactions were further analyzed by electron microscopy in order to determine whether they bore a structure consistent with that of strand exchange products. Several types of joint molecules were observed. α-Forms, consisting of a partially duplex circle attached to a duplex linear branch and a single-stranded tail, constituted 6% of the molecules observed in a number of randomly selected fields (Fig. 6b; Table 2). These are typical joint molecules in which a single strand has been transferred from a double-stranded duplex over to a single-stranded circle. Various degrees of strand transfer were observed among different α-forms (Fig. 6b, c, and d), but no fully duplex circles were seen, and it is unlikely that the enzyme preparation catalyzes complete strand exchange under these experimental conditions. A second type of joint molecule was seen bearing a "lollipop-like" structure. These σ-forms consisted of a full-length linear double strand attached to a single-stranded circle (Fig. 6a) and comprised 2% of all the molecules (Table 2). No displaced strand was visible in σ-forms at this level of resolution. Finally, a third type of joint molecule was seen, consisting of a partial-length linear duplex attached to a partially duplex circle (Fig. 6e) and composing 9% of all molecules on the grid. Displaced single strands were not seen in these α′-forms, which probably arose through nucleolytic degradation of the substrates and/or products.

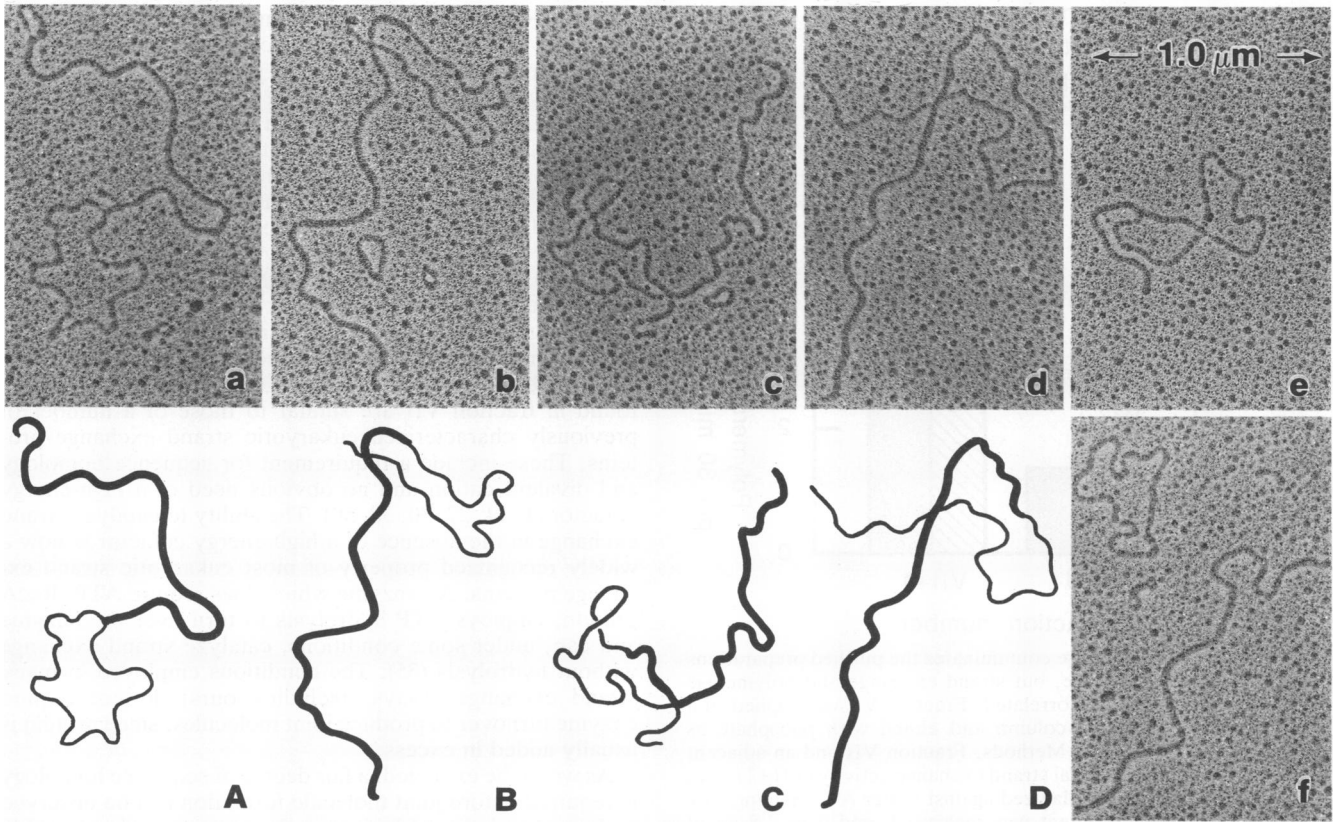

FIG. 6. Electron microscopic analysis of joint molecules formed by v-SEP. Unfractionated v-SEP reaction products were prepared for examination by electron microscopy as described in Methods and Materials. (a) Typical σ-form; (b to d) different types of α-forms; (e) α′-form; (f) single-stranded circular and linear duplex DNAs. Schematic interpretations of these structures (panels A to D), in which hybrid double-stranded regions are indicated by heavily inked lines, are also provided. Only the single-stranded circular and linear duplex substrates (as in panel f) were seen when the two DNAs were incubated without protein, phenol extracted, and spread in an identical manner (data not shown).

The v-SEP activity is not encoded by the D5R gene, nor is it vaccinia virus DNA polymerase. Analysis of the proteins present in our best preparations of v-SEP showed that a protein of ~110,000 M_r was present in fractions containing strand exchange activity (Fig. 3). Such a protein could be the 90-kDa D5R gene product, a gene essential for vaccinia virus replication (19, 46), or the 117-kDa DNA polymerase. In order to test whether v-SEP was the D5R gene product we obtained, temperature-sensitive vaccinia virus strains carrying three different D5R mutations (C17, C24, or E69) and infected BSC40 cells with the mutants, or with wild-type virus, at the permissive temperature. Extracts were prepared from infected monolayers (10 150-cm^2 dishes per stock), fractionated, and purified by chromatography through single-stranded DNA cellulose columns (fraction III) to remove a BSC40-specific nuclease. Compared with wild-type extracts, no obvious difference in the amount of v-SEP activity was seen in any of the mutant fractions either at 30 or at 42°C. Kinetic experiments showed no differences in the rate of joint molecule formation, and electron microscopy showed no obvious differences in the types of joint molecules formed in these reactions (data not shown). We conclude that this activity is probably not encoded by the D5R gene, nor does the D5R gene product modify this activity in any obvious way.

TABLE 2. Electron microscopic analysis of strand exchange products[a]

Product	Distribution	
	No. scored	%
Linear duplex	110	26
α-Form	26	6
α′-Form	37	9
σ-Form	8	2
Other[b]	236	57
Σ	417	100

[a] Unfractionated strand-exchange products were prepared for electron microscopic analysis as described in Materials and Methods. Photographs of several randomly chosen fields were prepared, and the different structures were assigned as indicated.

[b] Circular, degraded, and more-complex forms.

Fractions eluting off the final cellulose-phosphate column were also assayed for DNA polymerase, and it was discovered that fraction VII does contain DNA polymerase activity (Fig. 7). This supports the hypothesis that the 110-kDa polypeptide is vaccinia virus polymerase and raised the possibility that the polymerase might be capable of catalyzing strand exchange. To test this hypothesis, fraction VII

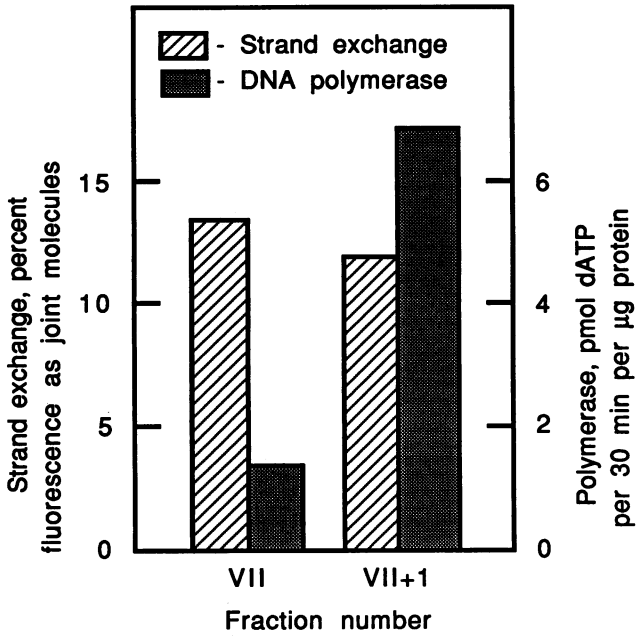

FIG. 7. DNA polymerase contaminates the purified preparations of strand exchange enzyme, but strand exchange and polymerase specific activities are not correlated. Fraction VI was applied to a 20-ml cellulose-phosphate column and eluted with phosphate as described in Materials and Methods. Fraction VII and an adjacent fraction containing additional strand exchange activity (VII+1) were recovered and separately dialyzed against buffer A containing 50% glycerol. The protein content was measured, and 0 to 2.5 μg of protein was assayed to determine the mean DNA polymerase specific activity in each fraction (solid grey bars). The two fractions were also reassayed for strand exchange activity under conditions in which activity was proportional to protein concentration (0.25 μg of protein per 30-μl reaction mixture) and quantitated by using scanning densitometry as previously described (hatched bars).

and an adjacent cellulose-phosphate fraction containing additional v-SEP were reassayed to measure both polymerase and v-SEP specific activities (Fig. 7). It was clear from this analysis that there was no correlation between polymerase and v-SEP specific activities (the adjacent fraction contained somewhat less v-SEP activity than fraction VII but five times the amount of polymerase), and therefore, the polymerase is not responsible for catalyzing strand exchange. Further experiments will be needed to determine whether the association of vaccinia virus DNA polymerase with v-SEP, through five chromatographic steps, is a coincidence or has some functional significance. These experiments also suggest that one of the two smaller polypeptides is the strand exchange protein.

DISCUSSION

Strand exchange proteins have now been isolated from a variety of sources, including prokaryotes, eukaryotes, and bacteriophage-infected cells. This communication shows that poxvirus infection induces synthesis of a strand exchange protein and further extends the range of organisms from which this activity can be isolated. It also marks the first time that a virus-associated recombinase has been isolated, and although it is formally possible that poxviruses induce expression of a host strand exchange protein, it is most likely that the gene is virus encoded. At present we cannot identify which of the hundred or so unidentified vaccinia virus genes (23) might encode the enzyme, but our data suggest it is not the D5R gene product or vaccinia virus DNA polymerase but an early gene product with a molecular mass of 52 ± 5 or 32 ± 3 kDa. We are currently attempting to purify the protein to homogeneity in order to identify the gene by protein sequencing. This has been harder than was expected, because the activity is labile and difficult to assay at high dilutions. Some evidence that eukaryotic strand exchange proteins form multienzyme complexes exists (1, 20), and it remains to be seen whether the different polypeptides in our preparations form any specific association.

Although v-SEP appears to be smaller than most eukaryotic enzymes and is not recovered from nuclear fractions (38), the basic enzymological properties of the preparation found in fraction VII are similar to those of a number of previously characterized eukaryotic strand exchange proteins. These include a requirement for sequence homology and divalent cation and no obvious need of a high-energy cofactor (1, 14, 27, 30, 38, 50). The ability to catalyze strand exchange in the absence of a high-energy cofactor is now a widely recognized property of most eukaryotic strand exchange proteins. An enzyme which does require ATP, RecA protein, employs ATP hydrolysis to turn over the enzyme and can, under some conditions, catalyze strand exchange without hydrolysis (35). The conditions employed in many strand exchange assays (including ours) do not require enzyme turnover to produce joint molecules, since protein is usually added in excess.

As would be expected, a fair degree of sequence homology is required before joint molecule formation can be observed in these reactions. A block of nonhomologous sequences 149 bp in length was sufficient to prevent pairing, although approximately 10% base mismatches did not prevent pairing. The ability to produce heteroduplex joint molecules in vitro is interesting, because the reaction reproduces an experiment, previously performed in vivo, which showed that heteroduplex molecules can be recovered from cells infected with Shope fibroma virus (21). In those experiments, as with these, heteroduplex appearance coincided with the onset of viral DNA replication, which is about 10 to 14 h postinfection in Shope fibroma virus-infected cells. Presumably the leporipoxvirus analog of v-SEP, which can be detected in Shope fibroma virus-infected cell extracts at 14 h postinfection, was responsible for forming these molecules. How much, or little, homology is required for the formation of joint molecules remains to be determined, although 6-bp insertions can influence the frequency of poxvirus-mediated recombination in vivo (43). Further experiments are worth pursuing, since understanding this feature of the reaction could provide clues as to the origin of recombinant poxviruses, such as malignant rabbit virus (4), and could indicate whether sequence divergence alone might isolate a poxviral species.

Under our experimental conditions, the reaction proceeded with a unique polarity because nonhomologous sequences on the 3′ end of the minus strand prevented joint molecule formation (Fig. 5). This shows that the reaction starts by pairing the 3′ end of the minus strand with the single-stranded plus strand and, since α-forms can be recovered from v-SEP-catalyzed reactions (Fig. 6), it follows that the duplex plus strand is then displaced in a 5′-to-3′ direction. The polarity by which joints are extended by v-SEP thus appears to be the same as that catalyzed by *E. coli* RecA protein and T4 UvsX protein but opposite to that of *U. maydis* Rec1 protein and the human strand exchange protein

called HPP-1 (13). Whether v-SEP has an end annealling and branch extension bias which permits the reaction to proceed in only this way requires further analysis. This is because the polarity of these reactions can be determined by the way in which duplex substrates have been resected by exonucleases. For example, depending on the assay and substrates that are used, RecA protein can pair 3′ or 5′ ends (31), while yeast Sep1 is capable of joint extension in both 5′-to-3′ and 3′-to-5′ directions (28). This question can be resolved by using substrates prepared with appropriate exonucleases, but under the conditions used here, v-SEP catalyzes joint formation and heteroduplex extension with a unique polarity which most closely resembles that of the T4 UvsX protein (54). The observation that v-SEP catalyzes strand transfer with a polarity opposite that of HPP-1 also supports the hypothesis that v-SEP is virus encoded.

Electron microscopy showed that at least three types of joint molecules could be recovered from v-SEP-catalyzed reactions. These were σ-forms consisting of a full-length duplex attached to a single-stranded circle with no visible displaced single strand, α-forms consisting of a partially duplex circle attached to a duplex linear molecule, and α′-forms which are probably nucleolytically degraded α-forms. About 6% of the molecules visible in these fields were intact α-forms (Table 2), which is an amount consistent with the amounts of joint molecules seen in agarose gel assays. Most importantly, the presence of α-forms shows that true strand exchange and not just a pairing reaction mediated by annealing of exonuclease degraded DNAs takes place in these reactions. The low abundance (2%) of σ-forms relative to α- and α′-forms and the unique polarity of pairing described above suggest that σ-forms may be kinetic precursors of the α-forms. We can also estimate the amount of DNA transferred in vitro by examining different α-forms and comparing the lengths of displaced single strands relative to the length of a single-stranded circle (Fig. 6). This ratio suggests that up to ~3,000 bases can be transferred between molecules and matches (28, 30, 35, 38, 50) or exceeds (14, 27) the extent of transfer seen in a number of non-ATP-driven strand exchange reactions. We saw no fully duplex circles in the electron microscope, but other assays (9) may be required to detect what might be very rare reaction products.

Finally, an obvious feature of the reaction described here is that the joint molecule it generates bears a 3′ end annealed to a single-stranded template. These molecules could serve as polymerase primers much as joint molecules synthesized in vitro by the action of T4 UvsX protein are T4 DNA polymerase substrates (22). We have suggested a derivative of the T4 replication-primed recombination model wherein, after DNA polymerase has replicated to the end of the telomere, recombinational invasion back into duplicated telomeric sequences permits reinitiation of replication and propagation of a replication fork out into the viral genome (21). Figure 8 shows the parallels between the in vitro reaction described here and our hypothetical replication model. Such a model accounts for many features of poxviral biology, including the structure of early-replication intermediates (16) and the ability of poxvirus-infected cells to catalyze nonspecific replication of transfected DNA (11), and may also explain why the viral polymerase seems to be copurifying with a strand exchange protein. Two predictions are (i) that in vitro-synthesized strand exchange products should be substrates for vaccinia virus DNA polymerase and (ii) that intramolecular strand invasions might be catalyzed by v-SEP. We are currently testing these hypotheses.

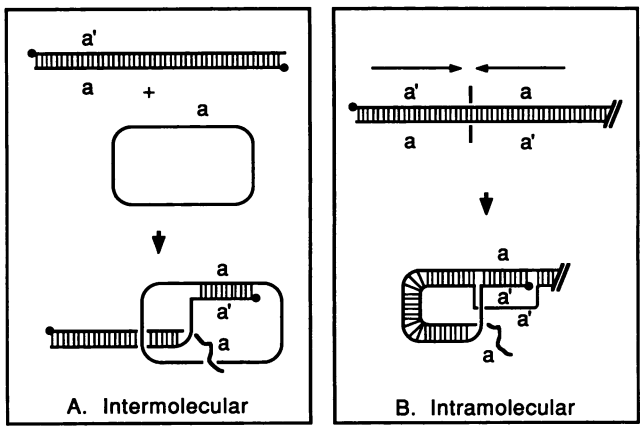

FIG. 8. Mechanism by which 3′ ends can prime the initiation of telomere replication. (A) Strand exchange assay used to detect v-SEP and the deduced invasion polarity; (B) Illustration of how this reaction might be employed to prime DNA synthesis on a telomeric double-stranded substrate containing inverted-repeat sequences (arrows). Filled dots (●) mark the 3′ ends of these molecules, and complementary homologous sequences (α and α′) are indicated.

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