Enzymatic processing of replication and recombination intermediates by the vaccinia virus DNA polymerase

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ABSTRACT

Poxvirus DNA polymerases play a critical role in promoting virus recombination. To test if vaccinia polymerase (E9L) could mediate this effect by catalyzing the post-synaptic processing of recombinant joint molecules, we prepared substrates bearing a nick, a 3′-unpaired overhang, a 5′ overhang, or both 3′ and 5′ overhangs. The sequence of the 5′ overhang was also modified to permit or preclude branch migration across the joint site. These substrates were incubated with E9L, and the fate of the primer strand characterized under steady-state reaction conditions. E9L rapidly excises a mispaired 3′ strand from a DNA duplex, producing a meta-stable nicked molecule that is a substrate for ligase. The reaction was not greatly affected by adding an unpaired 5′ strand, but since such molecules cannot be processed into nicked intermediates, the 3′-ended strand continued to be subjected to exonucleolytic attack. Incorporating homology into the 5′ overhang prevented this and permitted some strand assimilation, but such substrates also promoted strand-displacement DNA synthesis of a type predicted by the 1981 Moyer and Graves model for poxvirus replication. Single-strand annealing reactions are used by poxviruses to produce recombinant viruses and these data show that virus DNA polymerases can process DNA in such a manner as to both generate single-stranded substrates for such reactions and to facilitate the final processing of the reaction products.

INTRODUCTION

The *Poxviridae* comprise a diverse group of large double-stranded DNA viruses of which vaccinia virus represents the best-studied and prototypic member. A feature of poxvirus biology is that viral replication is intimately linked to reactions that promote high frequencies of homologous genetic recombination between co-infecting viruses (1), between duplicated virus sequences (2), and amongst DNAs co-transfected into poxvirus-infected cells (3,4). Because these reactions can also promote intermolecular recombination between virus-encoded and transfected DNAs (5,6), they have facilitated the development of poxviruses as a vector for recombinant gene expression and vaccine production. This link between DNA replication and recombination is not, of course, unique to poxviruses. Many other DNA viruses and bacteriophage exhibit a similar phenotype wherein replication and recombination are intertwined processes, but only in a few situations [e.g. T4 recombination priming of replication (7)] have detailed mechanistic explanations for the phenomenon been proposed.

Recombination reactions are usually catalyzed by collectives of proteins that must recognize and bind DNA ends (or single-stranded DNAs), synapse these DNAs with other homologous molecules, and ultimately process the resulting paired intermediates into stable duplex recombinants. How poxviruses accomplish all of these steps while replicating in the cytoplasm of infected cells is still unclear, although it is apparent that virus DNA polymerases are key participants in these reactions. These are large enzymes belonging to the 'B' family of DNA polymerases (8,9), possessing both DNA polymerase and 3′-to-5′ proofreading exonuclease activities (10,11). The linkage between replication and recombination was first detected using genetic (12,13) and inhibitor-based (2,3) methods. This led to the general conclusion that, besides catalyzing DNA synthesis, the vaccinia virus DNA polymerase (the E9L gene product) played some other and uncertain role in promoting recombinant formation. Some of what these role(s) could be are outlined in Figure 1.

Given the known enzymat\ic properties of poxvirus DNA polymerases, and polymerases in general, one can envision at least three ways of linking enzyme activity to recombinant

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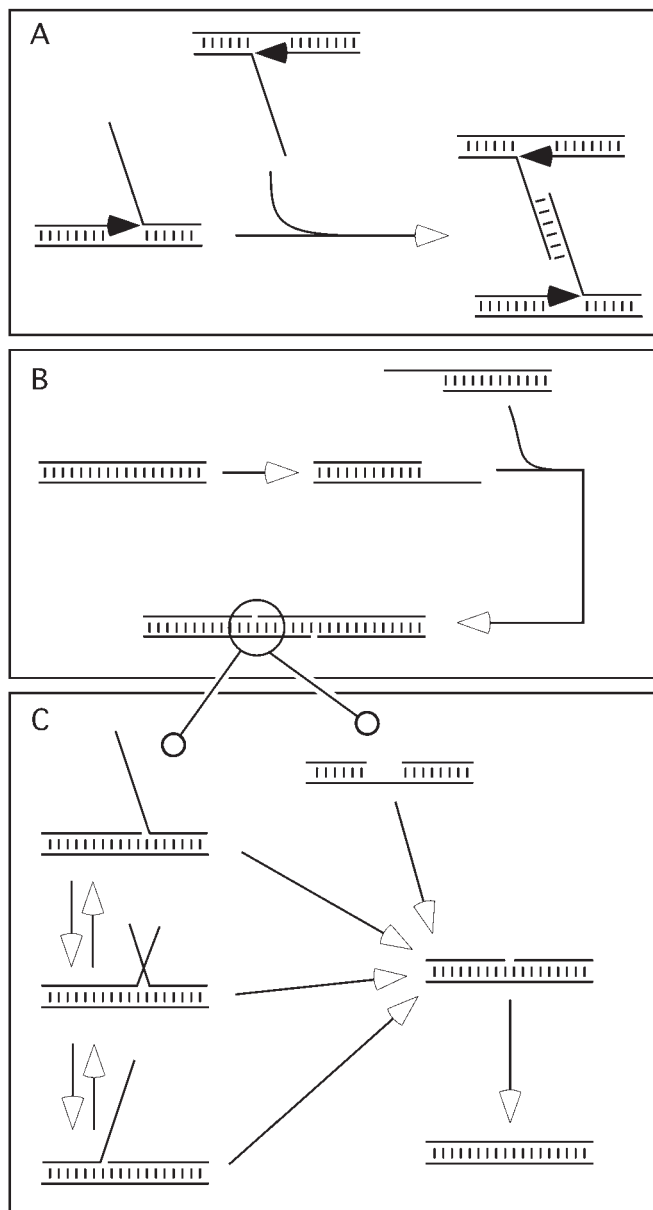
Figure 1. Reactions linking DNA polymerase activity and recombinant production in poxvirus-infected cells. (**A**) Displacement of single-stranded DNAs by DNA synthesis permits formation of recombinants through single-strand annealing reactions. (**B**) DNA excision catalyzed by the 3′-to-5′ proof-reading exonuclease exposes complementary DNA sequences and permits duplex-by-duplex joining reactions (15). (**C**) Some of the recombinant joints formed by single-strand annealing reactions will bear gaps or strand extensions that must be excised or repaired prior to DNA ligation. See text for details.

formation. First, poxviruses are thought to use a 'rolling hairpin' scheme for DNA synthesis that would cause single-strand displacement ahead of a virus replication fork (14). The spontaneous annealing of any complementary single strands displaced by DNA synthesis could initiate the process of recombinant formation. Any interference in polymerase activity would block this recombinant forming process (Figure 1A). Second, we have shown that vaccinia DNA polymerase catalyzes duplex joining reactions, both *in vivo* and *in vitro*, which concatenate molecules sharing ≥12 bp of terminal homology (15–17). These homology-dependent single-strand annealing

(SSA) reactions depend upon the exposure of complementary ends by the 3′-to-5′ proofreading exonuclease activity of the virus DNA polymerase (Figure 1B), and interfering with this activity would also block this recombination pathway. Finally, any recombination scheme that depends upon random strand annealing would not necessarily form structures that comprise a perfect duplex joint; that is, a joint that need only be ligated to generate a mature recombinant. Instead, many of the joint molecules formed by SSA would bear single-stranded gaps and/or unpaired strands that will require post-synaptic processing before they could be converted into stable recombinants by DNA ligase (Figure 1C). Since the DNA polymerase gene encodes the only polymerase and exonuclease functions known to be accessible to replicating poxviruses, any interference in either activity could also inhibit virus recombination.

A common theme that emerges from a consideration of Figure 1 is that one might gain further insights into the links between poxvirus replication and recombination, through a closer examination of what happens to different kinds of branched molecules when they are exposed to the activities of the viral DNA polymerase. We show here that vaccinia virus DNA polymerase can process some of these imperfect duplex joints into simple nicked products that are substrates for DNA ligase. We have also fortuitously discovered that under other conditions the enzyme can catalyze the strand displacement reactions (Figure 1A) that to date have been only a matter of speculation. These results identify at least two more mechanisms (besides catalyzing concatemer formation) by which poxvirus DNA polymerases could promote recombinant formation.

MATERIALS AND METHODS

Radioactive labeling of oligonucleotides

Oligonucleotides were purchased from either Sigma-Genosys (Mississauga, Canada) or the DNA synthesis core facility, University of Alberta, with gel purification of primer strands. Oligonucleotides were 5′ end-labeled using T4 polynucleotide kinase (New England Biolabs). A typical labeling reaction contained 0.6 nmol of oligonucleotide, 9 μCi of [γ-^{32}P]ATP (Amersham), and 20 U of T4 polynucleotide kinase in a final volume of 30 μl. Reactions were incubated at 37°C for 30 min and stopped by adding 1 μl of 0.5 M EDTA (pH 8.0). The labeled DNA was purified by passage through a Sephadex-25 microspin column (Amersham). Figure 2 illustrates the sequences and structures of the DNAs used in these experiments.

Preparation of double-stranded DNA joint substrates

Duplex substrates were prepared by mixing 0.3 nmol of labeled oligonucleotide primer with 0.4 nmol of unlabeled oligonucleotide template in reverse transcriptase buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl and 3 mM MgCl$_2$] in a total volume of 40 μl. This mixture was boiled for one minute and then cooled slowly to 35°C.

One end of these duplex substrates is protected from exonuclease attack by a hairpin structure. However, there remains another unprotected 3′ end on the unlabeled hairpin strand (Figure 2) and we were concerned that nuclease attack on the 3′ end of this strand, combined with attack on the 'top' strand, could sufficiently destabilize the duplex structure to

A. Oligonucleotide sequences

Primer strands:

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20-mer  5' GTCTAGTTGTTTCTTGACTG 3'

30-mer  5' GTCTAGTTGTTTCTTGACTGACGACGCGAA 3'
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Template strands:

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65-mer                        5' GATGGAGGGCCTGTTACTCATTT ⌐
        3' AGATCAACAAAGAACTGACCTACCTCCCGGACAATGAGTATT ⌐

                  5' ACGACGCGAA ⌐
75-mer                        GATGGAGGGCCTGTTACTCATTT ⌐
        3' AGATCAACAAAGAACTGACCTACCTCCCGGACAATGAGTATT ⌐

                  5' ACGAC ⌐
75-mer(h)                     GACTGGATGGAGGGCCTGTTACTCATTT ⌐
        3' AGATCAACAAAGAACTGACCTACCTCCCGGACAATGAGTATT ⌐


70-mer(h)                5' GACTGGATGGAGGGCCTGTTACTCATTT ⌐
        3' AGATCAACAAAGAACTGACCTACCTCCCGGACAATGAGTATT ⌐
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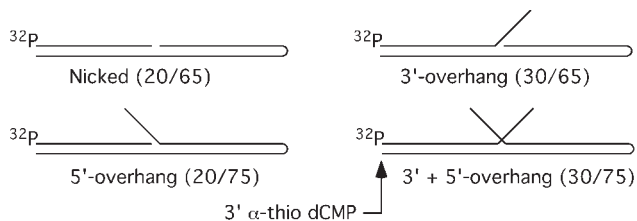
B. Annealed substrates

Figure 2. Oligonucleotide substrates. (**A**) The oligonucleotides used in these studies are shown. These DNAs (usually the primer strand) were labeled with polynucleotide kinase and [γ-^{32}P]ATP. The template strand contained a T$_4$ hairpin loop (29) designed to protect one end from exonucleolytic attack. An α-thio dCMP residue was incorporated into the other end of this strand by incubating the primer and template strands together with α-thio dCTP and reverse transcriptase. (**B**) The structures formed by combining primer and template strands are shown.

collapse these molecules into single-stranded oligonucleotides. Since thioester linkages can inhibit the activities of some proofreading exonucleases (18), we incorporated an α-thio-dCMP residue into this 3′ end using 20 U of MMLV reverse transcriptase (Invitrogen) in the presence of reverse transcriptase buffer, 0.1 M DTT and 1 mM α-thio-dCTP (Glen Research). The reaction was incubated at 37°C for 60 min, heated to 70°C for 10 min to inactivate the reverse transcriptase, and then the DNA was purified by passage through a Sephadex-25 spin column. This modification reduced the activity of the proofreading exonuclease at the template 3′ end by ∼2-fold, relative to molecules terminated by a one-nucleotide gap (data not shown), and ensured that fewer than 5 nt were removed from the 3′ end of the template strands during 1 h incubation in reactions containing dNTPs.

Recombinant protein

Vaccinia virus DNA polymerase was purified from BSC-40 cells co-infected with recombinant vaccinia vTMPOL and VTF7.5 viruses using the five-step procedure of McDonald and Traktman (19). The protein appeared >95% pure as judged by silver staining of SDS–PAGE gels (20,21).

Exonuclease assays

Each 65 μl of reaction mixture contained 50 pmol joint substrate, 110 ng vaccinia virus DNA polymerase (∼1 pmol), 15 μM dATP, 15 μM dCTP, 5 μM dGTP, 10 μM TTP, 30 mM Tris–HCl (pH 7.9), 5 mM MgCl$_2$, 70 mM NaCl, 1.8 mM DTT and 80 μg/ml acetylated bovine serum albumin (BSA) (15). Under these conditions, and in the absence of the A20R processivity factor, the enzyme interacts with DNA in a highly distributive manner (22,23). The reaction was started by adding the polymerase and incubated at 37°C with periodic sampling. Each aliquot was mixed immediately with an equal volume of 2× gel loading buffer [80% deionized formamide, 0.1 μg/ml bromophenol blue, 0.1 μg/ml xylene cyanol and 20 mM EDTA (pH 8.0)]. The samples were then denatured by heating to 56°C and fractionated through a 15% denaturing polyacrylamide gel containing 8 M urea and a 19:1 acrylamide:bis-acrylamide ratio. The gel was fixed with 10% (v/v) acetic acid and 10% (v/v) methanol in water, and dried on 3 mm chromatography paper (Fisher Scientific). The distribution of the radiolabel was determined using a Typhoon 8600 phosphoimager, and the image analysis and quantitation performed using ImageQuant software (Amersham). DNA size markers were prepared by ^{32}P-end-labeling a mixture of oligonucleotides of defined sizes. Reaction parameters were calculated using Prism 4.0 software running on a Macintosh G4 personal computer. The analysis used a non-linear curve-fitting algorithm to fit normalized data (N_0 at t_0 set equal to 100% where 100% = c.p.m. present in the labeled strand at $t = 0$) to a simple exponential association or decay curve. Rate constants are reported as plus or minus the standard error.

Ligation reactions

T4 polynucleotide kinase and unlabeled ATP were used to phosphorylate the larger (hairpin containing) template strand. The purified DNA was then mixed with ^{32}P-labeled 20mer or 30mer strand in 50 mM NaCl at the ratios indicated above, heated to 95°C for 1 min and cooled slowly to room temperature. The annealed substrates were treated with DNA polymerase, as described in the text, and 10 μl samples removed at intervals and mixed with an equal volume of a solution containing 100 mM Tris–HCl (pH 8.0) and 5 mM EDTA on ice. Each sample was phenol extracted, and a fixed quantity of each DNA (10^5 c.p.m.) was dried under vacuum and resuspended in 5 μl aliquots of 10 mM Tris–HCl (pH 8.0). The reactions were adjusted to 10 μl with 10× DNA ligase buffer, DNA ligase (1 U, Fermentas) and water, and then incubated at 37°C for 15 min. The reactions were stopped by adding 2× gel loading buffer and then the products were analyzed by denaturing gel electrophoresis (using a 10% polyacryamide gel) and autoradiography as described above.

RESULTS

Enzyme substrates

We used synthetic oligonucleotides to construct some of the different kinds of branched structures that might be formed during the course of virus replication and then examined the fate of these structures when exposed to vaccinia DNA

polymerase. These substrates consisted of a primer strand annealed to a hairpin-forming template strand (Figure 2). The hairpin served the purpose of reducing the number of $3'$ ends that are substrates for the exonuclease and thus helped to simplify the interpretation of these experiments. As noted above, the other $3'$ end of the template strand was partially protected from nuclease attack by filling in a single-nucleotide gap with α-thio-dCMP. The resulting structures are 40 bp duplex hairpin molecules with a $3'$-ended target located near the middle of the duplex (Figure 2). These structures were modified by annealing different combinations of primers and templates to generate a nicked duplex (20/65), a $3'$ over-hang (30/65), a $5'$ overhang (20/75), and both $3'$ and $5'$ over-hangs (30/75). In addition, the sequence of the $5'$ overhang was modified to allow homology-dependent branch migration at the joint site (molecules 20/70h and 30/75h, structures data not shown). In general, the primer strand was $5'$ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and the fate of the primer was followed as described below. An M-fold algorithm predicts a $\Delta G = -22.9$ kcal·mol^{-1} and T_m of 86°C for the 65mer DNA hairpin under the reaction conditions described in the Materials and Methods, thus only a vanishingly small fraction of the DNA could be single stranded at 37°C.

Effect of enzyme and dNTP concentrations on exonuclease activity

Most of the steady-state experiments described in this paper used 45 μM (total) dNTP concentration and ∼50:1 mole ratio of substrate to enzyme. These conditions were established by comparing the effects of varying the dNTP concentration on reactions containing 110 ng of polymerase, and varying the polymerase concentration in reactions containing 45 μM dNTP (data not shown). All of the reactions were incubated for 10 min at 37°C and stopped by adding loading buffer. Generally, the observed effects were rather insensitive to these two reaction variables, except at the extremes of conditions. For example, little net DNA synthesis and extensive degradation were seen when the concentration of dNTPs dropped below 25 μM. Increased amounts of dNTPs favored synthesis over degradation, but even the highest concentrations (180 μM) failed to drive further conversion of primer strands into longer products. We selected 45 μM dNTP as a suitable nucleotide concentration because it created conditions that permitted a mix of both synthesis and degradation while also being similar to what is thought to be the physiological dNTP concentration in vaccinia-infected cells (24). The reaction trend was relatively insensitive to polymerase concentration, except that more primer extension was detected at higher polymerase concentrations. At the very highest enzyme concentrations, we observed the destruction of the substrate, probably due to the turnover of all of the dNTPs in a 10 min incubation period. A 50:1 mol ratio of substrate to enzyme, with 10 min incubation time and 45 μM dNTP avoided this potential problem.

Relative exonuclease activity on single- and double-stranded substrates

Previous investigations have shown that the $3'$-to-$5'$ exo-nuclease exhibits greatly varied reaction rates depending upon whether the $3'$-ended strand is in a single- or

double-stranded state (10). To confirm this point using these new substrates, we compared the rate at which a ^{32}P-labeled strand is attacked by the enzyme, when either single-stranded or when base-paired within the nicked duplex structure designated 20/65 (Figure 2B). We also examined what effects dNTPs would have on the rate of exonuclease activity. The results of these experiments are shown in Figure 3.

We observed that the presence of a complementary strand greatly increased the stability of a ^{32}P-labeled primer strand within a nicked duplex substrate. This is best illustrated by comparing the relative rate constants for loss of the labeled 20mer by exonucleolytic conversion into other smaller species
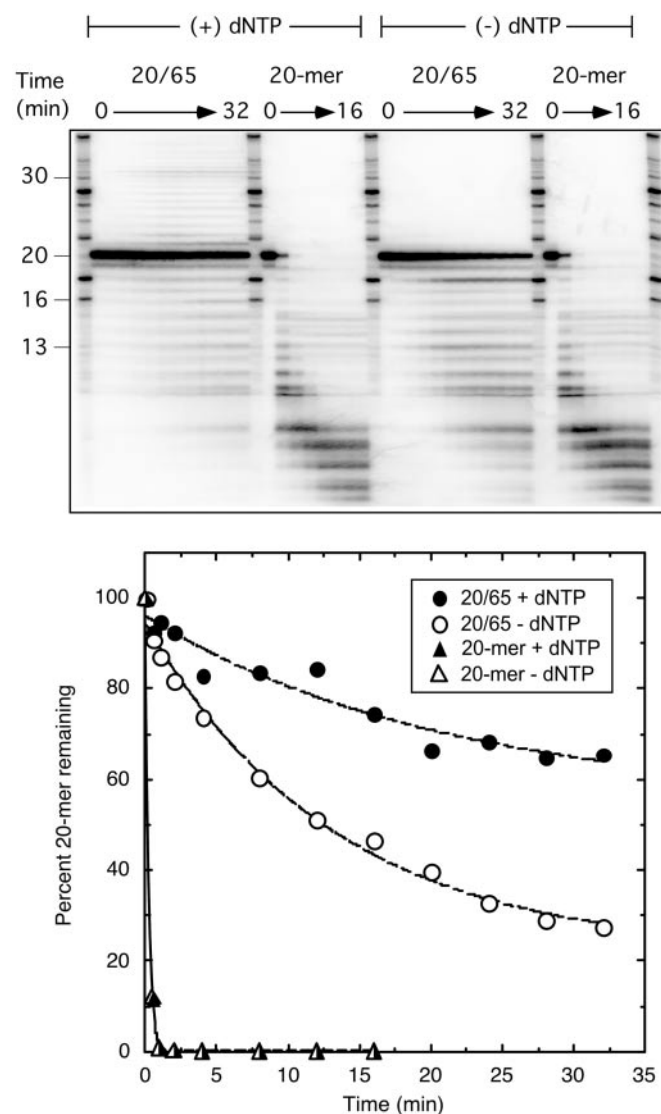
Figure 3. Effect of dNTPs and strandedness on the kinetics of primer strand processing. A ^{32}P-labeled 20mer primer strand was incubated for varying lengths of time with vaccinia DNA polymerase under standard reaction conditions, with or without a complementary 65mer template strand. Phosphorimaging was used to determine the amount of label remaining in the 20mer band at each time point (upper panel), and curve-fitting software was used to determine reaction rate constants assuming a simple exponential rate of decay for the 20mer species (lower panel). Because the single-stranded 20mer was degraded so quickly, the zero time point was assayed before adding DNA polymerase.

in the absence of dNTPs. We observed that the single-stranded DNA is degraded at least 60 times faster ($K \geqslant 4.3 \pm 0.1$ min^{-1}) than the same strand in a nicked duplex state ($K = 0.076 \pm 0.010$ min^{-1}) under otherwise identical reaction conditions (Figure 3). We also observed that the stability of the labeled strand is further increased, as would be expected, by adding dNTPs at normal physiological concentrations (24). Adding a total of 45 μM dNTP decreased the rate of loss of the primer strand within a 20/65 duplex substrate by ~2-fold ($K = 0.076 \pm 0.010$ min^{-1} versus 0.05 ± 0.03 min^{-1} for reactions without and with dNTP, respectively). Adding dNTPs had no significant effect on the rate of degradation of the single-stranded 20mer. These observations are consistent with the known behavior of proofreading B-family DNA polymerases. Analysis of these data using the exonuclease event counting method described by Cheng and Kuchta (25) yielded similar results (data not shown).

Closer inspection of Figure 3 showed that the ^{32}P-labeled 20mer is not just subject to exonuclease attack in the presence of dNTPs and a template strand, but can also prime some net DNA synthesis. We observed that ~5% of the radioactivity could be extended up to 20 nt beyond the location of the original nick by vaccinia DNA polymerase. A major fraction of the products was 31 nt long, but the longest showed evidence of termination within 2–3 nt of the point where the hairpin end is encountered (Figure 3). This reaction is rather surprising and we comment further on this matter below and in the Discussion.

Inspection of Figure 3 also suggests that the proofreading exonuclease may exhibit some sequence specificity, at least in this sequence context. This is most apparent when one compares the appearance and disappearance of different products over time. Both 13mer and 18mer species were seen to be unusually persistent molecules especially in the absence of dNTPs (Figure 3, upper panel). With few exceptions, the more stable species correspond to DNAs where the 3′-to-5′ exonuclease encounters a cytosine residue at the 3′-terminus, suggesting that deoxycytidylate residues are less preferred substrates. We have reproduced this effect with other unrelated oligonucleotides (e.g. Figure 4 and data not shown) and it cannot be attributed to the presence of a G:C base pair since pauses are not seen when the polymerase encounters a deoxyguanylate residue in the primer. Although these data do not provide compelling evidence of sequence specificity, we have noted the effect because it necessitates some caution in interpreting the data presented in the following sections.

Processing of the 3′-unpaired strand

To test the selectivity of the 3′ proofreading exonuclease on substrates bearing a mix of single- and double-stranded DNA structures, we prepared an oligonucleotide duplex encoding an unpaired 10 nt extension on the 3′ end of the primer strand (molecule 30/65, Figure 2B). The terminal portion of this 30mer was very rapidly degraded by the exonuclease, generating a series of products ranging from 30 to 20 nt long (Figure 4, right upper panel). The rate of depletion of the 30mer was perhaps only slightly reduced by the presence of dNTPs (~20%, data not shown), but ~3-fold faster than the rate of attack upon nicked duplex DNA in a 20/65 complex
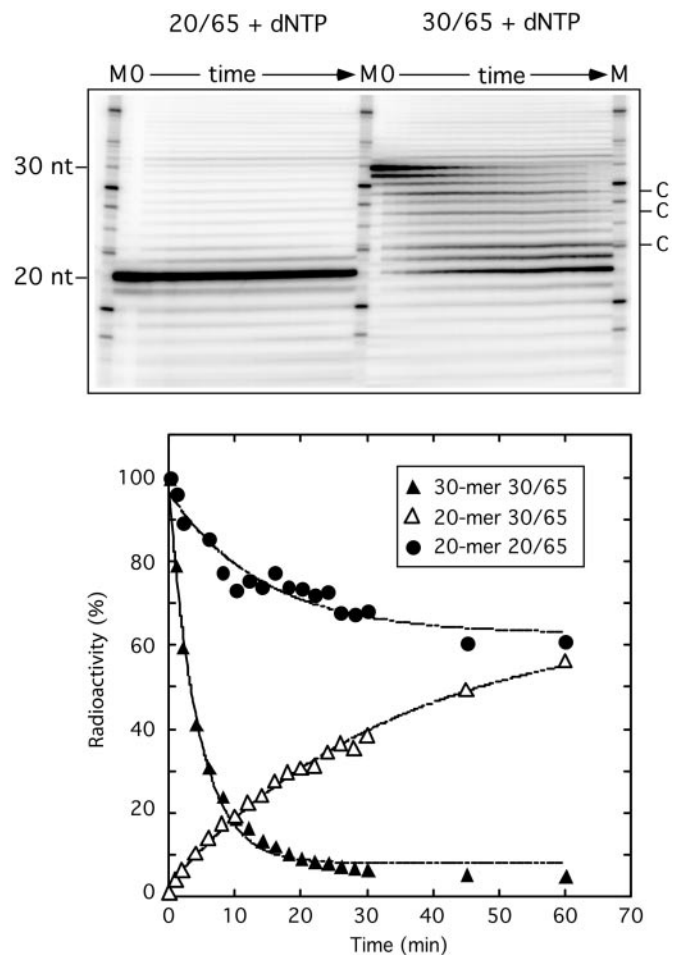

Figure 4. Comparison of the substrate properties of 20/65 and 30/65 (3′-mismatched) duplexes. Molecules containing 20mer or 30mer primer strands (Figure 2) were prepared as described in Materials and Methods, and incubated with vaccinia DNA polymerase in the presence of dNTPs. The 20mer strand and 30mer strand substrates are indicated. Also indicated are the 27mer, 25mer and 22mer bands that bear a 3′-terminal dCMP ('C', upper panel, at right).

($K = 0.23 \pm 0.01$ min^{-1} versus 0.07 ± 0.01 min^{-1}). Evidence is again seen of slower excision of single-stranded DNA whenever the DNA bears a 3′-deoxycytidylate terminal residue, as judged by the persistence and relative excess abundance of 27mer, 25mer and 22mer species (Figure 4, upper panel). The most noteworthy feature of these reactions is the efficiency with which the enzyme can process a 30/65mer mismatched substrate into a 20/65mer nicked substrate. At later time points, nearly half of the 30mer strands were processed into 20mer molecules, and a significant portion of the remaining strands were processed into 21mer or 22mers. The 22mer molecule incorporates 3′-deoxycytidylate residues (Figure 2), and thus its persistence might be explained by sequence effects. This effect cannot explain the stability of the 21mer and 20mers (which bear 3′-deoxyadenylate and 3′-deoxyguanylate residues respectively), and suggests that structural context is also an important criterion in regulating the excision of unpaired 3′-ended strands. Some delay in the production of longer extension products (>30 nt) was also noted when compared with the 20/65 substrate.

Processing of molecules bearing 5′-unpaired strands

Vaccinia DNA polymerase lacks any detectable 5′-to-3′ exonuclease activity (10) and this raises the question of what effect a mispaired 5′-ended strand might have on the stability of these substrates. To explore this question, we annealed a ^{32}P-labeled 20mer primer to a 75mer hairpin strand, thus generating a molecule bearing a 10 nt 5′-unpaired strand (molecule 20/75, Figure 2B). The addition of this 5′-mispaired strand significantly decreased the stability of the primer strand when compared with a 20/65 nicked duplex structure (Figure 5). In the absence of dNTPs, the rate of consumption of the 20mer was enhanced in the mismatched structure ∼3-fold relative to a nicked duplex molecule (data not shown). This difference in the stability was enhanced by adding dNTPs, where a primer in a nicked structure was 5-fold more stable than one where the 3′ end was located next to a 5′-mispaired strand ($K = 0.092 \pm 0.014$ min^{-1} versus 0.44 ± 0.06 min^{-1}). The altered stability of the 20mer primer strand reflects the fact that it is both a better exonuclease substrate, as well as promoting relatively more DNA synthesis when annealed to the 75mer (Figure 5). This enhanced activity
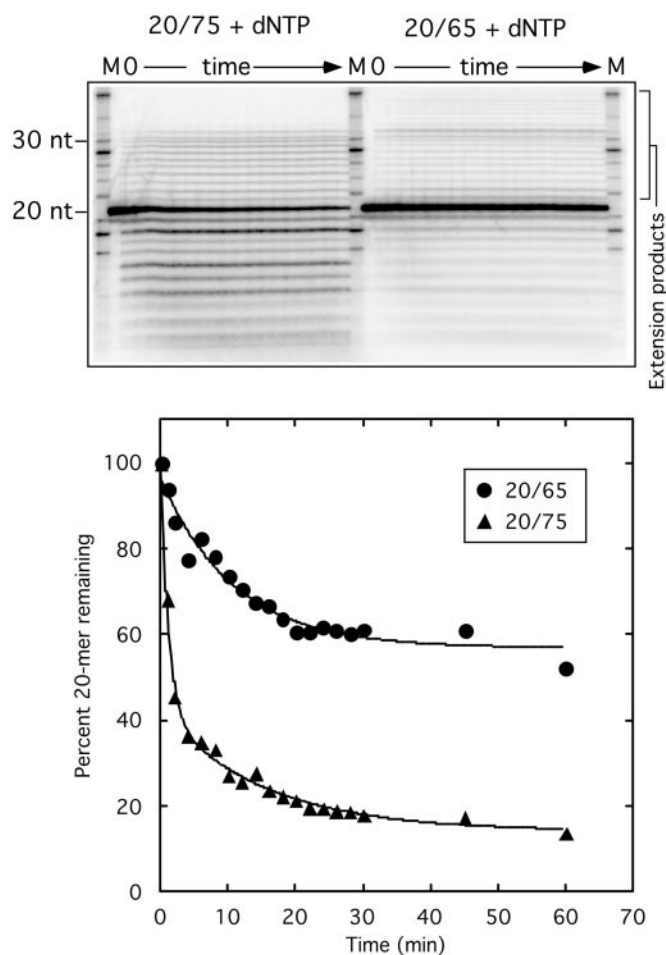
as a polymerase substrate is even more pronounced using a substrate bearing some homology in the unpaired 5′ strand and is discussed in more detail below (Figure 8).

Processing of molecules bearing 3′- and 5′-unpaired strands

Duplex substrates were also prepared by annealing the 30mer primer to a 75mer hairpin template. This created molecules encoding 10 nt mismatched strands on both sides of the joint region (Figure 2B, 30/75). This modification had little effect on the stability of the 3′ end of the 30mer strand regardless of whether it was annealed to a 65mer or 75mer template strand ($K = 0.17 \pm 0.01$ min^{-1} versus 0.23 ± 0.02 min^{-1} for 30/65 and 30/75 substrates, respectively). However, as this strand was processed into progressively smaller oligonucleotides, the meta-stable 20mer, 21mer and 22mer strands that appeared so prominently during exonuclease processing of a 30/65 substrate (Figures 4 and 6), were not seen (Figure 6, upper panel).
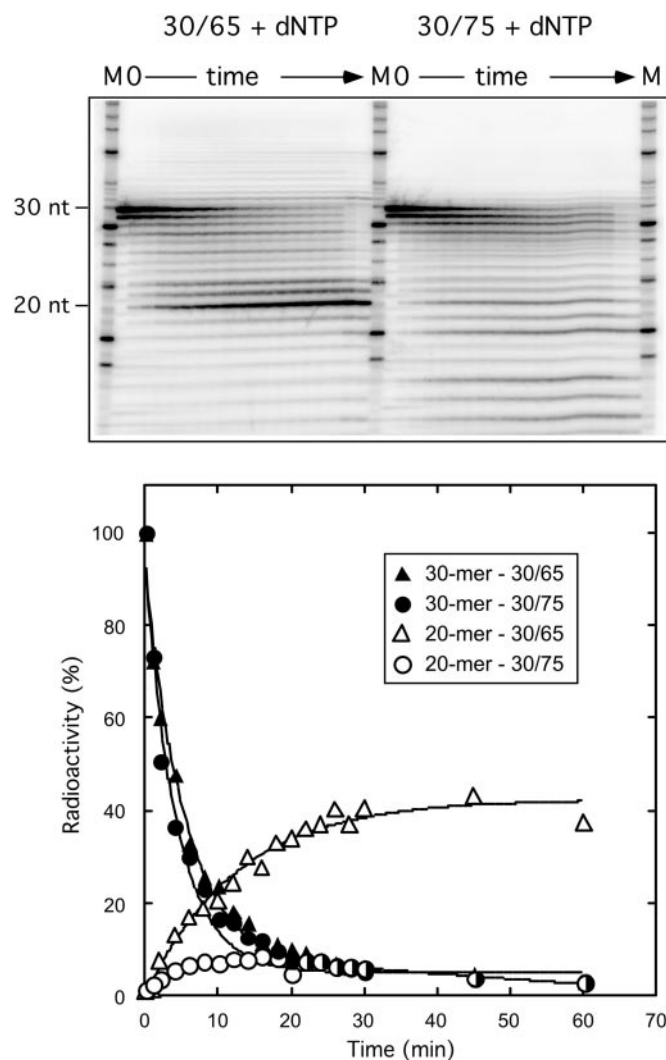

Figure 5. Comparison of the substrate properties of 20/65 and 20/75 (5′-mismatched) duplexes. Molecules containing 65mer or 75mer template strands (Figure 2) were prepared as described in Materials and Methods, and incubated with vaccinia DNA polymerase in the presence of dNTPs.

Figure 6. Comparison of the substrate properties of 30/65 (3′-mismatched) and 30/75 (3′ and 5′-mismatched) duplexes. Molecules containing 65mer or 75mer hairpin template strands (Figure 2) were prepared as described in Materials and Methods, and incubated with vaccinia DNA polymerase in the presence of dNTPs.

For example, >40% of the label was chased into a 20mer using a 30/65mer substrate whereas <10% of the label appeared as 20mer starting with a 30/75 substrate (Figure 6, bottom panel). Probably, as a consequence of the limited yield and instability of the 20mer product in 30/75-containing reactions, little if any subsequent chain extension synthesis was detected (represented by strands >30 nt in length, Figure 6).

Effect of sequence homology on joint processing

One explanation for the behavior of the 30/75 substrates is that these are static structures wherein the base-pairing properties of the two oligonucleotides used in their assembly fixes the point where the two unpaired strands must cross (Figure 2). We introduced new structural flexibility into these substrates by altering the sequence of 5 nt in the 5′ end of the 75mer strand, and therefore created additional opportunities for the strand to base-pair with the template. These changes then permit some branch migration over a 5 bp region, which is common to both the 30mer 3′ end and the 75mer 5′ end [Figure 2, '75mer (h)']. Adding this structural flexibility had a small effect upon the stability of the 30mer strand in that it was attacked by the exonuclease ∼2-fold faster in a 30/75h substrate ($K = 0.43 \pm 0.01$ min^{-1}) than in a 30/75 substrate ($K = 0.26 \pm 0.01$ min^{-1}) (Figure 7). However, this added structural flexibility otherwise had no effect on the kinds and relative abundance of the degradation products generated in reactions containing either the 30/75 or 30/75h substrates (Figure 7). In particular, no kinetic pauses were seen near points in the sequence marking the boundaries of these branch migrating regions, nor did the 20mer enjoy any special status when processed from a 30/75 substrate as it did when processed from a 30/65 substrate (Figures 4 and 6). Adding sequence homology to the branch point in 30/75h structures thus renders the 3′ end of the primer strand a somewhat better exonuclease substrate. However, the continued presence of a mismatched strand on the 5′ end of the hairpin template strand still destabilizes the 3′ end of the adjacent 20mer primer.

The 20/70h substrate produced different results. Like the 20/75 substrate, adding sequences now fully capable of branch migration greatly increased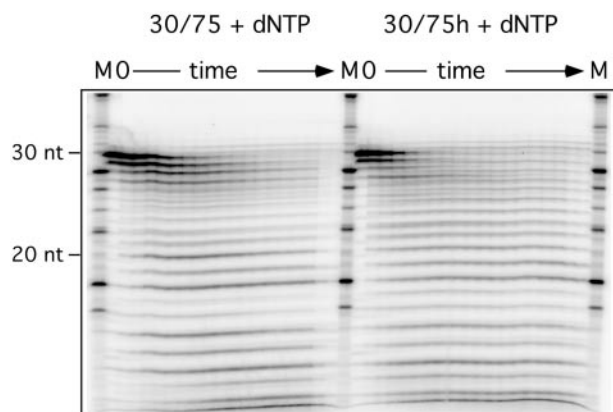 the susceptibility of the 3′ end of the 20mer to enzymatic attack, relative to that end in a 20/65 substrate (Figures 5 and 8). The 20mer strand was consumed 15-fold faster in the 20/70h substrate ($K = 0.25 \pm 0.01$ min^{-1}) than in the 20/65 substrate ($K = 0.017 \pm 0.005$ min^{-1}) (Figure 8, bottom panel). This had the expected effect of generating a ladder of smaller reaction products each of which typically appeared more rapidly where the substrate was capable of branch migration (Figure 9). One notable difference between the two reactions was that the 15mer reaction product seemed to be produced in greater quantities, compared with the yields of the other exonucleolytic degradation products [Figure 8, top (arrowed) and Figure 9]. The effect was seen both with and without dNTPs, although it was most obvious when reactions were conducted in the presence of dNTPs (Figure 9 and data not shown).
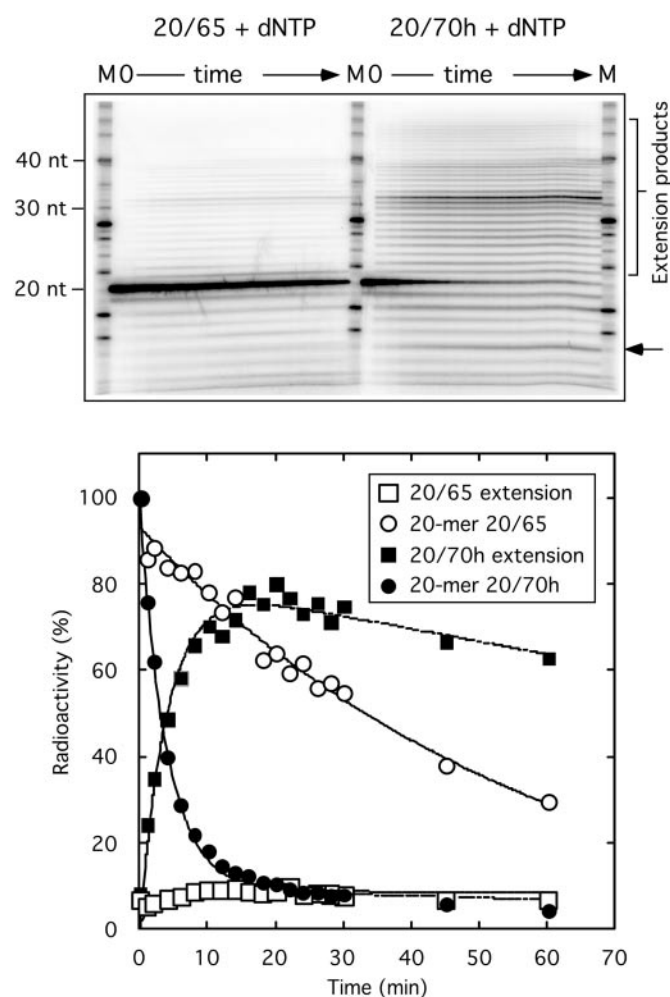

Figure 8. Comparison of the substrate properties of 20/65 and 20/70h duplexes. Molecules containing a 20mer primer strand and either a 65mer template strand, or a 70h template strand capable of a limited amount of branch migration (Figure 2), were prepared as described in Materials and Methods and incubated with vaccinia DNA polymerase in the presence of dNTPs. We also normalized the amount of label to the amount detected at $t = 0$ in the 20mer bands (6.5×10^5 and 5.3×10^5 c.p.m. for 20/65 and 20/70h substrates, respectively) and then calculated the amount of ^{32}P-label distributed in either the 20mer bands (open and closed circles) or migrating as the sum of all chain extension products (open and closed squares). The 15mer reaction product is indicated with an arrow along with the extension products.
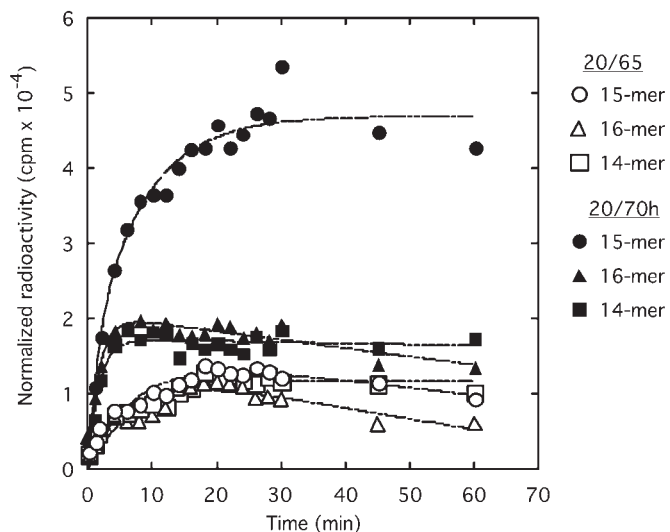

Figure 7. Effect of partial sequence homology located on the 5′ side of a 30/75 mismatched substrate. Molecules containing 75mer hairpin template strands (Figure 2) and either capable (30/75h) or incapable (30/75) of a limited amount of branch migration were incubated with vaccinia DNA polymerase in the presence of dNTPs.

Figure 9. Analysis of the excision products produced by treatment of 20/65 and 20/70 h duplexes with vaccinia DNA polymerase. The amount of ^{32}P-label present at each time point was determined from the phosphorimage shown in Figure 10 for the 14mer, 15mer and to 16mer reaction products. The data are plotted after normalization for the amount of 20mer label initially present in each reaction.

Figure 10. Vaccinia DNA polymerase processes 30/65 (3′-mismatched) substrates into nicked duplexes, which are substrates for T4 DNA ligase. A series of control reactions were performed to show that a 20/65 duplex is a substrate for T4 DNA ligase (lanes 11–18) but only if the 65mer hairpin template is treated first with polynucleotide kinase (lanes 2–9). If this phosphorylation reaction is omitted, only a small amount of strand joining is observed that is presumably due to intermolecular joining of a 5′ ^{32}P-labeled 20mer strand to an unlabeled 65mer template strand on another duplex (lanes 6–8).

Perhaps the most striking difference was the capacity of the 20/70h substrate to support qualitatively much more primer extension than did the 20/65 substrate. By the 15 min time point, 75% of the label in a 20/70h substrate was distributed among a ladder of extension products up to ∼40 nt in length, whereas only 9% of the primer stand label was extended in the 20/65 substrate (Figure 8). The same effect was also seen using the 20/75 substrate (Figure 5), but the amount of strand extension is greater using the substrate fully capable of branch migration.

Ligation of nicked intermediates

The 30/65 substrate is unique in that the 3′-unpaired strand is excised rapidly, generating a meta-stable 20mer product in ∼40% yields that should bear a simple nick (Figure 6) and thus be a substrate for a DNA ligase. To prove that these are nicked products, we added an unlabeled 5′-phosphate to the 65mer strand and annealed this DNA to a ^{32}P-labeled 30mer strand. These DNAs were then used as substrates in polymerase processing reactions (Figure 6). The reaction products were phenol extracted, ethanol precipitated and treated with T4 DNA ligase. Control reactions were also prepared containing the 20/65 nicked duplex substrate, both with and without a 5′-phosphoryl group on the 5′ end of the 65mer template strand.

The control experiments showed that the 20/65 nicked duplex molecule was a very good substrate for DNA ligase which, even after treatment with vaccinia DNA polymerase, could be converted with good yield into 85mer ligation products (Figure 10, lanes 15–18). Phenol extraction was necessary because the DNA polymerase seemed to interfere with ligation reactions (data not shown). That these were primarily intramolecular ligation products, and not formed by intermolecular blunt-end joining reactions, was suggested by the
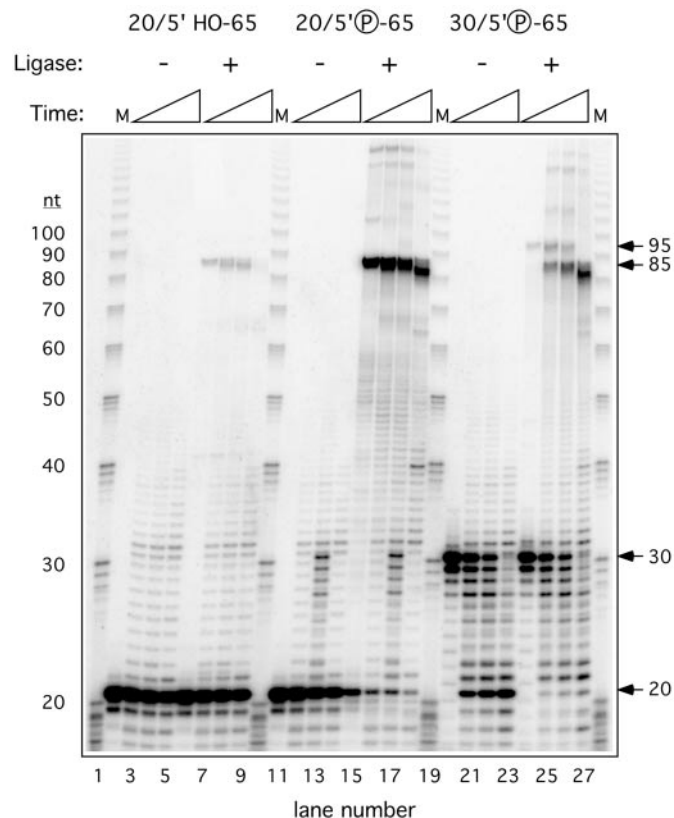
fact that only small quantities of 85mer products were formed if the 5′-phosphoryl group was left off the 65mer strand (Figure 10, lanes 6–9). In contrast, the intact 30/65 molecule was not a good substrate for DNA ligase (Figure 10, lane 24) until vaccinia DNA polymerase had an opportunity to generate significant quantities of the 20mer excision product (lanes 21–23). This 20mer reaction product was readily converted into 85mer joint molecules in the presence of DNA ligase (lanes 25–27). These species can be more readily differentiated from intermolecular ligation products than in the control experiments because, at least in the early stages of the polymerase processing reaction, intermolecular ligation products will be composed of 65mer strands ligated to molecules ≤30 nt in length and thus these products migrate more slowly than do 85mer strands (lanes 24–26). The ligation reactions consumed primarily the 20mer excision species although the 19mer excision product is also a substrate. This is explained by the fact that T4 DNA ligase can join strands separated by small gaps (26). No depletion of the longer (>30 nt) strand-extension products was detected, consistent with the likelihood that these are products of strand-displacement or strand switch synthesis and such DNAs would not be suitable substrates for DNA ligases. We concluded that the proofreading

exonuclease of vaccinia DNA polymerase can process 3′-branched structures into nicks that are substrates for DNA ligases.

DISCUSSION

Poxviruses replicate in the cytoplasm of even enucleated cells and must presumably replicate, repair and recombine virus DNAs using a far more limited repertoire of enzymes than are available to cellular organisms. Indeed, aside from a Holliday junction endonuclease, poxvirus genomes do not encode any other obvious 'classical' recombination functions. Nevertheless, one can detect induced levels of recombination activity in poxvirus-infected cells comparable to that seen in T4-infected *E.coli* (4). In this communication, we have examined how the properties of vaccinia virus DNA polymerase might endow the enzyme with a secondary function allied to recombinant production. These studies illustrate several possible reasons for why polymerase function may link viral replication and recombination.

These studies first confirm and extend the original work of Challberg and Englund (10,11). They noted the high level of 3′-to-5′ exonuclease activity on single-stranded DNA and an absence of any 5′-to-3′ exonuclease. When we compared the rate of degradation of the 20mer strand either alone, or accessible via a nick in a complex with the 65mer strand (20/65, Figure 3), our steady-state data suggests a ≥60-fold preference for single-stranded versus nicked double-stranded DNA. Fully single-stranded DNAs are also more rapidly degraded than are mispaired single strands within otherwise duplex structures, as can be seen by comparing the rate of degradation of the single-stranded 20mer (Figure 3) with a single-stranded mispaired portion of the 30mer (30/65, Figure 4). A general and expected feature of all these reactions is that adding dNTPs favors DNA polymerase activity and limits the proofreading exonuclease activity if there is also a template strand, although it is notable that what are believed to be physiological concentrations of dNTPs in vaccinia-infected cells (∼45 μM) (24) support a mix of both activities (e.g. Figures 3 and 4). These observations cannot be compared directly with the pre-steady-state analyses that have been applied to the proofreading exonucleases of other B-family DNA polymerases, like T4 DNA polymerase, but an enzymatic preference for single-stranded DNA ≫ mispaired DNA termini ≪ duplex DNA are all well-established features of these enzymes.

With regards to a possible role for vaccinia DNA polymerase in promoting post-synaptic steps in virus recombination, we noted several reaction features that are of relevance. In particular, vaccinia polymerase can rapidly excise the unpaired 3′ single-stranded tail from the 30mer strand in a 30/65 substrate. This reaction generates a metastable 20mer product in high yield, with nicks that can be sealed by a DNA ligase (Figure 10). Thus, if mispaired joint molecules of this type are formed by single-strand annealing reactions, vaccinia DNA polymerase plus the viral DNA ligase (27) can facilitate the conversion of such molecules into nick-free duplex products.

These reactions are modified in different ways by the presence of a single strand adjacent to, but positioned on, the 5′ side of the primer terminus. Under these conditions, the 20mer strand in a 20/75 substrate is depleted far more rapidly than in

the 20/65 substrate by a combination of both DNA synthesis (in the presence of dNTPs) and by excision (Figure 5). These observations show that although the enzyme cannot attack a 5′-ended strand, the presence of such 5′-branched substrates renders the 3′ end of the primer strand more accessible to the enzyme in than it is in a nicked duplex substrate. The 20/70h substrate permitted a demonstration that vaccinia DNA polymerase can use this reaction feature to catalyze strand assimilation reactions, excising sequence from the now more accessible 3′ end of the primer strand and simultaneously annealing any unpaired homologous sequences on the 5′ end of the longer template strand. In particular, we observed the more rapid production of a range of smaller excision products in reactions containing 20/70h substrates than in 20/65-containing substrates (Figure 8), and small amounts of the 15mer species (which would be formed by strand assimilation to generate a nicked duplex) were produced in excess relative to 14mer and 16mer sister excision products (Figure 9). In effect, vaccinia DNA polymerase can catalyze the same kinds of strand processing/annealing reactions that the yeast POL δ 3′-to-5′ exonuclease has been proposed to catalyze as a complement to Rad27/Fen1 activity (28).

Molecules containing discontinuities flanked by single-stranded extensions on both 3′ and 5′ ends (30/75 and 30/75h) exhibited more complex behaviors. The 3′-mismatch was initially degraded with equal efficiency in both 30/65 and 30/75 substrates (Figure 6), but while the former reaction produced a stable nicked 20mer excision product, the primer strand continued to be degraded beyond the 20mer point in the presence of a 5′-mismatched strand. Similarly, the nicked duplex produced during exonuclease attack on the 20/70h substrate appeared to be relatively more stable than other reaction intermediates. The simplest explanation for these data is that nicked duplexes represent a kinetically stable reaction intermediate under steady-state reaction conditions. However, when the substrate structure precludes formation of nicked products, the 3′-to-5′ exonuclease can continue to attack the 3′-ended strand even in the presence of dNTPs.

An unanticipated enzymatic property of vaccinia virus DNA polymerase was discovered when we examined the DNA synthesis occurring on each of the different substrates. Most of these molecules permitted small amounts of DNA synthesis with the particular exception of the 20/70h substrate. In this case, the majority (∼75%) of the input strands were quickly chased into a series of synthetic products extending as far as the location of the hairpin in the template strand (Figure 8). Although further studies are needed to explore the details of this reaction, it would seem to be of significance for two reasons. First, the 20/70h substrate mimics the hypothetical replication fork in the Moyer and Graves 'rolling hairpin' model for poxvirus DNA replication (14). The DNA synthesis we detect in these and other reactions (e.g. Figures 5 and 8) could thus be an example of the strand displacement reactions that have long been a key, but unproven, feature of the model. Second, as we have pointed out above (Figure 1), the single strands that are presumably displaced in such reactions could serve as substrates for SSA reactions. This would provide another 'pre-synaptic' mechanism for inextricably linking E9L function to poxvirus recombination reactions.

The reaction conditions employed in these experiments cause the polymerase to act in a distributive manner. These

DNA-binding properties would be altered by adding the A20R processivity factor (23), but how this protein might affect the reactions described here is difficult to predict a priori. The effect of the processivity factor on DNA polymerase function in other systems is varied, increasing proofreading activity in some and decreasing it in others. We are currently assembling multi-component reactions to investigate the effects of proteins like A20R on joint processing, and to characterize better the strand displacement reaction.

In conclusion, our observations provide insights into how the activities of vaccinia virus DNA polymerase suffice to catalyze several critical aspects of DNA metabolism in virus-infected cells. The 3′-to-5′ proofreading exonuclease can catalyze a reaction on duplex DNA that processes misaligned annealed DNA structures into metastable molecules containing simple nicks, and which are substrates for the repair activity of DNA ligases. Single-strand annealing reactions could also generate a variety of branched DNA duplexes during virus replication, and the strand assimilation reactions we have documented illustrate a potential role for the enzyme in 'tidying up' at the post-synaptic stage of these recombination reactions. Finally, an unanticipated property of branched hairpin molecules is that some seem to be good substrates for primer extension reactions. This is especially true if they bear a 5′ extension that, due to homology (20/70h), has some freedom to adopt multiple pairing arrangements. We are presently studying the substrate properties of such molecules with the expectation that it may provide further insights into the role poxvirus DNA polymerases play in promoting genetic recombination and into the still poorly understood structure of a poxvirus DNA replication fork.

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