

# DNA Branch Nuclease Activity of Vaccinia A22 Resolvase\*<sup>§</sup>

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DNA replication, recombination, and repair can result in formation of diverse branched DNA structures. Many large DNA viruses are known to encode DNA branch nucleases, but several of the expected activities have not previously been found among poxvirus enzymes. Vaccinia encodes an enzyme, A22 resolvase, which is known to be active on four-stranded DNA junctions (Holliday junctions) or Holliday junction-like structures containing three of the four strands. Here we report that A22 resolvase in fact has a much wider substrate specificity than previously appreciated. A22 resolvase cleaves Y-junctions, single-stranded DNA flaps, transitions from double strands to unpaired single strands (“splayed duplexes”), and DNA bulges *in vitro*. We also report site-directed mutagenesis studies of candidate active site residues. The results identify the likely active site and support a model in which a single active site is responsible for cleavage on Holliday junctions and splayed duplexes. Lastly, we describe possible roles for the A22 resolvase DNA-branch nuclease activity in DNA replication and repair.

Branched DNA molecules can be formed during many DNA transactions. For example, DNA 5'-flap structures (Fig. 1A) can be formed by strand displacement DNA synthesis during DNA replication. Double strand break repair may proceed by resection of DNA strands, then annealing of complementary regions. In this pathway, any potential unpaired strands would form DNA flaps (Fig. 1, A and B). DNA Y-junctions (Fig. 1C) or related structures with a nick at the branch point can be formed at stalled replication forks. Homologous recombination between partially complementary DNA strands can yield a variety of DNA flaps, bulges, and splayed duplexes (Fig. 1, A, B, E, and F). Holliday junctions (HJs)<sup>3</sup> (Fig. 1G) are formed at crossovers during homologous recombination and by extrusion of DNA cruciforms. Given the ubiquity of branched DNA structures, it is not surprising that many large DNA viruses encode enzymes for processing branched DNAs.

Poxviruses are large DNA viruses of animals that replicate in the cytoplasm, independent of the cell nucleus (1, 2). Thus poxviruses encode most of the enzymes required for replicating their DNA. Early steps of poxvirus DNA replication result in the formation of DNA concatemers (3). The concatemer junctions contain a conserved inverted repeat sequence that can be extruded as a DNA cruciform (4). At its base, this structure resembles a HJ (5), which is known to be a substrate for the vaccinia virus-encoded A22 resolvase *in vitro* (Fig. 1G). Cleavage of the HJ present at each concatemer junction, followed by ligation of the cleaved product, would yield genome monomers with the DNA hairpin termini characteristic of poxviruses. Consistent with the above model, inactivation of a conditional A22R mutant resulted in accumulation of the uncleaved concatemers *in vivo* (6), indicating that A22 is required for concatemer resolution. A reduction in overall DNA synthesis was also observed for the A22-mutant virus (6), suggesting that A22 resolvase may perform additional roles in DNA replication (discussed below). However, previous studies of A22 resolvase substrate specificity have suggested that the types of substrates accepted by the enzyme were fairly restrictive, with known substrates including only the HJ and related molecules in Fig. 1, G and H (6).

A22 resolvase is a member of the RNase H superfamily of enzymes, which includes HJ resolving enzymes identified in bacteria (RuvC) (7, 8) and yeast (Cce1) (9), as well as retroviral integrases, bacterial transposases, and the RISC nuclease argonaute (10–12). These enzymes have a conserved fold in the catalytic domain and are dependent on divalent metal ions (Mg<sup>2+</sup> or Mn<sup>2+</sup>) for activity. Each enzyme contains three or four conserved acidic residues that come together in space at the active site to bind the metal atoms, which are critical for catalysis (13–18). Purified A22 protein binds HJ substrates as a dimer (19) and cleaves at symmetric positions to yield two free nicked duplex DNAs (19–21). A22 is relatively sequence non-specific, displaying only a weak preference for cleavage at the consensus dinucleotide 5'-(G/C) ↓ (A/T)-3' (19, 21).

Here we report that A22 resolvase has a much wider substrate specificity for DNA branches than previously appreciated. We document cleavage of Y-junctions (Fig. 1C), both 5' and 3' DNA flaps (Fig. 1, A and B), DNA bulges (Fig. 1E), and “splayed duplexes” (Fig. 1F). These activities support models for a much wider role of A22 resolvase in poxvirus replication, more resembling the bacteriophage enzymes lambda Rap, T7 endonuclease I, and T4 endonuclease VII (22). We have also investigated the active site of vaccinia virus A22 resolvase using site-directed mutagenesis. A total of eleven amino acid substitutions in seven different residues were generated and analyzed for activity on HJ substrates, revealing some novel features of

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1.

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<sup>3</sup> The abbreviations used are: HJ, Holliday junction; Me<sub>2</sub>SO, dimethyl sulfoxide; nt, nucleotide.

highly conserved residues. Additional tests of mutant enzymes on branched DNA substrates supported the idea that a single active site is responsible for cleavage on all the substrates analyzed. We describe possible pathways of recombinational priming of replication and double strand break repair, which may utilize the A22 branch nuclease activity.

## EXPERIMENTAL PROCEDURES

**Mutagenesis and Purification of Recombinant Proteins**—The A22 resolvase open reading frame was amplified from vaccinia DNA (strain WR) and cloned into the pDuetMxe vector as previously described (21) to make pDuet-A22R-Mxe. pDuet-A22R-Mxe has the A22R gene in-frame with a C-terminal dual affinity tag and places this A22R tag fusion under the control of a T7 RNA polymerase promoter. Plasmids conferring single amino acid substitutions to the A22R gene product were constructed using the QuikChange (Stratagene) site-directed mutagenesis kit using pDuet-A22R-Mxe as the template and specific DNA oligonucleotide primer pairs to introduce each mutation (see supplemental Table S1 for oligonucleotide sequences). The presence of the mutations was confirmed by DNA sequencing. The proteins were overexpressed in *Escherichia coli* BL21(DE3)pLysS cells and purified by dual affinity chromatography as previously described (21). Protein amounts and purity were assessed by SDS-PAGE next to known standards.

**Preparation of DNA Substrates and Oligonucleotide Markers**—DNA substrates were prepared by annealing together the separate oligonucleotide strands that comprise each (see supplemental Table S1 for oligonucleotide sequences). Before the annealing step, one oligonucleotide was 5'-end-labeled using T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol). Unincorporated label was removed by passing the labeling reaction through a G-50 spin column (Amersham Biosciences). Equimolar amounts of the three unlabeled oligonucleotide strands were then added to the labeled strand. Next, sodium chloride was added to a concentration of 100 mM, the solution was heated to 95 °C for 3 min in a heat block, the heat source was removed from the block and the annealing reaction was allowed to cool to room temperature over 1.5–2 h. The substrates were then stored at 4 °C.

**Resolvase Cleavage Reactions**—Cleavage reactions were started by adding 0.4–0.5  $\mu$ g (18–22 pmol) of purified enzyme to a solution containing 0.2 pmol of  $^{32}$ P-labeled substrate DNA to achieve a final reaction volume of 20  $\mu$ l. The final solution conditions for the reactions were 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 20% Me<sub>2</sub>SO, 100  $\mu$ g/ml bovine serum albumin, 3–6 mM dithiothreitol, 0.02% Nonidet P-40, and 1% glycerol.

To examine duplex products, reactions were terminated after incubation at 37 °C for 25 min by the addition of a 5 $\times$  stop solution to give final concentrations of 20 mM EDTA, 0.2% SDS, 5% glycerol, and 0.02% bromphenol blue. The products of the reaction were resolved by electrophoresis in a 10% polyacrylamide gel containing 1 $\times$  TBE. Then, the wet gel was exposed to a phosphor screen and the bands were visualized using a Storm PhosphorImager (Molecular Dynamics).

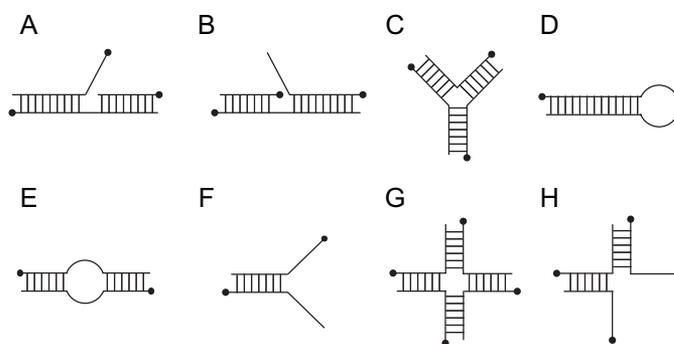


FIGURE 1. Branched DNA substrates used in this study. Black circles indicate DNA 5'-ends. A, 5'-flap. B, 3'-flap. C, Y-junction. D, hairpin. E, bulge. F, splayed duplex. G, HJ. H, Holliday-like junction with 3 of the 4 strands.

To examine single-stranded products, reactions were terminated by  $\geq 2$ -fold dilution in a solution containing 80% formamide, 10 mM EDTA, and 0.02% bromphenol blue. After heating at 95 °C for 3 min, the reaction mixtures were placed on ice and then resolved by electrophoresis in a 14% polyacrylamide (19:1) gel containing 7.5 M urea and 1 $\times$  TBE. The gel was then dried, exposed to a phosphor screen and the bands were visualized using a Storm PhosphorImager (Molecular Dynamics). Maxam-Gilbert chemical cleavage reactions were carried out as previously described (39, 40).

**Effect of Me<sub>2</sub>SO**—Cleavage reactions were started by adding 1.2–1.5  $\mu$ g ( $\sim 60$  pmol) of purified enzyme to a solution containing 0.6 pmol of  $^{32}$ P-labeled substrate DNA to achieve a final reaction volume of 60  $\mu$ l. The final solution conditions were the same as above except some reactions did not contain Me<sub>2</sub>SO. 12- $\mu$ l aliquots were stopped at 3, 6, 9, and 12 min. For some substrates, the 3-min time point was repeated using 20- $\mu$ l reaction volumes with the same enzyme and substrate concentrations and reaction conditions. The reaction products from each aliquot were resolved by electrophoresis in a 10% polyacrylamide gel containing 1 $\times$  TBE. Then, the wet gel was exposed to a phosphor screen, and the bands were quantified using a Storm PhosphorImager and ImageQuant software (Molecular Dynamics). For the 3-min time points, the concentration of product was calculated by multiplying the initial substrate concentration by the fraction of product formed, as determined by the phosphorimager analysis. Rates were estimated by dividing the product concentration by 180 s.

## RESULTS

**In Vitro Assays of A22 Specificity**—To investigate the substrate specificity of A22 resolvase, we prepared oligonucleotide substrates matching the structures in Fig. 1, A–H. For all substrates, each of the component DNA strands was separately labeled on the 5'-end with  $^{32}$ P so possible activity could be monitored on each DNA strand. The sequences of the oligonucleotides used are presented in supplemental Table S1. Substrates were incubated with purified A22 resolvase, then reactions were stopped and products were analyzed on both native gels and denaturing DNA sequencing-type gels. Products of Maxam-Gilbert chemical cleavage of each labeled DNA strand were included on the denaturing gels as size markers.

As a control, the E81Q mutant (Ref. 20, and discussed below) was compared with wild type for each substrate. The E81Q

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substitution eliminates one of the conserved acidic residues expected to form part of the active site. For all the substrates studied, the E81Q mutants were inactive, indicating that contaminating *E. coli* nucleases were not responsible for the observed cleavage activities.

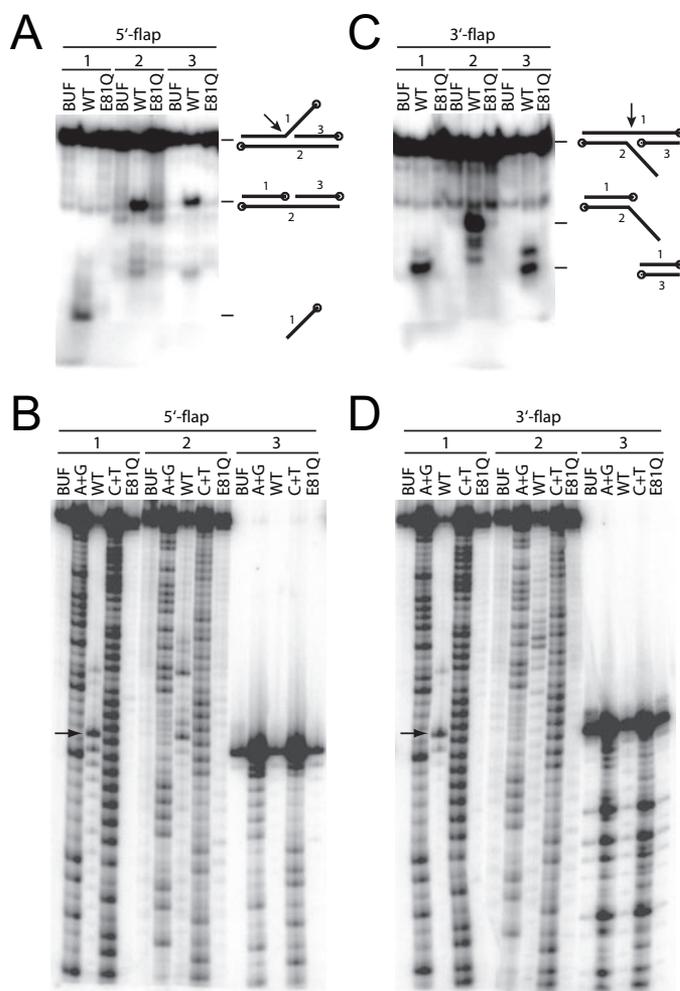
As expected from previous studies (20), little cleavage was seen on single-stranded DNA or completely complementary double-stranded DNA. In addition, we observed little activity on DNAs containing simple transitions from double to single-stranded regions, such as 5'- or 3'-recessed duplexes (data not shown). Efficient cleavage was seen on HJ substrates (see below) and on the HJ analogs with only three strands out of four (Fig. 1*H* and data not shown). Below we discuss cleavage on each of the substrates in Fig. 1, A–F in turn, and then return to the implications under “Discussion.”

**Flap Nuclease Activity of A22 Resolvase**—Incubation of the 5'-flap substrate with A22 resolvase, followed by analysis on native DNA gels (Fig. 2*A*), resulted in the appearance of faster migrating DNA forms. Cleavage of substrates labeled on strand 1 yielded the fastest migrating form, while cleavage of substrates labeled on strand 2 or 3 yielded a more slowly migrating product. Cleavage of substrates labeled on strands 2 and 3 also yielded faster migrating forms present in lower abundance.

Denaturing gel analysis (Fig. 2*B*) showed that the most prominent cleavage product was on strand 1, at the point where the 5'-single-stranded flap protrudes from the double helix (marked with *arrow* in Fig. 2, *A* and *B*), yielding a nicked duplex and a free single strand. Less abundant cleavages were also seen on strand 2 opposite the branch point. No significant cleavage was seen on strand 3. Thus the main product was the duplex with the flap removed.

On the 3'-flap substrate (Fig. 2*C*), in contrast, cleavage of reactions with the label attached to strand 2, after native gel electrophoresis, yielded a relatively slower migrating product, while reactions with labeled strands 1 and 3 yielded faster migrating cleavage products. The denaturing gel analysis (Fig. 2*D*) showed that the most prominent cleavage was on the continuous strand (strand 1), opposite the 3'-flap (marked with *arrow* in Fig. 2, *C* and *D*), thus accounting for the relative mobility of the products on the native gel. Less prominent but detectable cleavage was seen on strand 2, the protruding 3'-strand. No cleavage was detected on strand 3. Thus the polarity of the DNA strands in the flap substrates strongly affected the location of preferred cleavage sites (compare Fig. 2, *A* and *B* versus *C* and *D*).

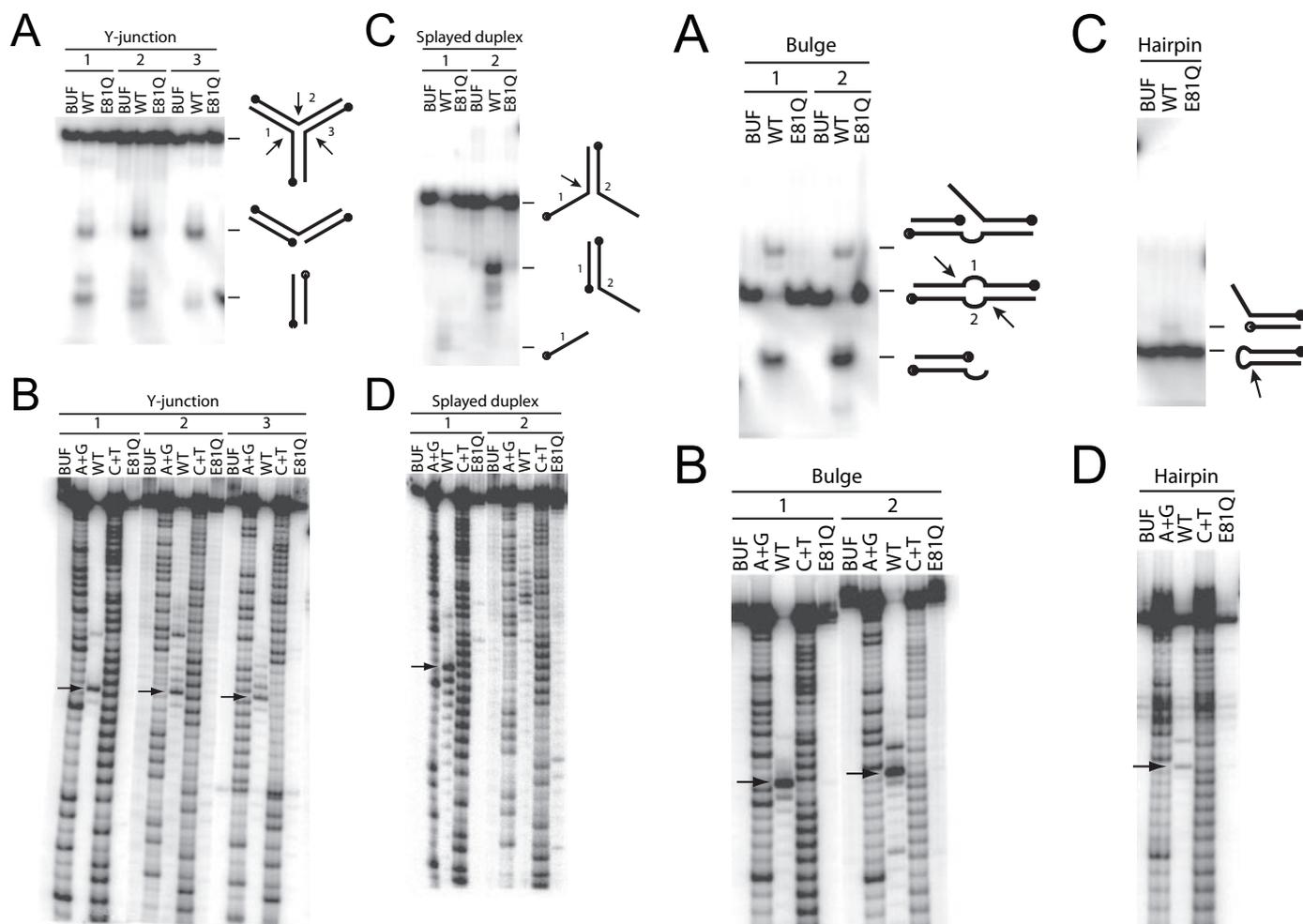
**Cleavage of DNA Y-junctions and Splayed Duplexes by A22 Resolvase**—A previous study of A22 resolvase did not detect cleavage on a DNA Y-junction (Fig. 1*C*) (20), but we obtained a different result with the Y-junction substrate studied here. Native gel analysis (Fig. 3*A*) showed that incubation of the Y-junction with A22 resolvase yielded two faster migrating forms that were seen regardless of which of the three strands was labeled. Analysis of the positions of cleavage on the three strands on a DNA sequencing-type gel (Fig. 3*B*) showed that each strand was cleaved by A22 resolvase, and that the major positions of cleavage were near the point where the three DNA arms come together. Additional positions of minor cleavage were seen at bases adjacent to the major cleavage position, and



**FIGURE 2. Cleavage of DNA flaps by A22 resolvase.** *A*, analysis of 5'-flap cleavage products by native PAGE. *BUF*, buffer control; *WT*, wild-type enzyme; *E81Q*, active site mutant enzyme. The identity of the 5'-end  $^{32}\text{P}$ -labeled strand in each set of reactions is indicated above the gel with a numeral. The deduced structures of DNA products are shown beside the gel with numbers indicating strand identities (see supplemental Table S1 for strand sequences). *Black circles* indicate DNA 5'-ends. *B*, analysis of 5'-flap cleavage products by denaturing PAGE. *A+G*, purine cleavage reaction; *C+T*, pyrimidine cleavage reaction. The identity of the 5'-end  $^{32}\text{P}$ -labeled strand in each set of reactions is indicated above the gel. The major cleavage product band is indicated with an *arrow*. *C*, analysis of 3'-flap cleavage products by native PAGE. *D*, analysis of 3'-flap cleavage products by denaturing PAGE.

for strands 1 and 2 weak cleavage was seen five bases 5' of the major position. Thus the two products seen on the native gel (Fig. 3*A*) can be inferred to be the products of a double cleavage, which would yield a nicked duplex as well as a double-stranded form equivalent to a single arm of the Y-junction.

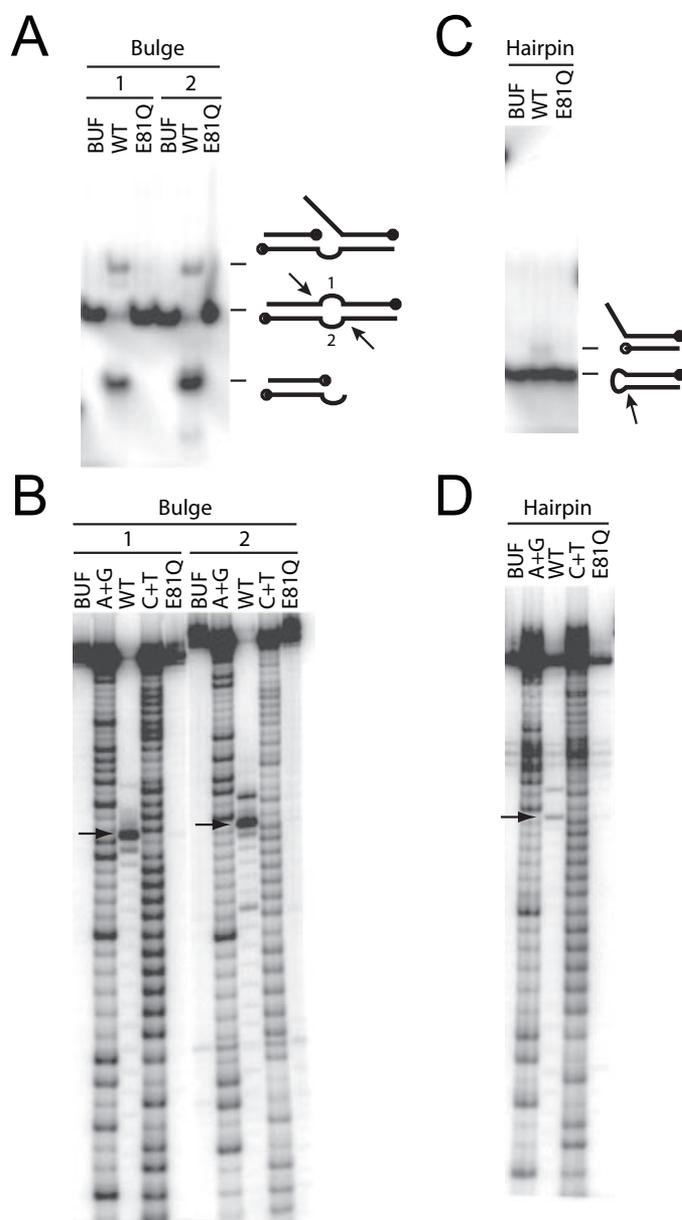
Resolvase was also active on a “splayed duplex” substrate, in which two strands are annealed along part of their length, so the annealed molecule contains a transition from double-stranded DNA to single-stranded DNA (Fig. 3*C*). Such molecules may be formed *in vivo* by annealing of partially complementary DNA regions. Analysis of cleavage products by native gel electrophoresis (Fig. 3*C*) showed that labeling of strand 1 yielded products that were low molecular weight and formed a diffuse smear. Labeling of strand 2 followed by cleavage with resolvase yielded a discrete higher molecular weight form. Analysis of the cleavage sites on denaturing DNA gels showed predominant



**FIGURE 3. Cleavage of DNA Y-junctions and splayed duplexes by A22 resolvase.** *A*, analysis of Y-junction cleavage products by native PAGE. Labels are as in Fig. 2. Deduced structures with strand numbers left unassigned indicate the structure can be the resultant product of more than one strand cleavage pathway. *B*, analysis of Y-junction cleavage products by denaturing PAGE. *C*, analysis of splayed duplex cleavage products by native PAGE. *D*, analysis of splayed duplex cleavage products by denaturing PAGE.

cleavage on strand 1 at the junction between the single- and double-stranded DNA regions, thereby releasing the single-stranded labeled 5'-DNA. Cleavage was less efficient on strand 2, but also took place near the single-to-double strand transition. Thus A22 resolvase primarily cleaves the splayed duplex to release the single-stranded region terminating with a 5'-end.

**Cleavage of DNA Bulges by A22 Resolvase**—DNA bulges are regions of unpaired bases between double helix-forming segments in a DNA duplex (Fig. 4A). A22 resolvase was highly active in cleaving such a structure, where 20 bp of duplex flank both sides of an unpaired region of 10 nt on each strand. The patterns of products were similar on native DNA gels for substrates labeled on either strand 1 or strand 2 (Fig. 4A). Most of the starting labeled DNA was converted to a faster migrating form, though, unexpectedly, a lower proportion was actually converted to a slower migrating form. Analysis of the cleavage products on denaturing DNA gels showed a major cleavage point on each strand, located at the transition point from single- to double-stranded DNA. The position of cleavage on each strand was on the 3'-side of the bulge region. Thus the polarity

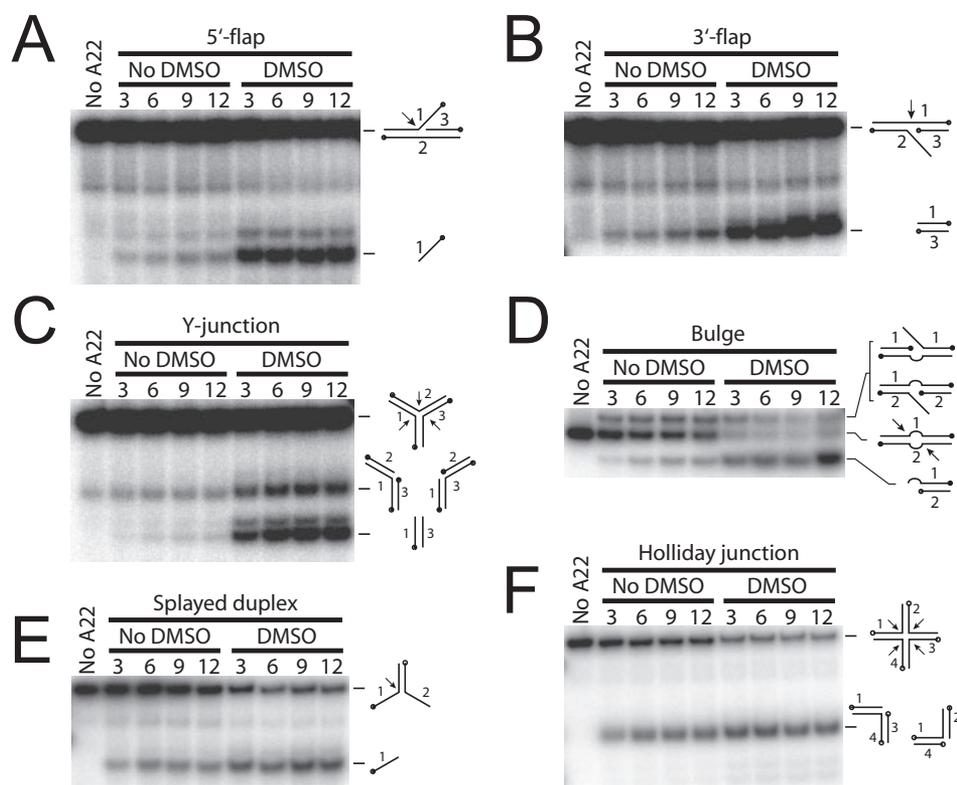


**FIGURE 4. Cleavage of DNA bulges and hairpins by A22 resolvase.** *A*, analysis of bulge cleavage products by native PAGE. Labels are as in Fig. 2. Deduced structures with strand numbers left unassigned indicate the structure can be the resultant product of more than one strand cleavage pathway. *B*, analysis of bulge cleavage products by denaturing PAGE. *C*, analysis of hairpin cleavage products by native PAGE. *D*, analysis of hairpin cleavage products by denaturing PAGE.

of cleavage of the DNA bulge resembled that seen on the splayed duplex.

There was no evidence for longer covalent DNA forms on the denaturing gel, indicating that the slower migrating form produced by the action of resolvase, seen on the native gel, is not a DNA joining product. The slower migrating DNA is likely to be a bulge substrate with one strand cleaved. This yields a branched DNA, with two double helical regions and a single-stranded flap. Branched DNAs are known to migrate more slowly than duplex DNAs, likely accounting for the observed band.

The activity of A22 resolvase was also tested on a DNA hairpin substrate (Fig. 4, C and D). The poxvirus telomeres consist



**FIGURE 5. Effect of dimethyl sulfoxide (DMSO) on A22 resolvase branch nuclease activity.** *A*, analysis of 5'-flap cleavage by native PAGE. *No DMSO*, no  $\text{Me}_2\text{SO}$  present in reaction; *DMSO*, 20%  $\text{Me}_2\text{SO}$  present in reaction; *No A22*, No enzyme added to substrate. Numbers above gel lanes indicate the reaction time in minutes. *B*, analysis of 3'-flap cleavage by native PAGE. *C*, analysis of Y-junction cleavage by native PAGE. *D*, analysis of bulge cleavage by native PAGE. *E*, analysis of splayed duplex cleavage by native PAGE. *F*, analysis of HJ cleavage by native PAGE. All substrates were 5'-end-labeled with  $^{32}\text{P}$  on strand 1. Drawings to the right of each gel only indicate the products that retain the  $^{32}\text{P}$  label (see Figs. 2–4 for analysis on all strands). Black circles indicate DNA 5'-ends.

**TABLE 1**

**Cleavage rates of branched DNA substrates by A22 resolvase and the effect of  $\text{Me}_2\text{SO}$**

Rate and error values are given as [product (pm)]/s and represent the mean and standard deviation, respectively, of *n* independent replicates.

DNA substrate	No $\text{Me}_2\text{SO}$	20% $\text{Me}_2\text{SO}$
5'-recess	0.41 ± 0.15 ( <i>n</i> = 2)	0.26 ± 0.038 ( <i>n</i> = 2)
5'-flap	1.4 ± 0.22 ( <i>n</i> = 3)	11 ± 0.67 ( <i>n</i> = 3)
3'-flap	1.6 ± 0.11 ( <i>n</i> = 3)	11 ± 0.51 ( <i>n</i> = 3)
Y-junction	0.57 ± 0.24 ( <i>n</i> = 3)	11 ± 0.44 ( <i>n</i> = 3)
Bulge	8.5 ± 2.2 ( <i>n</i> = 3)	33 ± 2.6 ( <i>n</i> = 2)
Splayed duplex	6.9 ± 0.99 ( <i>n</i> = 3)	23 ( <i>n</i> = 1)
Holliday junction	12 ± 0.50 ( <i>n</i> = 3)	33 ( <i>n</i> = 1)

of DNA hairpins, and a nuclease is hypothesized to cleave the termini to provide a free DNA 3'-end for replication initiation. The hairpin studied contained a duplex stem region of 20 bp and a single-stranded loop region of 10 nt. A small amount of slightly slower migrating material was seen in the native gel in the presence of the A22 resolvase. On the denaturing DNA gel, a slight amount of cleavage was seen, mapping to the base of the loop connecting the two strands in the DNA hairpin. Thus cleavage is detectable but weak in the hairpin studied. We return to these data and models for replication initiation under "Discussion."

**Effect of  $\text{Me}_2\text{SO}$  on DNA Branch Nuclease Activity**—In previous work, we reported enhancement of A22 resolvase cleavage activity on a HJ substrate in the presence of 20% dimethyl sulf-

oxide ( $\text{Me}_2\text{SO}$ ) (21). All the reactions discussed above were also performed under the conditions of 20%  $\text{Me}_2\text{SO}$ .  $\text{Me}_2\text{SO}$  enhances the activity of many DNA phosphoryl transferases, however, for some enzymes,  $\text{Me}_2\text{SO}$  has an additional effect of relaxing substrate specificity.

To investigate the effect of  $\text{Me}_2\text{SO}$  on substrate specificity for A22 resolvase, we examined product formation as a function of time for 6 different branched DNA substrates in the presence and absence of 20%  $\text{Me}_2\text{SO}$  (Fig. 5, A–F). Reaction aliquots were stopped at 3, 6, 9, and 12 min and then analyzed on native polyacrylamide gels (Fig. 5, A–F). For the 6 branched DNAs studied, we observed time-dependent product formation in the absence of  $\text{Me}_2\text{SO}$ . Cleavage rates were enhanced in the presence of  $\text{Me}_2\text{SO}$  for these substrates.

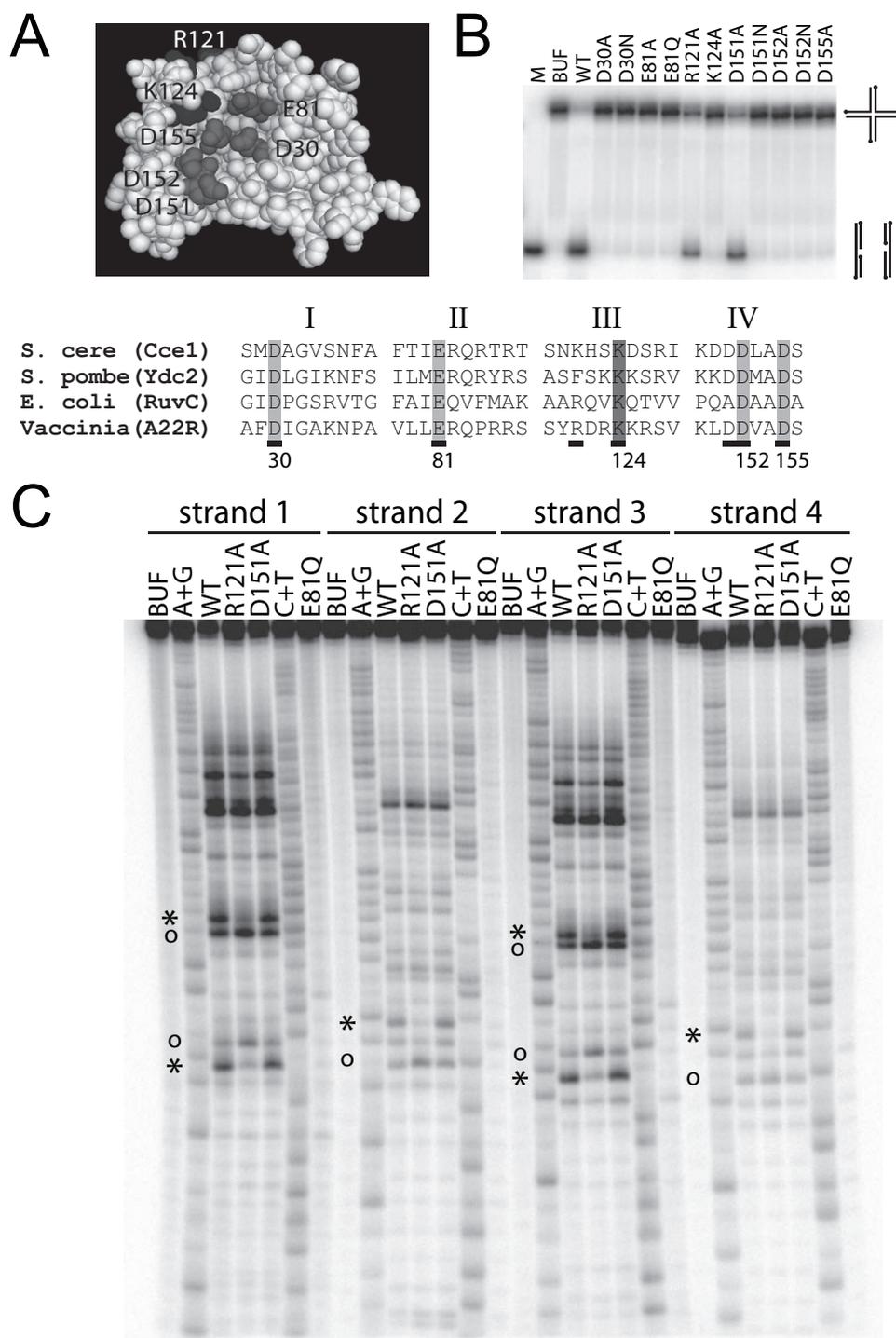
Table 1 shows the estimated rates after 3 min.  $\text{Me}_2\text{SO}$  significantly enhanced the rate for all 6 branched substrates but not the non-branched 5'-recess substrate. In the presence of  $\text{Me}_2\text{SO}$ , we observed the highest levels of activity with the HJ, bulge, and splayed duplex substrates and lower levels with the

5'-flaps, 3'-flap, and Y-junction. Five out of the six branched substrates show the same relative trends in cleavage rate in the absence of  $\text{Me}_2\text{SO}$ . The notable exception is the Y-junction, which exhibited the strongest rate enhancement by  $\text{Me}_2\text{SO}$  and was a DNA structure tested in previous work from Garcia *et al.* (20). They did not observe activity on their DNA Y-junction substrate; however, their reactions did not contain  $\text{Me}_2\text{SO}$ . Thus, this finding helps us to understand how our work diverged from previous studies.

We conclude that  $\text{Me}_2\text{SO}$  is not required for A22 resolvase to act on branched DNAs. Consistent with this assessment, our laboratory has begun studying a more soluble poxvirus resolvase which, in the absence of  $\text{Me}_2\text{SO}$ , cleaves the 5'-flap, 3'-flap, Y-junction, bulge, splayed duplex, and HJ to completion,<sup>4</sup> further supporting the idea that the cleavage observed in  $\text{Me}_2\text{SO}$  is not an *in vitro* artifact.

**Analysis of Conserved Residues by Mutagenesis**—To explore the A22 resolvase active site(s) responsible for the catalytic activities detected above, we prepared 11 derivatives of A22 resolvase with amino acid substitutions at candidate active site residues. These sites were identified by their homology with active site residues identified in previous studies of related

<sup>4</sup> F. D. Bushman, unpublished data.



**FIGURE 6. Cleavage activity of A22 resolvase derivatives with active site substitutions on HJ substrates.** *A*, structure: threading model of A22 based on the crystal structure of RuvC. *Highlighted positions* indicate residues examined in this study. *Light gray*, acidic residue; *dark gray*, basic residue. The model was constructed using SWISS-MODEL (41). *Alignment*: protein sequences of Cce1, Ydc2, RuvC, and A22 showing four RNase H-like HJ resolving enzyme conserved motifs (modified from Lilley and White (27)). *Highlighted positions* indicate absolutely conserved residues within the RNase H-like HJ resolvase family (*light gray*, acidic residue; *dark gray*, basic residue). *Black bars* indicate positions of the amino acid substitutions in A22 resolvase studied here. *S. cere*, *Saccharomyces cerevisiae*; *S. pombe*, *Schizosaccharomyces pombe*; *E. coli*, *Escherichia coli*; *Vaccinia*, *Vaccinia virus*. *B*, each purified enzyme was incubated with  $^{32}\text{P}$ -labeled HJ substrate HJ4  $\times$  20 and the products of the reaction were separated by native PAGE. The amino acid substitution present in each purified enzyme is indicated *above* the gel lane. *M*, 50-bp duplex marker matching the expected cleavage product; *BUF*, buffer control; *WT*, wild-type enzyme. The substrate and product bands are indicated by the diagrams to the *right* of the gel. *C*, cleavage specificity of A22 resolvase R121A and D151A. Each purified enzyme was incubated with the mobile HJ substrate HJ4  $\times$  20, and cleavage products were analyzed by denaturing PAGE. The amino acid substitutions studied are indicated *above* the gel lanes. *Circles* and *asterisks* indicate positions where enhanced and diminished cleavage, respectively, was observed for R121A.

RNase H-family enzymes (23–26). Eight charged residues were selected, because acidic residues can participate in metal binding, and basic residues in DNA binding (Asp<sup>30</sup>, Glu<sup>81</sup>, Arg<sup>86</sup>, Arg<sup>121</sup>, Lys<sup>124</sup>, Asp<sup>151</sup>, Asp<sup>152</sup>, and Asp<sup>155</sup>). Modified A22R genes were overexpressed in *E. coli* and the encoded proteins purified by affinity chromatography. Each protein tested behaved similarly to the wild type during expression and purification, suggesting that the modified proteins are similarly folded, except R86A and D155N, which showed reduced solubility (data not shown). The D30N and E81Q enzymes have been previously studied in A22 resolvase (20) and were used here as controls. D30N and E81Q are inactive for cleavage but have no apparent defect in HJ binding.

Fig. 6A shows a model of A22 resolvase, which was constructed using the crystal structure of *E. coli* RuvC as a template together with a sequence alignment of conserved motifs. In the model the four conserved acidic residues (Asp<sup>30</sup>, Glu<sup>81</sup>, Asp<sup>152</sup>, and Asp<sup>155</sup>) come together in space to form a potential active site. By analogy to other RNase H-like family members, we hypothesize that these four acidic residues bind two divalent metal ions that activate hydrolysis.

Each mutant enzyme was tested for cleavage of a HJ substrate (Fig. 6B). The control wild-type A22 resolvase cleaved the substrate to form duplex products of the expected mobility on a native DNA gel (Fig. 6B, lane labeled WT). No product formation was observed for enzymes containing amino acid substitutions at any of the five absolutely conserved charged residues (D30A/N, E81A/Q, K124A, D152A/N, D155A). The R121A and D151A enzymes retained cleavage activity. In contrast to D151A, however, the D151N enzyme displayed no detectable cleavage activity. Evidently the negative charge of the wild-type Asp does not play a direct role in catalysis, but the amide substitution introduces a dominant defect, an

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effect seen previously for related enzymes (26, 27), though the Ala and Asn substitutions were not previously compared.

To determine whether the active R121A or D151A enzymes had different cleavage specificities, we analyzed the positions of cleavage on the mobile HJ, HJ4 × 20. HJ4 × 20 contains a 20-bp region of sequence homology that is symmetric across the junction, thus allowing the crossover point to travel along the junction by branch migration. Unique sequences at the end of each arm prevent branch migration through the ends of the junction arms. Thus many different nucleotide sequences are available for cleavage within the homology region. Each of the four strands was separately <sup>32</sup>P-labeled and analyzed. The products of the reactions were separated by electrophoresis on a denaturing DNA-sequencing type gel adjacent to products of Maxam-Gilbert chemical cleavage of the same DNAs (Fig. 6C).

R121A displayed a detectably altered pattern of cleavage. In repeated experiments, three sites showed cleavage enhancement (Fig. 6C, circles) and another three sites exhibited diminished cleavage (Fig. 6C, asterisks) compared with wild-type A22 resolvase. Thus the R121A enzyme shows a distinctive cleavage pattern; consistent with a model in which R121 makes a DNA contact important for DNA sequence discrimination. The D151A enzyme showed a cleavage pattern indistinguishable from wild-type.

**Activities of A22 Mutant Proteins on the Splayed Duplex Substrate**—One of the A22 resolvase mutants, E81Q, was tested against all the DNA substrates in Fig. 1, A–H. The mutant enzyme was inactive on all substrates, indicating that the functional deficit was similar on all types of branched DNAs. To investigate the generality of this observation, each mutant enzyme was tested on the splayed duplex substrate (Fig. 7). Resolvase derivatives with substitutions that were inactive on the HJ substrate were also inactive on the splayed duplex substrate. Similarly, the two resolvase derivatives that retained activity on the HJ substrate (R121A or D151A) also retained activity on the splayed duplex. These findings are consistent with the idea that A22 resolvase uses a single active site for cleaving HJs and other branched DNAs.

## DISCUSSION

DNA branches are produced in many pathways of DNA replication, recombination, and repair. As expected from the ubiquity of branched DNAs, many DNA viruses are known to encode DNA branch endonucleases that remove branches to allow packaging of viral genomes. For poxviruses, however, viral-encoded nucleases active at DNA 5'-flaps, Y-junctions, bulges, or splayed duplexes have not previously been reported. Here we report that A22 resolvase of vaccinia virus is active on all these substrates. These data indicate that the activities of A22 resolvase are actually more similar to the previously described bacteriophage enzymes lambda Rap, T4 endonuclease VII, and T7 endonuclease I, each of which cleaves HJs and a variety of branched DNAs (22). In addition, we report mutagenesis studies of conserved charged residues, which likely comprise part of the enzyme active site. The mutant A22 derivatives showed similar deficits for cleavage of HJs and splayed

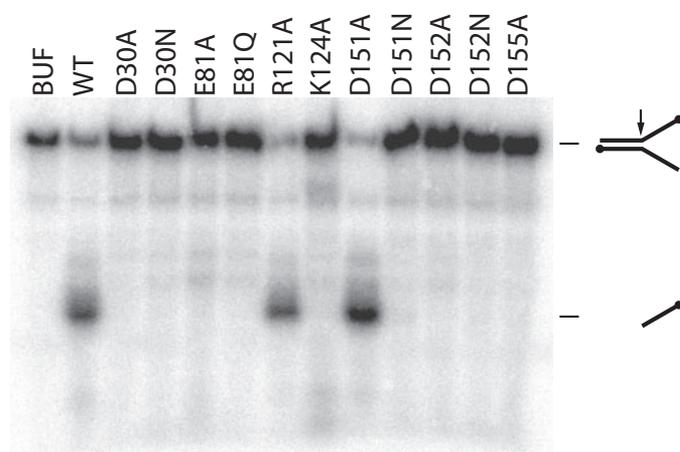


FIGURE 7. Cleavage activity of A22 resolvase derivatives with active site substitutions on splayed duplex substrates. Labels are as in Fig. 6B.

duplexes, indicating that a common active site is likely responsible for cleavage of each.

**Resolvase Cleavage Products as Substrates for Subsequent Steps**—On HJ substrates (Fig. 1G), cleavage by A22 resolvase is offset by one base from the DNA branch point (19).<sup>4</sup> Because the cleavage is symmetric across the junction, the product nicked duplexes are continuous, and so can be ligated closed by action of the poxvirus-encoded DNA ligase. However, data reported here, shows that the one base offset is a general feature of A22 resolvase cleavage on branched DNAs. For all the molecules shown in Fig. 1, cleavage was offset one base 3' to the branch point. On the 5'-flap branched substrate, this resulted in a 1-nt gap—the cleavage product could therefore not be sealed by ligase without further processing to fill in the gap. On the 3'-branched substrate (Fig. 1B), cleavage yielded a DNA double strand break, which would require several further steps to become rejoined (discussed below). Thus the products of cleavage at these branched substrates are expected to be intermediates in pathways involving several additional enzymatic steps.

**Possible Roles for A22 Resolvase in Priming DNA Replication**—The favored model for initiation of poxvirus DNA replication holds that a DNA endonuclease provides a nick near the hairpin termini of the genome to create a 3'-hydroxyl for initiation, and that replication then proceeds by a “rolling hairpin” mechanism (28). Poxviruses are not known to encode primase enzymes and while there is some evidence for RNA priming of replication from early reports (29–32) this has not been strengthened using modern methods.

Viruses depleted for A22 resolvase activity were reported to be reduced for total viral DNA synthesis in a single round infection, suggesting that A22 resolvase cleavage may be involved in generating 3'-ends for priming DNA synthesis (6). In the rolling hairpin replication model, cleavage of HJs by A22 resolvase yields DNA 3'-ends, which may then serve as primers for continued replication. However, two additional pathways potentially involving A22 resolvase may also yield free 3'-ends that may serve as primers.

One possibility is that A22 is the nuclease that provides the first nick to initiate rolling hairpin replication. In this model, A22 resolvase would supply the free 3'-end by cleaving near the DNA hairpin structure at the termini of the viral DNA. Our

initial tests of such a substrate yielded a somewhat intermediate result; the hairpin was cleaved, but with relatively low efficiency. We note, however, that the authentic viral telomeres have unusual structures. The sequences are highly A/T-rich, and there are extra-helical bases extruded at the terminal regions (33). It will be of interest to test whether A22 resolvase is able to cleave DNA hairpins that better match the authentic telomeres. Possibly additional poxvirus-encoded proteins assist such a reaction.

Another pathway of replication initiation, recombinational priming, could potentially take advantage of the A22 resolvase branch nuclease activity. After a vaccinia genome enters a cell and at least some of the entering DNA genome has been replicated, DNA strands formed by copying one template could be released and used to prime DNA replication on another molecule. Here we report that A22 resolvase cleavage at several types of branched DNAs could produce free single strands that might act as primers. Recombinational priming may involve pairing of single DNA strands, because poxvirus E9 DNA polymerase readily anneals single strands but is much less active in invading single strands into double-stranded regions (34). Depending on the replication pathway, branched DNAs could be produced by strand displacement synthesis, which would then need to be cleaved by A22 resolvase to allow DNA packaging. Branch nuclease activities of several phage enzymes have been suggested to play such roles (35, 36). Thus the branch nuclease activities of A22 resolvase may be useful both for generating single strands to serve as primers for replication, and for processing branched replication products prior to packaging.

*Possible Involvement of A22 in Double Strand Break Repair*—Double strand breaks in the poxvirus DNA could be generated by 1) A22 cleavage at stalled replication forks, 2) by A22 cleavage at DNA 3'-flaps (Fig. 1B), 3) by DNA synthesis across a nicked DNA strand, or 4) by various forms of DNA damage. The poxvirus E9 DNA polymerase is highly active in fusing DNA double strand ends that contain regions of sequence homology, thereby initiating repair of the double strand break and allowing the molecule to serve as a template for replication (37). The polymerase contains a 3' to 5' exonuclease activity, which resects single strands to expose complementary sequences. The presence of the E9 polymerase then accelerates pairing between complementary strands (37). Any protruding single stranded 5'-ends that did not participate in duplex formation would form DNA branches that would then be substrates for A22 resolvase. Protruding 3'-ends could be removed either by endonucleolytic cleavage by A22 resolvase or exonucleolytic cleavage by E9 polymerase (37). Thus A22 resolvase has the needed activities to remove unpaired regions and allow repair to proceed.

A22 resolvase may channel stalled replication forks into the above pathway for restarting DNA synthesis. Stalled replication forks resemble DNA three way junctions as in Fig. 1C, though potentially with a nick in one of the arms at the junction. Cleavage as in Fig. 3 yields a nicked duplex and a DNA duplex corresponding to one arm of the Y-junction. Repair of such structures by the pathway related to the double strand break repair described above could reactivate the replication fork for continued DNA synthesis.

*The A22 Resolvase Active Site and Implications for Inhibitor Development*—The mutagenesis study of A22 resolvase expands the collection of amino acid residues known to be required for catalysis and extends the parallels with other members of the RNase H superfamily. As is illustrated in Fig. 6A, a model for A22 resolvase structure can be readily generated based on the structure of the *E. coli* RuvC protein. In the model, the acidic residues that are sensitive to mutation are brought close together in space, likely forming a binding site for two metal atoms. The effects of amino acid substitutions on this putative active site were similar on the various DNA substrates, supporting the idea that a single active site is probably responsible for all the observed nuclease activities.

These findings have implications for development of antiviral agents against poxviruses. Another member of the RNase H superfamily, the HIV integrase enzyme, has been the topic of extensive drug development efforts, and integrase inhibitors are currently performing well in clinical trials (38). We have found that some of the inhibitors of HIV integrase are also active against poxvirus resolvase,<sup>4</sup> suggesting that this compound family may provide a feasible starting point for development of poxvirus antivirals.

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